EUROPEAN PHARMACOPOEIA

Free access to supportive pharmacopoeial texts in the field of vaccines for human use during the coronavirus disease (COVID-19) pandemic

Updated package - October 2020

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edqm
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Free access to supportive pharmacopoeial texts in the field of vaccines for human use during the coronavirus disease (COVID-19) pandemic

Updated package

The EDQM is committed to supporting users during the coronavirus disease (COVID-19) pandemic – as well as contributing to the wider global effort to combat the virus – by openly sharing knowledge and providing access to relevant guidance/standards.

To support organisations involved in the development, manufacture or testing of COVID-19 vaccines worldwide, many of which are universities and small and medium-sized enterprises, the EDQM is offering temporary free access to texts of the European Pharmacopoeia (Ph. Eur.) in the field of vaccines.

This package includes quality standards for vaccines which developers can take into account to help build appropriate analytical control strategies for their COVID-19 candidate vaccines and ensure the quality and safety of the final product. Application of such quality requirements may ultimately help to facilitate regulatory acceptance of a subsequent marketing authorisation application.

For ease of reading, a summary table listing the pharmacopoeial texts, with information regarding the vaccine types or vaccine platforms concerned (e.g. live attenuated viral vaccine, recombinant viral-vectored vaccines) is provided. The list of texts is not exhaustive and will be reviewed regularly and updated as required.

The pharmacopoeial texts comprise overarching general texts (general notices, general monographs, dosage form monographs and general chapters) as well as selected individual vaccine monographs and analytical methods. The texts are from the 10th Edition of the Ph. Eur., including Supplement 10.4.

This publication will be available on the EDQM website (https://go.edqm.eu/Pheurvaccinespackage) until further notice. It will be withdrawn when appropriate.

This in no way affects the existing legal status of the European Pharmacopoeia, nor does it imply or confer any demonstrated effectiveness of a particular vaccine type or vaccine platform for the prevention of COVID-19. This is confirmed by the inclusion of the following text at the bottom of pharmacopoeial texts reproduced in this document: "Not official text. Please refer to the current legally effective version of the Pharmacopoeia to ensure compliance."

The package was updated on 25 October 2020 to include additional Ph. Eur. texts.

If you have any questions or comments please contact the EDQM via the mailbox: VaccinesTF@edqm.eu.

The European Pharmacopoeia is published by the European Directorate for the Quality of Medicines & HealthCare of the Council of Europe (EDQM).

TEXT N°	TITLE	PRODUCT TYPE(S)
General Notices		
1.	General Notices	
General monographs		
2034	Substances for pharmaceutical use	All vaccines.
2619	Pharmaceutical preparations	All vaccines.
0153	Vaccines for human use	All vaccines.
0784	Recombinant DNA technology, products of	Recombinant protein-based vaccines.
1483	Products with risk of transmitting agents of animal spongiform encephalopathies	Vaccines produced using material of animal origin.
Dosage form monographs		
0520	Parenteral preparations	Vaccines for parenteral administration.
0676	Nasal preparations	Vaccines for nasal administration.
General chapters		
5.2.1	Terminology used in monographs on biological products	All vaccines.
5.2.2	Chicken flocks free from specified pathogens for the production and quality control of vaccines	Vaccines produced in specified pathogen-free primary avian tissues.
5.2.3	Cell substrates for the production of vaccines for human use	Vaccines using cell cultures for production.
2.6.16	Tests for extraneous agents in viral vaccines for human use	Live attenuated viral vaccines, inactivated viral vaccines, recombinant viral vectored vaccines.
5.1.7	Viral safety	Vaccines produced using material of human or animal origin.
5.2.8	Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products	Vaccines produced using material of animal origin.
5.14	Gene transfer medicinal products for human use	*Certain considerations may be relevant to recombinant viral vectored vaccines using adenovirus or poxvirus as backbone, and to DNA vaccines.*

TEXT N°	TITLE	PRODUCT TYPE(S)
	METHODS OF ANALYSIS	
2.6.1	Sterility	All vaccines[1].
5.1.6	Alternative methods for control of microbiological quality	All vaccines[2].
2.6.2	Mycobacteria	Vaccines using cell cultures or primary avian tissues for production.
2.6.7	Mycoplasmas	Vaccines using cell cultures or primary avian tissues for production.
5.1.3	Efficacy of antimicrobial preservation	Multi-dose vaccines containing a preservative.
2.6.14	Bacterial endotoxins	All vaccines[1].
5.1.10	Guidelines for using the test for bacterial endotoxins	All vaccines[1].
2.7.2	Microbiological assay of antibiotics	Where antibiotics are used during the production process[3], the residual antibiotic concentration may be determined by a microbiological assay adapted from chapter 2.7.2 or by other suitable methods (e.g. liquid chromatography).
2.6.34	Host-cell protein assays	Recombinant protein-based vaccines and purified recombinant viral vectored vaccines.
2.5.33	Total protein	Certain live attenuated viral vaccines and recombinant viral vectored vaccines that are less amenable to purification.
2.6.35	Quantification and characterisation of residual host-cell DNA	Vaccines produced in continuous cell lines[4].
2.2.29	Liquid chromatography	When the method is selected.
2.6.21	Nucleic acid amplification techniques	When the method is selected.
2.7.1	Immunochemical methods	When the method is selected.
2.7.24	Flow cytometry	When the method is selected.
2.2.1	Clarity and degree of opalescence of liquids	Vaccines in liquid form, lyophilised vaccines after reconstitution.
2.2.2	Degree of coloration of liquids	Vaccines in liquid form, lyophilised vaccines after reconstitution.
2.2.3	Potentiometric determination of pH	Vaccines in liquid form, lyophilised vaccines after reconstitution.
2.2.35	Osmolality	Vaccines for parenteral administration.
2.5.12	Water: semi-micro determination	Lyophilised vaccines.
2.9.17	Test for extractable volume of parenteral preparations	Vaccines for parenteral administration.
2.9.20	Particulate contamination: visible particles	Vaccines in liquid form, lyophilised vaccines after reconstitution.

1 Unless otherwise justified and authorised, as described in the General Monograph *Vaccines for human use (0153)*.

2 A comprehensive validation package, including the demonstration of equivalence with the compendial test, is a prerequisite when opting to use alternative microbiological methods for sterility.

3 It is preferable to have a production free from antibiotics. Unless otherwise justified, at no stage during production is penicillin or streptomycin used.

4 See also General Chapter *5.2.3. Cell substrates for the production of vaccines for human use.*

TEXT N°	TITLE	PRODUCT TYPE(S)
INDIVIDUAL VACCINE MONOGRAPHS		
Examples of individual monographs for various types of vaccines. *The following examples demonstrate the applicable requirements for specific products. They can be taken as guidance and should be considered for vaccines in a similar class without a specific monograph.*		
2441	Human papillomavirus vaccine (rDNA)	Recombinant protein-based vaccines. Production in an insect cell / baculovirus expression vector system.
1056	Hepatitis B vaccine (rDNA)	Recombinant protein-based vaccines. Production in CHO cells.
0214	Poliomyelitis vaccine (inactivated)	Inactivated viral vaccines.
0537	Yellow fever vaccine (live)	Live attenuated viral vaccines. *Certain considerations may be relevant to recombinant viral vectored vaccines using yellow fever virus or other viruses as backbone.*
0213	Measles vaccine (live)	Live attenuated viral vaccines. *Certain considerations may be relevant to recombinant viral vectored vaccines using measles virus or other viruses as backbone.*
2772	Influenza vaccine (live, nasal)	Live attenuated viral vaccines for nasal administration. *Certain considerations may be relevant to recombinant viral vectored vaccines using influenza virus or other viruses as backbone.*
MONOGRAPHS ON ADJUVANTS		
1664	Aluminium hydroxide, hydrated, for adsorption	Vaccines containing aluminium hydroxide as adjuvant.
2805	Squalene	Vaccines containing a squalene-based adjuvant.

General Notices

TEXT N°	TITLE	PRODUCT TYPE(S)
1.	General Notices	

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1. GENERAL NOTICES

1.1. GENERAL STATEMENTS

The General Notices apply to all monographs and other texts of the European Pharmacopoeia.

The official texts of the European Pharmacopoeia are published in English and French. Translations in other languages may be prepared by the signatory States of the European Pharmacopoeia Convention. In case of doubt or dispute, the English and French versions are alone authoritative.

In the texts of the European Pharmacopoeia, the word 'Pharmacopoeia' without qualification means the European Pharmacopoeia. The official abbreviation Ph. Eur. may be used to indicate the European Pharmacopoeia.

The use of the title or the subtitle of a monograph implies that the article complies with the requirements of the relevant monograph. Such references to monographs in the texts of the Pharmacopoeia are shown using the monograph title and reference number in *italics*.

A preparation must comply throughout its period of validity; a distinct period of validity and/or specifications for opened or broached containers may be decided by the competent authority. The subject of any other monograph must comply throughout its period of use. The period of validity that is assigned to any given article and the time from which that period is to be calculated are decided by the competent authority in light of experimental results of stability studies.

Unless otherwise indicated in the General Notices or in the monographs, statements in monographs constitute mandatory requirements. General chapters become mandatory when referred to in a monograph, unless such reference is made in a way that indicates that it is not the intention to make the text referred to mandatory but rather to cite it for information.

The active substances, excipients, pharmaceutical preparations and other articles described in the monographs are intended for human and veterinary use (unless explicitly restricted to one of these uses).

Quality systems. The quality standards represented by monographs are valid only where the articles in question are produced within the framework of a suitable quality system. The quality system must assure that the articles consistently meet the requirements of the Pharmacopoeia.

Alternative methods. The tests and assays described are the official methods upon which the standards of the Pharmacopoeia are based. With the agreement of the competent authority, alternative methods of analysis may be used for control purposes, provided that the methods used enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. In the event of doubt or dispute, the methods of analysis of the Pharmacopoeia are alone authoritative.

Demonstration of compliance with the Pharmacopoeia

(1) An article is not of Pharmacopoeia quality unless it complies with all the requirements stated in the monograph. This does not imply that performance of all the tests in a monograph is necessarily a prerequisite for a manufacturer in assessing compliance with the Pharmacopoeia before release of a product. The manufacturer may obtain assurance that a product is of Pharmacopoeia quality on the basis of its design, together with its control strategy and data derived, for example, from validation studies of the manufacturing process.

(2) An enhanced approach to quality control could utilise process analytical technology (PAT) and/or real-time release testing (including parametric release) strategies as alternatives to end-product testing alone. Real-time release testing in circumstances deemed appropriate by the competent authority is thus not precluded by the need to comply with the Pharmacopoeia.

(3) Reduction of animal testing: the European Pharmacopoeia is dedicated to phasing out the use of animals for test purposes, in accordance with the 3Rs (Replacement, Reduction, Refinement) set out in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. In demonstrating compliance with the Pharmacopoeia as indicated above (1), manufacturers may consider establishing additional systems to monitor consistency of production. With the agreement of the competent authority, the choice of tests performed to assess compliance with the Pharmacopoeia when animal tests are prescribed is established in such a way that animal usage is minimised as much as possible.

Grade of materials. Certain materials that are the subject of a pharmacopoeial monograph may exist in different grades suitable for different purposes. Unless otherwise indicated in the monograph, the requirements apply to all grades of the material. In some monographs, particularly those on excipients, a list of functionality-related characteristics that are relevant to the use of the substance may be appended to the monograph for information. Test methods for determination of one or more of these characteristics may be given, also for information.

General monographs. Substances and preparations that are the subject of an individual monograph are also required to comply with relevant, applicable general monographs. Cross-references to applicable general monographs are not normally given in individual monographs.

General monographs apply to all substances and preparations within the scope of the Definition section of the general monograph, except where a preamble limits the application, for example to substances and preparations that are the subject of a monograph of the Pharmacopoeia.

General monographs on dosage forms apply to all preparations of the type defined. The requirements are not necessarily comprehensive for a given specific preparation and requirements additional to those prescribed in the general monograph may be imposed by the competent authority.

General monographs and individual monographs are complementary. If the provisions of a general monograph do not apply to a particular product, this is expressly stated in the individual monograph.

Validation of pharmacopoeial methods. The test methods given in monographs and general chapters have been validated in accordance with accepted scientific practice and current recommendations on analytical validation. Unless otherwise stated in the monograph or general chapter, validation of the test methods by the analyst is not required.

Implementation of pharmacopoeial methods. When implementing a pharmacopoeial method, the user must assess whether and to what extent the suitability of the method under the actual conditions of use needs to be demonstrated according to relevant monographs, general chapters and quality systems.

Conventional terms. The term 'competent authority' means the national, supranational or international body or organisation vested with the authority for making decisions concerning the issue in question. It may, for example, be a national pharmacopoeia authority, a licensing authority or an official control laboratory.

The expression 'unless otherwise justified and authorised' means that the requirements have to be met, unless the

competent authority authorises a modification or an exemption where justified in a particular case.

Statements containing the word 'should' are informative or advisory.

In certain monographs or other texts, the terms 'suitable' and 'appropriate' are used to describe a reagent, micro-organism, test method etc.; if criteria for suitability are not described in the monograph, suitability is demonstrated to the satisfaction of the competent authority.

Medicinal product. (a) Any substance or combination of substances presented as having properties for treating or preventing disease in human beings and/or animals; or (b) any substance or combination of substances that may be used in or administered to human beings and/or animals with a view either to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis.

Herbal medicinal product. Any medicinal product, exclusively containing as active ingredients one or more herbal drugs or one or more herbal drug preparations, or one or more such herbal drugs in combination with one or more such herbal drug preparations.

Active substance. Any substance intended to be used in the manufacture of a medicinal product and that, when so used, becomes an active ingredient of the medicinal product. Such substances are intended to furnish a pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease, or to affect the structure and function of the body.

Excipient (auxiliary substance). Any constituent of a medicinal product that is not an active substance. Adjuvants, stabilisers, antimicrobial preservatives, diluents, antioxidants, for example, are excipients.

Interchangeable methods. Certain general chapters contain a statement that the text in question is harmonised with the corresponding text of the Japanese Pharmacopoeia and/or the United States Pharmacopeia and that these texts are interchangeable. This implies that if a substance or preparation is found to comply with a requirement using an interchangeable method from one of these pharmacopoeias it complies with the requirements of the European Pharmacopoeia. In the event of doubt or dispute, the text of the European Pharmacopoeia is alone authoritative.

References to regulatory documents. Monographs and general chapters may contain references to documents issued by regulatory authorities for medicines, for example directives and notes for guidance of the European Union. These references are provided for information for users for the Pharmacopoeia. Inclusion of such a reference does not modify the status of the documents referred to, which may be mandatory or for guidance.

1.2. OTHER PROVISIONS APPLYING TO GENERAL CHAPTERS AND MONOGRAPHS

Quantities. In tests with numerical limits and assays, the quantity stated to be taken for examination is approximate. The amount actually used, which may deviate by not more than 10 per cent from that stated, is accurately weighed or measured and the result is calculated from this exact quantity. In tests where the limit is not numerical, but usually depends upon comparison with the behaviour of a reference substance in the same conditions, the stated quantity is taken for examination. Reagents are used in the prescribed amounts.

Quantities are weighed or measured with an accuracy commensurate with the indicated degree of precision. For weighings, the precision corresponds to plus or minus 5 units after the last figure stated (for example, 0.25 g is to be interpreted as 0.245 g to 0.255 g). For the measurement of volumes, if the figure after the decimal point is a zero or ends in a zero (for example, 10.0 mL or 0.50 mL), the volume is measured using a pipette, a volumetric flask or a burette, as appropriate; otherwise, a graduated measuring cylinder or a graduated pipette may be used. Volumes stated in microlitres are measured using a micropipette or microsyringe.

It is recognised, however, that in certain cases the precision with which quantities are stated does not correspond to the number of significant figures stated in a specified numerical limit. The weighings and measurements are then carried out with a sufficiently improved accuracy.

Apparatus and procedures. Volumetric glassware complies with Class A requirements of the appropriate International Standard issued by the International Organisation for Standardisation.

Unless otherwise prescribed, analytical procedures are carried out at a temperature between 15 °C and 25 °C.

Unless otherwise prescribed, comparative tests are carried out using identical tubes of colourless, transparent, neutral glass with a flat base; the volumes of liquid prescribed are for use with tubes having an internal diameter of 16 mm, but tubes with a larger internal diameter may be used provided the volume of liquid used is adjusted (*2.1.5*). Equal volumes of the liquids to be compared are examined down the vertical axis of the tubes against a white background, or if necessary against a black background. The examination is carried out in diffuse light.

Any solvent required in a test or assay in which an indicator is to be used is previously neutralised to the indicator, unless a blank test is prescribed.

Water-bath. The term 'water-bath' means a bath of boiling water unless water at another temperature is indicated. Other methods of heating may be substituted provided the temperature is near to but not higher than 100 °C or the indicated temperature.

Drying and ignition to constant mass. The terms 'dried to constant mass' and 'ignited to constant mass' mean that 2 consecutive weighings do not differ by more than 0.5 mg, the 2nd weighing following an additional period of drying or of ignition respectively appropriate to the nature and quantity of the residue.

Where drying is prescribed using one of the expressions 'in a desiccator' or '*in vacuo*', it is carried out using the conditions described in chapter *2.2.32. Loss on drying*.

Reagents. The proper conduct of the analytical procedures described in the Pharmacopoeia and the reliability of the results depend, in part, upon the quality of the reagents used. The reagents are described in general chapter *4*. It is assumed that reagents of analytical grade are used; for some reagents, tests to determine suitability are included in the specifications.

Solvents. Where the name of the solvent is not stated, the term 'solution' implies a solution in water.

Where the use of water is specified or implied in the analytical procedures described in the Pharmacopoeia or for the preparation of reagents, water complying with the requirements of the monograph *Purified water (0008)* is used, except that for many purposes the requirements for bacterial endotoxins (*Purified water in bulk*) and microbial contamination (*Purified water in containers)* are not relevant. The term 'distilled water' indicates purified water prepared by distillation.

The term 'ethanol' without qualification means anhydrous ethanol. The term 'alcohol' without qualification means ethanol (96 per cent). Other dilutions of ethanol are indicated by the term 'ethanol' or 'alcohol' followed by a statement of the percentage by volume of ethanol (C_2H_6O) required.

Expression of content. In defining content, the expression 'per cent' is used according to circumstances with one of 2 meanings:

– per cent *m/m* (percentage, mass in mass) expresses the number of grams of substance in 100 g of final product;

– per cent *V/V* (percentage, volume in volume) expresses the number of millilitres of substance in 100 mL of final product.

The expression 'parts per million' (or ppm) refers to mass in mass, unless otherwise specified.

Temperature. Where an analytical procedure describes temperature without a figure, the general terms used have the following meaning:

– in a deep-freeze: below – 15 °C;

– in a refrigerator: 2 °C to 8 °C;

– cold or cool: 8 °C to 15 °C;

– room temperature: 15 °C to 25 °C.

1.3. GENERAL CHAPTERS

Containers. Materials used for containers are described in general chapter *3.1*. General names used for materials, particularly plastic materials, each cover a range of products varying not only in the properties of the principal constituent but also in the additives used. The test methods and limits for materials depend on the formulation and are therefore applicable only for materials whose formulation is covered by the preamble to the specification. The use of materials with different formulations, and the test methods and limits applied to them, are subject to agreement by the competent authority.

The specifications for containers in general chapter *3.2* have been developed for general application to containers of the stated category, but in view of the wide variety of containers available and possible new developments, the publication of a specification does not exclude the use, in justified circumstances, of containers that comply with other specifications, subject to agreement by the competent authority.

Reference may be made within the monographs of the Pharmacopoeia to the definitions and specifications for containers provided in chapter *3.2. Containers*. The general monographs for pharmaceutical dosage forms may, under the heading Definition/Production, require the use of certain types of container; certain other monographs may, under the heading Storage, indicate the type of container that is recommended for use.

1.4. MONOGRAPHS

TITLES

Monograph titles are in English and French in the respective versions and there is a Latin subtitle.

RELATIVE ATOMIC AND MOLECULAR MASSES

The relative atomic mass (A_r) or the relative molecular mass (M_r) is shown, as and where appropriate, at the beginning of each monograph. The relative atomic and molecular masses and the molecular and graphic formulae do not constitute analytical standards for the substances described.

CHEMICAL ABSTRACTS SERVICE (CAS) REGISTRY NUMBER

CAS registry numbers are included for information in monographs, where applicable, to provide convenient access to useful information for users. CAS Registry Number® is a registered trademark of the American Chemical Society.

DEFINITION

Statements under the heading Definition constitute an official definition of the substance, preparation or other article that is the subject of the monograph.

Limits of content. Where limits of content are prescribed, they are those determined by the method described under Assay.

Herbal drugs. In monographs on herbal drugs, the definition indicates whether the subject of the monograph is, for example, the whole drug or the drug in powdered form. Where a monograph applies to the drug in several states, for example both to the whole drug and the drug in powdered form, the definition states this.

PRODUCTION

Statements under the heading Production draw attention to particular aspects of the manufacturing process but are not necessarily comprehensive. They constitute mandatory requirements for manufacturers, unless otherwise stated. They may relate, for example, to source materials; to the manufacturing process itself and its validation and control; to in-process testing; or to testing that is to be carried out by the manufacturer on the final article, either on selected batches or on each batch prior to release. These statements cannot necessarily be verified on a sample of the final article by an independent analyst. The competent authority may establish that the instructions have been followed, for example, by examination of data received from the manufacturer, by inspection of manufacture or by testing appropriate samples.

The absence of a Production section does not imply that attention to features such as those referred to above is not required.

Choice of vaccine strain, Choice of vaccine composition. The Production section of a monograph may define the characteristics of a vaccine strain or vaccine composition. Unless otherwise stated, test methods given for verification of these characteristics are provided for information as examples of suitable methods. Subject to approval by the competent authority, other test methods may be used without validation against the method shown in the monograph.

POTENTIAL ADULTERATION

Due to the increasing number of fraudulent activities and cases of adulteration, information may be made available to Ph. Eur. users to help detect adulterated materials (i.e. active substances, excipients, intermediate products, bulk products and finished products).

To this purpose, a method for the detection of potential adulterants and relevant limits, together with a reminder that all stages of production and sourcing are subjected to a suitable quality system, may be included in this section of monographs on substances for which an incident has occurred or that present a risk of deliberate contamination. The frequency of testing by manufacturers or by users (e.g. manufacturers of intermediate products, bulk products and finished products, where relevant) depends on a risk assessment, taking into account the level of knowledge of the whole supply chain and national requirements.

This section constitutes requirements for the whole supply chain, from manufacturers to users (e.g. manufacturers of intermediate products, bulk products and finished products, where relevant). The absence of this section does not imply that attention to features such as those referred to above is not required.

CHARACTERS

The statements under the heading Characters are not to be interpreted in a strict sense and are not requirements.

Solubility. In statements of solubility in the Characters section, the terms used have the following significance, referred to a temperature between 15 °C and 25 °C.

Descriptive term	Approximate volume of solvent in millilitres per gram of solute			
Very soluble	less than	1		
Freely soluble	from	1	to	10
Soluble	from	10	to	30
Sparingly soluble	from	30	to	100
Slightly soluble	from	100	to	1000
Very slightly soluble	from	1000	to	10 000
Practically insoluble	more than			10 000

The term 'partly soluble' is used to describe a mixture where only some of the components dissolve. The term 'miscible' is used to describe a liquid that is miscible in all proportions with the stated solvent.

IDENTIFICATION

Scope. The tests given in the Identification section are not designed to give a full confirmation of the chemical structure or composition of the product; they are intended to give confirmation, with an acceptable degree of assurance, that the article conforms to the description on the label.

First and second identifications. Certain monographs have subdivisions entitled 'First identification' and 'Second identification'. The test or tests that constitute the 'First identification' may be used in all circumstances. The test or tests that constitute the 'Second identification' may be used in pharmacies provided it can be demonstrated that the substance or preparation is fully traceable to a batch certified to comply with all the other requirements of the monograph.

Certain monographs give two or more sets of tests for the purpose of the first identification, which are equivalent and may be used independently. One or more of these sets usually contain a cross-reference to a test prescribed in the Tests section of the monograph. It may be used to simplify the work of the analyst carrying out the identification and the prescribed tests. For example, one identification set cross-refers to a test for enantiomeric purity while the other set gives a test for specific optical rotation: the intended purpose of the two is the same, that is, verification that the correct enantiomer is present.

Powdered herbal drugs. Monographs on herbal drugs may contain schematic drawings of the powdered drug. These drawings complement the description given in the relevant identification test.

TESTS AND ASSAYS

Scope. The requirements are not framed to take account of all possible impurities. It is not to be presumed, for example, that an impurity that is not detectable by means of the prescribed tests is tolerated if common sense and good pharmaceutical practice require that it be absent. See also below under Impurities.

Calculation. Where the result of a test or assay is required to be calculated with reference to the dried or anhydrous substance or on some other specified basis, the determination of loss on drying, water content or other property is carried out by the method prescribed in the relevant test in the monograph. The words 'dried substance' or 'anhydrous substance' etc. appear in parentheses after the result. Where a quantitative determination of a residual solvent is carried out and a test for loss on drying is not carried out, the content of residual solvent is taken into account for the calculation of the assay content of the substance, the specific optical rotation and the specific absorbance. No further indication is given in the specific monograph.

Limits. The limits prescribed are based on data obtained in normal analytical practice; they take account of normal analytical errors, of acceptable variations in manufacture and compounding and of deterioration to an extent considered acceptable. No further tolerances are to be applied to the limits prescribed to determine whether the article being examined complies with the requirements of the monograph.

In determining compliance with a numerical limit, the calculated result of a test or assay is first rounded to the number of significant figures stated, unless otherwise prescribed. The limits, regardless of whether the values are expressed as percentages or as absolute values, are considered significant to the last digit shown (for example 140 indicates 3 significant figures). The last figure of the result is increased by one when the part rejected is equal to or exceeds one half-unit, whereas it is not modified when the part rejected is less than a half-unit.

Indication of permitted limit of impurities. The acceptance criteria for related substances are expressed in monographs either in terms of comparison of peak areas (comparative tests) or as numerical values. For comparative tests, the approximate content of impurity tolerated, or the sum of impurities, may be indicated in brackets for information only. Acceptance or rejection is determined on the basis of compliance or non-compliance with the stated test. If the use of a reference substance for the named impurity is not prescribed, this content may be expressed as a nominal concentration of the substance used to prepare the reference solution specified in the monograph, unless otherwise described.

Herbal drugs. For herbal drugs, the sulfated ash, total ash, water-soluble matter, alcohol-soluble matter, water content, content of essential oil and content of active principle are calculated with reference to the drug that has not been specially dried, unless otherwise prescribed in the monograph.

Equivalents. Where an equivalent is given, for the purposes of the Pharmacopoeia only the figures shown are to be used in applying the requirements of the monograph.

Culture media. The culture media described in monographs and general chapters have been found to be satisfactory for the intended purpose. However, the components of media, particularly those of biological origin, are of variable quality, and it may be necessary for optimal performance to modulate the concentration of some ingredients, notably:

- peptones and meat or yeast extracts, with respect to their nutritive properties;
- buffering substances;
- bile salts, bile extract, deoxycholate, and colouring matter, depending on their selective properties;
- antibiotics, with respect to their activity.

STORAGE

The information and recommendations given under the heading Storage do not constitute a pharmacopoeial requirement but the competent authority may specify particular storage conditions that must be met.

The articles described in the Pharmacopoeia are stored in such a way as to prevent contamination and, as far as possible, deterioration. Where special conditions of storage are recommended, including the type of container (see section 1.3. General chapters) and limits of temperature, they are stated in the monograph.

The following expressions are used in monographs under Storage with the meaning shown.

In an airtight container means that the product is stored in an airtight container (*3.2*). Care is to be taken when the container is opened in a damp atmosphere. A low moisture content may be maintained, if necessary, by the use of a desiccant in the container provided that direct contact with the product is avoided.

Protected from light means that the product is stored either in a container made of a material that absorbs actinic light sufficiently to protect the contents from change induced by such light, or in a container enclosed in an outer cover that provides such protection, or is stored in a place from which all such light is excluded.

LABELLING

In general, labelling of medicines is subject to supranational and national regulation and to international agreements. The statements under the heading Labelling are not therefore comprehensive and, moreover, for the purposes of the Pharmacopoeia only those statements that are necessary to demonstrate compliance or non-compliance with the monograph are mandatory. Any other labelling statements are included as recommendations. When the term 'label' is used in the Pharmacopoeia, the labelling statements may appear on the container, the package, a leaflet accompanying the package, or a certificate of analysis accompanying the article, as decided by the competent authority.

WARNINGS

Materials described in monographs and reagents specified for use in the Pharmacopoeia may be injurious to health unless adequate precautions are taken. The principles of good quality control laboratory practice and the provisions of any appropriate regulations are to be observed at all times. Attention is drawn to particular hazards in certain monographs by means of a warning statement; absence of such a statement is not to be taken to mean that no hazard exists.

IMPURITIES

A list of all known and potential impurities that have been shown to be detected by the tests in a monograph may be given. See also chapter *5.10. Control of impurities in substances for pharmaceutical use*. The impurities are designated by a letter or letters of the alphabet. Where a letter appears to be missing, the impurity designated by this letter has been deleted from the list during monograph development prior to publication or during monograph revision.

FUNCTIONALITY-RELATED CHARACTERISTICS OF EXCIPIENTS

Monographs on excipients may have a section on functionality-related characteristics. The characteristics, any test methods for determination and any tolerances are not mandatory requirements; they may nevertheless be relevant for use of the excipient and are given for information (see also section 1.1. General statements).

REFERENCE STANDARDS

Certain monographs require the use of reference standards (chemical reference substances, herbal reference standards, biological reference preparations, reference spectra). See also chapter *5.12. Reference standards*. The European Pharmacopoeia Commission establishes the official reference standards, which are alone authoritative in case of arbitration. These reference standards are available from the European Directorate for the Quality of Medicines & HealthCare (EDQM). Information on the available reference standards and a batch validity statement can be obtained via the EDQM website.

1.5. ABBREVIATIONS AND SYMBOLS

A	Absorbance
$A_{1\ \mathrm{cm}}^{1\ \mathrm{per\ cent}}$	Specific absorbance
A_r	Relative atomic mass
$[\alpha]_D^{20}$	Specific optical rotation
bp	Boiling point
BRP	Biological reference preparation
CRS	Chemical reference substance
d_{20}^{20}	Relative density
λ	Wavelength
HRS	Herbal reference standard
IU	International Unit
M	Molarity
M_r	Relative molecular mass
mp	Melting point
n_D^{20}	Refractive index
Ph. Eur. U.	European Pharmacopoeia Unit
ppb	Parts per billion (micrograms per kilogram)
ppm	Parts per million (milligrams per kilogram)
R	Substance or solution defined under *4. Reagents*
R_F	Retardation factor (see chapter *2.2.46*)
R_{st}	Used in chromatography to indicate the ratio of the distance travelled by a substance to the distance travelled by a reference substance
RV	Substance used as a primary standard in volumetric analysis (chapter *4.2.1*)

Abbreviations used in the monographs on immunoglobulins, immunosera and vaccines

CFU	Colony-forming units
LD_{50}	The statistically determined quantity of a substance that, when administered by the specified route, may be expected to cause the death of 50 per cent of the test animals within a given period
MLD	Minimum lethal dose
L+/10 dose	The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, causes the death of the test animals within a given period
L+ dose	The smallest quantity of a toxin that, in the conditions of the test, when mixed with 1 IU of antitoxin and administered by the specified route, causes the death of the test animals within a given period
lr/100 dose	The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.01 IU of antitoxin and injected intracutaneously causes a characteristic reaction at the site of injection within a given period
Lp/10 dose	The smallest quantity of toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, causes paralysis in the test animals within a given period
Lo/10 dose	The largest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, does not cause symptoms of toxicity in the test animals within a given period
Lf dose	The quantity of toxin or toxoid that flocculates in the shortest time with 1 IU of antitoxin

$CCID_{50}$	The statistically determined quantity of virus that may be expected to infect 50 per cent of the cell cultures to which it is added
EID_{50}	The statistically determined quantity of virus that may be expected to infect 50 per cent of the fertilised eggs into which it is inoculated
ID_{50}	The statistically determined quantity of a virus that may be expected to infect 50 per cent of the animals into which it is inoculated
PD_{50}	The statistically determined dose of a vaccine that, in the conditions of the test, may be expected to protect 50 per cent of the animals against a challenge dose of the micro-organisms or toxins against which it is active
ED_{50}	The statistically determined dose of a vaccine that, in the conditions of the test, may be expected to induce specific antibodies in 50 per cent of the animals for the relevant vaccine antigens
PFU	Pock-forming units or plaque-forming units
SPF	Specified-pathogen-free

Collections of micro-organisms

ATCC	American Type Culture Collection 10801 University Boulevard Manassas, Virginia 20110-2209, USA
C.I.P.	Collection de Bactéries de l'Institut Pasteur B.P. 52, 25 rue du Docteur Roux 75724 Paris Cedex 15, France
IMI	International Mycological Institute Bakeham Lane Surrey TW20 9TY, Great Britain
I.P.	Collection Nationale de Culture de Microorganismes (C.N.C.M.) Institut Pasteur 25, rue du Docteur Roux 75724 Paris Cedex 15, France
NCIMB	National Collection of Industrial and Marine Bacteria Ltd 23 St Machar Drive Aberdeen AB2 1RY, Great Britain
NCPF	National Collection of Pathogenic Fungi London School of Hygiene and Tropical Medicine Keppel Street London WC1E 7HT, Great Britain
NCTC	National Collection of Type Cultures Central Public Health Laboratory Colindale Avenue London NW9 5HT, Great Britain
NCYC	National Collection of Yeast Cultures AFRC Food Research Institute Colney Lane Norwich NR4 7UA, Great Britain
NITE	Biological Resource Center Department of Biotechnology National Institute of Technology and Evaluation 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba, 292-0818 Japan
S.S.I.	Statens Serum Institut 80 Amager Boulevard, Copenhagen, Denmark

1.6. UNITS OF THE INTERNATIONAL SYSTEM (SI) USED IN THE PHARMACOPOEIA AND EQUIVALENCE WITH OTHER UNITS

INTERNATIONAL SYSTEM OF UNITS (SI)

The International System of Units comprises 2 main classes of units, namely base units and derived units[(1)]. The base units are the metre, the kilogram, the second, the ampere, the kelvin, the mole and the candela.

The derived units are formed as products of powers of the base units according to the algebraic relationships linking the corresponding quantities. Some of these derived units have special names and symbols. The derived units used in the Pharmacopoeia are shown in Table 1.6.-1.

Some important and widely used units outside the International System are shown in Table 1.6.-2.

The prefixes shown in Table 1.6.-3 are used to form the names and symbols of the decimal multiples and submultiples of SI units.

Table 1.6.-1. – *Derived units used in the European Pharmacopoeia and equivalence with other units*

Quantity		Unit				Conversion of other units into SI units
Name	Symbol	Name	Symbol	Expression in SI base units	Expression in other SI units	
Wave number	ν	one per metre	1/m	m^{-1}		
Wavelength	λ	micrometre nanometre	µm nm	10^{-6}m 10^{-9}m		
Area	A, S	square metre	m^2	m^2		
Volume	V	cubic metre	m^3	m^3		1 mL = 1 cm^3 = 10^{-6} m^3
Frequency	ν	hertz	Hz	s^{-1}		
Density	ρ	kilogram per cubic metre	kg/m^3	$kg \cdot m^{-3}$		1 g/mL = 1 g/cm^3 = 10^3 $kg \cdot m^{-3}$
Velocity, speed	v	metre per second	m/s	$m \cdot s^{-1}$		

(1) The definitions of the units used in the International System are given in the booklet 'Le Système International d'Unités (SI)', published by the Bureau International des Poids et Mesures, Pavillon de Breteuil, F-92310 Sèvres.

Quantity		Unit				Conversion of other units into SI units
Name	**Symbol**	**Name**	**Symbol**	**Expression in SI base units**	**Expression in other SI units**	
Force	*F*	newton	N	$m \cdot kg \cdot s^{-2}$		1 dyne = 1 $g \cdot cm \cdot s^{-2} = 10^{-5}$ N 1 kp = 9.806 65 N
Pressure, stress	*p*	pascal	Pa	$m^{-1} \cdot kg \cdot s^{-2}$	$N \cdot m^{-2}$	1 dyne/$cm^2 = 10^{-1}$ Pa = 10^{-1} $N \cdot m^{-2}$ 1 atm = 101 325 Pa = 101.325 kPa 1 bar = 10^5 Pa = 0.1 MPa 1 mm Hg = 133.322 387 Pa 1 Torr = 133.322 368 Pa 1 psi = 6.894 757 kPa
Dynamic viscosity	η	pascal second	Pa·s	$m^{-1} \cdot kg \cdot s^{-1}$	$N \cdot s \cdot m^{-2}$	1 P = 10^{-1} Pa·s = 10^{-1} $N \cdot s \cdot m^{-2}$ 1 cP = 1 mPa·s
Kinematic viscosity	ν	square metre per second	m^2/s	$m^2 \cdot s^{-1}$	$Pa \cdot s \cdot m^3 \cdot kg^{-1}$ $N \cdot m \cdot s \cdot kg^{-1}$	1 St = 1 $cm^2 \cdot s^{-1} = 10^{-4}$ $m^2 \cdot s^{-1}$
Energy	*W*	joule	J	$m^2 \cdot kg \cdot s^{-2}$	N·m	1 erg = 1 $cm^2 \cdot g \cdot s^{-2}$ = 1 dyne·cm = 10^{-7} J 1 cal = 4.1868 J
Power, radiant flux	*P*	watt	W	$m^2 \cdot kg \cdot s^{-3}$	$N \cdot m \cdot s^{-1}$ $J \cdot s^{-1}$	1 erg/s = 1 dyne·cm·s^{-1} = 10^{-7} W = 10^{-7} $N \cdot m \cdot s^{-1} = 10^{-7}$ $J \cdot s^{-1}$
Absorbed dose (of radiant energy)	*D*	gray	Gy	$m^2 \cdot s^{-2}$	$J \cdot kg^{-1}$	1 rad = 10^{-2} Gy
Electric potential difference, voltage	*U*	volt	V	$m^2 \cdot kg \cdot s^{-3} \cdot A^{-1}$	$W \cdot A^{-1}$	
Electric resistance	*R*	ohm	Ω	$m^2 \cdot kg \cdot s^{-3} \cdot A^{-2}$	$V \cdot A^{-1}$	
Electric charge	*Q*	coulomb	C	A·s		
Activity referred to a radionuclide	*A*	becquerel	Bq	s^{-1}		1 Ci = $37 \cdot 10^9$ Bq = $37 \cdot 10^9$ s^{-1}
Concentration (of amount of substance), molar concentration	*c*	mole per cubic metre	mol/m^3	$mol \cdot m^{-3}$		1 mol/L = 1 M = 1 $mol/dm^3 = 10^3$ $mol \cdot m^{-3}$
Mass concentration	ρ	kilogram per cubic metre	kg/m^3	$kg \cdot m^{-3}$		1 g/L = 1 g/dm^3 = 1 $kg \cdot m^{-3}$
Catalytic activity	*Z*	katal	kat	$mol \cdot s^{-1}$		

NOTES

1. In the Pharmacopoeia, the Celsius temperature is used (symbol *t*). This is defined by the following equation:

$$t = T - T_0$$

where T_0 = 273.15 K by definition. The Celsius or centigrade temperature is expressed in degrees Celsius (symbol °C). The unit 'degree Celsius' is equal to the unit 'kelvin'.

2. The practical expressions of concentrations used in the Pharmacopoeia are defined in the General Notices.

3. The radian is the plane angle between two radii of a circle that cut off on the circumference an arc equal in length to the radius.

4. In the Pharmacopoeia, conditions of centrifugation are defined by reference to the acceleration due to gravity (*g*):

$$g = 9.806\ 65\ m \cdot s^{-2}$$

5. Certain quantities without dimensions are used in the Pharmacopoeia: relative density (*2.2.5*), absorbance (*2.2.25*), specific absorbance (*2.2.25*) and refractive index (*2.2.6*).

6. The microkatal is defined as the enzymic activity that, under defined conditions, produces the transformation (e.g. hydrolysis) of 1 micromole of the substrate per second.

Table 1.6.-2. – *Non-SI units accepted for use with the SI units*

Quantity	Unit		Value in SI units
	Name	Symbol	
Time	minute	min	1 min = 60 s
	hour	h	1 h = 60 min = 3600 s
	day	d	1 d = 24 h = 86 400 s
Plane angle	degree	°	1° = (π/180) rad
Volume	litre	L	1 L = 1 dm^3 = 10^{-3} m^3
Mass	tonne	t	1 t = 10^3 kg
	dalton	Da	1 Da = 1.660539040(20) × 10^{-27} kg
Rotational frequency	revolution per minute	r/min	1 r/min = (1/60) s^{-1}
Energy	electronvolt	eV	1eV=1.602176634 × 10^{-19}J

Table 1.6.-3. – *Decimal multiples and sub-multiples of SI units*

Factor	Prefix	Symbol	Factor	Prefix	Symbol
10^{18}	exa	E	10^{-1}	deci	d
10^{15}	peta	P	10^{-2}	centi	c
10^{12}	tera	T	10^{-3}	milli	m
10^{9}	giga	G	10^{-6}	micro	µ
10^{6}	mega	M	10^{-9}	nano	n
10^{3}	kilo	k	10^{-12}	pico	p
10^{2}	hecto	h	10^{-15}	femto	f
10^{1}	deca	da	10^{-18}	atto	a

General monographs

TEXT N°	TITLE	PRODUCT TYPE(S)
2034	Substances for pharmaceutical use	All vaccines.
2619	Pharmaceutical preparations	All vaccines.
0153	Vaccines for human use	All vaccines.
0784	Recombinant DNA technology, products of	Recombinant protein-based vaccines.
1483	Products with risk of transmitting agents of animal spongiform encephalopathies	Vaccines produced using material of animal origin.

01/2021:2034

SUBSTANCES FOR PHARMACEUTICAL USE

Corpora ad usum pharmaceuticum

DEFINITION

Substances for pharmaceutical use are any organic or inorganic substances that are used as active substances or excipients for the production of medicinal products for human or veterinary use. They may be obtained from natural sources or produced by extraction from raw materials, fermentation or synthesis.

This general monograph does not apply to herbal drugs, herbal drugs for homoeopathic preparations, herbal drug preparations, herbal drug extracts, or mother tinctures for homoeopathic preparations, which are the subject of separate general monographs (*Herbal drugs (1433)*, *Herbal drugs for homoeopathic preparations (2045)*, *Herbal drug preparations (1434)*, *Herbal drug extracts (0765)*, *Mother tinctures for homoeopathic preparations (2029)*). It does not apply to raw materials for homoeopathic preparations, except where there is an individual monograph for the substance in the non-homoeopathic part of the Pharmacopoeia.

This monograph does not apply to chemical precursors for radiopharmaceutical preparations which are the subject of a separate monograph (*Chemical precursors for radiopharmaceutical preparations (2902)*).

Where a substance for pharmaceutical use not described in an individual monograph of the Pharmacopoeia is used in a medicinal product prepared for the special needs of individual patients, the need for compliance with the present general monograph is decided in the light of a risk assessment that takes account of the available quality of the substance and its intended use.

Where medicinal products are manufactured using substances for pharmaceutical use of human or animal origin, the requirements of chapter *5.1.7. Viral safety* apply.

Substances for pharmaceutical use may be used as such or as starting materials for subsequent formulation to prepare medicinal products. Depending on the formulation, certain substances may be used either as active substances or as excipients. Solid substances may be compacted, coated, granulated, powdered to a certain fineness, or processed in other ways. A monograph is applicable to a substance processed with an excipient only where such processing is mentioned in the definition section of the monograph.

Substance for pharmaceutical use of special grade. Unless otherwise indicated or restricted in the individual monographs, a substance for pharmaceutical use is intended for human and veterinary use, and is of appropriate quality for the manufacture of all dosage forms in which it can be used.

Polymorphism. Individual monographs do not usually specify crystalline or amorphous forms, unless bioavailability is affected. All forms of a substance for pharmaceutical use comply with the requirements of the monograph, unless otherwise indicated.

PRODUCTION

Substances for pharmaceutical use are manufactured by procedures that are designed to ensure a consistent quality and comply with the requirements of the individual monograph or approved specification.

The manufacture of active substances must take place under conditions of good manufacturing practice.

The provisions of general chapter *5.10* apply to the control of impurities in substances for pharmaceutical use.

Whether or not it is specifically stated in the individual monograph that the substance for pharmaceutical use:

- is a recombinant protein or another substance obtained as a direct gene product based on genetic modification, where applicable, the substance also complies with the requirements of the general monograph *Products of recombinant DNA technology (0784)*;
- is obtained from animals susceptible to transmissible spongiform encephalopathies other than by experimental challenge, where applicable, the substance also complies with the requirements of the general monograph *Products with risk of transmitting agents of animal spongiform encephalopathies (1483)*;
- is a substance derived from a fermentation process, whether or not the micro-organisms involved are modified by traditional procedures or recombinant DNA (rDNA) technology, where applicable, the substance also complies with the requirements of the general monograph *Products of fermentation (1468)*.

If solvents are used during production, they are of suitable quality. In addition, their toxicity and their residual level are taken into consideration (*5.4*). If water is used during production, it is of suitable quality.

The identity of elemental impurities derived from intentionally added catalysts and reagents is known, and strategies for controlling them should be established using the principles of risk management.

If substances are produced or processed to yield a certain form or grade, that specific form or grade of the substance complies with the requirements of the monograph. Certain functionality-related tests may be described to control properties that may influence the suitability of the substance and subsequently the properties of dosage forms prepared from it.

Powdered substances may be processed to obtain a certain degree of fineness (*2.9.35*).

Compacted substances are processed to increase the particle size or to obtain particles of a specific form and/or to obtain a substance with a higher bulk density.

Coated active substances consist of particles of the active substance coated with one or more suitable excipients.

Granulated active substances are particles of a specified size and/or form produced from the active substance by granulation directly or with one or more suitable excipients.

If substances are processed with excipients, these excipients comply with the requirements of the relevant monograph or, where no such monograph exists, the approved specification.

Where active substances have been processed with excipients to produce, for example, coated or granulated substances, the processing is carried out under conditions of good manufacturing practice and the processed substances are regarded as intermediates in the manufacture of a medicinal product.

CHARACTERS

The statements under the heading Characters (e.g. statements about the solubility or a decomposition point) are not to be interpreted in a strict sense and are not requirements. They are given for information.

Where a substance may show polymorphism, this may be stated under Characters in order to draw this to the attention of the user who may have to take this characteristic into consideration during formulation of a preparation.

IDENTIFICATION

Where under Identification an individual monograph contains subdivisions entitled 'First identification' and 'Second identification', the test or tests that constitute the 'First identification' may be used in all circumstances. The test or tests that constitute the 'Second identification' may be

used in pharmacies only, provided it can be demonstrated that the substance or preparation is fully traceable to a batch certified to comply with all the other requirements of the monograph. The implementation of the tests under the second identification is subject to national regulation.

Certain monographs give two or more sets of tests for the purpose of the first identification, which are equivalent and may be used independently. One or more of these sets usually contain a cross-reference to a test prescribed in the Tests section of the monograph. It may be used to simplify the work of the analyst carrying out the identification and the prescribed tests. For example, one identification set cross-refers to a test for enantiomeric purity while the other set gives a test for specific optical rotation: the intended purpose of the two is the same, that is, verification that the correct enantiomer is present.

TESTS

Polymorphism (*5.9*). If the nature of a crystalline or amorphous form imposes restrictions on its use in preparations, the nature of the specific crystalline or amorphous form is identified, its morphology is adequately controlled and its identity is stated on the label.

Related substances. Unless otherwise prescribed or justified and authorised, organic impurities in active substances are to be reported, identified wherever possible, and qualified as indicated in Table 2034.-1 or in Table 2034.-2 for peptides obtained by chemical synthesis.

Table 2034.-1. – *Reporting, identification and qualification of organic impurities in active substances*

Use	Maximum daily dose	Reporting threshold	Identification threshold	Qualification threshold
Human use or human and veterinary use	≤ 2 g/day	> 0.05 per cent	> 0.10 per cent or a daily intake of > 1.0 mg (whichever is the lower)	> 0.15 per cent or a daily intake of > 1.0 mg (whichever is the lower)
Human use or human and veterinary use	> 2 g/day	> 0.03 per cent	> 0.05 per cent	> 0.05 per cent
Veterinary use only	Not applicable	> 0.10 per cent	> 0.20 per cent	> 0.50 per cent

Table 2034.-2. – *Reporting, identification and qualification of organic impurities in peptides obtained by chemical synthesis*

Reporting threshold	Identification threshold	Qualification threshold
> 0.1 per cent	> 0.5 per cent	> 1.0 per cent

Specific thresholds may be applied for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects.

For DNA reactive impurities, the requirements of ICH Guideline M7 *Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk* must be complied with for active substances to be used in medicinal products for human use, in cases defined in the scope of the guideline.

If the individual monograph does not provide suitable control for a new impurity, a suitable test for control must be developed and included in the specification for the substance.

The requirements above do not apply to biological and biotechnological products, oligonucleotides, products of fermentation and semi-synthetic products derived therefrom, to crude products of animal or plant origin or herbal products.

Elemental impurities. Permitted daily exposures for elemental impurities (e.g. as included in the ICH Q3D guideline, the principles of which are reproduced in general chapter *5.20. Elemental impurities*) apply to the medicinal product. Individual monographs on substances for pharmaceutical use therefore do not contain specifications for elemental impurities unless otherwise prescribed.

Residual solvents are limited according to the principles defined in chapter *5.4*, using general method *2.4.24* or another suitable method. Where a quantitative determination of a residual solvent is carried out and a test for loss on drying is not carried out, the content of residual solvent is taken into account for calculation of the assay content of the substance, the specific optical rotation and the specific absorbance.

Microbiological quality. Individual monographs give acceptance criteria for microbiological quality wherever such control is necessary. Table 5.1.4.-2. – *Acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use* in chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use* gives recommendations on microbiological quality that are of general relevance for substances subject to microbial contamination. Depending on the nature of the substance and its intended use, different acceptance criteria may be justified.

Sterility (*2.6.1*). If intended for use in the manufacture of sterile dosage forms without a further appropriate sterilisation procedure, or if offered as sterile grade, the substance for pharmaceutical use complies with the test for sterility.

Bacterial endotoxins (*2.6.14*). The substance for pharmaceutical use complies with the test for bacterial endotoxins if it is labelled as a bacterial endotoxin-free grade or if it is intended for use in the manufacture of parenteral preparations or preparations for irrigation without a further appropriate procedure for the removal of bacterial endotoxins. The limit, when not indicated in the individual monograph, is determined in accordance with the recommendations of general chapter *5.1.10. Guidelines for using the test for bacterial endotoxins*.

Pyrogens (*2.6.8*). If the test for pyrogens is justified rather than the test for bacterial endotoxins and if a pyrogen-free grade is offered, the substance for pharmaceutical use complies with the test for pyrogens. The limit and test method are stated in the individual monograph or approved by the competent authority. Based on appropriate test validation for bacterial endotoxins and pyrogens, the test for bacterial endotoxins may replace the test for pyrogens.

Additional properties. Control of additional properties (e.g. physical characteristics, functionality-related characteristics) may be necessary for individual manufacturing processes or formulations. Grades (such as sterile, endotoxin-free, pyrogen-free) may be produced with a view to manufacture of preparations for parenteral administration or other dosage forms and appropriate requirements may be specified in an individual monograph.

ASSAY

Unless justified and authorised, contents of substances for pharmaceutical use are determined. Suitable methods are used.

LABELLING

In general, labelling is subject to supranational and national regulation and to international agreements. The statements under the heading Labelling therefore are not comprehensive and, moreover, for the purposes of the Pharmacopoeia only those statements that are necessary to demonstrate compliance or non-compliance with the monograph are mandatory. Any other labelling statements are included as recommendations. When the term 'label' is used in the Pharmacopoeia, the labelling statements may appear on the container, the package,

a leaflet accompanying the package or a certificate of analysis accompanying the article, as decided by the competent authority.

Where appropriate, the label states that the substance is:

- intended for a specific use;
- of a distinct crystalline form;
- of a specific degree of fineness;
- compacted;
- coated;
- granulated;
- sterile;
- free from bacterial endotoxins;
- free from pyrogens;
- containing gliding agents.

Where applicable, the label states:

- the degree of hydration;
- the name and concentration of any excipient.

04/2019:2619

PHARMACEUTICAL PREPARATIONS

Pharmaceutica

INTRODUCTION

This monograph is intended to be a reference source of standards in the European Pharmacopoeia on active substances, excipients and dosage forms, which are to be applied in the manufacture/preparation of pharmaceuticals, but not a guide on how to manufacture as there is specific guidance available covering methods of manufacture and associated controls.

It does not cover investigational medicinal products, but competent authorities may refer to pharmacopoeial standards when authorising clinical trials using investigational medicinal products.

DEFINITION

Pharmaceutical preparations are medicinal products generally consisting of active substances that may be combined with excipients, formulated into a dosage form suitable for the intended use, where necessary after reconstitution, presented in a suitable and appropriately labelled container.

Pharmaceutical preparations may be licensed by the competent authority, or unlicensed and made to the specific needs of patients according to legislation. There are 2 categories of unlicensed pharmaceutical preparations:

- extemporaneous preparations, i.e. pharmaceutical preparations individually prepared for a specific patient or patient group, supplied after preparation;
- stock preparations, i.e. pharmaceutical preparations prepared in advance and stored until a request for a supply is received.

In addition to this monograph, pharmaceutical preparations also comply with the General Notices and with the relevant general chapters of the Pharmacopoeia. General chapters are normally given for information and become mandatory when referred to in a general or specific monograph, unless such reference is made in a way that indicates that it is not the intention to make the text referred to mandatory but rather to cite it for information.

Where relevant, pharmaceutical preparations also comply with the dosage form monographs (e.g. *Capsules (0016)*, *Tablets (0478)*) and general monographs relating to pharmaceutical preparations (e.g. *Allergen products (1063)*, *Herbal teas (1435)*, *Homoeopathic preparations (1038)*, *Homoeopathic pillules, coated (2786)*, *Homoeopathic pillules, impregnated (2079)*, *Immunosera for human use, animal (0084)*, *Immunosera for veterinary use (0030)*, *Live biotherapeutic products for human use (3053)*, *Monoclonal antibodies for human use (2031)*, *Radiopharmaceutical preparations (0125)*, *Vaccines for human use (0153)*, *Vaccines for veterinary use (0062)*).

ETHICAL CONSIDERATIONS AND GUIDANCE IN THE PREPARATION OF UNLICENSED PHARMACEUTICAL PREPARATIONS

The underlying principle of legislation for pharmaceutical preparations is that, subject to specific exemptions, no pharmaceutical preparation may be placed on the market without an appropriate marketing authorisation. The exemptions from the formal licensing requirement allow the supply of unlicensed products to meet the special needs of individual patients. However, when deciding to use an unlicensed preparation all health professionals involved (e.g. the prescribing practitioners and/or the preparing pharmacists) have, within their area of responsibilities, a duty of care to the patient receiving the pharmaceutical preparation.

In considering the preparation of an unlicensed pharmaceutical preparation, a suitable level of risk assessment is undertaken.

The risk assessment identifies:

- the criticality of different parameters (e.g. quality of active substances, excipients and containers; design of the preparation process; extent and significance of testing; stability of the preparation) to the quality of the preparation; and
- the risk that the preparation may present to a particular patient group.

Based on the risk assessment, the person responsible for the preparation must ensure, with a suitable level of assurance, that the pharmaceutical preparation is, throughout its shelf-life, of an appropriate quality and suitable and fit for its purpose. For stock preparations, storage conditions and shelf-life have to be justified on the basis of, for example, analytical data or professional judgement, which may be based on literature references.

PRODUCTION

Manufacture/preparation must take place within the framework of a suitable quality system and be compliant with the standards relevant to the type of product being made. Licensed products must comply with the requirements of their licence. For unlicensed products a risk assessment as outlined in the section 'Ethical considerations and guidance in the preparation of unlicensed pharmaceutical preparations' is of special importance, as these products are not previously assessed by the competent authority.

Where pharmaceutical preparations are manufactured/prepared using materials of human or animal origin, the general requirements of general chapters *5.1.7. Viral safety* and *5.2.6. Evaluation of safety of veterinary vaccines and immunosera* and of the general monograph *Products with risk of transmitting agents of animal spongiform encephalopathies (1483)* apply, where appropriate.

Formulation. During pharmaceutical development or prior to manufacture/preparation, suitable ingredients, processes, tests and specifications are identified and justified in order to ensure the suitability of the product for the intended purpose. This includes consideration of the properties required in order to identify whether specific ingredient properties or process steps are critical to the required quality of the pharmaceutical preparation.

Active substances and excipients. Active substances and excipients used in the formulation of pharmaceutical preparations comply with the requirements of the relevant general monographs, e.g. *Substances for pharmaceutical use (2034)*, *Essential oils (2098)*, *Herbal drug extracts (0765)*, *Herbal drugs (1433)*, *Herbal drug preparations (1434)*, *Herbal drugs for homoeopathic preparations (2045)*, *Mother tinctures for homoeopathic preparations (2029)*, *Methods of preparation of homoeopathic stocks and potentisation (2371)*, *Products of fermentation (1468)*, *Products of recombinant DNA technology (0784)*, *Vegetable fatty oils (1579)*.

In addition, where specific monographs exist, the quality of the active substances and excipients used complies with the corresponding monographs.

Where no specific monographs exist, the required quality must be defined, taking into account the intended use and the involved risk.

When physicochemical characteristics of active substances and functionality-related characteristics (FRCs) of excipients (e.g. particle-size distribution, viscosity, polymorphism) are critical in relation to their role in the manufacturing process and quality attributes of the pharmaceutical preparation, they must be identified and controlled.

Detailed information on FRCs is given in general chapter *5.15. Functionality-related characteristics of excipients*.

Microbiological quality. The formulation of the pharmaceutical preparation and its container must ensure that the microbiological quality is suitable for the intended use.

During development, it shall be demonstrated that the antimicrobial activity of the preparation as such or, if necessary, with the addition of a suitable preservative or preservatives, or by the selection of an appropriate container, provides adequate protection from adverse effects that may arise from microbial contamination or proliferation during the storage and use of the preparation. A suitable test method together with criteria for evaluating the preservative properties of the formulation are provided in general chapter *5.1.3. Efficacy of antimicrobial preservation*.

If preparations do not have adequate antimicrobial efficacy and do not contain antimicrobial preservatives they are supplied in single-dose containers, or in multidose containers that prevent microbial contamination of the contents after opening.

In the manufacture/preparation of non-sterile pharmaceutical preparations, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapters *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use* and *5.1.8. Microbiological quality of herbal medicinal products for oral use and extracts used in their preparation*.

Sterile preparations are manufactured/prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in general chapter *5.1.1. Methods of preparation of sterile products*.

Containers. A suitable container is selected. Consideration is given to the intended use of the preparation, the properties of the container, the required shelf-life, and product/container incompatibilities. Where applicable, containers for pharmaceutical preparations comply with the requirements for containers (*3.2* and subsections) and materials used for the manufacture of containers (*3.1* and subsections).

Stability. Stability requirements of pharmaceutical preparations are dependent on their intended use and on the desired storage time.

Where applicable, the probability and criticality of possible degradation products of the active substance(s) and/or reaction products of the active substance(s) with an excipient and/or the immediate container must be assessed. Depending on the result of this assessment, limits of degradation and/or reaction products are set and monitored in the pharmaceutical preparation. Licensed products require a stability exercise.

Methods used for the purpose of stability testing for all relevant characteristics of the preparation are validated as stability indicating, i.e. the methods allow the quantification of the relevant degradation products and physical characteristic changes.

TESTS

Relevant tests to apply in order to ensure the appropriate quality of a particular dosage form are described in the specific dosage form monographs.

Where it is not practical, for unlicensed pharmaceutical preparations, to carry out the tests (e.g. batch size, time restraints), other suitable methods are implemented to ensure that the appropriate quality is achieved in accordance with the risk assessment carried out and any local guidance or legal requirements.

Stock preparations are normally tested to a greater extent than extemporaneous preparations.

The following tests are applicable to many preparations and are therefore listed here.

Appearance. The appearance (e.g. size, shape and colour) of the pharmaceutical preparation is controlled.

Identity and purity tests. Where applicable, the following tests are carried out on the pharmaceutical preparation:

- identification of the active substance(s);
- identification of specific excipient(s), such as preservatives;
- purity tests (e.g. investigation of degradation products, residual solvents (*2.4.24*) or other related impurities, sterility (*2.6.1*));
- safety tests (e.g. safety tests for biological products).

Elemental impurities. General chapter *5.20. Elemental impurities* applies to pharmaceutical preparations except products for veterinary use, unlicensed preparations and other products that are excluded from the scope of this chapter.

For pharmaceutical preparations outside the scope of general chapter *5.20*, manufacturers of these products remain responsible for controlling the levels of elemental impurities using the principles of risk management.

If appropriate, testing is performed using suitable analytical procedures according to general chapter *2.4.20. Determination of elemental impurities*.

Uniformity (*2.9.40* or *2.9.5*/*2.9.6*). Pharmaceutical preparations presented in single-dose units comply with the test(s) as prescribed in the relevant specific dosage form monograph. If justified and authorised, general chapter *2.9.40* can be applicable only at the time of release.

Special uniformity requirements apply in the following cases:

- for herbal drugs and herbal drug preparations, compliance with general chapter *2.9.40* is not required;
- for homoeopathic preparations, the provisions of general chapters *2.9.6* and *2.9.40* are normally not appropriate, however in certain circumstances compliance with these chapters may be required by the competent authority;
- for single- and multivitamin and trace-element preparations, compliance with general chapters *2.9.6* and *2.9.40* (*content uniformity only*) is not required;
- in justified and authorised circumstances, for other preparations, compliance with general chapters *2.9.6* and *2.9.40* may not be required by the competent authority.

Reference standards. Reference standards may be needed at various stages for quality control of pharmaceutical preparations. They are established and monitored taking due account of general chapter *5.12. Reference standards*.

ASSAY

Unless otherwise justified and authorised, contents of active substances and specific excipients such as preservatives are determined in pharmaceutical preparations. Limits must be defined and justified.

Suitable and validated methods are used. If assay methods prescribed in the respective active substance monographs are used, it must be demonstrated that they are not affected by the presence of the excipients and/or by the formulation.

Reference standards. See Tests.

LABELLING AND STORAGE

The relevant labelling requirements given in the general dosage form monographs apply. In addition, relevant European Union or other applicable regulations apply.

GLOSSARY

Formulation: the designing of an appropriate formula (including materials, processes, etc.) that will ensure that the patient receives the suitable pharmaceutical preparation in an appropriate form that has the required quality and that will be stable and effective for the required length of time.

Licensed pharmaceutical preparation: a medicinal product that has been granted a marketing authorisation by a competent authority. Synonym: authorised pharmaceutical preparation.

Manufacture: all operations of purchase of materials and products, Production, Quality Control, release, storage, distribution of medicinal products and the related controls.

Preparation (of an unlicensed pharmaceutical preparation): the 'manufacture' of unlicensed pharmaceutical preparations by or at the request of pharmacies or other healthcare establishments (the term 'preparation' is used instead of 'manufacture' in order clearly to distinguish it from the industrial manufacture of licensed pharmaceutical preparations).

Reconstitution: manipulation to enable the use or application of a medicinal product with a marketing authorisation in accordance with the instructions given in the summary of product characteristics or the patient information leaflet.

Risk assessment: the identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards.

Unlicensed pharmaceutical preparation: a medicinal product that is exempt from the need of having a marketing authorisation issued by a competent authority but is made for specific patients' needs according to legislation.

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VACCINES FOR HUMAN USE

Vaccina ad usum humanum

DEFINITION

Vaccines for human use are preparations containing antigens capable of inducing a specific and active immunity in man against an infecting agent or the toxin or antigen elaborated by it. Immune responses include the induction of the innate and the adaptive (cellular, humoral) parts of the immune system. Vaccines for human use shall have been shown to have acceptable immunogenic activity and safety in man with the intended vaccination schedule.

Vaccines for human use may contain: whole micro-organisms (bacteria, viruses or parasites), inactivated by chemical or physical means that maintain adequate immunogenic properties; whole live micro-organisms that are naturally avirulent or that have been treated to attenuate their virulence whilst retaining adequate immunogenic properties; antigens extracted from the micro-organisms or secreted by the micro-organisms or produced by genetic engineering or chemical synthesis. The antigens may be used in their native state or may be detoxified or otherwise modified by chemical or physical means and may be aggregated, polymerised or conjugated to a carrier to increase their immunogenicity. Vaccines may contain an adjuvant. Where the antigen is adsorbed on a mineral adjuvant, the vaccine is referred to as 'adsorbed'.

Terminology used in monographs on vaccines for human use is defined in general chapter *5.2.1*.

Bacterial vaccines containing whole cells are suspensions of various degrees of opacity in colourless or almost colourless liquids, or may be freeze-dried. They may be adsorbed. The concentration of living or inactivated bacteria is expressed in terms of International Units of opacity or, where appropriate, is determined by direct cell count or, for live bacteria, by viable count.

Bacterial vaccines containing bacterial components are suspensions or freeze-dried products. They may be adsorbed. The antigen content is determined by a suitable validated assay.

Bacterial toxoids are prepared from toxins by diminishing their toxicity to an acceptable level or by completely eliminating it by physical or chemical procedures whilst retaining adequate immunogenic properties. The toxins are obtained from selected strains of micro-organisms. The method of production is such that the toxoid does not revert to toxin. The toxoids are purified. Purification is performed before and/or after detoxification. Toxoid vaccines may be adsorbed.

Viral vaccines are prepared from viruses grown in animals, in fertilised eggs, in suitable cell cultures or in suitable tissues, or by culture of genetically engineered cells. They are liquids that vary in opacity according to the type of preparation or may be freeze-dried. They may be adsorbed. Liquid preparations and freeze-dried preparations after reconstitution may be coloured if a pH indicator such as phenol red has been used in the culture medium.

Synthetic antigen vaccines are generally clear or colourless liquids. The concentration of the components is usually expressed in terms of specific antigen content.

Combined vaccines are multicomponent preparations formulated so that different antigens are administered simultaneously. The different antigenic components are intended to protect against different strains or types of the same organism and/or against different organisms. A combined vaccine may be supplied by the manufacturer either as a single liquid or freeze-dried preparation or as several constituents with directions for admixture before use. Where there is no monograph to cover a particular combination, the vaccine complies with the monograph for each individual component, with any necessary modifications approved by the competent authority.

Adsorbed vaccines are suspensions and may form a sediment at the bottom of the container.

PRODUCTION

General provisions. The production method for a given product must have been shown to yield consistently batches comparable with the batch of proven clinical efficacy, immunogenicity and safety in man. Product specifications including in-process testing should be set. Specific requirements for production including in-process testing are included in individual monographs. Where justified and authorised, certain tests may be omitted where it can be demonstrated, for example by validation studies, that the production process consistently ensures compliance with the test.

Unless otherwise justified and authorised, vaccines are produced using a seed-lot system. The methods of preparation are designed to maintain adequate immunogenic properties, to render the preparation harmless and to prevent contamination with extraneous agents.

Where vaccines for human use are manufactured using materials of human or animal origin, the general requirements of general chapter *5.1.7. Viral safety* apply in conjunction with the more specific requirements relating to viral safety in this monograph, in general chapters *5.2.2. Chicken flocks free from specified pathogens for the production and quality control of vaccines*, *5.2.3. Cell substrates for the production of vaccines for human use* and *2.6.16. Tests for extraneous agents in viral vaccines for human use*, and in individual monographs.

Unless otherwise justified and authorised, in the production of a final lot of vaccine, the number of passages of a virus, or the number of subcultures of a bacterium, from the master seed lot shall not exceed that used for production of the vaccine shown to be satisfactory in clinical trials with respect to safety and efficacy or immunogenicity.

Vaccines are as far as possible free from ingredients known to cause toxic, allergic or other undesirable reactions in man. Suitable additives, including stabilisers and adjuvants may be incorporated. Penicillin and streptomycin are neither used at any stage of production nor added to the final product; however, master seed lots prepared with media containing penicillin or streptomycin may, where justified and authorised, be used for production.

Consistency of production is an important feature of vaccine production. Monographs on vaccines for human use give limits for various tests carried out during production and on the final lot. These limits may be in the form of maximum values, minimum values, or minimum and maximum tolerances around a given value. While compliance with these limits is required, it is not necessarily sufficient to ensure consistency of production for a given vaccine. For relevant tests, the manufacturer must therefore define for each product a suitable action or release limit or limits to be applied in view of the results found for batches tested clinically and those used to demonstrate consistency of production. These limits may subsequently be refined on a statistical basis in light of production data.

Substrates for propagation. Substrates for propagation comply with the relevant requirements of the Pharmacopoeia (*5.2.2*, *5.2.3*) or in the absence of such requirements with those of the competent authority. Processing of cell banks and subsequent cell cultures is done under aseptic conditions in an area where no other cells are being handled. Serum and trypsin used in the preparation of cell suspensions shall be shown to be free from extraneous agents.

Seed lots/cell banks. The master seed lot or cell bank is identified by historical records that include information on its origin and subsequent manipulation. Suitable measures are taken to ensure that no extraneous agent or undesirable substance is present in a master or working seed lot or a cell bank.

Culture media. Culture media are as far as possible free from ingredients known to cause toxic, allergic or other undesirable reactions in man; if inclusion of such ingredients is necessary, it shall be demonstrated that the amount present in the final lot is reduced to such a level as to render the product safe. Approved animal (but not human) serum may be used in the growth medium for cell cultures but the medium used for maintaining cell growth during virus multiplication shall not contain serum, unless otherwise stated. Cell culture media may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration, although it is preferable to have a medium free from antibiotics during production.

Propagation and harvest. The seed cultures are propagated and harvested under defined conditions. The purity of the harvest is verified by suitable tests as defined in the monograph.

Control cells. For vaccines produced in cell cultures, control cells are maintained and tested as prescribed. In order to provide a valid control, these cells must be maintained in conditions that are essentially equivalent to those used for the production cell cultures, including use of the same batches of media and media changes.

Control eggs. For live vaccines produced in eggs, control eggs are incubated and tested as prescribed in the monograph.

Purification. Where applicable, validated purification procedures may be applied.

Inactivation. Inactivated vaccines are produced using a validated inactivation process whose effectiveness and consistency have been demonstrated. Where it is recognised that extraneous agents may be present in a harvest, for example in vaccines produced in eggs from healthy, non-SPF flocks, the inactivation process is also validated with respect to a panel of model extraneous agents representative of the potential extraneous agents. A test for effectiveness of the inactivation process is carried out as soon as possible after the inactivation process.

Carrier proteins. Bacterial polysaccharide antigens may be conjugated with carrier proteins to improve their immunogenicity to enable the induction of a protective response in infants. Carrier proteins comply with the relevant requirements of general chapter *5.2.11. Carrier proteins for the production of conjugated polysaccharide vaccines for human use*.

Test for sterility of intermediates prior to final bulk. Individual monographs on vaccines for human use may prescribe a test for sterility for intermediates.

In agreement with the competent authority, replacement of the sterility test by a bioburden test with a low bioburden limit based on batch data and process validation may be acceptable for intermediates preceding the final bulk, provided that a sterilising filtration is performed later in the production process.

It is a prerequisite that the intermediate is filtered through a bacteria-retentive filter prior to storage, that authorised pre-filtration bioburden limits have been established for this filtration, and that adequate measures are in place to avoid contamination and growth of micro-organisms during storage of the intermediate.

Final bulk. The final bulk is prepared by aseptically blending the ingredients of the vaccine. For non-liquid vaccines for administration by a non-parenteral route, the final bulk is prepared by blending the ingredients of the vaccine under suitable conditions.

Adjuvants. One or more adjuvants may be included in the formulation of a vaccine to potentiate and/or modulate the immune response to the antigen(s). Adjuvants may be included in the formulation of the final vaccine or presented separately. Suitable characterisation and quality control of the adjuvant(s), alone and in combination with the antigen(s), is essential for consistent production. Quality specifications are established for each adjuvant, alone and in combination with the antigen(s).

Adsorbents as adjuvants. Vaccines may be adsorbed on aluminium hydroxide, aluminium phosphate, calcium phosphate or other suitable adsorbents. The adsorbents are prepared in special conditions that confer the appropriate physical form and adsorptive properties.

Where an adsorbent is used as an adjuvant and is generated *in situ* during production of the vaccine, quality specifications are established for each of the ingredients and for the generated adsorbent in the vaccine. Quality specifications are intended to control, in particular:

- qualitative and quantitative chemical composition;
- physical form and associated adsorptive properties, where relevant, and particularly where the adjuvant will be present as an adsorbent;
- interaction between adjuvant and antigen;
- purity, including bacterial endotoxin content and microbiological quality;
- any other parameters identified as being critical for functionality.

The stability of each adjuvant, alone and in combination with the antigen(s), particularly for critical parameters, is established during development studies.

Antimicrobial preservatives. Antimicrobial preservatives are used to prevent spoilage or adverse effects caused by microbial contamination occurring during the use of a vaccine. Antimicrobial preservatives are not included in freeze-dried products. For single-dose liquid preparations, inclusion of antimicrobial preservatives is not normally acceptable. For multidose liquid preparations, the need for effective antimicrobial preservation is evaluated taking into account likely contamination during use and the maximum recommended period of use after broaching of the container. If an antimicrobial preservative is used, it shall be shown that it does not impair the safety or efficacy of the vaccine. Addition of antibiotics as antimicrobial preservatives is not normally acceptable.

During development studies, the effectiveness of the antimicrobial preservative throughout the period of validity shall be demonstrated to the satisfaction of the competent authority.

The efficacy of the antimicrobial preservative is evaluated as described in general chapter *5.1.3*. If neither the A criteria nor the B criteria can be met, then in justified cases the following criteria are applied to vaccines for human use: bacteria, no increase at 24 h and 7 days, 3 $\log_{10}$ reduction at 14 days, no increase at 28 days; fungi, no increase at 14 days and 28 days.

Stability of intermediates. During production of vaccines, intermediates are obtained at various stages and are stored, sometimes for long periods. Such intermediates include:

- seed lots and cell banks;
- live or inactivated harvests;
- purified harvests that may consist of toxins or toxoids, polysaccharides, bacterial or viral suspensions;
- purified antigens;
- adsorbed antigens;

- conjugated polysaccharides;
- final bulk vaccine;
- vaccine in the final closed container stored at a temperature lower than that used for final-product stability studies and intended for release without re-assay.

Except where they are used within a short period of time, stability studies are carried out on the intermediates in the intended storage conditions to establish the expected extent of degradation. For final bulk vaccine, stability studies may be carried out on representative samples in conditions equivalent to those intended to be used for storage. For each intermediate (except for seed lots and cell banks), a period of validity applicable for the intended storage conditions is established, where appropriate in light of stability studies.

Final lot. The final lot is prepared by aseptically distributing the final bulk into sterile, tamper-evident containers, which, after freeze-drying where applicable, are closed so as to exclude contamination. For non-liquid vaccines for administration by a non-parenteral route, the final lot is prepared by distributing the final bulk under suitable conditions into sterile, tamper-evident containers. Where justified and authorised, certain tests prescribed for the final lot may be carried out on the final bulk, if it has been demonstrated that subsequent manufacturing operations do not affect compliance.

Appearance. Unless otherwise justified and authorised, each container (vial, syringe or ampoule) in each final lot is inspected visually or mechanically for acceptable appearance.

Degree of adsorption. For an adsorbed vaccine, unless otherwise justified and authorised, a release specification for the degree of adsorption is established in light of results found for batches used in clinical trials. From the stability data generated for the vaccine it must be shown that at the end of the period of validity the degree of adsorption is not less than for batches used in clinical trials.

Thermal stability. When the thermal stability test is prescribed in a monograph for a live attenuated vaccine, the test is carried out on the final lot to monitor the lot-to-lot consistency in heat-sensitivity of viral/bacterial particles in the product. Suitable conditions are indicated in the individual monograph. The test may be omitted as a routine test for a given product once the consistency of the production process has been demonstrated, in agreement with the competent authority, using relevant parameters, such as consistency in yield, ratio of infectious viruses (viable bacteria) before and after freeze-drying, potency at release and real-time stability under the prescribed conditions as well as thermal stability. Where there is a significant change in the manufacturing procedure of the antigen(s) or formulation, the need for re-introduction of the test is considered.

Stability. During development studies, maintenance of potency of the final lot throughout the period of validity shall be demonstrated; the loss of potency in the recommended storage conditions is assessed. Excessive loss even within the limits of acceptable potency may indicate that the vaccine is unacceptable.

Expiry date. Unless otherwise stated, the expiry date is calculated from the beginning of the assay or from the beginning of the first assay for a combined vaccine. For vaccines stored at a temperature lower than that used for stability studies and intended for release without re-assay, the expiry date is calculated from the date of removal from cold storage. If, for a given vaccine, an assay is not carried out, the expiry date for the final lot is calculated from the date of an approved stability-indicating test or, failing this, from the date of freeze-drying or the date of filling into the final containers. For a combined vaccine where components are presented in separate containers, the expiry date is that of the component which expires first.

The expiry date applies to vaccines stored in the prescribed conditions.

Animal tests. In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. The criteria for judging tests in monographs must be applied in light of this. For example, if it is indicated that an animal is considered to be positive, infected, etc. when typical clinical signs or death occur, then as soon as sufficient indication of a positive result is obtained the animal in question shall be either euthanised or given suitable treatment to prevent unnecessary suffering. In accordance with the General Notices, alternative test methods may be used to demonstrate compliance with the monograph and the use of such tests is particularly encouraged when this leads to replacement or reduction of animal use or reduction of suffering. Guidance on how to substitute *in vivo* methods by *in vitro* methods, in cases where a direct head-to-head comparison is not possible, can be found in general chapter *5.2.14*.

TESTS

Vaccines comply with the tests prescribed in individual monographs including, where applicable, the following:

pH (*2.2.3*). Liquid vaccines, after reconstitution where applicable, comply with the limits for pH approved for the particular preparation.

Adjuvant. If the vaccine contains an adjuvant, the amount is determined and shown to be within acceptable limits with respect to the expected amount (see also the tests for aluminium and calcium below).

Aluminium (*2.5.13*): maximum 1.25 mg of aluminium (Al) per single human dose where an aluminium adsorbent has been used in the vaccine, unless otherwise stated.

Calcium (*2.5.14*): maximum 1.3 mg of calcium (Ca) per single human dose where a calcium adsorbent has been used in the vaccine, unless otherwise stated.

Free formaldehyde (*2.4.18*): maximum 0.2 g/L of free formaldehyde in the final product where formaldehyde has been used in the preparation of the vaccine, unless otherwise stated.

Phenol (*2.5.15*): maximum 2.5 g/L in the final product where phenol has been used in the preparation of the vaccine, unless otherwise stated.

Water (*2.5.12*): maximum 3.0 per cent *m/m* for freeze-dried vaccines, unless otherwise stated.

Extractable volume (*2.9.17*). Unless otherwise justified and authorised, it complies with the requirement for extractable volume.

Bacterial endotoxins. Unless otherwise justified and authorised, a test for bacterial endotoxins is carried out on the final product. Where no limit is specified in the individual monograph, the content of bacterial endotoxins determined by a suitable method (*2.6.14*) is less than the limit approved for the particular product.

STORAGE

Store protected from light. Unless otherwise stated, the storage temperature is 5 ± 3 °C; liquid adsorbed vaccines must not be allowed to freeze.

LABELLING

The label states:

- the name of the preparation;
- a reference identifying the final lot;
- the recommended human dose and route of administration;
- the storage conditions;
- the expiry date;
- the name and amount of any antimicrobial preservative;

- the name of any antibiotic, adjuvant, flavour or stabiliser present in the vaccine;
- where applicable, that the vaccine is adsorbed;
- the name of any constituent that may cause adverse reactions and any contra-indications to the use of the vaccine;
- for freeze-dried vaccines:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the vaccine is to be used after reconstitution.

04/2019:0784

RECOMBINANT DNA TECHNOLOGY, PRODUCTS OF

Producta ab arte ADN recombinandorum

This monograph provides general requirements for the manufacture and control of finished products derived from recombinant DNA (rDNA) technology, and includes requirements for the active substance in these products. Active substances produced by rDNA technology are expected to meet the requirements given in the corresponding section of the present monograph. These requirements are not necessarily comprehensive in a given case and complementary or additional requirements may be prescribed in an individual monograph or imposed by the competent authority.

The monograph is also applicable to vaccine antigens produced by rDNA technology, with more detailed requirements provided in the general monograph Vaccines for human use (0153) and in individual vaccine monographs.

Certain aspects of the monograph may apply to products produced in transgenic animals and plants.

The monograph is not applicable to recombinant organisms that are intended to be used directly in man and animals, for example as live recombinant vectors or vaccines.

DEFINITION

Products of rDNA technology are produced by genetic modification in which DNA coding for the required product is introduced, usually by means of a plasmid or a viral vector, into suitable micro-organisms such as bacteria and yeast, or a suitable cell line of mammalian (including human), insect or plant origin. The DNA introduced can then be expressed as a protein.

The desired product is then recovered by extraction and purification. The cell or micro-organism used to harbour the vector is referred to as the host cell, and the stable association of the two used in the manufacturing process is referred to as the host-vector system.

Products of rDNA technology can also undergo intentional modifications such as pegylation or conjugation.

PRODUCTION

GENERAL PROVISIONS

Production of rDNA products is based on a validated seed-lot system using a host-vector combination that has been shown suitable.

The seed-lot system typically uses a master cell bank and a working cell bank. The host cell, the vector, the host-vector system, the master cell bank and the working cell bank, as well as their establishment, maintenance and cultivation are to be described in detail.

Where products of rDNA technology are manufactured using materials of human or animal origin, the requirements of general chapter *5.1.7. Viral safety* apply.

The production process is validated for the following aspects:

- process consistency, including cell-culture/fermentation, purification and any subsequent intentional modification of the active substance where applicable;
- removal or inactivation of extraneous agents;
- removal of product- and process-related impurities (e.g. unwanted variants, host-cell proteins and DNA, antibiotics, cell-culture components);
- removal of pyrogenic substances where applicable.

A batch shown to be stable and representative of batches tested clinically is used as a reference preparation for identification, tests and assay. The reference preparation is appropriately characterised.

EXPRESSION VECTOR AND HOST CELL

The starting materials to be used for the production of the rDNA product, including the expression vector, the host-cell and the pool of transformed or transfected cells from which the initial clone will be derived, are documented.

Information on the source and history of the host cell and of the expression vector is recorded. The gene coding for the protein of interest as well as other functional sequences of the vector should be characterised (e.g. nucleotide sequence of the gene of interest and its flanking regions, restriction map).

The suitability of the host-vector system, particularly with regard to microbiological purity, is demonstrated by:

- phenotypic and genotypic characterisation of the host cell;
- characterisation of the expression vector within the host cell (e.g. integration, copy number, nucleotide sequence);
- documentation on the raw materials, the transformation/transfection of the host cell by the expression vector and the strategy for deriving the clone used to establish the cell bank system.

CELL BANKS

The master cell bank (MCB) is a homogeneous suspension of the original cells already transformed or transfected by the expression vector containing the desired gene, distributed as equal volumes in a single operation into individual containers for storage (e.g. in liquid nitrogen). In some cases, it may be necessary to establish separate master cell banks for the expression vector and the host cells.

The working cell bank (WCB) is a homogeneous suspension of cells derived from the MCB(s) at a finite passage level, distributed as equal volumes in a single operation into individual containers for storage (e.g. in liquid nitrogen).

For each type of cell bank, all containers are treated identically during storage and once removed from storage, are not returned to the cell stock.

The characterisation and testing of eukaryotic and prokaryotic cell banks are critical components of the control of rDNA-derived biological products. Cell banks are tested to confirm identity, purity and suitability of the cell substrate for the intended manufacturing use. The strategy for testing the cell banks may vary according to the nature and biological properties of the cells (e.g. growth properties) and their cultivation history (e.g. use of animal-derived raw material). Molecular methods are used to analyse the expression vector for copy number, insertions or deletions, and the number of integration sites. The nucleotide sequences of the coding region, flanking regions and promoters are shown to be identical to those determined for the expression vector. The coding region corresponds to that expected for the protein sequence. If any differences in nucleic acid sequences are identified, these must be clearly defined and the expression vector shown to be stable and capable of expressing the expected product consistently.

Cell banks must be characterised and tested at different stages including the MCB, the WCB and cells at or beyond the maximum population doubling level used for production.

For prokaryotic and yeast cell banks, characterisation and testing includes molecular identity of the gene being expressed, identity and purity of the cells including strain identification (e.g. by biochemical, genetic, or proteomic methods), phenotypic and genotypic strain characterisation, viability, presence of the plasmid (e.g. sequence, copy number, restriction map, percentage of cells retaining the plasmid), microbiological purity and, if appropriate, a test for bacteriophage. Additional tests may be relevant and shall be addressed on a case-by-case basis.

For animal cell banks, characterisation and testing includes morphology, identity, viability, genetic stability of the cells (e.g. copy number, integrity of the expression cassette) and testing for extraneous agents. Animal cell substrates have the capacity to propagate extraneous agents such as mycoplasma and viruses. In addition, animal cells may contain endogenous agents such as endogenous retroviruses. Consequently, a testing strategy with regard to extraneous agents should be developed based on a risk assessment taking into account the nature and the history of the cell line.

CULTURE MEDIA AND OTHER RAW MATERIALS

The quality of media and other raw materials used in the production of rDNA proteins is controlled, with consideration given to the impact of the raw material on the quality, safety and efficacy of the final medicinal product. In particular, the origin of the materials must be known and their traceability documented.

CULTURE AND HARVEST

The cell bank (e.g. 1 or more vials(s) of the WCB) is used to start the culture process. In-process controls (e.g. for population doubling levels, cell concentration, volumes, pH, cultivation times, temperature, microbial tests) are established to ensure suitable performance and consistency with regard to the culture process.

Criteria for harvesting and terminating the production must be defined by the manufacturer, and take into account the limit for *in vitro* cell age (e.g. limited number of passages or population doublings) for which the cell substrate has been demonstrated as stable and capable of producing the desired product/intermediate. Each harvest is tested to ensure absence of contamination.

When prokaryotic or other microbial cells are used to manufacture the product, absence of contamination of the harvest is demonstrated primarily by establishing microbial purity. When animal cells are used, the absence of extraneous agents is verified using appropriate *in vitro* cell culture methods and/or molecular methods.

PURIFICATION

The extraction and purification procedure must be shown to yield the desired intermediates at a suitable purity. In this respect, an analysis of the step(s) taken to control the product-related impurities, and to remove or inactivate process-related impurities or contaminants is conducted.

In-process controls (e.g. for yield, volume, pH, processing time, temperature, elution profile and fraction selection, microbial tests) are established to ensure suitable performance and consistency with regard to the purification process.

Holding times and storage conditions of intermediates are to be defined where appropriate.

CHARACTERISATION

During development studies, the active substance is characterised extensively in order to determine its structure, physico-chemical properties, biological activity, immunochemical properties and purity.

Characterisation is necessary to identify the quality attributes that may be important for the safety and efficacy of the product. It provides the basis for release specifications, stability evaluation and for any testing that may have to be performed in support of process changes.

Structure. The characterisation should determine, as far as possible, the primary and higher-order structure of the protein, any post-translational modifications such as glycosylation, and any other intentional modification.

The amino-acid sequence is deduced from the DNA sequence of the expression vector and confirmed by analysis of the produced protein. The amino-acid sequence and the disulfide bridges of the substance are determined using a combination of techniques such as peptide mapping (*2.2.55*) and mass spectrometry (*2.2.43*).

The analysis of pegylated proteins should include, but not be limited to, the location of modification and the degree of site occupancy.

Glycosylation is characterised, and covers overall monosaccharide composition (neutral sugars, amino sugars and sialic acids), glycosylation site(s) and type (e.g. N- or O-linked), degree of site occupancy, and oligosaccharide structures of glycan chains (extensions, branching and linkage) using, for example, the principles and techniques described in general chapter *2.2.59. Glycan analysis of glycoproteins*.

Due to their possible immunogenicity, particular attention is paid to glycan structures that are not known to be present in natural human proteins.

The higher-order structure of the substance is examined using physico-chemical methods such as circular dichroism spectroscopy, Fourier transform infrared spectroscopy, fluorescence, differential scanning calorimetry, proton nuclear magnetic resonance spectrometry and/or hydrogen-deuterium exchange mass spectrometry.

Biological assays based on functional activity may also serve as additional confirmation of the higher-order structure.

Biological activity. The biological activity (i.e. the specific ability of a product to achieve a defined biological effect) is assessed by biological, biochemical (including immunochemical assays) or physico-chemical assays, as appropriate.

The mechanism of action is investigated and preferably reflected in the potency assay.

Immunochemical properties. Where relevant for the mechanism of action, e.g. in the case of antibodies, immunochemical properties (such as Fc-effector functions) are characterised extensively.

Product-related substances and impurities. Products of rDNA technology commonly display several sources of heterogeneity (e.g. N- or C-terminal processing, N-terminal pyroglutamation, deamidation, oxidation, isomerisation, fragmentation, disulfide bond mismatch, N-linked and O-linked glycosylation, glycation, aggregation), which leads to a complex product profile consisting of several molecular entities or variants. When the activity, safety and efficacy of these variants are comparable to those of the desired product, the variants are regarded as product-related substances. Other variants are considered as product-related impurities.

The methods used to assess product-related substances and impurities should be capable of detecting structural variants with different physico-chemical properties, e.g. charge, size and hydrophobicity. Typically, a combination of orthogonal methods is applied, e.g. chromatographic, electrophoretic and spectroscopic techniques.

Characterisation of charged variants, such as differentially sialylated or deamidated variants, is performed using appropriate methods (e.g. capillary electrophoresis, isoelectric focusing, ion-exchange chromatography), which may be coupled to other techniques such as mass spectrometry.

High-molecular-mass forms such as dimers and higher oligomers can be resolved and quantified by size-based separation procedures (e.g. size-exclusion chromatography, field flow fractionation, analytical ultracentrifugation) coupled with suitable detection methods (e.g. ultraviolet, fluorescence, light scattering).

Process-related impurities and contaminants.

Process-related impurities derived from the upstream process may include host-cell proteins, host-cell DNA, or other media components (e.g. inducers, antibiotics, serum). They are to be evaluated qualitatively and quantitatively.

Host-cell proteins are investigated using a sensitive assay capable of detecting a wide range of protein impurities, and taking into account the recommendations given in general chapter *2.6.34. Host-cell protein assays*.

Residual host-cell DNA is determined using a suitably sensitive assay.

Process-related impurities derived from the downstream process may include enzymes, processing reagents (e.g. guanidine, dyes, oxidising and reducing agents), salts (e.g. heavy metals, non-metallic ions), solvents, carriers, ligands (e.g. protein A) and other leachables.

Contaminants include all adventitiously introduced materials not intended to be part of the manufacturing process (e.g. micro-organisms, microbial proteases, bacterial endotoxins).

Process-related impurities and contaminants are controlled using appropriate strategies based on the principles of risk management.

Content. Content is determined by use of an appropriate physico-chemical or immunochemical assay. The total protein content (expressed in mass units) can be determined as described in general chapter *2.5.33. Total protein*. The protein content is determined using several methods, including absolute method(s).

CONTROL STRATEGY

Based on the understanding of the product and process, a planned set of controls that ensures process performance and product quality (i.e. a control strategy) is established. This control strategy includes the control of process parameters, in-process controls, control of raw materials, intermediates, active substance and finished product, as well as the methods used and the frequency of control.

The control strategy should ensure that the quality attributes relevant to the safety and efficacy of the product are within the appropriate range, limit or distribution to ensure the desired product quality.

The selection of tests and the stages at which they are performed are based on knowledge of the manufacturing process and extensive characterisation of the active substance and finished product. Some characterisation tests may be selected to become part of the specifications. The specifications for the active substance and finished product are only 1 part of the overall control strategy.

ACTIVE SUBSTANCE

The active substance is tested for appearance, identity, microbial quality and bacterial endotoxins, product-related substances, product- and process-related impurities, structural integrity, protein content and biological activity, with comparison to suitable reference standards where appropriate. When the active substance is a conjugated or chemically modified protein, e.g. a pegylated protein, appropriate tests must be performed on both the modified and unmodified protein. Tests for product-related variants (e.g. proportion of modified and unmodified protein) and process-related impurities derived from the modification procedure (e.g. by-products of the modification reaction, reagents) are carried out and acceptance criteria should be established.

FINISHED PRODUCT

One or more batches of active substance may be combined to obtain the finished product. Suitable stabilisers and excipients may be added.

The finished product complies with the relevant dosage form monographs, with the relevant individual monograph as appropriate, and with the following considerations.

IDENTIFICATION

The identity test(s) must be specific and must be based on unique aspects of the product's molecular structure or other specific properties such as the size of the molecule, its primary sequence, its isoelectric profile, its chromatographic properties and its functional conformation, with comparison of the product to a suitable reference standard where appropriate. Methods used in the determination of potency or purity may also contribute to the identification.

TESTS

Methods capable of determining process-related impurities and product-related substances and impurities (e.g. resulting from product truncation, fragmentation, aggregation, oxidation, deamidation) are applied and acceptance criteria established, as appropriate. Where impurities are controlled at a suitable earlier stage, the tests may be omitted at the finished product stage.

Tests applied to modified proteins. Suitable tests are carried out depending on the type of modification and the product complies with the approved limits.

ASSAY

Content. It complies with the limits approved for the particular product. It is usually based on protein content and expressed in mass units. The procedures described in general chapter *2.5.33. Total protein* may be applied. When appropriate, other methods using a suitable reference standard such as liquid chromatography (*2.2.29*) may also be used. For modified proteins, the content refers to the protein part of the molecule.

Potency. A potency assay is established using a suitable reference standard and carried out against this reference standard. General chapter *5.3. Statistical analysis of results of biological assays and tests* may be used to design the assay and calculate the results.

01/2008:1483

PRODUCTS WITH RISK OF TRANSMITTING AGENTS OF ANIMAL SPONGIFORM ENCEPHALOPATHIES

Producta cum possibili transmissione vectorium enkephalopathiarum spongiformium animalium

DEFINITION

Products with risk of transmitting agents of animal spongiform encephalopathies are those derived from tissues or secretions of animals susceptible to transmissible spongiform encephalopathies other than by experimental challenge. This monograph applies to all substances or preparations obtained from such animals and to all substances or preparations where products obtained from such animals are included as active substances or excipients or have been used during production, for example as raw or source materials, starting materials or reagents.

PRODUCTION

Production complies with chapter *5.2.8. Minimising the risk of transmitting animal spongiform encephalophathy agents via human and veterinary medicinal products*.

Dosage form monographs

TEXT N°	TITLE	PRODUCT TYPE(S)
0520	Parenteral preparations	Vaccines for parenteral administration.
0676	Nasal preparations	Vaccines for nasal administration.

04/2015:0520
corrected 10.0

PARENTERAL PREPARATIONS

Parenteralia

The requirements of this monograph do not necessarily apply to products derived from human blood, to immunological preparations, or radiopharmaceutical preparations. Special requirements may apply to preparations for veterinary use depending on the species of animal for which the preparation is intended.

DEFINITION

Parenteral preparations are sterile preparations intended for administration by injection, infusion or implantation into the human or animal body.

Parenteral preparations may require the use of excipients, for example to make the preparation isotonic with respect to blood, to adjust the pH, to increase solubility, to prevent deterioration of the active substances or to provide adequate antimicrobial properties, but not to adversely affect the intended medicinal action of the preparation or, at the concentrations used, to cause toxicity or undue local irritation.

Containers for parenteral preparations are made as far as possible from materials that are sufficiently transparent to permit the visual inspection of the contents, except for implants and in other justified and authorised cases.

Where applicable, the containers for parenteral preparations comply with the requirements for *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

Parenteral preparations intended for chronic use or total parenteral nutrition should have appropriate limits for specific components or elements, taking long-term toxicity into account.

Parenteral preparations are supplied in glass containers (*3.2.1*) or in other containers such as plastic containers (*3.2.2*, *3.2.2.1* and *3.2.9*) and prefilled syringes. The tightness of the container is ensured by suitable means. Closures ensure a good seal, prevent the access of micro-organisms and other contaminants and usually permit the withdrawal of a part or the whole of the contents without removal of the closure. The plastic materials or elastomers (*3.2.9*) used to manufacture the closures are sufficiently firm and elastic to allow the passage of a needle with the least possible shedding of particles. Closures for multidose containers are sufficiently elastic to ensure that the puncture is resealed when the needle is withdrawn.

Several categories of parenteral preparations may be distinguished:

- injections;
- infusions;
- concentrates for injections or infusions;
- powders for injections or infusions;
- gels for injections;
- implants.

PRODUCTION

During the development of a parenteral preparation, the formulation for which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided under *Efficacy of antimicrobial preservation* (*5.1.3*).

Parenteral preparations are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms. Recommendations on this aspect are provided in the text *Methods of preparation of sterile products* (*5.1.1*).

Water used in the manufacture of parenteral preparations complies with the requirements of water for injections in bulk stated in the monograph *Water for injections (0169)*.

TESTS

Particulate contamination: sub-visible particles (*2.9.19*). For preparations for human use, solutions for infusion or solutions for injection comply with the test.

In the case of preparations for subcutaneous or intramuscular injection, higher limits may be appropriate. Radiopharmaceutical preparations are exempt from these requirements. Preparations for which the label states that the product is to be used with a final filter are exempt from these requirements, providing it has been demonstrated that the filter delivers a solution that complies with the test.

For preparations for veterinary use, when supplied in containers with a nominal content of more than 100 mL and when the content is equivalent to a dose of more than 1.4 mL per kilogram of body mass, solutions for infusion or solutions for injection comply with the test for particulate contamination: sub-visible particles.

Sterility (*2.6.1*). Parenteral preparations comply with the test for sterility.

STORAGE

In a sterile, airtight, tamper-evident container.

LABELLING

The label states:

- the name and concentration of any added antimicrobial preservative;
- where applicable, that the solution is to be used in conjunction with a final filter;
- where applicable, that the preparation is free from bacterial endotoxins or that it is apyrogenic.

Injections

DEFINITION

Injections are sterile solutions, emulsions or suspensions. They are prepared by dissolving, emulsifying or suspending the active substance(s) and any added excipients in water, in a suitable non-aqueous liquid, that may be non-sterile where justified, or in a mixture of these vehicles.

Solutions for injection, examined under suitable conditions of visibility, are clear and practically free from particles.

Emulsions for injection do not show any evidence of phase separation. Suspensions for injection may show a sediment which is readily dispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be withdrawn.

Multidose preparations. Multidose aqueous injections contain a suitable antimicrobial preservative at an appropriate concentration except when the preparation itself has adequate antimicrobial properties. When a preparation for parenteral administration is presented in a multidose container, the precautions to be taken for its administration and more particularly for its storage between successive withdrawals are given.

Antimicrobial preservatives. Aqueous preparations which are prepared using aseptic precautions and which cannot be terminally sterilised may contain a suitable antimicrobial preservative in an appropriate concentration.

No antimicrobial preservative is added when:

- the volume to be injected in a single dose exceeds 15 mL, unless otherwise justified;
- the preparation is intended for administration by routes where, for medical reasons, an antimicrobial preservative is not acceptable, such as intracisternally, epidurally, intrathecally or by any route giving access to the cerebrospinal fluid, or intra- or retro-ocularly.

Such preparations are presented in single-dose containers.

PRODUCTION

In the manufacture of injections containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

Single-dose preparations. The volume of the injection in a single-dose container is sufficient to permit the withdrawal and administration of the nominal dose using a normal technique (*2.9.17*).

TESTS

Uniformity of dosage units. Single-dose suspensions for injection comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the test for uniformity of content shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

Uniformity of content (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose suspensions for injection with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A for uniformity of content of single-dose preparations. If the preparation contains more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

Bacterial endotoxins - pyrogens. A test for bacterial endotoxins (*2.6.14*) is carried out or, where justified and authorised, the test for pyrogens (*2.6.8*). Recommendations on the limits for bacterial endotoxins are given in general chapter *5.1.10*.

Preparations for human use. The preparation complies with a test for bacterial endotoxins (*2.6.14*) or with the test for pyrogens (*2.6.8*).

Preparations for veterinary use. When the volume to be injected in a single dose is 15 mL or more and is equivalent to a dose of 0.2 mL or more per kilogram of body mass, the preparation complies with a test for bacterial endotoxins (*2.6.14*) or with the test for pyrogens (*2.6.8*).

Any preparation. Where the label states that the preparation is free from bacterial endotoxins or apyrogenic, respectively, the preparation complies with a test for bacterial endotoxins (*2.6.14*) or with the test for pyrogens (*2.6.8*), respectively.

Infusions

DEFINITION

Infusions are sterile, aqueous solutions or emulsions with water as the continuous phase. They are usually made isotonic with respect to blood. They are principally intended for administration in large volume. Infusions do not contain any added antimicrobial preservative.

Solutions for infusion, examined under suitable conditions of visibility, are clear and practically free from particles.

Emulsions for infusion do not show any evidence of phase separation.

PRODUCTION

In the manufacture of infusions containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

The volume of the infusion in the container is sufficient to permit the withdrawal and administration of the nominal dose using a normal technique (*2.9.17*).

TESTS

Bacterial endotoxins - pyrogens. They comply with a test for bacterial endotoxins (*2.6.14*) or, where justified and authorised, with the test for pyrogens (*2.6.8*). For the latter test inject 10 mL per kilogram of body mass into each rabbit, unless otherwise justified and authorised.

Concentrates for injections or infusions

DEFINITION

Concentrates for injections or infusions are sterile solutions intended for injection or infusion after dilution. They are diluted to a prescribed volume with a prescribed liquid before administration. After dilution, they comply with the requirements for injections or for infusions.

TESTS

Bacterial endotoxins - pyrogens. They comply with the requirements prescribed for injections or for infusions, after dilution to a suitable volume.

Powders for injections or infusions

DEFINITION

Powders for injections or infusions are solid, sterile substances distributed in their final containers and which, when shaken with the prescribed volume of a prescribed sterile liquid rapidly form either clear and practically particle-free solutions or uniform suspensions. After dissolution or suspension, they comply with the requirements for injections or for infusions.

Freeze-dried products for parenteral administration are considered as powders for injections or infusions.

PRODUCTION

The uniformity of content and uniformity of mass of freeze-dried products for parenteral administration are ensured by the in-process control of the amount of the solution prior to freeze-drying.

TESTS

Uniformity of dosage units. Powders for injections or infusions comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

Uniformity of content (*2.9.6*). Unless otherwise prescribed or justified and authorised, powders for injections or infusions with a content of active substance less than 2 mg or less than 2 per cent of the total mass, or with a unit mass equal to or less than 40 mg comply with test A for uniformity of content of single-dose preparations. If the preparation contains more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

Uniformity of mass (*2.9.5*). Powders for injections or infusions comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

Bacterial endotoxins - pyrogens. They comply with the requirements prescribed for injections or for infusions, after dissolution or suspension in a suitable volume of liquid.

LABELLING

The label states the instructions for the preparation of injections and infusions.

Gels for injections

DEFINITION

Gels for injections are sterile gels with a viscosity suitable to guarantee a modified release of the active substance(s) at the site of injection.

Implants

DEFINITION

Implants are sterile, solid preparations of a size and shape suitable for parenteral implantation and release of the active substance(s) over an extended period of time. Each dose is provided in a sterile container.

TESTS

A suitable test is carried out to demonstrate the appropriate release of the active substance(s).

01/2021:0676

NASAL PREPARATIONS

Nasalia

DEFINITION

Nasal preparations are preparations intended for administration to the nasal cavities to deliver active substances for a local or systemic effect. They are liquid, semi-solid or solid preparations containing one or more active substances in a suitable vehicle. They may contain excipients, for example to adjust the tonicity or viscosity of the preparation, to adjust or stabilise the pH, to increase the solubility of the active substances, to stabilise the preparation or to provide adequate antimicrobial properties. The excipients do not adversely affect the intended medicinal action of the preparation or, at the concentrations used, cause toxicity or undue local irritation.

Nasal preparations are supplied in multidose or single-dose containers, if necessary provided with a suitable administration device that is designed to avoid the introduction of contaminants.

Unless otherwise justified and authorised, aqueous nasal preparations supplied in multidose containers contain a suitable preservative at a suitable concentration, except where the preparation itself has adequate antimicrobial properties.

Preparations for administration to the injured nose, particularly when the mucosa is damaged, or prior to surgery are sterile and, unless otherwise justified and authorised, free from preservatives and supplied in single-dose containers.

Where applicable, containers for nasal preparations comply with the requirements for *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

Several categories of nasal preparations may be distinguished:

- nasal drops;
- nasal sprays;
- nasal powders;
- semi-solid nasal preparations;
- nasal washes;
- nasal sticks.

PRODUCTION

During the development of nasal preparations whose formulation contains a preservative, the need for and the efficacy of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in general chapter *5.1.3. Efficacy of antimicrobial preservation*.

In the manufacture, packaging, storage and distribution of nasal preparations, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

Sterile nasal preparations are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in general chapter *5.1.1. Methods of preparation of sterile products*.

In the manufacture of nasal preparations containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

During manufacture of metered-dose nasal sprays and metered-dose nasal powders supplied in multidose containers, the uniformity of delivered dose must be ensured within a container (intra-container) and between containers (inter-container).

For intra-container testing of the uniformity of delivered dose, a test is given in the Tests section.

For inter-container testing of the uniformity of delivered dose, see below.

Uniformity of delivered dose, inter-container testing. Unless otherwise justified and authorised, the test applies. *Prepare and use the containers as directed in the instructions to the patient*.

An example of a suitable procedure is to take 10 containers and collect a single dose from each container, collecting the dose at the beginning (from 3 containers), middle (from 4 containers) and end (from 3 containers) of the number of doses stated on the label. Other inter-container testing procedures are possible, where justified.

A suitable dose collection apparatus is described in the monograph *Preparations for inhalation (0671)*.

TESTS

Sterility (*2.6.1*). Where the label states that the preparation is sterile, it complies with the test.

STORAGE

If the preparation is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

- the name of any added preservative;
- where applicable, that the preparation is sterile;
- for multidose containers:
  - the period within which the preparation is to be used after opening;
  - where applicable, the number of deliveries per container;
  - where applicable, the delivered dose or, if justified and authorised (e.g. where the dose has been established as a metered dose or as a pre-metered dose), the metered dose or the pre-metered dose.

Nasal drops

DEFINITION

Nasal drops are liquid preparations intended for instillation into the nasal cavities. They are solutions, emulsions or suspensions.

Nasal drops that are emulsions may show evidence of phase separation but are readily redispersed on shaking. Nasal drops that are suspensions may show a sediment that is readily dispersed on shaking to give a suspension that remains sufficiently stable to enable the correct dose to be delivered.

Nasal drops are usually supplied in multidose containers of glass or a suitable plastic material that are fitted with an integral dropper or with a screw cap of suitable materials incorporating a dropper and rubber or plastic teat. This cap assembly may also be supplied separately.

PRODUCTION

During the development of nasal drops supplied in single-dose containers, it must be demonstrated that the nominal content can be withdrawn from the container.

TESTS

Unless otherwise prescribed or justified and authorised, nasal drops supplied in single-dose containers comply with the following tests.

Uniformity of dosage units (*2.9.40*). Nasal drops supplied in single-dose containers comply with the test or, where justified and authorised, with the test for uniformity of mass or uniformity of content shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

Uniformity of mass. Nasal drops that are solutions comply with the following test. Weigh individually the contents of 10 containers emptied as completely as possible, and determine the average mass. Not more than 2 of the individual masses deviate by more than 10 per cent from the average mass, and none deviate by more than 20 per cent.

Uniformity of content (*2.9.6*). Nasal drops that are suspensions or emulsions comply with the requirements under Test B. Empty each container as completely as possible and carry out the test on the individual contents.

Nasal sprays

DEFINITION

Nasal sprays are liquid preparations intended for spraying into the nasal cavities. They are solutions, emulsions or suspensions.

Nasal sprays that are emulsions may show evidence of phase separation but are readily redispersed on shaking. Nasal sprays that are suspensions may show a sediment that is readily dispersed on shaking to give a suspension that remains sufficiently stable to enable the correct dose to be delivered.

Nasal sprays are usually supplied in multidose containers with atomising devices or in pressurised containers fitted with a suitable adapter, with or without a metering dose valve. Pressurised containers comply with the requirements of the monograph *Pressurised pharmaceutical preparations (0523)*.

The size of the droplets generated and the resulting spray pattern are such that spray deposition is localised in the nasal cavity.

TESTS

Unless otherwise prescribed or justified and authorised, metered-dose nasal sprays comply with the following tests.

Prepare and use the container as directed in the instructions to the patient.

Uniformity of delivered dose, intra-container testing. Metered-dose nasal sprays supplied in multidose containers comply with the following test. If justified and authorised, for preparations that are solutions, the test for uniformity of delivered dose can be replaced by the test for uniformity of delivered mass.

Use an apparatus capable of quantitatively retaining the dose leaving the metered-dose container. A suitable apparatus is described in the monograph *Preparations for inhalation (0671)*.

Take 1 container and discharge the contents into the apparatus until the number of deliveries that constitute the minimum recommended dose have been sampled. Quantitatively collect the contents of the apparatus and determine the amount of active substance. Repeat the procedure for a further 2 doses.

Discharge the container to waste until $(n/2) + 1$ deliveries remain, where n is the number of deliveries stated on the label. Collect 4 doses using the procedure described above. Discharge the container to waste until 3 doses remain. Collect these 3 doses using the procedure described above.

For preparations containing more than one active substance, carry out the test for uniformity of delivered dose for each active substance.

For pressurised metered-dose nasal sprays, prevent excessive cooling by waiting not less than 5 s between each actuation.

Unless otherwise justified and authorised, the preparation complies with the test if 9 out of the 10 results lie between 75 per cent and 125 per cent of the mean value and all lie between 65 per cent and 135 per cent.

If 2 or 3 values lie outside the limits of 75 per cent to 125 per cent but within the limits of 65 per cent to 135 per cent, repeat the test for 2 more containers. Not more than 3 of the 30 values lie outside the limits of 75 per cent to 125 per cent and no value lies outside the limits of 65 per cent to 135 per cent. Unless otherwise authorised, the mean value must be between 85 per cent and 115 per cent of the label claim for delivered dose.

Uniformity of delivered mass, multidose containers. Metered-dose nasal sprays that are solutions and are supplied in multidose containers comply with the following test.

Take 1 container, weigh and discharge the contents to waste until the number of deliveries that constitute the minimum recommended dose has been discharged. Weigh the container again. Calculate the difference between the 2 masses. Repeat the procedure for a further 2 doses.

Discharge the container to waste until $(n/2) + 1$ deliveries remain, where n is the number of deliveries stated on the label. Determine the mass of 4 doses using the procedure described above.

Discharge the container to waste until 3 doses remain. Determine the mass of these 3 doses using the procedure described above.

Unless otherwise justified and authorised, the preparation complies with the test if 9 out of the 10 results lie between 75 per cent and 125 per cent of the mean value and all lie between 65 per cent and 135 per cent.

If 2 or 3 values lie outside the limits of 75 per cent to 125 per cent but within the limits of 65 per cent to 135 per cent, repeat the test for 2 more containers. Not more than 3 of the 30 values lie outside the limits of 75 per cent to 125 per cent and no value lies outside the limits of 65 per cent to 135 per cent. Unless otherwise authorised, the mean value must be between 85 per cent and 115 per cent of the target delivered mass.

Uniformity of delivered mass, single-dose containers. Metered-dose nasal sprays that are solutions and are supplied in single-dose containers comply with the following test.

Take 1 container, weigh and discharge the contents to waste until the number of deliveries that constitute the minimum recommended dose has been discharged. Weigh the container again. Calculate the difference between the 2 masses. Repeat the procedure for a further 9 containers.

The preparation complies with the test if 8 out of the 10 results lie between 75 per cent and 125 per cent of the mean value and all lie between 65 per cent and 135 per cent of the mean value.

Number of deliveries per container. Nasal sprays supplied in multidose containers comply with the following test.

Take 1 container and discharge the contents to waste until empty. Record the number of deliveries discharged. The total number of deliveries discharged from the container is not less than the number stated on the label.

Leak rate. Metered-dose nasal sprays supplied in pressurised containers comply with the following test.

Take a suitable number of containers, for example 1 container. Remove any labels and record the date and time to the nearest half hour. Weigh the container(s) to the nearest milligram and record the mass (m_1) in milligrams. Allow the container(s) to stand in an upright position at a temperature of 25.0 ± 2.0 °C for not less than 3 days, and again weigh the container(s), recording the mass (m_2) in milligrams, and recording the date and time to the nearest half hour. Determine the time (t), in hours, during which the container was under test.

Calculate the total loss of mass, in milligrams, over the entire shelf-life of the container (D), in months, using the following expression:

General chapters

TEXT N°	TITLE	PRODUCT TYPE(S)
5.2.1	Terminology used in monographs on biological products	All vaccines.
5.2.2	Chicken flocks free from specified pathogens for the production and quality control of vaccines	Vaccines produced in specified pathogen-free primary avian tissues.
5.2.3	Cell substrates for the production of vaccines for human use	Vaccines using cell cultures for production.
2.6.16	Tests for extraneous agents in viral vaccines for human use	Live attenuated viral vaccines, inactivated viral vaccines, recombinant viral vectored vaccines.
5.1.7	Viral safety	Vaccines produced using material of human or animal origin.
5.2.8	Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products	Vaccines produced using material of animal origin.
5.14	Gene transfer medicinal products for human use	*Certain considerations may be relevant to recombinant viral vectored vaccines using adenovirus or poxvirus as backbone, and to DNA vaccines.*

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corrected 6.0

5.2.1. TERMINOLOGY USED IN MONOGRAPHS ON BIOLOGICAL PRODUCTS

For some items, alternative terms commonly used in connection with veterinary vaccines are shown in parenthesis.

Seed-lot system. A seed-lot system is a system according to which successive batches of a product are derived from the same master seed lot. For routine production, a working seed lot may be prepared from the master seed lot. The origin and the passage history of the master seed lot and the working seed lot are recorded.

Master seed lot. A culture of a micro-organism distributed from a single bulk into containers and processed together in a single operation in such a manner as to ensure uniformity and stability and to prevent contamination. A master seed lot in liquid form is usually stored at or below – 70 °C. A freeze-dried master seed lot is stored at a temperature known to ensure stability.

Working seed lot. A culture of a micro-organism derived from the master seed lot and intended for use in production. Working seed lots are distributed into containers and stored as described above for master seed lots.

Cell-bank system (Cell-seed system). A system whereby successive final lots (batches) of a product are manufactured by culture in cells derived from the same master cell bank (master cell seed). A number of containers from the master cell bank (master cell seed) are used to prepare a working cell bank (working cell seed). The cell-bank system (cell-seed system) is validated for the highest passage level achieved during routine production.

Master cell bank (Master cell seed). A culture of cells distributed into containers in a single operation, processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination. A master cell bank (master cell seed) is usually stored at – 70 °C or lower.

Working cell bank (Working cell seed). A culture of cells derived from the master cell bank (master cell seed) and intended for use in the preparation of production cell cultures. The working cell bank (working cell seed) is distributed into containers, processed and stored as described for the master cell bank (master cell seed).

Primary cell cultures. Cultures of cells obtained by trypsination of a suitable tissue or organ. The cells are essentially identical to those of the tissue of origin and are no more than 5 *in vitro* passages from the initial preparation from the animal tissue.

Cell lines. Cultures of cells that have a high capacity for multiplication *in vitro*. In diploid cell lines, the cells have essentially the same characteristics as those of the tissue of origin. In continuous cell lines, the cells are able to multiply indefinitely in culture and may be obtained from healthy or tumoral tissue. Some continuous cell lines have oncogenic potential under certain conditions.

Production cell culture. A culture of cells intended for use in production; it may be derived from one or more containers of the working cell bank (working cell seed) or it may be a primary cell culture.

Control cells. A quantity of cells set aside, at the time of virus inoculation, as uninfected cell cultures. The uninfected cells are incubated under similar conditions to those used for the production cell cultures.

Single harvest. Material derived on one or more occasions from a single production cell culture inoculated with the same working seed lot or a suspension derived from the working seed lot, incubated, and harvested in a single production run.

Monovalent pooled harvest. Pooled material containing a single strain or type of micro-organism or antigen and derived from a number of eggs, cell culture containers etc. that are processed at the same time.

Final bulk vaccine. Material that has undergone all the steps of production except for the final filling. It consists of one or more monovalent pooled harvests, from cultures of one or more species or types of micro-organism, after clarification, dilution or addition of any adjuvant or other auxiliary substance. It is treated to ensure its homogeneity and is used for filling the containers of one or more final lots (batches).

Final lot (Batch). A collection of closed, final containers or other final dosage units that are expected to be homogeneous and equivalent with respect to risk of contamination during filling or preparation of the final product. The dosage units are filled, or otherwise prepared, from the same final bulk vaccine, freeze-dried together (if applicable) and closed in one continuous working session. They bear a distinctive number or code identifying the final lot (batch). Where a final bulk vaccine is filled and/or freeze-dried on several separate sessions, there results a related set of final lots (batches) that are usually identified by the use of a common part in the distinctive number or code; these related final lots (batches) are sometimes referred to as sub-batches, sub-lots or filling lots.

Combined vaccine. A multicomponent preparation formulated so that different antigens are administered simultaneously. The different antigenic components are intended to protect against different strains or types of the same organism and/or different organisms. A combined vaccine may be supplied by the manufacturer either as a single liquid or freeze-dried preparation or as several constituents with directions for admixture before use.

07/2010:50202

5.2.2. CHICKEN FLOCKS FREE FROM SPECIFIED PATHOGENS FOR THE PRODUCTION AND QUALITY CONTROL OF VACCINES

Where specified, chickens, embryos or cell cultures used for the production or quality control of vaccines are derived from eggs produced by chicken flocks free from specified pathogens (SPF). The SPF status of a flock is ensured by means of the system described below. The list of micro-organisms given is based on current knowledge and will be updated as necessary.

A flock is defined as a group of birds sharing a common environment and having their own caretakers who have no contact with non-SPF flocks. Once a flock is defined, no non-SPF birds are added to it.

Each flock is housed so as to minimise the risk of contamination. The facility in which the flock is housed must not be sited near to any non-SPF flocks of birds with the exception of flocks that are in the process of being established as SPF flocks and that are housed in facilities and conditions appropriate to SPF flocks. The SPF flock is housed within an isolator or in a building with filtered air under positive pressure. Appropriate measures are taken to prevent entry of rodents, wild birds, insects and unauthorised personnel.

Personnel authorised to enter the facility must have no contact with other birds or with agents potentially capable of infecting the flock. It is advisable for personnel to shower and change clothing or to wear protective clothing before entering the controlled facility.

Wherever possible, items taken into the facility are sterilised. In particular it is recommended that the feed is suitably treated to avoid introduction of undesirable micro-organisms and that water is at least of potable quality, for example from a chlorinated supply. No medication is administered to birds within the flock that might interfere with detection of any disease.

A permanent record is kept of the general health of the flock and any abnormality is investigated. Factors to be monitored include morbidity, mortality, general physical condition, feed consumption, daily egg production and egg quality, fertility and hatchability. Records are maintained for a period of at least 5 years. Details of any deviation from normal in these performance parameters or detection of any infection are notified to the users of the eggs as soon as practicable.

The tests or combination of tests described below must have suitable specificity and sensitivity with respect to relevant serotypes of the viruses. Samples for testing are taken at random.

A positive result for chicken anaemia virus (CAV) does not necessarily exclude use of material derived from the flock, but live vaccines for use in birds less than 7 days old shall be produced using material from CAV-negative flocks. Inactivated vaccines for use in birds less than 7 days old may be produced using material from flocks that have not been shown to be free from CAV, provided it has been demonstrated that the inactivation process inactivates CAV.

ESTABLISHMENT OF AN SPF FLOCK

A designated SPF flock is derived from chickens shown to be free from vertically-transmissible agents listed in Table 5.2.2-1. This is achieved by testing of 2 generations prior to the designated SPF flock. A general scheme for the procedure to be followed in establishing and maintaining an SPF flock is shown diagrammatically in Table 5.2.2.-2. In order to establish a new SPF flock, a series of tests must be conducted on 3 generations of birds. All birds in the 1st generation must be tested at least once before the age of 20 weeks for freedom from avian leucosis group-antigen and tested by an enzyme immunoassay (EIA) or by virus neutralisation (VN) for freedom of antibodies to avian leucosis virus subtypes A, B and J. All birds must also be tested for freedom from antibodies to the vertically-transmissible agents listed in Table 5.2.2-1. From the age of 8 weeks the flock is tested for freedom from *Salmonella*. Clinical examination is carried out on the flock from 8 weeks of age and the birds must not exhibit any signs of infectious disease. The test methods to be used for these tests are given in the table and further guidance is also given in the section below on routine testing of designated SPF flocks. From 20 weeks of age, the flock is tested as described under Routine testing of designated SPF flocks. All stages of this testing regime are also applied to the subsequent 2 generations, except the testing of every bird before lay for vertically-transmissible agents. All test results must indicate freedom from pathogens in all 3 generations for the flock consisting of the 3rd generation to be designated as SPF.

SPF embryos derived from another designated SPF flock contained within a separate facility on the same site may be introduced. From 8 weeks of age, these replacement birds are regarded as a flock and are tested in accordance with test procedures described above.

INITIAL TESTING REQUIREMENTS FOR SUBSEQUENT GENERATIONS DERIVED FROM A DESIGNATED SPF FLOCK

Where a replacement flock is derived exclusively from a fully established SPF flock the new generation is tested prior to being designated as SPF. In addition to the tests for *Salmonella* and monitoring of the general health and performance of the flock, further specific testing from the age of 8 weeks is required. Tests are performed on two 5 per cent samples of the flock (minimum 10, maximum 200 birds) taken with an interval of at least 4 weeks between the ages of 12-16 weeks and 16-20 weeks.

All samples are collected and tested individually. Blood samples for antibody tests and suitable samples for testing for leucosis antigen are collected. The test methods to be used are as described under Routine testing of designated SPF flocks. Only when all tests have confirmed the absence of infection may the new generation be designated as SPF.

ROUTINE TESTING OF DESIGNATED SPF FLOCKS

General examination and necropsy. Clinical examination is carried out at least once per week throughout the life of the flock in order to verify that the birds are free from fowl-pox virus and signs of any other infection. In the event of mortality exceeding 0.2 per cent per week, necropsy is performed on all available carcasses to verify that there is no sign of infection. Where appropriate, histopathological and/or microbiological/virological studies are performed to confirm diagnosis. Specific examination for tuberculosis lesions is carried out and histological samples from any suspected lesions are specifically stained to verify freedom from *Mycobacterium avium*. Caecal contents of all available carcasses are examined microbiologically for the presence of *Salmonella* spp. using the techniques described below. Where appropriate, caecal samples from up to 5 birds may be pooled.

Cultural testing for Salmonella spp. Cultural testing for *Salmonella* spp. is performed either by testing samples of droppings or cloacal swabs or by testing of drag swabs. Where droppings or cloacal swabs are tested, a total of 60 samples within each 4-week period is tested throughout the entire life of the flock. Tests may be performed on pools of up to 10 samples. Where drag swabs are tested, a minimum of 2 drag swabs are tested during each 4-week period throughout the entire life of the flock. Detection of *Salmonella* spp. in these samples is performed by pre-enrichment of the samples followed by culture using *Salmonella*-selective media.

Tests for avian leucosis antigen. Prior to the commencement of laying, cloacal swabs or blood samples (using buffy coat cultivation) are tested for the presence of group-specific leucosis antigen. A total of 5 per cent (minimum 10, maximum 200) of the flock is sampled during each 4-week period. During lay, albumen samples from 5 per cent (minimum 10, maximum 200) of the eggs are tested in each 4-week period. Tests are performed by EIA for group-specific antigen using methods that are capable of detecting antigen from subgroups A, B and J.

Test for antibodies to other agents. Tests for antibodies to all agents listed in Table 5.2.2.-1 are performed throughout the laying period of the flock. In each 4-week period, samples are taken from 5 per cent (minimum 10, maximum 200) of the flock. It is recommended that 1.25 per cent of the flock is sampled each week since some test methods for some agents must be conducted on a weekly basis. Table 5.2.2.-1 classifies the agents into those that spread rapidly through the flock and those that spread slowly or may not infect the entire flock. For those agents listed as slowly spreading, each sample is tested individually. For those agents listed as rapidly spreading, at least 20 per cent of the samples collected in each 4-week period are tested individually or, where serum neutralisation or ELISA tests are employed, all of the samples may be tested individually or by preparing pools of 5 samples, collected at the same time.

Suitable methods to be used for detection of the agents are shown in Table 5.2.2.-1. Subject to agreement by the competent authority, other test methods may be used provided they are shown to be at least as sensitive as those indicated and of appropriate specificity.

Table 5.2.2.-1

Agent	Test to be used**	Vertical transmission	Rapid/slow spread
Avian adenoviruses, group 1	AGP, EIA	yes	slow
Avian encephalomyelitis virus	AGP, EIA	yes	rapid
Avian infectious bronchitis virus	HI, EIA	no	rapid
Avian infectious laryngotracheitis virus	VN, EIA	no	slow
Avian leucosis viruses	EIA for virus, VN, EIA for antibody	yes	slow
Avian nephritis virus	IS	no	slow
Avian orthoreoviruses	IS, EIA	yes	slow
Avian reticuloendotheliosis virus	AGP, IS, EIA	yes	slow
Chicken anaemia virus	IS, EIA, VN	yes	slow
Egg drop syndrome virus	HI, EIA	yes	slow
Infectious bursal disease virus	Serotype 1: AGP, EIA, VN Serotype 2: VN	no	rapid
Influenza A virus	AGP, EIA, HI	no	rapid
Marek's disease virus	AGP	no	rapid
Newcastle disease virus	HI, EIA	no	rapid
Turkey rhinotracheitis virus	EIA	no	slow
Mycoplasma gallisepticum	Agg and HI to confirm a positive test, EIA, HI	yes	slow
Mycoplasma synoviae	Agg and HI to confirm a positive test, EIA, HI	yes	rapid
Salmonella pullorum	Agg	yes	slow

Agg: agglutination
AGP: agar gel precipitation; the technique is suitable where testing is carried out weekly
EIA: enzyme immunoassay
HI: haemagglutination inhibition
IS: immunostaining
VN: virus neutralisation

**Subject to agreement by the competent authority, other types of test may be used provided they are at least as sensitive as those indicated and of appropriate specificity.

Table 5.2.2-2. – *Schematic description of the establishment and maintenance of SPF flocks*

NEW STOCK	Establish freedom from vertically-transmissible agents
	Test all birds for avian leucosis antigen and antibodies prior to 20 weeks of age
	Test for *Salmonella* spp. and perform general clinical observation from 8 weeks of age
	Carry out routine testing for specified agents from 20 weeks of age
2nd GENERATION	Test all birds for avian leucosis antigen and antibodies prior to 20 weeks of age
	Test for *Salmonella* spp. and perform general clinical observation from 8 weeks of age
	Carry out routine testing for specified agents from 20 weeks of age
3rd GENERATION	Test all birds for avian leucosis antigen and antibodies prior to 20 weeks of age
	Test for *Salmonella* spp. and perform general clinical observation from 8 weeks of age

	DESIGNATE FLOCK AS SPF IF ALL TESTS ARE SATISFACTORY
3rd GENERATION	Carry out routine testing for specified agents from 20 weeks of age
	Carry out post-lay testing for vertically-transmissible agents
SUBSEQUENT GENERATIONS	Test two 5 per cent samples for avian leucosis antigen and for antibodies against specified agents between 12 and 20 weeks of age
	Test for *Salmonella* spp. and perform general clinical observation from 8 weeks of age
	Carry out routine testing for specified agents from 20 weeks of age
	Carry out post-lay testing for vertically-transmissible agents

TESTS TO BE CONDUCTED AT THE END OF THE LAYING PERIOD

Following the last egg collection, final testing to confirm the absence of vertically-transmissible agents indicated in Table 5.2.2.-1 is performed. After the last egg collection, a minimum of 5 per cent of the flock (minimum 10, maximum 200) is retained for at least 4 weeks. Blood samples are collected from every bird in the group during the 4-week period with at least 1.25 per cent of the birds (25 per cent of the sample) being bled not earlier than 4 weeks after the final egg collection. Serum samples are tested for vertically-transmissible agents (as defined by Table 5.2.2.-1) using the methods indicated. Where sampling is performed on a weekly basis, at least 1.25 per cent of the birds (25 per cent of the sample) are tested each week during this period. Alternatively, within 4 weeks of the final egg collection blood and/or other suitable sample materials are collected from at least 5 per cent of the flock and tested for the presence of vertically-transmissible agents using validated nucleic acid amplification techniques (*2.6.21*).

ACTION TO BE TAKEN IN THE EVENT OF DETECTION OF A SPECIFIED AGENT

If evidence is found of contamination of the flock by an agent listed as slowly spreading in Table 5.2.2.-1, all materials derived from the flock during the 4-week period immediately preceding the date on which the positive sample was collected are considered unsatisfactory. Similarly, if evidence is found of contamination of the flock by an agent listed as rapidly spreading in Table 5.2.2.-1, all materials derived from the flock during the 2-week period immediately preceding the date on which the positive sample was collected are considered unsatisfactory. Any product manufactured with such materials, and for which the use of SPF materials is required, is considered unsatisfactory and must be discarded; any quality control tests conducted using the materials are invalid.

Producers must notify users of all eggs of the evidence of contamination as soon as possible following the outbreak.

Any flock in which an outbreak of any specified agent is confirmed may not be redesignated as an SPF flock. Any progeny derived from that flock during or after the 4-week period prior to the last negative sample being collected may not be designated as SPF.

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5.2.3. CELL SUBSTRATES FOR THE PRODUCTION OF VACCINES FOR HUMAN USE

This general chapter deals with diploid cell lines and continuous cell lines used as cell substrates for the production of vaccines for human use; additional issues specifically related to vaccines prepared by recombinant DNA technology are covered by the monograph *Products of recombinant DNA technology (0784)*. The testing to be carried out at the various stages (cell seed, master cell bank (MCB), working cell bank (WCB), end of productions cells (EOPC) or extended cell bank (ECB) corresponding to cells at or beyond the maximum population doubling level used for production) is indicated in Table 5.2.3.-1. General provisions for the use of cell lines and test methods are given below. Where primary cells or cells that have undergone a few passages without constitution of a cell bank are used for vaccine production, requirements are given in the individual monograph for the vaccine concerned.

Diploid cell lines. A diploid cell line has a high but finite capacity for multiplication *in vitro*.

Continuous cell lines. A continuous cell line has the capacity to multiply indefinitely *in vitro*; the cells often have differences in karyotype compared to the original cells; they may be obtained from healthy or tumour tissue either from mammals or from insects.

There are perceived theoretical risks associated with the use of continuous cell lines, especially if their tumorigenic potential has been demonstrated experimentally. These risks are linked to the potential biological activity of the residual host-cell DNA present in the vaccine. The residual host-cell DNA may be associated with an infectivity risk if the genome of a DNA virus or a provirus is present in the cellular DNA (either integrated or extra chromosomal). In addition, there is a potential risk of oncogenicity if the cell substrate is tumorigenic.

For vaccines produced in continuous cell lines, whether tumorigenic or not, risk assessment and risk mitigation must be performed to evaluate the suitability of the cell substrate, to define the acceptable criteria for residual host-cell DNA in the final product and to evaluate the consistency of host-cell proteins.

Cell-bank system. Production of vaccines in diploid or continuous cell lines is based on a cell-bank system. The *in vitro* age of the cells is counted from the MCB. Each WCB is prepared from one or more containers of the MCB. The use, identity and inventory control of the containers is carefully documented.

Media and substances of human or animal origin. The composition of media used for isolation and all subsequent culture is recorded in detail, and if substances of human or animal origin are used they must be free from extraneous agents (*2.6.16*) and must comply with general chapter *5.1.7. Viral safety*.

If human albumin is used, it complies with the monograph *Human albumin solution (0255)*.

If bovine serum is used, it complies with the monograph *Bovine serum (2262)*.

Unless of recombinant origin, trypsin used for the preparation of cell cultures is tested by suitable methods and shown to be sterile and free from mycoplasmas and viruses.

Cell seed. The data used to assess the suitability of the cell seed comprises information, where available, on source, history and characterisation.

Source of the cell seed. For human cell lines, the following information concerning the donor is recorded: ethnic and geographical origin; age; sex; general physiological condition; tissue or organ used; results of any tests for pathogens.

For animal cell lines, the following information concerning the source of the cells is recorded: species; strain; breeding conditions; geographical origin; age; sex; general physiological condition; tissue or organ used; results of any tests for pathogens.

Cells of neural origin, such as neuroblastoma and P12 cell lines are not used for vaccine production since they may contain substances that concentrate agents of spongiform encephalopathies.

History of the cell seed. The following information is recorded: the method used to isolate the cell seed; culture methods; any other procedures used to establish the MCB, notably any that might expose the cells to extraneous agents.

Full information may not be available on the media ingredients used in the past for cultivation of cells, for example on the source of substances of animal origin; where justified and authorised, cell banks already established using such media may be used for vaccine production.

Characterisation of the cell seed. The following properties are investigated:

(1) the identity of the cells, using methods such as isoenzyme analysis, *in vitro* immunochemical assays, nucleic acid fingerprinting and nucleic acid amplification techniques (NAT);

(2) the growth characteristics of the cells and their morphological properties (optical and electron microscopy);

(3) for diploid cell lines, karyotype;

(4) for diploid cell lines, the *in vitro* life span in terms of population doubling level.

Cell substrate stability. Suitable viability of the cell line in the intended storage conditions must be demonstrated. For a given product to be prepared in the cell line, it is necessary to demonstrate that consistent production can be obtained with cells at passage and/or population doubling levels at the beginning and end of the intended period of use.

Infectious extraneous agents. For cell lines for vaccine production, the testing for infectious extraneous agents must be carried out based on a risk assessment. The origin of the cell substrate as well as the potential extraneous agents that may be inadvertently introduced during production processes or through the use of animal or plant derived raw materials must be taken into account in the choice of suitable permissive cells. One such strategy is given in Table 5.2.3.-1, but alternative strategies could focus on more extensive testing at the MCB or WCB level. In any case, any strategy must be justified and lead to the same level of safety as outlined in Table 5.2.3-1. New, sensitive molecular techniques with broad detection capabilities are available, including massive parallel sequencing (MPS) methods, degenerate polymerase chain reaction (PCR) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays and mass spectrometry. These methods may be used either as an alternative to *in vivo* or specific NAT tests or as a supplement/alternative to *in vitro* culture tests, in agreement with the competent authority. The capacity of the process to remove/inactivate specific viruses must take into account the origin and culture history of the cell line and adventitious viruses that are known to persistently infect the species of origin, for example, simian virus 40 in rhesus monkeys, Flock house virus in insect cells or viruses that may inadvertently be introduced during production processes or through the use of raw materials of animal or plant origin. For cell lines of insect origin, tests for specific

viruses relevant to the species of origin of the insect cells and for arboviruses (arthropod-borne viruses) are carried out. The panel of viruses tested is chosen according to the current state of scientific knowledge. For cell lines shown to express endogenous retroviral particles (e.g. rodent cells), the test for reverse transcriptase is not needed because it is expected to be positive, and thus infectivity tests must be performed to determine whether these endogenous retroviral particles are infectious or not.

Cell lines that show the presence of infectious retroviruses are not acceptable for production of vaccines, unless otherwise justified and authorised.

Table 5.2.3.-1. – *Testing of cell lines*

Test	Cell seed	Master cell bank (MCB)	Working cell bank (WCB)	EOPC/ECB (Cells at or beyond the maximum population doubling level used for production)
1. IDENTITY AND PURITY				
Morphology	+	+	+	+
Identification	+	+	+	+
Karyotype (diploid cell lines)	+	+	+(1)	+(1)
Life span (diploid cell lines)	–	+	+	–
2. EXTRANEOUS AGENTS				
Bacterial and fungal contamination	–	+	+	–
Mycobacteria	–	+(2)	+(2)	–
Mycoplasmas	–	+	+	–
Spiroplasmas(3)	–	+	+	–
Electron microscopy	–	+(4)	–	+(4)
Tests for extraneous agents in cell cultures (with viable cells or equivalent cell lysate)	–	+	+	+
Tests in suckling mice and eggs	–	–	+(5)	+(5)
Test for specific viruses by NAT	–	+(6)	+(6)	+(6)
Test for viruses using broad molecular methods	+(7)	+(7)	+(7)	+(7)
Retroviruses	–	+(4)	–	+(4)
3. TUMORIGENICITY				
Tumorigenicity	+(8, 9)	–	–	+(8)

(1) The diploid character is established for each WCB but using cells at or beyond the maximum population doubling level used for production.

(2) If the cells are susceptible to infection with *Mycobacterium tuberculosis* or other species.

(3) If insect cells or raw materials of plant origin are used.

(4) Testing is carried out for the MCB, but using cells at or beyond the maximum population doubling level used for production.

(5) Testing is carried out for each WCB, but using cells at or beyond the maximum population doubling level used for production.

(6) Specific tests for possible contaminants (e.g. viruses) defined according to a risk assessment based on the origin of the cells and on the potential extraneous agents inadvertently introduced during production processes or through the use of animal or plant derived raw materials. The appropriate testing stages should be selected based on the risk assessment.

(7) These methods may be used either as alternative to *in vivo* tests and specific NAT or as supplement or alternative to *in vitro* culture tests based on the risk assessment and in agreement with the competent authority. The appropriate testing stages should be selected based on the risk assessment.

(8) The MRC-5, WI-38 and FRhL-2 cell lines are recognised as being non-tumorigenic and they do not need to be tested. Tests are not carried out on cell lines that are known or assumed to be tumorigenic, for example CHO and BHK-21.

(9) Testing is carried out on the cell seed, but using cells at or beyond the maximum population doubling level used for production.

Tumorigenicity. Tumorigenicity is defined as the potential of a given cell line to induce a tumour after injection of intact live cells into immunodeficient/immunosuppressed animals (usually rodents). The tumorigenicity test is carried out using cells at or beyond the maximum population doubling level that will be used for vaccine production.

The MRC-5, WI-38 and FRhL-2 cell lines are recognised as being non-tumorigenic and further testing is not necessary. Known tumorigenic cell lines (e.g. CHO) do not need to be documented further.

When a previously uncharacterised cell line is tumorigenic, an oncogenicity study must be performed using purified DNA from the cell line and/or cell line lysate to demonstrate the absence of oncogenic components. The results are used as part of the risk analysis performed to support the use of the cell line for vaccine production. The determination of the TPD_{50} (tumour-producing dose in 50 per cent of animals) and the capacity to form metastases are characteristic properties that must be determined as part of the risk analysis.

Despite the difficulty in demonstrating a perfect and conclusive correlation with a tumorigenic phenotype, additional *in vitro* characterisation tests may be performed to document other cell substrate properties, such as the ability to grow in soft agar gels, the ability to induce invasive cell growth in muscle and/or the ability of the cell substrate to induce transformation of 3T3 cells.

Residual host-cell DNA. For each particular vaccine produced on continuous cell lines, residual host-cell DNA content must be tested and an acceptable upper limit, based on a risk assessment, must be established in the final product taking into consideration:

(1) the nature of the cell substrate (non-tumorigenic, level of tumorigenicity) and its origin (human/non-human);

(2) the presence in the production process of any steps to inactivate the potential biological activity (oncogenicity, infectivity) of the residual host-cell DNA (e.g. chemical agents such as betapropiolactone and/or DNase treatment);

(3) the capacity of the process to reduce the amount and size of the contaminating residual host-cell DNA;

(4) the intended use of the vaccine (e.g. route of administration);

(5) the method used to measure the residual host-cell DNA.

In general, a purification process for parenteral vaccines is able to reduce residual host-cell DNA in final products to less than 10 ng per dose, but the acceptance limits must be approved by the competent authority.

Once validation studies (e.g. spiking studies using an adequate size distribution of DNA) have been performed and the reproducibility of the production process in reducing residual host-cell DNA to the level expected has been demonstrated, residual host-cell DNA testing may be omitted after agreement from the competent authority.

Chromosomal characterisation. Diploid cell lines shall be shown to be diploid. More extensive characterisation of a diploid cell line by karyotype analysis is required if the removal of intact cells during post-harvest processing has not been validated. Samples from 4 passage levels evenly spaced over the life span of the cell line are examined. A minimum of 200 cells in metaphase are examined for exact chromosome count and for the frequency of hyperploidy, hypoploidy, polyploidy, breaks and structural abnormalities.

The MRC-5, WI-38 and FRhL-2 cell lines are recognised as being diploid and well characterised; where they are not genetically modified, further characterisation is not necessary.

TEST METHODS FOR CELL CULTURES

Morphology. The morphology of the cells is adequately described and documented.

Identification. Nucleic acid fingerprint analysis and a relevant selection of the following are used to establish the identity of the cells:

(1) biochemical characteristics (isoenzyme analysis);

(2) immunological characteristics (histocompatibility antigens, *in vitro* immunochemical assays);

(3) cytogenetic markers;

(4) NAT.

Contaminating cells. The nucleic acid fingerprint analysis carried out for identification also serves to demonstrate freedom from contaminating cells.

Bacterial and fungal contamination. The MCB and each WCB comply with the test for sterility (*2.6.1*), carried out using, for each medium, 10 mL of supernatant from cell cultures. Carry out the test on 1 per cent of the containers, with a minimum of 2 containers.

Mycobacteria. If the cells are susceptible to infection with *Mycobacterium tuberculosis* or other species, the MCB and each WCB comply with the test for mycobacteria (*2.6.2*). NAT (*2.6.21*) may be used as an alternative to this culture method provided such an assay is validated and shown to be comparable to the culture method.

Mycoplasmas (*2.6.7*). The MCB and each WCB comply with the test for mycoplasmas. Use one or more containers for the test.

Spiroplasmas. Spiroplasmas may be introduced into cell substrates through contamination of raw materials of plant origin or when insect cell lines are used. When appropriate, the MCB and each WCB are demonstrated to be free of spiroplasmas using a validated method approved by the competent authority. NAT methods for detection of mycoplasmas (*2.6.7*) may be used to detect spiroplasmas after validation and agreement from the competent authority. Use one or more containers for the test.

Electron microscopy. The MCB is examined by electron microscopy for the presence of extraneous agents. Cell lines are maintained at the temperature routinely used for production and taken at or beyond the maximum population doubling level used for production. In addition, insect cell lines are maintained at temperatures above and below those routinely used for production and may also be subjected to other treatments such as exposure to chemical stressors. For insect cell lines the maintenance temperatures and treatments used are agreed with the competent authority, along with the number of sectioned cells to be examined.

Test for extraneous agents in cell cultures. For mammalian cells, viable cells (at least 10^7 cells) or the equivalent cell lysate, in their culture supernatant, are either co-cultivated (for viable cells) or inoculated (for cell lysate) onto monolayer cultures of:

(1) human diploid cells;

(2) continuous simian kidney cells; and

(3) for cell substrates other than human or simian, cells of that species, from a separate batch.

For insect cell lines, cell lysates are inoculated onto monolayer cultures of other cell systems, including human, simian and, in addition, at least 1 cell line that is different from that used in production, is permissible to insect viruses and allows detection of human arboviruses (e.g. BHK-21).

The resulting co-cultivated cell culture (for viable cells) or inoculated cell cultures (for cell lysate) are observed for evidence of viruses by cytopathic effect for at least 2 weeks. If the cell line is known to be capable of supporting the growth of human or simian cytomegalovirus, the human diploid cultures are observed for at least 4 weeks. The extended 4-week cell culture of human diploid cells, for the purpose of detecting human or simian cytomegalovirus, can be replaced by the use of NAT (*2.6.21*). In cases where it is difficult to keep the cell cultures healthy for the additional 2 weeks, it may be necessary to introduce fresh medium or to subculture after 2 weeks onto fresh cultures in order to be able to detect viral agents. At the end of the observation period, carry out tests on the cell culture supernatants for haemagglutinating viruses, or on the viable cells for haemadsorbing viruses using guinea-pig red blood cells. If the guinea-pig red blood cells have been stored, they shall have been stored at 5 ± 3 °C for not more than 7 days. Analyse half of the cultures after incubation at 5 ± 3 °C for 30 min and the other half after incubation at 20-25 °C for 30 min. The test for haemagglutinating viruses is not valid for arboviruses.

The test is not valid unless at least 80 per cent of the cell cultures remain viable. The cell substrate complies with the test if no evidence of any extraneous agent is found.

Retroviruses

If the cell line is not known to produce retroviral particles, examine for the presence of retroviruses using:

(1) product-enhanced reverse transcriptase (PERT) assay (*2.6.21*) carried out for cell bank supernatants using cells at or beyond the maximum population doubling level that will be used for production;

(2) transmission electron microscopy.

If tests (1) and/or (2) give a positive result, infectivity assays are carried out on permissible human cells with a PERT assay end-point on the supernatant.

If the cell line is shown to produce retroviral particles (e.g. rodent cell lines), examine for the presence of retroviruses using:

- transmission electron microscopy;
- infectivity assays carried out on permissible human cells and on relevant additional cells (e.g. *Mus dunni* cells or SC-1 cells for CHO cell substrate) with a PERT assay end-point on the supernatant, except when the amplification cells are positive for reverse transcriptase, in which case the readout is performed using plaque assay or a fluorescent focus assay.

Since the sensitivity of PERT assays is very high, interpretation of a positive signal may be equivocal and a decision on the acceptability of a cell substrate is based on all available data.

Tests in suckling mice. The test is carried out if a risk assessment indicates that it provides a risk mitigation taking into account the overall testing package applied to a given cell substrate.

Inject 10^7 viable cells or the equivalent cell lysate, in their culture supernatant into 2 litters of suckling mice less than 24 h old, comprising not fewer than 10 animals;

Inject at least 0.1 mL intraperitoneally and 0.01 mL intracerebrally.

Observe the suckling mice for at least 4 weeks. Investigate suckling mice that become sick or show any abnormality to establish the cause of illness. The cell substrate complies with the test if no evidence of any extraneous agent is found. The test is invalid if fewer than 80 per cent of the suckling mice in each group remain healthy and survive to the end of the observation period.

Tests in eggs (only required for avian cell substrates). The test is carried out if a risk assessment indicates that it provides a risk mitigation taking into account the overall testing package applied to a given cell substrate. Inject an inoculum of 10^6 viable cells or the equivalent cell lysate, in their culture supernatant, into the allantoic cavity of ten 9- to 11-day-old SPF embryonated hens' eggs (*5.2.2*) and into the yolk sac of ten 5- to 7-day-old SPF embryonated hens' eggs. Incubate for not less than 5 days. Test the allantoic fluids for the presence of haemagglutinins using mammalian and avian red blood cells; carry out the test at 5 ± 3 °C and 20-25 °C and read the results after 30-60 min. The cell substrate complies with the test if no evidence of any extraneous agent is found. The test is invalid if fewer than 80 per cent of the embryos remain healthy and survive to the end of the observation period.

Tests for specific viruses. The list of specific viruses to be tested is defined based on a viral contamination risk assessment in accordance with the principles detailed in general chapter *5.1.7. Viral Safety*, and takes into account (but is not limited to) the origin of the cells and the potential sources of viral contamination (e.g. raw material of animal or plant origin). NAT tests (*2.6.21*) are carried out with or without prior amplification in cells. For cell lines of rodent origin, NAT (*2.6.21*) or antibody production tests in mice, rats or hamsters are used to detect species-specific viruses.

Tests for viruses using broad molecular methods. In agreement with the competent authority, broad molecular methods (e.g. High Throughput Sequencing) may be used either as an alternative to *in vivo* tests and specific NAT or as a supplement or alternative to *in vitro* culture tests based on the risk assessment.

For both NAT (*2.6.21*) and broad molecular methods, the stage at which testing is to be conducted (e.g. MCB, WCB, EOPC/ECB) is also based on the risk assessment and depends on the steps where viral contaminants may be introduced. In case of positive results with either broad molecular methods or NAT tests, a follow-up investigation must be conducted to determine whether detected nucleic acids are due to the presence of infectious extraneous agents and/or are known to constitute a risk to human health.

Tests for tumorigenicity *in vivo*. The test establishes a comparison between the continuous cell line and a suitable positive control cell line as reference (for example, HeLa or Hep2 cells).

Animal systems that have been shown to be suitable for this test include:

(1) athymic mice (Nu/Nu genotype);

(2) newborn mice, rats or hamsters that have been treated with antithymocyte serum or globulin;

(3) thymectomised and irradiated mice that have been reconstituted (T^-, B^+) with bone marrow from healthy mice.

Whichever animal system is selected, the cell line and the positive control cells are injected into separate groups of 10 animals each. In both cases, the inoculum for each animal is 10^7 cells suspended in a volume of 0.2 mL, and the injection may be given by the intramuscular or the subcutaneous route. Newborn animals are treated with 0.1 mL of antithymocyte serum or globulin on days 0, 2, 7 and 14 after birth. A potent serum or globulin is one that suppresses the immune mechanisms of growing animals to the extent that the subsequent inoculum of 10^7 positive control cells regularly produces tumours and metastases. Severely affected animals showing evident, progressively growing tumours are euthanised before the end of the test to avoid unnecessary suffering.

At the end of the observation period all animals, including the positive control group, are euthanised and examined for gross and microscopic evidence of the proliferation of inoculated cells at the site of injection and in other organs (for example, lymph nodes, lungs, kidneys and liver).

In all test systems, the animals are observed and palpated at regular intervals for the formation of nodules at the sites of injection. Any nodules formed are measured in 2 perpendicular directions, the measurements being recorded regularly to determine whether there is progressive growth of the nodule. Animals showing nodules that begin to regress during the period of observation are euthanised before the nodules are no longer palpable, and processed for histological examination. Animals with progressively growing nodules are observed for 1-2 weeks. Among those without nodule formation, half are observed for 3 weeks and half for 12 weeks before they are euthanised and processed for histological examination. A necropsy is performed on each animal and includes examination for gross evidence of tumour formation at the site of injection and in other organs such as lymph nodes, lungs, brain, spleen, kidneys and liver. All tumour-like lesions and the site of injection are examined histologically. In addition, since some cell lines may give rise to metastases without evidence of local tumour growth, any detectable regional lymph nodes and the lungs of all animals are examined histologically.

The test is invalid if fewer than 9 of the 10 animals injected with the reference positive-control cells show progressively growing tumours.

For a new tumorigenic cell line, in order to document the level of tumorigenicity, a dose range of cell substrate (e.g. dose of cells in the range of 10^5, 10^6 and 10^7) is injected in different groups of 10 animals. The number of animals showing progressively growing nodules within the animal groups is monitored to calculate the TPD_{50}.

07/2020:20616

2.6.16. TESTS FOR EXTRANEOUS AGENTS IN VIRAL VACCINES FOR HUMAN USE

INTRODUCTION

A strategy for testing extraneous agents in viral vaccines must be developed based on a risk assessment following the principles of viral contamination risk detailed in general chapter *5.1.7. Viral safety*. This strategy includes a full package of suitable tests that are able to detect different families of extraneous agents that may infect the source of virus strains including cell substrates and raw material of animal or plant origin. It also takes into account the capacity of the manufacturing process to remove or inactivate viruses. The list of tests summarised in Table 2.6.16.-1 must be adapted depending on the extraneous agents that have the potential to contaminate the product: for *in vitro* tests, the risk assessment may allow, with the agreement of the competent authority, the use of other permissive cell lines or molecular biology methods depending on the manufacturing process and the incubation temperature for the growth of particular viruses. If *in vivo* tests are more relevant than *in vitro* tests for the detection of some adventitious viruses (e.g. suckling mice for the vesicular stomatitis virus and fertilised SPF eggs for the influenza virus) the decision to maintain or to introduce such *in vivo* assays in a testing strategy must be justified by the risk assessment.

New, sensitive molecular methods with broad detection capabilities are available. These new approaches include high-throughput sequencing (HTS) methods, nucleic acid amplification techniques (NAT) (e.g. polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), product-enhanced reverse transcriptase (PERT) assays) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays, and mass spectrometry with broad-spectrum PCR. These methods may be used either as an alternative to *in vivo* tests and specific NAT or as a supplement/alternative to *in vitro* culture tests based on the risk assessment and with the agreement of the competent authority.

In tests that require prior neutralisation of the virus, use specific antibodies of non-human, non-simian origin; if the virus has been propagated in avian tissues, the antibodies must also be of non-avian origin. To prepare antiserum, use an immunising antigen produced in cell cultures from a species different from that used for the production of the vaccine and free from extraneous agents. Where the use of SPF eggs is prescribed, the eggs are obtained from a flock free from specified pathogens (*5.2.2*).

TEST METHODS

Relevant tests for extraneous agents to be carried out at various production stages are indicated in Table 2.6.16.-1 using the methods described below, based on the risk assessment.

Take samples at the time of harvesting, and if not tested immediately, keep at a temperature below – 40 °C.

Table 2.6.16.-1. – *Relevant tests for extraneous agents at various production stages*

	Virus seed lots	Virus harvests	Production of culture substrates	
			Control cells	Control eggs
Bacterial and fungal contamination	+	+	-	-
Mycoplasmas	+	+	-	-
Spiroplasmas(1)	+	-	-	-
Mycobacteria	+	+	-	-
Test in suckling mice(2)	+	-	-	-
Avian viruses(3)	+	+	-	-
Test for extraneous agents in cell cultures(4)	+	+	+	+
Insect viruses(5)	+	+	-	-
Test on control cells (microscopic examination)	-	-	+	-
Haemadsorbing viruses	-	-	+	-
Test on control eggs (haemagglutinating agents)	-	-	-	+
Avian leucosis viruses(6)	-	-	+	+
Test for specific viruses by NAT(7)	+	+	-	-
Test for viruses using broad molecular methods(8)	+	+	-	-

(1) If insect cells or raw materials of plant origin are used.

(2) If the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package.

(3) If the virus is propagated in avian or primary avian tissues. If the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package.

(4) Test performed in suitable permissive cell cultures. Based on a risk assessment.

(5) If the virus is propagated in insect cells.

(6) If the virus is propagated in primary avian tissues or in eggs.

(7) Based on a risk assessment.

(8) These methods may be used either as an alternative to *in vivo* tests and specific NAT or as a supplement/alternative to *in vitro* culture tests based on the risk assessment and in agreement with the competent authority.

Bacterial and fungal contamination. Each virus seed lot and virus harvest complies with the test for sterility (*2.6.1*).

Mycoplasmas (*2.6.7*). Each virus seed lot and virus harvest complies with the test for mycoplasmas.

Spiroplasmas. Spiroplasmas may be introduced into virus seed lots through contamination of raw materials of plant origin or when insect cell lines are used for virus propagation. When appropriate, virus seed lots are demonstrated to be free of spiroplasmas using a validated method approved by the competent authority. NAT methods for detection of mycoplasmas (*2.6.7*) may be used to detect spiroplasmas after validation and agreement from the competent authority.

Mycobacteria (*2.6.2*). A 2.7 mL sample of each virus seed lot and each virus harvest is tested for the presence of *Mycobacterium* spp. by culture methods known to be sensitive

for the detection of these organisms. NAT (*2.6.21*) may be used as an alternative, provided such an assay is validated and shown to be comparable to the culture method.

Test in suckling mice. Each virus seed lot is tested in suckling mice if the risk assessment indicates that this test provides a risk mitigation, taking into account the overall testing package. Inoculate no fewer than 20 suckling mice, each less than 24 h old, intracerebrally with 0.01 mL and intraperitoneally with at least 0.1 mL of the virus seed lot. Observe the suckling mice daily for at least 4 weeks. Carry out an autopsy of all suckling mice that die after the first 24 h of the test or that show signs of illness, and examine for evidence of viral infection by direct macroscopical observation. The virus seed lot passes the test if no suckling mice show evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the original inoculated suckling mice survive the observation period.

Avian viruses. Each virus seed lot propagated in avian tissues and each virus harvest propagated in primary avian tissues is tested for avian viruses if the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package. Neutralise a sample equivalent to 100 human doses of vaccine or 10 mL, whichever is the greater. Using 0.5 mL per egg, inoculate a group of fertilised SPF eggs, 9-11 days old, by the allantoic route and a second group, 5-7 days old, into the yolk sac. Incubate for 7 days. The virus seed lot or harvest complies with the test if the allantoic and yolk sac fluids show no sign of the presence of any haemagglutinating agent and if all embryos and chorio-allantoic membranes examined for gross pathology, are normal. The test is not valid unless at least 80 per cent of the inoculated eggs survive for 7 days.

Test for extraneous agents in cell cultures. For each virus seed lot, each virus harvest and each production cell culture (control cells or control eggs), tests for other extraneous agents must be carried out based on a risk assessment. The origin of the cell substrate and virus strain, as well as the potential extraneous agents that may be inadvertently introduced during production processes or through the use of animal- or plant-derived raw materials, must be taken into account when choosing suitable permissive cells.

For each virus seed lot and virus harvest, neutralised samples, equivalent (unless otherwise prescribed) to 500 human doses of vaccine or 50 mL, whichever is the greater, are tested for the presence of extraneous agents by inoculation into continuous simian and human cell cultures. If the virus is grown in simian or human cells, the neutralised virus harvest is tested on a separate culture of these cells. If the virus is grown in a mammalian cell system other than simian or human, or in avian cells, cells of that species, but from a separate batch, are also inoculated. The cells are incubated at 36 ± 1 °C and observed for a period of 14 days. If the production cell culture is maintained at a temperature other than 36 ± 1 °C, a supplementary test for extraneous agents is carried out at the production temperature using the same type of cells used for growth of the virus. A subculture of 14 days is carried out followed by a haemadsorbing test. The virus seed lot or harvest passes the tests if none of the cell cultures show evidence of the presence of any extraneous agents after 14 and 28 days of incubation, and no evidence of any haemadsorbing viruses after 28 days. The test is not valid unless at least 80 per cent of the cell cultures remain viable.

Insect viruses. Each virus seed lot and virus harvest propagated in insect cells is tested for insect viruses. Neutralised samples, equivalent (unless otherwise prescribed) to 500 human doses of vaccine or 50 mL, whichever is the greater, are tested for the presence of extraneous agents by inoculation into at least 1 cell culture different from that used in production and permissible to insect viruses, and that also allows detection of human arboviruses (e.g. BHK-21). The choice of cells is approved by the competent authority and takes into account the origin of the production cells and the likely contaminants that may be detected by the chosen cells. The cells are incubated at an appropriate temperature and observed for a period of 14 days. A subculture of 14 days is carried out followed by a haemadsorbing test. The virus seed lot or harvest passes the tests if none of the cell cultures show evidence of the presence of any extraneous agents after 14 and 28 days of incubation, and no evidence of any haemadsorbing virus after 28 days. The test is not valid unless at least 80 per cent of the cell cultures remain viable.

Tests on control cells. Where cell cultures are used for virus production, examine the control cells microscopically for the absence of any virus causing cytopathic degeneration throughout the incubation time of the inoculated production cell cultures or for no less than 14 days beyond the time of inoculation of the production vessels, whichever is the longer. The test is not valid unless at least 80 per cent of the control cell cultures survive to the end of the observation period.

At 14 days or at the time of the last virus harvest, whichever is the longer, pool the supernatant fluids from the control cells and examine for the presence of extraneous agents over a period of 14 days as described above for the virus seed lot and the virus harvest by inoculation of relevant cell cultures depending on the type of cells used for virus growth.

Haemadsorbing viruses. Where cell cultures are used for virus production, a microscopic examination of the control cells is carried out as described above for the test for extraneous agents in cell cultures. At 14 days or at the time of the last virus harvest, whichever is the longer, examine no fewer than 25 per cent of the control cultures for the presence of haemadsorbing viruses by the addition of guinea-pig red blood cells. If the test for haemadsorbing viruses is not feasible, carry out a test for haemagglutinating viruses. If the guinea-pig red blood cells have been stored, they shall have been stored at 5 ± 3 °C for not more than 7 days. Read half of the cultures after incubation at 5 ± 3 °C for 30 min and the other half after incubation at 20-25 °C for 30 min. No evidence of haemadsorbing agents is found.

Tests on control eggs. Where eggs are used for virus production, examine 0.25 mL of the allantoic fluid from each control egg for haemagglutinating agents by mixing directly with chicken red blood cells and after a passage in SPF eggs carried out as follows: inoculate a 5 mL sample of the pooled amniotic fluids from the control eggs in 0.5 mL volumes into the allantoic cavity and into the amniotic cavity of SPF eggs. The control eggs comply with the test if no evidence of the presence of haemagglutinating agents is found in either test.

In addition, inoculate 5 mL samples of the pooled amniotic fluids from the control eggs into suitable permissive cells including human, simian and avian cells. Observe the cell cultures for 14 days at a suitable incubation temperature. The control eggs comply with the test if no evidence of the presence of extraneous agents is found. The test is not valid unless 80 per cent of the inoculated cultures survive to the end of the observation period.

Avian leucosis viruses. For each virus propagated in primary avian cell tissues or in eggs, the production cell culture (control cells or control eggs) is tested for avian leucosis viruses. When cell cultures are used for virus production, a microscopic examination of the control cells is carried out as described above for the test for extraneous agents prior to the test for avian leucosis viruses. At 14 days or at the time of the last virus harvest, whichever is the longer, carry out the test for avian leucosis viruses on DF-1 cells or leucosis-free chick-embryo cell cultures with amplification through 5 passages using at least 5 mL of the supernatant fluid from the control cells or at least 10 mL of a sample of the pooled amniotic fluids from the

control eggs. PERT assay end-point can be used after DF-1 amplification for detection of exogenous avian retroviruses (including avian leucosis virus). For specific detection of avian leucosis virus, several end-points can be used such as immunostaining, enzyme-linked immunosorbent assay (ELISA) or complement fixation for avian leucosis (COFAL). Control cells or control eggs comply with the test if there is no evidence of the presence of any avian leucosis virus.

Tests for specific viruses by NAT. Based on a risk assessment related to the manufacturing process, each virus seed lot and each virus harvest may be tested by NAT (*2.6.21*) for specific viruses that are not detected by conventional *in vivo* or cell culture assays.

Test for viruses using broad molecular methods. With the agreement of the competent authority, broad molecular methods (e.g. HTS) may be used either as an alternative to *in vivo* tests and specific NAT, or as a supplement/alternative to *in vitro* culture tests based on the risk assessment.

Both NAT (*2.6.21*) and broad molecular methods are carried out with or without prior amplification in suitable permissive cells. In cases of positive results with either broad molecular methods or NAT, a follow-up investigation must be conducted to determine whether detected nucleic acids are due to the presence of infectious extraneous agents and/or are known to constitute a risk to human health.

01/2008:50107

5.1.7. VIRAL SAFETY

This chapter provides general requirements concerning the viral safety of medicinal products whose manufacture has involved the use of materials of human or animal origin. Since viral safety is a complex issue, it is important that a risk assessment is carried out. Requirements to be applied to a specific medicinal product are decided by the competent authority.

Where the risk of viral contamination exists, complementary measures are used as appropriate to assure the viral safety of medicinal products, based on:

- selection of source materials and testing for viral contaminants;
- testing the capacity of the production process to remove and/or inactivate viruses;
- testing for viral contamination at appropriate stages of production.

Where appropriate, one or more validated procedures for removal or inactivation of viruses are applied.

Further detailed recommendations on viral safety, including validation studies, are provided, in particular, by the *Note for guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses (CPMP/BWP/268/95)* of the Committee for Proprietary Medicinal Products, and the *ICH guideline Q5A: Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin* (including any subsequent revisions of these documents).

Requirements concerning immunological products for veterinary use are dealt with in the monographs *Vaccines for veterinary use (0062)* and *Immunosera for veterinary use (0030)* and related general chapters.

Risk assessment

A risk assessment with respect to viral safety is carried out where materials of human or animal origin are used as ingredients of medicinal products or in the manufacture of active substances, excipients or medicinal products.

The principle of the risk assessment is to consider various factors that may influence the potential level of infectious particles in the medicinal product and factors related to the use of the medicinal product that determine or influence the viral risk to the recipients.

The risk assessment takes into consideration relevant factors, for example:

- the species of origin;
- the organ, tissue, fluid of origin;
- the potential contaminants in view of the origin of the raw material and the history of the donor(s), preferably including epidemiological data;
- the potential contaminants from the manufacturing process (for example, from risk materials used during manufacture);
- the infectivity and pathogenicity of the potential contaminants for the intended recipients of the medicinal product, taking account of the route of administration of the medicinal product;
- the amount of material used to produce a dose of medicinal product;
- controls carried out on the donor(s), on the raw material, during production and on the final product;
- the manufacturing process of the product and its capacity to remove and/or inactivate viruses.

The risk assessment can be based mainly on the manufacturing conditions if these include rigorous inactivation steps (for example, for gelatin etc., and products terminally sterilised by steam or dry heat as described in the general texts on sterility (*5.1*)).

07/2011:50208

5.2.8. MINIMISING THE RISK OF TRANSMITTING ANIMAL SPONGIFORM ENCEPHALOPATHY AGENTS VIA HUMAN AND VETERINARY MEDICINAL PRODUCTS

This chapter is identical with the Note for Guidance on Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products – Revision 3, (EMA/410/01 rev. 3).

Contents

1. INTRODUCTION

1-1. *SCIENTIFIC BACKGROUND*

Transmissible Spongiform Encephalopathies (TSEs) are chronic degenerative nervous diseases characterised by the accumulation of an abnormal isoform of a cellular glycoprotein (known as PrP or prion protein). The abnormal isoform of PrP (PrP^{TSE}) differs from normal PrP (PrPc) in being highly resistant to protease and heat denaturation treatments. PrP^{TSE} is considered to be the infective agent responsible for transmitting TSE disease.

TSE diseases in animals include:

- bovine spongiform encephalopathy (BSE) in cattle,
- scrapie in sheep and goats,
- chronic wasting disease (CWD) in cervids (deer and elk),
- transmissible mink encephalopathy (TME) in farmed mink,
- feline spongiform encephalopathy (FSE) in felids (specifically domestic cats and captive large cats), and
- spongiform encephalopathy of exotic ungulates in zoos.

In humans, spongiform encephalopathies include different forms of Creutzfeldt-Jakob Disease (CJD), Kuru, Gerstmann-Sträussler-Scheinker Syndrome (GSS), and Fatal Familial Insomnia (FFI).

Iatrogenic transmission of spongiform encephalopathies has been reported. In sheep, scrapie has been accidentally transmitted by the use of Louping Ill vaccine prepared from pooled, formaldehyde treated ovine brain and spleen in which material from scrapie-infected sheep had been inadvertently incorporated. Also, transmission of scrapie to sheep and goats occurred following use of a formol-inactivated vaccine against contagious agalactia, prepared with brain and mammary gland homogenates of sheep infected with *Mycoplasma agalactiae*. In man, cases of transmission of CJD have been reported which have been attributed to the parenteral administration of growth hormone and gonadotropin derived from human cadaveric pituitary glands. Cases of CJD have also been attributed to the use of contaminated instruments in brain surgery and with the transplantation of human dura mater and cornea.

Interspecies TSE transmission is restricted by a number of natural barriers, transmissibility being affected by the species of origin, the prion strain, dose, route of exposure and, in some species, the host allele of the PRNP gene. Species barriers can be crossed under appropriate conditions.

BSE was first diagnosed in the United Kingdom in 1986 and a large number of cattle and individual herds have been affected. It is clear that BSE is a food borne disease associated with feed (e.g. meat and bone meal) derived from TSE affected animals. Other countries have experienced cases of BSE, either in animals imported from the United Kingdom or in indigenous animals. There is convincing evidence to show that the variant form of CJD (vCJD) is caused by the agent which is responsible for BSE in cattle. Therefore, a cautious approach continues to be warranted if biological materials from species naturally affected by TSE diseases, especially bovine species, are used for the manufacture of medicinal products.

In the course of active surveillance programs, two previously unrecognized forms of atypical BSE (BSE-L, also named BASE, and BSE-H) have been identified in rare sporadic cases from Europe, North America, and Japan. The 'L' and 'H' identify the higher and lower electrophoretic positions of their protease-resistant PrP^{TSE} isoforms. It is noteworthy that atypical cases have been found in countries that did not experience classical BSE so far, like Sweden, or in which only few classical BSE cases have been found like Canada or USA. The atypical BSE agent has been experimentally transmitted to transgenic mice expressing the human prion protein and to a cynomolgus monkey.

Scrapie occurs worldwide and has been reported in most European countries. It has the highest incidence in Cyprus. While humans have been exposed to naturally occurring scrapie for over 250 years, there is no epidemiological evidence directly linking scrapie to spongiform encephalopathies in humans[(1)]. However, there remains a theoretical and currently unquantifiable risk that some BSE-contaminated protein supplement may have been fed to sheep. Further, it should also be assumed that any BSE agent introduced into the small ruminant population via contaminated feed is likely to be recycled and amplified[(2)].

(1) This is currently being assessed by EFSA and ECDC. For updated information, please refer to the following link: http://registerofquestions.efsa.europa.eu/roqFrontend/questionsListLoader?mandate=M-2009-0221

(2) In January 2005, after confirmation of BSE in a goat in France, additional legislative measures were taken related to monitoring and an increased testing of small ruminants. The increased surveillance did not identify additional cases of BSE in sheep and goats in the EU.

There is interest in infecting cells with TSE agents to develop assays and for basic scientific reasons. Some success has been reported, usually but not always with neural cell lines. The conditions needed to infect a cell are not well understood and the process is difficult requiring particular combinations of agent and cell. It is not considered appropriate to make specific recommendations in terms of cell substrates to be used for production of biological/biotechnology-derived substances. Nevertheless, the possibility of infection of cell lines with TSE agents should be taken into account in risk assessments.

1-2. *REGULATORY COMPLIANCE*

Risk assessment. Since the use of animal-derived materials is unavoidable for the production of some medicinal products and that complete elimination of risk at source is rarely possible, the measures taken to manage the risk of transmitting animal TSEs via medicinal products represent risk minimisation rather than risk elimination. Consequently, the basis for regulatory compliance should be based on a risk assessment, taking into consideration all pertinent factors as identified in this chapter (see below).

Legal basis. The note for guidance is published by the European Commission following

- Annex I, part I, module 3, section 3.2: *Content: basic principles and requirements*, point (9) of Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use[(3)], as amended, and
- Annex I, Title I, part 2, section C *Production and control of starting material* of Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products[(4)], as amended.

These directives require that applicants for marketing authorisation for human and veterinary medicinal products must demonstrate that medicinal products are manufactured in accordance with the latest version of this note for guidance published in the *Official Journal of the European Union*. This is a continuing obligation after the marketing authorisation has been granted.

By definition, the principle of Specified Risk Materials as defined in Regulation (EC) No 999/2001 of the European Parliament and of the Council[(5)] does not apply to medicinal products. However, Regulation (EC) No 1774/2002 of the European Parliament and of the Council[(6)], which applies since 1st May 2003, lays down health rules concerning animal by-products not intended for human consumption. As a general rule, and unless properly justified, all animal by-products used as starting materials in the manufacture of medicinal products should be 'Category 3 (i.e. safe) materials or equivalent', as defined in Regulation (EC) No 1774/2002. Justification for the use of substances derived from other, high infectivity materials must follow an appropriate benefit/risk evaluation (see further below).

The note for guidance should be read in conjunction with the various EU legal instruments including Commission decisions progressively implemented since 1991. Where appropriate, references to these decisions are given in the text. Position statements and explanatory notes made by the Committee for Medicinal Products for Human Use (CHMP) and Committee for Medicinal Products for Veterinary Use (CVMP) are still applicable for the purpose of regulatory compliance unless otherwise superseded by the note for guidance.

The general monograph *Products with risk of transmitting agents of animal spongiform encephalopathies* of the European Pharmacopoeia refers to this chapter, which is identical with the note for guidance. The monograph forms the basis for issuing Certificates of Suitability as a procedure for demonstrating TSE compliance for substances and materials used in the manufacture of human and veterinary medicinal products.

Clarification of note for guidance. As the scientific understanding of TSEs, especially the pathogenesis of the diseases, is evolving, from time to time CHMP and its Biologics Working Party in collaboration with CVMP and its Immunologicals Working Party may be required in the future to develop supplementary guidance in the form of position statements or explanatory notes for the purpose of clarifying the note for guidance. The supplementary guidance shall be published by the Commission and on the website of the European Medicines Agency and taken into consideration accordingly in the scope of the certification of the European Directorate for the Quality of Medicines & HealthCare (EDQM).

2. SCOPE

TSE-RELEVANT ANIMAL SPECIES

Cattle, sheep, goats and animals that are naturally susceptible to infection with transmissible spongiform encephalopathy agents or susceptible to infection through the oral route other than humans[(7)] and non-human primates are defined as "TSE-relevant animal species"[(8)].

MATERIALS

This chapter is concerned with materials derived from "TSE-relevant animal species" that are used for the preparation of:

- active substances,
- excipients and adjuvants, and
- raw and starting materials and reagents used in production (e.g. bovine serum albumin, enzymes, culture media including those used to prepare working cell banks, or new master cell banks for medicinal products which are subject to a new marketing authorisation).

This chapter is also applicable to materials that come into direct contact with the equipment used in manufacture of the medicinal product or that come in contact with the medicinal product and therefore have the potential for contamination.

Materials used in the qualification of plant and equipment, such as culture media used in media fill experiments to validate the aseptic filling process, shall be considered in compliance with this chapter provided that the constituent or constituents are derived from tissues with no detectable infectivity (category IC tissues), where the risk of cross-contamination with potentially infective tissues has been considered (see section 3-3) and where the materials are sourced from countries with negligible BSE risk or controlled BSE risk (Categories A and B, respectively – see section 3-2). Such information shall be provided in the dossier for a marketing authorisation and verified during routine inspection for compliance with Good Manufacturing Practice (GMP).

Other materials such as cleaning agents, softeners and lubricants that come into contact with the medicinal product during its routine manufacture or in the finishing stage or in the primary packaging are considered in compliance with this chapter if they are tallow derivatives prepared using the rigorous physicochemical processes as described in section 6.

(3) OJ L 311, 28.11.2001, p. 67.
(4) OJ L 311, 28.11.2001, p. 1.
(5) OJ L 147, 31.5.2001, p. 1.
(6) OJ L 273, 10.10.2002, p. 1. Regulation (EC) 1774/2002 has been repealed by Regulation (EC) 1069/2009 that will apply from 4 March 2011 (OJ L 300, 14.11.2009, p. 1).
(7) Regulatory guidance and position papers have been issued by the Committee for Medicinal Products for Human Use and its Biologics Working Party on human tissue derived medicinal products in relation to CJD and vCJD. Such guidance can be found on http://www.ema.europa.eu
(8) Pigs and birds, which are animal species of particular interest for the production of medicinal products, are not naturally susceptible to infection via the oral route. Therefore they are not TSE-relevant animal species within the meaning of this chapter. Also dogs, rabbits and fish are non TSE-relevant animal species within the meaning of this chapter.

SEED LOTS, CELL BANKS AND ROUTINE FERMENTATION/PRODUCTION[9]

For the purpose of regulatory compliance, master seeds or master cell banks in marketing authorisation applications lodged after 1 July 2000 (for human medicinal products) or 1 October 2000 (for veterinary medicinal products) shall be covered by the note for guidance.

Master seeds and master cell banks,

– for vaccine antigens,

– for a biotechnology-derived medicinal product as described in the Annex to Regulation (EC) No 726/2004 of the European Parliament and of the Council[10], and

– for other medicinal products using seed lots or cell banking systems in their manufacture,

that have already been approved for the manufacture of a constituent of an authorised medicinal product shall be considered in compliance with the note for guidance even if they are incorporated in marketing authorisation applications lodged after 1 July 2000 (for human medicinal products) or 1 October 2000 (for veterinary medicinal products).

Master cell banks and master seeds established before 1 July 2000 (for human medicinal products) or 1 October 2000 (for veterinary medicinal products), but not yet approved as a constituent of an authorised medicinal product shall demonstrate that they fulfil the requirements of the note for guidance. If, for some raw or starting materials or reagents used for the establishment of these cell banks or seeds, full documentary evidence is no longer available, the applicant should present a risk assessment as described in Section 4 of the note for guidance.

Established working seeds or cell banks used in the manufacture of medicinal products authorised before 1 July 2000 (human medicines) or 1 October 2000 (veterinary medicines), which have been subjected to a properly conducted risk assessment by a Competent Authority of the Member States or the European Medicines Agency and declared to be acceptable, shall also be considered compliant.

However, where materials derived from the "TSE-relevant animal species" are used in fermentation/routine production processes or in the establishment of working seeds and working cell banks, the applicant must demonstrate that they fulfil the requirements of the note for guidance.

3. GENERAL CONSIDERATIONS

3-1. *SCIENTIFIC PRINCIPLES FOR MINIMISING RISK*

When manufacturers have a choice, the use of materials from "non TSE-relevant animal species" or non-animal origin is preferred. The rationale for using materials derived from "TSE-relevant animal species" instead of materials from "non-TSE-relevant species" or of non-animal origin should be given. If materials from "TSE-relevant animal species" have to be used, consideration should be given to all the necessary measures to minimise the risk of transmission of TSE.

Readily applicable diagnostic tests for TSE infectivity *in vivo* are not yet available. Diagnosis is based on post-mortem confirmation of characteristic brain lesions by histopathology and/or detection of PrP^{TSE} by Western blot or immunoassay. The demonstration of infectivity by the inoculation of suspect tissue into target species or laboratory animals is also used for confirmation. However, due to the long incubation periods of all TSEs, results of *in vivo* tests are available only after months or years.

Several immunochemical tests have been developed for the detection of PrP^{TSE} in post-mortem samples and some are now considered to be extremely sensitive. However, their ability to detect an infected animal depends on the timing of sample collection in relation to timing of exposure, the type of tissue collected and infectious dose acquired, together with consequential timing of onset of clinical disease. There is currently insufficient information on how this might be affected by strain variations.

Although screening of source animals by *in vitro* tests may prevent the use of animals at late stages of incubation of the disease and may provide information about the epidemiological status of a given country or region, none of the tests are considered suitable to unambiguously confirm the negative status of an animal.

Minimising the risks of transmission of TSE is based upon three complementary parameters:

– the source animals and their geographical origin,

– nature of animal material used in manufacture and any procedures in place to avoid cross-contamination with higher risk materials,

– production process(es) including the quality assurance system in place to ensure product consistency and traceability.

3-2. *ANIMAL SOURCE*

The source materials used for the production of materials for the manufacture of medicinal products shall be derived from animals fit for human consumption following ante- and post-mortem inspection in accordance with EU or equivalent (third country) conditions, except for materials derived from live animals, which should be found healthy after clinical examination.

3-2-1. **Geographical sourcing**

3-2-1-1. *Bovine materials*

The World Organisation for Animal Health (OIE)[11] lays down the criteria for the assessment of the status of countries in the chapter of the International Animal Health Code on bovine spongiform encephalopathy. Countries or regions are classified as follows:

A. countries or regions with a negligible BSE risk;

B. countries or regions with a controlled BSE risk;

C. countries or regions with an undetermined BSE risk.

As stipulated in Commission Regulation (EC) No 999/2001, as amended[12], the classification of countries or regions thereof according to their BSE risk, based on the rules laid down by OIE, is legally binding in the EU since 1 July 2007. Commission Decision 2007/453/EC[13] as amended, provides the classification of countries or regions according to their BSE risk.

(9) See also: Position paper on the assessment of the risk of transmission of animal spongiform encephalopathy agents by master seed materials used in the production of veterinary vaccines (EMEA/CVMP/019/01-February 2001 adopted by the Committee for Medicinal Products for Veterinary Use (CVMP) in July 2001, (OJ C 286, 12.10.2001, p. 12)).
(10) OJ L 136, 30.4.2004, p. 1.
(11) http://www.oie.int/eng/Status/BSE/en_BSE_free.htm
(12) Regulation (EC) No 722/2007 (OJ L 164, 26.6.2007, p. 7)
(13) OJ L 172, 30.6.2007, p. 84

Previously, the European Commission Scientific Steering Committee (SSC)[14] had established a temporary system for classifying the countries according to their geographical BSE risk (GBR)[15].

For the purposes of this chapter the BSE classification based on the OIE rules should be used. If a country, which was previously classified in accordance to the SSC GBR criteria, has not been classified yet according to the OIE rules, the GBR classification can be used until OIE classification has taken place, provided that there is no evidence of significant change in its BSE risk[16].

Where there is a choice, animals should be sourced from countries with the lowest possible BSE risk (negligible BSE risk countries (Category A)) unless the use of material from countries with a higher BSE risk is justified. Some of the materials identified in Section 6, "Specific Conditions" can be sourced from countries with controlled BSE risk (Category B) and, in some cases, from countries with undetermined BSE risk (Category C), provided that the controls and requirements as specified in the relevant sections below are applied. Apart from these exceptions, animals must not be sourced from countries with undetermined BSE risk (Category C), and justifications for the use of animals from countries with undetermined BSE risk (Category C) must always be provided.

3-2-1-2. Sheep and goats (small ruminants)

Naturally occurring clinical scrapie cases have been reported in a number of countries worldwide. As BSE in sheep and goats could possibly be mistaken for scrapie, as a precautionary measure, sourcing of materials derived from small ruminants shall take into account the prevalence of both BSE and scrapie in the country and the tissues from which the materials are derived.

The principles related to "BSE negligible risk (closed) bovine herds" (see section 3-2-2) could equally be applied in the context of small ruminants in order to develop a framework to define the TSE status of a flock of small ruminants. For sheep, because of the concern over the possibility of BSE in sheep, the use of a genotype(s) showing resistance to BSE/scrapie infection could be considered in establishing TSE free flocks[17]. However, the possibility that genotypes resistant to scrapie could be susceptible to BSE (experimental oral exposure) or atypical scrapie (natural cases) should also be taken into account. Goats have not been studied sufficiently with regard to a genotype specific sensitivity.

Material of small ruminant origin should preferably be sourced from countries with a long history of absence of scrapie. Justification shall be required if the material is sourced from some other origin.

3-2-2. **BSE negligible risk (closed) bovine herds**. The safest sourcing is from countries or regions with a negligible risk (Category A countries). Other countries may have or have had cases of BSE at some point in time and the practical concept of "Negligible risk (closed) bovine herds" has been developed by the SSC and endorsed by the CHMP and CVMP. Criteria for establishing and maintaining a "BSE negligible risk (closed) bovine herd" can be found in the SSC opinion of 22-23 July 1999[18].

For the time being it is not possible to quantify the reduction of the geographical BSE risk for cattle from BSE 'negligible risk (closed) bovine herds'. However, it is expected that this risk reduction is substantial. Therefore, sourcing from such closed bovine herds shall be considered in the risk assessment in conjunction with the OIE classification of the country.

3-3. *ANIMAL PARTS, BODY FLUIDS AND SECRETIONS AS STARTING MATERIAL*

In a TSE infected animal, different organs and secretions have different levels of infectivity. If materials from 'TSE-relevant animal species' have to be used, consideration should be given to use materials of the lowest category of risk. The tables given in the Annex of this chapter[19] summarise current data about the distribution of infectivity and PrP^{TSE} in cattle with BSE, and in sheep and goats with scrapie[20].

The information in the tables is based exclusively upon observations of naturally occurring disease or primary experimental infection by the oral route (in cattle) but does not include data on models using strains of TSE that have been adapted to experimental animals, because passaged strain phenotypes can differ significantly and unpredictably from those of naturally occurring disease. Because immunohistochemical and/or Western blot detection of misfolded host protein (PrP^{TSE}) have proven to be a surrogate marker of infectivity, PrP^{TSE} testing results have been presented in parallel with bioassay data. Tissues are grouped into three major infectivity categories, irrespective of the stage of disease:

Category IA	**High-infectivity tissues** central nervous system (CNS) tissues that attain a high titre of infectivity in the later stages of all TSEs, and certain tissues that are anatomically associated with the CNS
Category IB	**Lower-infectivity tissues** peripheral tissues that have tested positive for infectivity and/or PrP^{TSE} in at least one form of TSE
Category IC	**Tissues with no detectable infectivity** tissues that have been examined for infectivity, without any infectivity detected, and/or PrP^{TSE}, with negative results

Category IA tissues and substances derived from them shall not be used in the manufacture of medicinal products, unless justified (see Section 5).

Although the category of lower risk tissues (category IB tissues) almost certainly includes some (e.g. blood) with a lower risk than others (e.g. lymphoreticular tissues), the data about infectivity levels in these tissues are too limited to

(14) The Scientific Steering Committee established by Commission Decision 97/404/EC (OJ L 169, 27.6.1997, p. 85) shall assist the Commission to obtain the best scientific advice available on matters relating to consumer health. Since May 2003, its tasks have been taken over by the European Food Safety Authority (EFSA): http://www.efsa.europa.eu

(15) The European Scientific Steering Committee classification for geographical BSE risk (GBR) gives an indication of the level of likelihood of the presence of one or more cattle clinically or pre-clinically infected with BSE in a given country or region. A definition of the four categories is provided in the following Table.

GBR level	Presence of one or more cattle clinically or pre-clinically infected with BSE in a geographical region/country
I	Highly unlikely
II	Unlikely but not excluded
III	Likely but not confirmed or confirmed at a lower level
IV	Confirmed at a higher level (≥ 100 cases/1 Million adult cattle per year)

Reports of the GBR assessment of the countries are available on the SSC website (http://ec.europa.eu/food/fs/sc/ssc/outcome_en.html)

(16) Experts consider that the GBR classification system is stable enough, so that it can continue to be used, during the interim period, for the demonstration of compliance with this chapter.

(17) Opinion of the Scientific Panel on Biological Hazards on 'the breeding programme for TSE resistance in sheep': http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620775678.htm

(18) SSC Scientific Opinion on the conditions related to "BSE Negligible Risk (Closed) Bovine Herds" adopted at the meeting of 22-23 July 1999. http://ec.europa.eu/food/fs/sc/ssc/out56_en.html

(19) The tissue classification tables are based upon the most recent WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2010) http://www.who.int/bloddproducts/tablestissueinfectivity.pdf

(20) A Scientific opinion on BSE/TSE infectivity in small ruminant tissues is currently being reviewed by EFSA (Question No EFSA-Q-2010-052). For updated information please follow this link: http://registerofquestions.efsa.europa.eu/roqFrontend/questionsListLoader?mandate=M-2010-0041

subdivide the category into different levels of risk. It is also evident that the placement of a given tissue in one or another category can be disease and species specific, and subject to revision as new data emerge.

For the risk assessment (see section 4), manufacturers and/or marketing authorisation holders/applicants shall take into account the tissue classification tables in the Annex to this chapter.

The categories in the tables are only indicative and it is important to note the following points.

- In certain situations there could be **cross-contamination** of tissues of different categories of infectivity. The potential risk will be influenced by the circumstances in which tissues were removed, especially by contact of tissues with lower-infectivity tissues or no detectable infectivity (categories IB and IC tissues) with high-infectivity tissues (category IA tissues). Thus, cross-contamination of some tissues may be increased if infected animals are slaughtered by brain stunning (penetrative or non penetrative) or if the brain and/or spinal cord is sawed. The risk of cross-contamination will be decreased if body fluids are collected with minimal damage to tissue and cellular components are removed, and if foetal blood is collected without contamination from other maternal or foetal tissues including placenta, amniotic and allantoic fluids. For certain tissues, it is very difficult or impossible to prevent cross-contamination with category IA tissues (e.g. skull). This has to be considered in the risk assessment.
- For certain classes of substances the **stunning/slaughtering techniques** used may be important in determining the potential risk[21] because of the likelihood of disseminating the brain particles into the peripheral organs, particularly to the lungs. Stunning/slaughtering techniques should be described as well as the procedures to remove high infectivity tissues. The procedures to collect the animal tissues/organs to be used and the measures in place to avoid cross-contamination with a higher risk material must also be described in detail.
- The risk of contamination of tissues and organs with BSE-infectivity potentially harboured in central nervous material as a consequence of the stunning method used for cattle slaughtering depends on the following factors:
  - the amount of BSE-infectivity in the brain of the slaughtered animal,
  - the extent of brain damage,
  - the dissemination of brain particles in the animal body.

  These factors must be considered in conjunction with the OIE/GBR classification of the source animals, the age of the animals in the case of cattle and the *post-mortem* testing of the cattle using a validated method.

  The underlying principles indicated above would be equally applicable to sheep and goats.

The risk posed by cross-contamination will be dependent on several complementary factors including:

- measures adopted to avoid contamination during collection of tissues (see above),
- level of contamination (amount of the contaminating tissue),
- amount and type of materials collected at the same time.

Manufacturers or the marketing authorisation holders/applicants should take into account the risk with respect to cross-contamination.

3-4. *AGE OF ANIMALS*

As the TSE infectivity accumulates in bovine animals over an incubation period of several years, it is prudent to source from young animals.

Presence of infectious material has essentially been reported in the central nervous system and related tissues, as well as in the lymphoreticular system, depending on the TSE agent (BSE in cattle or scrapie in sheep and goat). The exact time course of infectivity in the respective body parts and tissues, from the date of infection, is not known in both species and, as such, it is difficult to give clear guidance on the age above which the various tissues may be infected and should not be collected. The initial recommendation to collect tissues in the youngest age is still valid. In addition, it is noteworthy that the age criteria depend also on the geographical origin. Age is a more important parameter for materials from countries where the risk is higher (Category B and C countries), than from countries with a negligible BSE risk (Category A countries).

3-5. *MANUFACTURING PROCESS*

The assessment of the overall TSE risk reduction of a medicinal product shall take into account the control measures instituted with respect to:

- sourcing of the raw/starting materials, and
- the manufacturing process.

Controlled sourcing is a very important criterion in achieving acceptable safety of the product, due to the documented resistance of TSE agents to most inactivation procedures.

A quality assurance system, such as ISO 9000 certification, HACCP[22] or GMP, must be put in place for monitoring the production process and for batch delineation (i.e. definition of batch, separation of batches, cleaning between batches). Procedures shall be put in place to ensure traceability as well as self-auditing and to auditing suppliers of raw/starting materials.

Certain production procedures may contribute considerably to the reduction of the risk of TSE contamination, e.g. procedures used in the manufacture of tallow derivatives (see section 6). As such rigorous processing cannot be applied to many products, processes involving physical removal, such as precipitation and filtration to remove prion-rich material, are likely to be more appropriate than chemical treatments. A description of the manufacturing process, including in-process controls applied, shall be presented and the steps that might contribute to reduction or elimination of TSE contamination should be discussed. Whenever different manufacturing sites are involved, the steps performed at each site shall be clearly identified. The measures in place in order to ensure traceability of every production batch to the source material should be described.

Cleaning process. Cleaning of process equipment may be difficult to validate for the elimination of TSE agents. It is reported that after exposure to high titre preparations of TSE agent, detectable infectivity can remain bound to the surface of stainless steel. The removal of all adsorbed protein by the use of 1 M sodium hydroxide or chlorine releasing disinfectants (e.g. 20 000 ppm chlorine for 1 h) have been considered acceptable approaches where equipment that cannot be replaced has been exposed to potentially contaminated material. Milder treatments with limited concentrations of alkali or stabilized bleach, when properly formulated with detergents and used at specified temperatures, have been shown to exhibit similar efficiency for removing prions as did classical NaOH or chlorine treatments. A system based on vaporised hydrogen peroxide also appeared to be efficient

(21) SSC opinion on stunning methods and BSE risk (The risk of dissemination of brain particles into the blood and carcass when applying certain stunning methods), adopted at the meeting of 10-11 January 2002. http://ec.europa.eu/food/fs/sc/ssc/out245_en.pdf. Report of the EFSA Working group on BSE risk from dissemination of brain particles in blood and carcass. Question No EFSA-Q-2003-122, adopted on 21 October 2004, http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620777397.htm
(22) Hazard Analysis Critical Control Point.

for inactivating TSE agents. These new treatments are more compatible with delicate materials and may be suitable for practical use[23].

If risk materials are used in the manufacture of a product, cleaning procedures, including control measures, shall be put in place in order to minimise the risk of cross-contamination between production batches. This is especially important if materials from different risk categories are handled in the same plant with the same equipment. In the case of using category IA materials in the manufacture of a product, dedicated equipment shall be used, unless otherwise justified.

Further research is needed to develop and validate new decontamination procedures to lower the risk of cross-contamination for material and devices which are not compatible with WHO-recommended procedures.

Removal/Inactivation validation. Validation studies of removal/inactivation procedures for TSEs can be difficult to interpret. It is necessary to take into consideration the nature of the spiked material and its relevance to the natural situation, the design of the study (including scaling-down of processes) and the method of detection of the agent (*in vitro* or *in vivo* assay). Further research is needed to develop an understanding of the most appropriate "spike preparation" for validation studies. Therefore, validation studies are currently not generally required. However, if claims are made for the safety of the product with respect to TSEs based on the ability of manufacturing processes to remove or inactivate TSE agents, they must be substantiated by appropriate investigational studies[24].

In addition to appropriate sourcing, manufacturers are encouraged to continue their investigations into removal and inactivation methods to identify steps/processes that would have benefit in assuring the removal or inactivation of TSE agents. In any event, a production process wherever possible shall be designed taking account of available information on methods which are thought to inactivate or remove TSE agents.

For certain types of products (see section 6-3 Bovine blood and blood derivatives), where validated removal/inactivation is not readily applicable, process evaluation might be required. This should be based on the starting material and any published data on TSE risk.

4. RISK ASSESSMENT OF MATERIALS OR SUBSTANCES USED IN THE MANUFACTURE AND PREPARATION OF A MEDICINAL PRODUCT IN THE CONTEXT OF REGULATORY COMPLIANCE

The assessment of the risk associated with TSE needs careful consideration of all of the parameters as outlined in section 3-1 (Scientific Principles for Minimising Risk).

As indicated in the introduction to this chapter, regulatory compliance is based on a favourable outcome from a risk assessment. The risk assessments, conducted by the manufacturers and/or the marketing authorisation holders or applicants for the different materials or substances from "TSE-relevant animal species" used in the manufacture of a medicinal product shall show that all TSE risk factors have been taken into account and, where possible, risk has been minimised by application of the principles described in this chapter. TSE Certificates of suitability issued by the EDQM may be used by the marketing authorisation holders or applicants as the basis of the risk assessments.

An overall risk assessment for the medicinal product, conducted by the marketing authorisation holders or applicants, shall take into account the risk assessments for all the different materials from "TSE-relevant animal species" and, where appropriate, TSE reduction or inactivation by the manufacturing steps of the active substance and/or finished product.

The final determination of regulatory compliance rests with the competent authority.

It is incumbent upon the manufacturers and/or the marketing authorisation holders or applicants for both human and veterinary medicinal products to select and justify the control measures for a given "TSE-relevant animal species" derivative, taking into account the latest scientific and technical progress.

5. BENEFIT/RISK EVALUATION

In addition to the parameters as mentioned in sections 3 (that may be covered by a TSE Certificate of Suitability issued by the EDQM) and 4, the acceptability of a particular medicinal product containing materials derived from a "TSE-relevant animal species", or which as a result of manufacture could contain these materials, shall take into account the following factors:

- route of administration of the medicinal product,
- quantity of animal material used in the medicinal product,
- maximum therapeutic dosage (daily dose and duration of treatment),
- intended use of the medicinal product and its clinical benefit,
- presence of a species barrier.

High-infectivity tissues (category IA tissues) and substances derived thereof shall not be used in manufacture of medicinal products, their starting materials and intermediate products (including active substances, excipients and reagents), unless justified. A justification why no other materials can be used shall be provided. In these exceptional and justified circumstances, the use of high-infectivity tissues could be envisaged for the manufacture of active substances, when, after performing the risk assessment as described in Section 4 of this chapter, and taking into account the intended clinical use, a positive benefit/risk assessment can be presented by the marketing authorisation applicant. Substances from category IA materials, if their use is justified, must be produced from animals of countries with negligible BSE risk (Category A).

6. SPECIFIC CONSIDERATIONS

The following materials prepared from "TSE-relevant animal species" are considered in compliance with this chapter provided that they meet at least the conditions specified below. The relevant information or a certificate of suitability granted by the EDQM shall be provided by the marketing authorisation applicant/holder.

6-1. *COLLAGEN*

Collagen is a fibrous protein component of mammalian connective tissue.

For collagen, documentation to demonstrate compliance with this chapter needs to be provided taking into account the provisions listed in sections 3 to 5. In addition, consideration should be given to the following.

- For collagen produced from bones, the conditions specified for gelatin are applicable (see below). Lower inactivation capacity is expected from the collagen manufacturing process than from that of gelatin. Therefore, sourcing becomes a more critical aspect to consider.
- Collagen produced from tissues such as hides, skins, tendons and sinews do not usually present a measurable TSE risk provided that contamination with potentially infected materials, for example spillage of blood and/or central nervous tissues, is avoided during procurement. Therefore, hides represent a safer raw material for

(23) WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2006) http://www.who.int/bloodproducts/tse/WHO%20TSE%20Guidelines%20FINAL-22%20JuneupdatedNL.pdf
(24) Guideline on the investigation of manufacturing process for plasma-derived medicinal products with regard to vCJD risk CPMP/BWP/5136/03

human implants derived from collagen. However, cross-contamination with brain material released during the slaughtering process that may have dried on the surface of hides would be difficult to eliminate. This is another aspect to consider in the evaluation of safety of this source material.

The collagen manufacturing process can have some steps in common with the manufacture of gelatin such as alkaline and sodium sulphate treatment, calcium hydroxide and sodium hydroxide treatments or enzyme treatment. However, even these common steps can differ in duration and pH condition which can result in significant differences in their inactivation capacity. Manufacturers should at least conduct a process evaluation based on the similarities of the collagen processing steps, as compared to known inactivation steps in the manufacture of gelatin, in order to support the safety of the product. In addition to processing, differences also exist in the final use of the material and, consequently, in their risk assessment, while gelatin is widely used for oral administration, many collagen applications are in the form of surgical implants. This aspect should also be considered in the final risk assessment.

6-2. *GELATIN*

Gelatin is a natural, soluble protein, gelling or non-gelling, obtained by the partial hydrolysis of collagen produced from bones, hides and skins of animals.

For gelatin, documentation to demonstrate compliance with this chapter needs to be provided taking into account the provisions listed in sections 3 to 5. In addition, consideration should be given to the following[(25)].

The source material used

Gelatin used in medicinal products can be manufactured from bones or hides.

Hides as the starting material. On the basis of current knowledge, hides used for gelatin production represent a safer source material as compared to bones. However, it is highly recommended that measures should be put in place to avoid cross-contamination with potentially infected materials during procurement.

Bones as the starting material. Where bones are used to manufacture gelatin, the quality of the starting materials needs to be controlled as an additional parameter to ensure the safety of the final product. Therefore, the following should be applied.

1. Skulls and spinal cord shall be removed from the collected bones (raw/starting material) independent of the age or the country of origin of the cattle.

2. Vertebrae shall be removed from the raw/starting materials from cattle over 30 months from countries with a controlled or an undetermined BSE risk (Categories B or C).

3. Gelatin for parenteral use should only be manufactured from bones coming from countries with a negligible or a controlled BSE risk (Category A and B, respectively). Gelatin for oral use can be manufactured from bones from countries with a negligible, a controlled or an undetermined BSE risk (Category A, B and C, respectively).

4. Gelatin shall be manufactured using one of the manufacturing methods described below.

Manufacturing methods

Hides. No specific measures with regard to the processing conditions are required for gelatin produced from hides provided that control measures are put in place to avoid cross-contamination both during the procurement of the hides and during the manufacturing process.

Bones. Where bones are used as the starting material, the mode of manufacture will be the second parameter that will ensure the safety of gelatin.

- Gelatin can be manufactured from bones from countries with a negligible, a controlled or an undetermined BSE risk (Categories A, B or C) sourced in accordance with the conditions described in section 6-2 under The source material used, using the acid, alkaline or heat/pressure manufacturing process.
- The manufacturing process shall be taken into consideration when performing the risk assessment as described in Section 4 of this chapter. Both the acid and the alkaline manufacturing methods have shown similar overall inactivation/removal of TSE infectivity in the gelatin validation experiments. Studies have shown that an additional alkaline treatment (pH 13, 2 h) of the bones/ossein further increases the TSE inactivation/removal capacity of the manufacturing process. Other processing steps such as filtration, ion-exchange chromatography and UHT sterilisation also contributes to the safety of gelatin.
- For a typical alkaline manufacturing process, bones are finely crushed, degreased with hot water and demineralised with dilute hydrochloric acid (at a minimum of 4 per cent and pH < 1.5) over a period of at least 2 days to produce the ossein. This is followed by an alkaline treatment with saturated lime solution (pH at least 12.5) for a period of at least 20 days.
- Bovine bones may also be treated by an acid process. The liming step is then replaced by an acid pre-treatment where the ossein is treated at pH < 3.5 for a minimum of 10 hours.
- A "flash" heat treatment (sterilisation) step at 138 °C minimum for 4 s at least is applied to both acid and alkaline manufacturing process.
- In the heat/pressure process, the dried degreased crushed bones are autoclaved with saturated steam at a pressure greater than 3 bar and a minimum temperature of 133 °C, for at least 20 min, followed by extraction of the protein with hot water.

The finishing steps are similar for the alkaline, acid and heat/pressure process and include extraction of the gelatine, washing, filtration and concentration.

6-3. *BOVINE BLOOD AND BLOOD DERIVATIVES*

Foetal bovine serum is commonly used in cell cultures. Foetal bovine serum should be obtained from foetuses harvested in abattoirs from healthy dams fit for human consumption and the womb should be completely removed and the foetal blood harvested in dedicated space or area by cardiac puncture into a closed collection system using aseptic technique.

Newborn calf serum is obtained from calves under 20 days old and calf serum from animals under the age of 12 months. In the case of donor bovine serum, given that it may be derived from animals less than 36 months old, the TSE negative status of the donor herd shall be well defined and documented. In all cases, serum shall be collected according to specified protocols by personnel trained in these procedures to avoid cross-contamination with higher risk tissues.

For bovine blood and blood derivatives, documentation to demonstrate compliance with this chapter needs to be provided taking into account the provisions listed in sections 3 to 5. In addition, consideration should be given to the following.

Traceability

Traceability to the slaughterhouse must be assured for each batch of serum or plasma. Slaughterhouses must have available lists of farms from which the animals are originated. If serum

(25) Based on the Opinion of the Scientific Panel on Biological Hazards of the European Food Safety Authority on the 'Quantitative assessment of the human BSE risk posed by gelatine with respect to residual BSE risk'. The EFSA Journal, 312, (1-28). http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620776107.htm
The requirements for source material selection and manufacture are appropriate for oral or parenteral gelatin for use in human and veterinary medicinal products.

is produced from living animals, records must be available for each serum batch which assures the traceability to the farms.

Geographical origin

Whilst tissue infectivity of BSE in cattle is more restricted than scrapie, as a precautionary measure bovine blood should be sourced from Category A countries. Bovine blood from Category B countries is also acceptable provided that there is no risk for cross-contamination of blood with brain material from the slaughter of animals over 21 months[(26)] of age.

Stunning methods

If it is sampled from slaughtered animals, the method of slaughter is of importance to assure the safety of the material. It has been demonstrated that stunning by captive bolt stunner with or without pithing as well as by pneumatic stunner, especially if it injects air, can destroy the brain and disseminate brain material into the blood stream. Non-penetrative stunning is no more considered as an alternative to penetrative stunning because contamination of blood with brain material has been demonstrated[(27)]. Negligible risk can be expected from electro-narcosis[(28)], but this even does not provide strict safety because, when unsuccessful, animals may have to be additionally stunned. The stunning methods must therefore be described for the bovine blood collection process.

Whenever a risk of cross-contamination of blood with brain cannot be avoided at routine slaughtering in countries with a controlled BSE risk (Category B), safety measures such as restriction of the age of the cattle and/or reduction of infectious agents during manufacture have to be applied.

Age

For countries with a controlled BSE risk (Category B), a precautionary age limit of 21 months shall apply for bovine blood or blood derivatives where no significant reduction of TSE agents can be assumed from manufacture. An age limit of 30 months is considered sufficient for blood derivatives where significant reduction of TSE agents can be demonstrated as described below.

Reduction of TSE agents during manufacture

For blood derivatives, the capacity of the manufacturing process to reduce/eliminate TSE agents should be estimated from investigational studies. The estimation may be based on published data or in house data whenever it can be shown that such data is relevant to the specific manufacturing process. If it cannot be concluded that the reduction capacity is comparable, it is recommended that manufacturers undertake product-specific investigational studies. Investigations using biochemical assay may be sufficient if there is scientific evidence that this assay correlates with infectivity data. General guidance for investigational studies on reduction of TSE agents has been outlined[(29)]. Brain-derived spike preparations are appropriate for studies investigating the risk from brain-contaminated blood.

6-4. *TALLOW DERIVATIVES*

Tallow is fat obtained from tissues including subcutaneous, abdominal and inter-muscular areas and bones. Tallow used as the starting material for the manufacture of tallow derivatives shall be 'Category 3 material or equivalent', as defined in Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption.

Tallow derivatives, such as glycerol and fatty acids, manufactured from tallow by rigorous processes are thought unlikely to be infectious and they have been the subject of specific consideration by CHMP and CVMP. For this reason, such materials manufactured under the conditions at least as rigorous as those given below shall be considered in compliance for this chapter, irrespective of the geographical origin and the nature of the tissues from which tallow derivatives are derived. Examples of rigorous processes are:

- trans-esterification or hydrolysis at not less than 200 °C for not less than 20 min under pressure (glycerol, fatty acids and fatty acid esters production),
- saponification with 12 M NaOH (glycerol and soap production):
  - batch process: at not less than 95 °C for not less than 3 h,
  - continuous process: at not less than 140 °C, under pressure for not less than 8 min, or equivalent,
- distillation at 200 °C.

Tallow derivatives manufactured according to these conditions are unlikely to present any TSE risk and shall therefore be considered compliant with this chapter.

Tallow derivatives produced using other conditions must demonstrate compliance with this chapter.

6-5. *ANIMAL CHARCOAL*

Animal charcoal is prepared by carbonisation of animal tissues, such as bones, using temperatures higher than 800 °C. Unless otherwise justified, the starting material for the manufacture of animal charcoal shall be Category 3 material or equivalent, as defined in Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002

Table 5.2.8.-1. – *Concept for acceptance of bovine blood/sera and derivatives*

Product	Foetal bovine serum	Donor calf serum	Adult bovine donor serum	Calf serum	Adult bovine serum / plasma	Adult bovine serum / plasma / serum derivative	Adult bovine serum derivative	Adult bovine serum derivative
Geographical origin of cattle	Cat. A and B	Cat. A and B	Cat. A and B[1]	Cat. A and B	Cat. A	Cat. B	Cat. A	Cat. B
Age of cattle	unborn	< 1 year	< 36 months	< 1 year	No limit	< 21 months[2]	No limit	< 30 months
Slaughtering/cross-contamination of blood with CNS material	No risk of cross-contamination			Risk of cross-contamination				
Demonstration of Prion reduction during manufacture	No			No				Yes[3]

1. When sourced in Category B countries, cattle should be from well-defined and documented herds.

2. A higher age may be allowed if cross-contamination of blood with CNS material can be clearly ruled out (e.g. halal slaughter).

3. Demonstration of prion reduction may not be required if cross-contamination of blood with CNS material can be clearly ruled out (e.g. halal slaughter).

(26) Opinion of the Scientific Panel on Biological Hazards on the assessment of the age limit in cattle for the removal of certain Specified Risk Materials (SRM). Question No EFSA-Q-2004-146, adopted on 28 April 2005

(27) The tissue classification tables are based upon the most recent WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2010) http://www.who.int/bloodproducts/tablestissueinfectivity.pdf

(28) Report of the EFSA Working Group on BSE risk from dissemination of brain particles in blood and carcass. Question No EFSA-Q-2003-112, adopted on 21 October 2004, http://www.efsa.europa.eu/en/sciencebiohaz/biohaz_opinions/opinion_annexes/733.html

(29) Guideline on the investigation of manufacturing process for plasma-derived medicinal products with regard to vCJD risk CPMP/BWP/5136/03.

laying down health rules concerning animal by-products not intended for human consumption. Irrespective of the geographical origin and the nature of the tissue, for the purpose of regulatory compliance, animal charcoal shall be considered in compliance with this chapter.

Charcoal manufactured according to these conditions is unlikely to present any TSE risk and shall therefore be considered compliant with this chapter. Charcoal produced using other conditions must demonstrate compliance with this chapter.

6-6. *MILK AND MILK DERIVATIVES*

In the light of the current scientific knowledge and irrespective of the geographical origin, bovine milk is unlikely to present any risk of TSE contamination[(30)].

Certain materials, including lactose, are extracted from whey, the spent liquid from cheese production following coagulation. Coagulation can involve the use of calf rennet, an extract from abomasum, or rennet derived from other ruminants. The CHMP/CVMP have performed a risk assessment for lactose and other whey derivatives produced using calf rennet and concluded that the TSE risk is negligible if the calf rennet is produced in accordance with the process described in the risk assessment report[(31)]. The conclusion was endorsed by the SSC[(32)] which has also performed an assessment of the TSE risk of rennet in general[(33)].

Bovine milk derivatives manufactured according to the conditions described below are unlikely to present any TSE risk and shall therefore be considered compliant with this chapter.

- The milk is sourced from healthy animals in the same conditions as milk collected for human consumption, and
- no other ruminant materials, with the exception of calf rennet, are used in the preparation of such derivatives (e.g. pancreatic enzyme digests of casein).

Milk derivatives produced using other processes or rennet derived from other ruminant species must demonstrate compliance with this chapter.

6-7. *WOOL DERIVATIVES*

Derivatives of wool and hair of ruminants, such as lanolin and wool alcohols derived from hair shall be considered in compliance with this chapter, provided the wool and hair are sourced from live animals.

Wool derivatives produced from wool which is sourced from slaughtered animals declared "fit for human consumption" and the manufacturing process in relation to pH, temperature and duration of treatment meets at least one of the stipulated processing conditions listed below are unlikely to present any TSE risk and shall therefore be considered compliant with this chapter.

- Treatment at pH ≥ 13 (initial; corresponding to a NaOH concentration of at least 0.1 M NaOH) at 60 °C for at least 1 h. This occurs normally during the reflux stage of the organic-alkaline treatment.
- Molecular distillation at ≥ 220 °C under reduced pressure.

Wool derivatives produced using other conditions must demonstrate compliance with this chapter.

6-8. *AMINO ACIDS*

Amino acids can be obtained by hydrolysis of materials from various sources.

Unless otherwise justified, the starting material for the manufacture of amino acids shall be 'Category 3 material or equivalent', as defined in Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption.

Amino acids prepared using the following processing conditions are unlikely to present any TSE risk and shall be considered compliant with this chapter:

- amino acids produced from hides and skins by a process which involves exposure of the material to a pH of 1 to 2, followed by a pH of > 11, followed by heat treatment at 140 °C for 30 min at 3 bar,
- the resulting amino acids or peptides must be filtered after production, and
- analysis is performed using a validated and sensitive method to control any residual intact macromolecules, with an appropriate limit set.

Amino acids prepared using other conditions must demonstrate compliance with this chapter.

6-9 PEPTONES

Peptones are partial hydrolysates of protein, achieved by enzymic or acid digestion. They are used in microbiological culture media to support the nutritional requirements of micro-organisms, which might be used as seed stocks or in industrial scale fermentations for the production of human and veterinary medicinal products, including vaccines. There is considerable interest in the use of vegetable protein as an alternative to animal sourced protein. However:

- where gelatin is used as the protein source material, reference is made to section 6-2 Gelatin, of this chapter,
- where casein is used as the protein source material, reference is made to section 6-6 Milk and milk derivatives, of this chapter,
- where tissue of TSE-relevant animal species is the protein source material, the tissue must be sourced from animals fit for consumption (see section 3-2 Source animals, of this chapter) with a maximum age of 30 months old for cattle from countries with a controlled BSE risk (Category B). The age of animals is of minimal concern for animals from countries with a negligible BSE risk (Category A).

Annex: major categories of infectivity

The tables below are taken from the *WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2010).*

Data entries are shown as follows:

+	=	Presence of infectivity or PrP^{TSE}
−	=	Absence of detectable infectivity or PrP^{TSE}
NT	=	Not tested
NA	=	Not applicable
?	=	Uncertain interpretation
()	=	Limited or preliminary data
[]	=	Infectivity or PrP^{TSE} data based exclusively on bioassays in transgenic (Tg) mice over-expressing the PrP-encoding gene or PRP^{TSE} amplification methods

(30) For milk and milk derivatives from small ruminants, please see EFSA opinion on Question No EFSA-Q-2008-310, adopted on 22 October 2008, http://www.efsa.europa.eu/en/scdocs/scdoc/849.htm

(31) Committee for Medicinal Products for Human Use and its Biologics Working Party conducted a risk and regulatory assessment of lactose prepared using calf rennet. The risk assessment included the source of the animals, the excision of the abomasums and the availability of well-defined quality assurance procedures. The quality of any milk replacers used as feed for the animals from which abomasums are obtained is particularly important. The report can be found on http://www.ema.europa.eu/pdfs/human/press/pus/057102.pdf

(32) Provisional statement on the safety of calf-derived rennet for the manufacture of lactose, adopted by the SSC at its meeting of 4-5 April 2002 (http://ec.europa.eu/food/fs/sc/ssc/out255_en.pdf)

(33) The SSC issued an opinion on the safety of animal rennet in regard to risks from animal TSE and BSE in particular, adopted at its meeting of 16 May 2002 (http://ec.europa.eu/food/fs/sc/ssc/out265_en.pdf)

Category IA: High-infectivity tissues

Tissue	Cattle BSE		Sheep and goats Scrapie		Elk and deer CWD	
	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}
Brain	+	+	+	+	+	+
Spinal cord	+	+	+	+	NT	+
Retina	+	NT	NT	+	NT	+
Optic nerve[2]	+	NT	NT	+	NT	+
Spinal ganglia	+	+	+	+	NT	+
Trigeminal ganglia	+	+	NT	+	NT	-
Pituitary gland[3]	−	NT	+	+	NT	+
Dura mater[3]	NT	NT	NT	NT	NT	NT

Category IB: Lower-infectivity tissues

Tissue	Cattle BSE		Sheep and goats Scrapie		Elk and deer CWD	
	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}
Peripheral nervous system						
Peripheral nerves	[+]	+	+	+	NT	+
Autonomic ganglia[4]	NT	+	NT	+	NT	+
Lymphoreticular tissues						
Spleen	−	−	+	+	NT	+
Lymph nodes	−	−	+	+	NT	+
Tonsil	+	−	+	+	NT	+
Nictitating membrane	+	−	[+]	+	NT	+
Thymus	−	NT	+	+	NT	−
Alimentary tract[5]						
Oesophagus	−	NT	[+]	+	NT	+
Fore-stomach[6] (ruminants only)	−	NT	[+]	+	NT	+
Stomach/abomasum	−	NT	[+]	+	NT	+
Duodenum	−	−	[+]	+	NT	+
Jejunum[7]	−	+	[+]	+	NT	NT
Ileum[7]	+	+	+	+	NT	+
Appendix	NA	NA	NA	NA	NA	NA
Colon/caecum[7]	−	−	+	+	NT	+
Rectum	NT	NT	NT	+	NT	+
Reproductive tissues						
Placenta[8]	−	NT	+	+	NT	−
Ovary[3]	−	NT	−	−	NT	−
Uterus[3]	−	NT	−	−	NT	−
Other tissues						
Mammary gland/udder[9]	−	NT	−	+	NT	NT
Skin[3, 10]	−	NT	−	+	[+]	[+]
Adipose tissue	−	NT	NT	NT	[+]	NT
Heart/pericardium	−	NT	−	NT	NT	+
Lung	−	NT	−	−	NT	+
Liver[3]	−	NT	+	−	NT	−
Kidney[3, 11]	−	−	[+]	+	NT	+
Adrenal	[+]	+	+	−	NT	+
Pancreas[3]	−	NT	+	NT	NT	+

Tissue	Cattle BSE		Sheep and goats Scrapie		Elk and deer CWD	
	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}
Bone marrow[12]	[+]	NT	+	NT	NT	−
Skeletal muscle[13]	[+]	NT	[+]	+	[+]	−
Tongue[14]	−	NT	[+]	+	NT	−
Blood vessels	−	NT	NT	+	NT	−
Nasal mucosa[15]	−	NT	+	+	NT	+
Salivary gland	−	NT	+	NT	−	−
Cornea[16]	NT	NT	NT	NT	NT	NT
Body fluids, secretion and excretions						
CSF	−	NT	+	−	NT	NT
Blood[17]	−	?	+	?	+	?
Saliva	NT	NT	−	NT	+	[−]
Milk[18]	−	−	+	[+]	NT	NT
Urine[19]	−	NT	−	−	−[+]	[+]
Feces[19]	−	NT	−	NT	−[+]	NT

Category IC: Tissues with no detectable infectivity

Tissue	Cattle BSE		Sheep and goats Scrapie		Elk and deer CWD	
	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}
Reproductive tissues						
Testis	−	NT	−	−	NT	−
Prostate/Epididymis/Seminal vesicle	−	NT	−	−	NT	−
Semen	−	NT	−	−	NT	NT
Placenta fluids	−	NT	NT	NT	NT	NT
Foetus[20]	−	NT	−	−	NT	(−)
Embryos[20]	−	NT	?	NT	NT	NT
Musculo-skeletal tissues						
Bone	−	NT	NT	NT	NT	NT
Tendon	−	NT	NT	NT	NT	NT
Other tissues						
Gingival tissues	NT	NT	NT	NT	NT	NT
Dental pulp	NT	NT	NT	NT	NT	NT
Trachea	−	NT	NT	NT	NT	−
Thyroid gland	NT	NT	−	NT	NT	−
Body fluids, secretions and excretions						
Colostrum[21]	(−)	−	(?)	NT	NT	NT
Cord blood[21]	−	NT	NT	NT	NT	NT
Sweat	NT	NT	NT	NT	NT	NT
Tears	NT	NT	NT	NT	NT	NT
Nasal mucus	NT	NT	NT	NT	NT	NT
Bile	NT	NT	NT	NT	NT	NT

1. Infectivity bioassays of human tissues have been conducted in either primates or mice (or both), bioassays of cattle tissues have been conducted in either cattle or mice (or both), and most bioassays of sheep and/or goat tissues have been conducted only in mice. In regard to sheep and goats not all results are consistent for both species, for example, two goats (but no sheep) have contracted BSE naturally [Eurosurveillance, 2005, Jeffrey et al., 2006]. Similarly, most of the results described for CWD were derived from studies in deer, and findings may not be identical in elk or other cervids.

2. In experimental models of TSE, the optic nerve has been shown to be a route of neuroinvasion, and contains high titres of infectivity.

3. No experimental data about infectivity in pituitary gland or dura mater in humans with all forms of human TSE have been reported, but cadaveric dura mater patches, and growth hormone derived from cadaveric pituitaries have transmitted disease to hundreds of people and therefore must be included in the category of high-risk tissues. PrP^{TSE} was detected by immunoblot in the dura mater of a vCJD patient who died in the US after an unusually long incubation period (see also Table IB for other positive tissues: skin, kidney, liver, pancreas, ovary and uterus) [Notari et al., 2010]. It must be mentioned that earlier studies of numerous cases examined in the UK reported all of these tissues to be negative [Ironside et al., 2002, Head et al., 2004].

4. In cattle, PrP^{TSE} is reported to be inconsistently present in the enteric plexus in the distal ileum, but immunohistochemical examination of tissues from a single 'fallen stock' case of BSE in Japan suggested (albeit equivocally) involvement of myenteric plexuses throughout the small and large intestine [Kimura and Haritani, 2008].

5. In vCJD, PrP^{TSE} is limited to gut-associated lymphoid and nervous tissue (mucosa, muscle, and serosa are negative).

6. Ruminant fore stomachs (reticulum, rumen, and omasum) are widely consumed, as is the true stomach (abomasum). The abomasum of cattle (and sometimes sheep) is also a source of rennet.

7. When a large BSE oral dose was used to infect cattle experimentally, infectivity was detected in the jejunum and the ileo-caecum junction in Tg mice overexpressing PrP [courtesy of Dr M Groschup]. PrP^{TSE} was detected at low incidence in lymphoid tissue of ileum [Terry et al., 2003] and has been detected at an even lower frequency in jejunal lymphoid tissue of cattle similarly infected by the oral route [EFSA, 2009].

8. A single report of transmission of sporadic CJD infectivity from human placenta has never been confirmed and is considered improbable.

9. PrP^{TSE} has been detected in scrapie-infected sheep with chronic mastitis, but not from infected sheep without mastitis [Ligios et al., 2005].

10. Studies in hamsters orally infected with scrapie revealed that PrP^{TSE} deposition in skin was primarily located within small nerve fibres. Also, apical skin 'velvet' from the antlers of CWD-infected deer is reported to contain PrP^{TSE} and infectivity [Angers et al., 2009].

11. PrP^{TSE} detected by immunocytochemistry in the renal pelvis of scrapie-infected sheep [Siso et al., 2006], and in lymphoid follicles within connective tissue adjacent to the renal pelvis in CWD-infected mule deer [Fox et al., 2006].

12. A single positive marrow in multiple transmission attempts from cattle orally dosed with BSE-infected brain [Wells et al., 1999, Wells et al., 2005, Sohn et al., 2009].

13. Muscle homogenates have not transmitted disease to primates from humans with sporadic CJD, or to cattle from cattle with BSE. However, intra-cerebral inoculation of a semitendinosus muscle homogenate (including nervous and lymphatic elements) from a single cow with clinical BSE has transmitted disease to transgenic mice that overexpress PrP at a rate indicative of trace levels of infectivity [Buschmann and Groschup, 2005]. Also, recent published and unpublished studies have reported the presence of PrP^{TSE} in skeletal muscle in experimental rodent models of scrapie and vCJD [Beekes et al., 2005], in experimental and natural scrapie infections of sheep and goats [Andreoletti et al., 2004], in sheep orally dosed with BSE [Andreoletti, unpublished data], and in humans with sporadic, iatrogenic, and variant forms of CJD [Glatzel et al., 2003, Kovacs et al., 2004, Peden et al., 2006]. Bioassays of muscle in transgenic mice expressing cervid PrP have documented infectivity in CWD-infected mule deer [Angers et al., 2006], and experiments are underway to determine whether detectable PrP^{TSE} in other forms of TSE is also associated with infectivity.

14. In cattle, bioassay of infectivity in the tongue was negative, but the presence of infectivity in palatine tonsil has raised concern about possible infectivity in lingual tonsillar tissue at the base of the tongue that may not be removed at slaughter [Wells et al., 2005, EFSA, 2008]. In sheep naturally infected with scrapie, 7 of 10 animals had detectable PrP^{TSE} in the tongue [Casalone et al., 2005, Corona et al., 2006].

15. Limited chiefly to regions involved in olfactory sensory reception.

16. Because only one case of iatrogenic CJD has been certainly attributed to a corneal transplant among hundreds of thousands of recipients (one additional case is considered probable, and another case only possible), cornea has been categorized as a lower-risk tissue, other anterior chamber tissues (lens, aqueous humour, iris, conjunctiva) have been tested with a negative result both in vCJD and other human TSEs, and there is no epidemiological evidence that they have been associated with iatrogenic disease transmission.

17. A wealth of data from studies of blood infectivity in experimental rodent models of TSE have been extended by recent studies documenting infectivity in the blood of sheep with naturally occurring scrapie and in sheep transfused with blood from BSE-infected cattle [Houston et al., 2008], of deer with naturally occurring CWD [Mathiason et al., 2006], and (from epidemiological observations) in the red cell fraction (which includes significant amounts of both plasma and leukocytes) of four blood donors in the pre-clinical phase of vCJD infections [reviewed in Brown, 2006, Hewitt et al., 2006]. Plasma Factor VIII administration has also been potentially implicated in a subclinical case of vCJD in a haemophilia patient [Peden et al., 2010]. Blood has not been shown to transmit disease from humans with any form of 'classical' TSE [Dorsey et al., 2009], or from cattle with BSE (including fetal calf blood). A number of laboratories using new, highly sensitive methods to detect PrP^{TSE} are reporting success in a variety of animal and human TSEs. However, several have experienced difficulty obtaining reproducible results in plasma, and it is not yet clear that positive results imply a potential for disease transmissibility, either because of false positives, or of 'true' positives that are due to sub-transmissible concentrations of PrP^{TSE}. Because of these considerations (and the fact that no data are yet available on blinded testing of specimens from naturally infected humans or animals) the expert group felt that it was still too early to evaluate the validity of these tests with sufficient confidence to permit either a negative or positive conclusion.

18. Evidence that infectivity is not present in milk from BSE-infected bovines includes temporo-spatial epidemiologic observations failing to detect maternal transmission to calves suckled for long periods, clinical observations of over a hundred calves suckled by infected cows that have not developed BSE, and experimental observations that milk from infected cows reared to an age exceeding the minimum incubation period has not transmitted disease when administered intra-cerebrally or orally to mice [Middleton and Barlow, 1993, Taylor et al., 1995]. Also, PrP^{TSE} has not been detected in milk from cattle incubating BSE following experimental oral challenge [SEAC, 2005]. However, low levels (µg to ng/L) of normal PrP have been detected in milk from both animals and humans [Franscini et al., 2006]. PrP^{TSE} has been detected in the mammary glands of scrapie-infected sheep with chronic mastitis [Ligios et al., 2005], and very recently it has been reported that milk (which in some cases also contained colostrum) from scrapie-infected sheep transmitted disease to healthy animals [Konold et al., 2008, Lacroux et al., 2008].

19. A mixed inoculum of urine and faeces from naturally infected CWD deer did not transmit disease during an 18-month observation period after inoculation of healthy deer with a heterozygous (96 G/S) PRNP genotype [Mathiason et al., 2006]. However, recent bioassays in Tg mice have transmitted disease from both urine [Haley et al., 2009] and faeces [Tamgüney et al., 2009]. In addition, mice with lymphocytic nephritis that were experimentally infected with scrapie shed both PrP^{TSE} and infectivity in urine, when bioassayed in Tg mice [Seegeret al., 2005]. Very low levels of infectivity have also been detected in the urine (and histologically normal kidneys) of hamsters experimentally infected with scrapie [Gregori and Rohwer, 2007, Gonzalez-Romero et al., 2008]. Finally, in an experimental scrapie-hamster model, oral dosing resulted in infectious faeces when bioassayed in Tg mice over-expressing PrP [Safar et al., 2008].

20. Embryos from BSE-affected cattle have not transmitted disease to mice, but no infectivity measurements have been made on fetal calf tissues other than blood (negative mouse bioassay) [Fraser and Foster, 1994]. Calves born of dams that received embryos from BSE- affected cattle have survived for observations periods of up to seven years, and examination of the brains of both the unaffected dams and their offspring revealed no spongiform encephalopathy or PrP^{TSE} [Wrathall et al., 2002].

21. Early reports of transmission of sporadic CJD infectivity from human cord blood and colostrum have never been confirmed and are considered improbable. A bioassay from a cow with BSE in transgenic mice over-expressing bovine PrP gave a negative result [Buschmann and Groschup, 2005], and PrP^{TSE} has not been detected in colostrum from cattle incubating BSE following experimental oral challenge [SEAC, 2005].

04/2019:51400

5.14. GENE TRANSFER MEDICINAL PRODUCTS FOR HUMAN USE

This general chapter is published for information.

This general chapter contains a series of texts on gene transfer medicinal products for human use. The texts provide a framework of requirements applicable to the production and control of these products. For a specific medicinal product, application of these requirements and the need for any further texts is decided by the competent authority. The texts are designed to be applicable to approved products; the need for application of part or all of the texts to products used during the different phases of clinical trials is decided by the competent authority. The provisions of the chapter do not exclude the use of alternative production and control methods that are acceptable to the competent authority.

Further detailed recommendations on gene transfer medicinal products for human use are provided by the Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products (CPMP/BWP/3088/99) and the Guideline on Development and Manufacture of Lentiviral Vectors (CHMP/BWP/2458/03) of the Committee for Medicinal Products for Human Use (including any subsequent revisions of these documents).

DEFINITION

For the purposes of this general chapter, gene transfer medicinal product (GTMP) shall mean a product obtained through a set of manufacturing processes aimed at the transfer, to be performed either *in vivo* or *ex vivo*, of a prophylactic, diagnostic or therapeutic gene (i.e. a piece of nucleic acid) to human/animal cells, and its subsequent expression *in vivo*. The gene transfer involves an expression system known as a vector, which can be of viral as well as non-viral origin. The vector can also be included in a human or animal cell.

Recombinant vectors, such as viral vectors and plasmids. Recombinant vectors are either injected directly into the patient's body (*in vivo* gene transfer) or transferred into host cells before administration of these genetically modified cells to the patient (*ex vivo* gene transfer). Viral vectors are derived from various viruses (for example, adenoviruses, poxviruses, retroviruses, lentiviruses, adeno-associated-viruses, herpesviruses). These vectors can be replicative, non-replicative or conditionally replicative. Plasmid vectors include nucleic acids in a simple formulation (for example, naked DNA) or complexed to various molecules (synthetic vectors such as lipids or polymers). Genetic material transferred by GTMPs consists of nucleotide sequences, which may notably encode gene products, antisense transcripts or ribozymes. Chemically synthesised oligonucleotides are not within the scope of this general chapter. After transfer, the genetic material may remain either cytoplasmic or episomal, or may be integrated into the host cell genome, depending on the integrating or non-integrating status of the vector.

Genetically modified cells. Genetically modified eukaryotic or bacterial cells are modified by vectors to express a product of interest.

PRODUCTION

Substances used in production. The raw materials used during the manufacturing process, including viral seed lot and cell bank establishment, where applicable, are qualified. Unless otherwise justified, all substances used are produced within a recognised quality management system using suitable production facilities. Suitable specifications are established to control notably their identity, potency (where applicable), purity and safety in terms of microbiological quality and bacterial endotoxin contamination. The quality of water used complies with the relevant corresponding monographs (*Purified water (0008)*, *Water for injections (0169)*). Where bovine serum is used, it complies with the monograph *Bovine serum (2262)*. The use of antibiotics is avoided wherever possible during production.

Viral safety. The requirements of chapter *5.1.7* apply.

Transmissible spongiform encephalopathies (*5.2.8*). A risk assessment of the product with respect to transmissible spongiform encephalopathies is carried out and suitable measures are taken to minimise such risk.

Recombinant vectors

PRODUCTION

GENERAL PROVISIONS

For viral vectors, production is based on a cell bank system and a virus seed-lot system, wherever possible.

For plasmid vectors, production is based on a bacterial cell bank system.

The production method shall have been shown to yield a vector of consistent quality. Unless otherwise justified and authorised, the vector in the final product shall have undergone no more passages or subcultures from the master seed lot than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

SUBSTRATE FOR VECTOR PROPAGATION

The substrates used comply with relevant requirements of the European Pharmacopoeia (*5.2.2*, *5.2.3*, and the section Bacterial cells used for the manufacture of plasmid vectors for human use).

CHARACTERISATION OF THE VECTOR

Historical records of vector construction are documented, including the origin of the vector and its subsequent manipulation, notably deleted or modified regions.

The vector is characterised using suitable and validated methods.

The genetic stability of the vector at or beyond the maximum passage level or the maximum number of cell doublings of the cell line used for production is assessed by suitable methods.

PROPAGATION AND HARVEST

All processing of the cell banks and subsequent cell cultures is done in an area where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The purity of the harvest is verified by suitable tests as defined in the corresponding specific sections.

PURIFIED HARVEST

The bulk of active substance is defined as a lot of purified recombinant vectors (viral vectors, or naked or complexed plasmids).

FINAL LOT

Unless otherwise justified and authorised, formulation and distribution of the final bulk is carried out under aseptic conditions using sterile containers (*3.2*).

The stability of the final lot is assessed using stability protocols including the duration, storage conditions, number of lots to be tested, test schedule and assays to be performed.

ASSAYS AND TESTS

The GTMPs comply with assays and tests described in the corresponding specific sections.

Genetically modified cells

For cells to be modified with a recombinant vector, the data related to the recombinant vector are documented above, under Recombinant vectors.

PRODUCTION

CELL SUBSTRATE

For xenogeneic cell lines, including bacterial cells, a cell bank system comprising a master cell bank and working cell banks is established.

For autologous and allogeneic cells, a cell banking system comprising a master cell bank and working cell banks is established wherever possible.

TRANSFECTION / TRANSDUCTION

Cells are transfected or transduced using a recombinant vector (plasmid or viral vector) qualified as described under Recombinant vectors; the process is validated. They are handled under aseptic conditions in an area where no other cells or vectors are handled at the same time. All reagents used during cell manipulation steps are fully qualified. Antibiotics are avoided unless otherwise justified and authorised. Transfection or transduction is carried out under aseptic conditions.

FINAL LOT

In the case of frozen storage, the viability of genetically modified cells is assessed before freezing and after thawing.

If the cells are not used within a short period, stability is determined by verifying cell viability and expression of the genetic insert.

In the case of genetically modified cells encapsulated before implantation in man, any encapsulating component used is considered as part of the final product, and is therefore quality-controlled and fully characterised (for example, physical integrity, selective permeability, sterility).

ASSAYS AND TESTS

Controls of xenogeneic, allogeneic or autologous cells include the following:

- identity, counting and viability of cells;
- overall integrity, functionality, copies per cell, transfer and expression efficiency of the genetic insert;
- microbiological controls (*2.6.1* or *2.6.27*), endotoxin content, mycoplasma contamination (*2.6.7*), adventitious virus contamination and, where applicable, replicative vector generation.

The competent authority may approve a reduced testing programme where necessary because of limited availability of cells. Where necessary because of time constraints, the product may be released for use before the completion of certain tests.

PLASMID VECTORS FOR HUMAN USE

DEFINITION

Plasmid vectors for human use are double-stranded circular forms of bacterial DNA that carry a gene of interest or a nucleotide sequence encoding antisense sequences or ribozymes and its expression cassette; they are amplified in bacteria extrachromosomally. They are used to transfer genetic material into human somatic cells *in vivo* or to genetically modify autologous, allogeneic, xenogeneic or bacterial cells before administration to humans. Plasmid vectors may be presented as naked DNA or may be formulated with synthetic delivery systems such as lipids (lipoplexes), polymers (polyplexes) and/or peptide ligands that facilitate transfer across the cell membrane and delivery to the cell, or that target delivery via specific receptors.

Plasmids formulated with synthetic delivery systems are not within the scope of this section.

PRODUCTION

PLASMID CONSTRUCTION

A typical plasmid vector is composed of:

- the plasmid vector backbone that contains multiple restriction endonuclease recognition sites for insertion of the genetic insert and the bacterial elements necessary for plasmid production, such as selectable genetic markers for the identification of cells that carry the recombinant vector;
- the required regulatory genetic elements to facilitate expression of the genetic insert;
- the genetic insert;
- a polyadenylation signal.

A complete description of the plasmid DNA, including its nucleotide sequence, is established with the identification, source, means of isolation and nucleotide sequence of the genetic insert. The source and function of component parts of the plasmid, such as the origin of replication, viral and eukaryotic promoters and genes encoding selection markers, are documented.

GENERAL PROVISIONS

Cell banks. Production of plasmid vectors is based on a bacterial cell-bank system with generation and characterisation of a master cell bank (MCB), working cell banks (WCBs) and end-of-production cells (EOPCs), which comply with the section Bacterial cells used for the manufacture of plasmid vectors for human use. The raw materials used during the manufacturing process, including cell bank establishment, are qualified.

Selection techniques. Unless otherwise justified and authorised, antibiotic-resistance genes used as selectable genetic markers, particularly for clinically useful antibiotics, are not included in the vector construct. Other selection techniques for the recombinant plasmid are preferred.

Reference standards. A suitable batch of the formulated plasmid, preferably one that has been clinically evaluated, is fully characterised and retained for use as a reference standard as necessary in routine control tests.

PROPAGATION AND HARVEST

Plasmid DNA is transferred to host strain bacterial cells and a single clone of transformed bacteria is expanded to create the MCB. The WCB is then derived from the MCB. The EOPCs are obtained from the WCB by fermentation in production conditions.

Plasmid DNA is isolated from harvested cells using an extraction step and is purified to obtain the bulk product.

Unless otherwise justified and authorised, caesium chloride-ethidium bromide density gradients are not used for production.

PURIFIED PLASMID

The production process is optimised to remove impurities consistently while retaining product activity. The requirement to test for a particular impurity depends on the following:

- the demonstrated capability of the manufacture and purification processes to remove or inactivate the impurity through process validation, using specific quantification methods;
- the potential toxicity associated with the impurity;
- the potential decrease of the efficacy of the genetic insert product associated with the impurity.

If selective resistance to specific antibiotics has been used for selection, data from validation studies of purification procedures are required to demonstrate the clearance capability for residual antibiotics.

Relevant in-process controls are performed to ensure that the process is continuously under control, for example, amount and form of plasmid after the extraction steps and amount of endotoxins after the extraction steps.

Only a batch of purified plasmid that complies with the following requirements may be used.

Identity and integrity of the purified plasmid. Identity and integrity of the purified plasmid are established by suitable methods such as sequencing or nucleic acid amplification techniques (NAT) (*2.6.21*); restriction enzyme analysis may be used where it is sufficient to detect potential critical modifications in the plasmid and confirm the plasmid identity.

Plasmid DNA. The following indications are given as examples.

DNA concentrations greater than 500 ng/mL may be determined using absorbance measurement at 260 nm. A 50 µg/mL double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/mL are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

DNA forms. Plasmid DNA is characterised in terms of the proportions of supercoiled, multimeric, relaxed monomer and linear forms, using suitable analytical methods, examples of which are given below. For quantification of supercoiled forms, anion-exchange high performance liquid chromatography (HPLC) or capillary electrophoresis may be used. Capillary electrophoresis is also suitable for the quantification of other forms.

Residual host-cell DNA. The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual RNA. The content of residual RNA is determined, unless the process has been validated to demonstrate suitable clearance. Reverse-phase HPLC (RP-HPLC) may be used, or quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) (*2.6.21*) when a lower limit of detection is required.

Residual host-cell protein. The concentration of residual host-cell protein is determined using standard protein assays (*2.5.33*), SDS-PAGE followed by silver staining, or specific immuno-assays such as western blot or ELISA, unless the process has been validated to demonstrate suitable clearance.

Microbiological control. Depending on the preparation concerned, it complies with the test for sterility (*2.6.1*) or the bioburden is determined (*2.6.12*).

Bacterial endotoxins (*2.6.14*): less than the limit approved for the particular preparation.

FINAL BULK

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*). It complies with the test for sterility.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

IDENTIFICATION

The plasmid vector is identified by restriction enzyme analysis or by sequencing. The test for biological activity also serves to identify the product.

TESTS

Tests carried out on the final lot include the following.

Appearance.

pH (*2.2.3*): within the limits approved for the particular preparation.

Extractable volume (*2.9.17*). It complies with the test for extractable volume.

Residual moisture (*2.5.12*): within the limits approved for the particular freeze-dried preparation.

DNA forms. The percentage of the specific monomeric supercoiled form is determined as described for the purified plasmid.

Sterility (*2.6.1*). It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*): less than the limit approved for the particular preparation.

ASSAY

Plasmid DNA: not less than the quantity stated on the label, determined, for example, by 1 of the following methods.

DNA concentrations greater than 500 ng/mL may be determined using absorbance measurement at 260 nm. A 50 µg/mL double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/mL are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

Biological activity. Wherever possible, biological activity is assessed through *in vitro* or *in vivo* bioassays. A well-defined, representative reference standard is required as a positive control for the assay. Bioassays employed to assay plasmid vectors generally involve transfection of a relevant cell line *in vitro*, followed by some functional measure of the expressed genetic insert. Such functional assays provide information about the activity of the product encoded by the genetic insert instead of the expression level of the genetic insert itself.

It may be necessary to supplement the bioassay with western-blot and ELISA assays to assess the integrity and quantity of the expressed product.

LABELLING

The label states:

- the plasmid DNA concentration;
- the recommended human dose;
- for freeze-dried preparations:
  - the name and volume of the liquid to be added;
  - the time within which the product is to be used after reconstitution.

BACTERIAL CELLS USED FOR THE MANUFACTURE OF PLASMID VECTORS FOR HUMAN USE

Production of plasmid vectors for human use is based on the use of a bacterial cell-bank system with generation and characterisation of a master cell bank (MCB), working cell banks (WCBs) and end-of-production cells (EOPCs). A bacterial cell bank for the manufacture of plasmid vectors is a collection of vials containing bacterial cells stored under defined conditions, with uniform composition, and obtained from pooled cells derived from a single clone of a transformed host strain. The MCB has a known, documented history; it is preferably derived from a qualified repository source. The

WCB is produced by expanding one or more vials of the MCB. Methods and reagents used to produce the bank and storage conditions are documented.

MCBs and WCBs are qualified by testing an aliquot of the banked material or testing a subculture of the cell bank.

The following table indicates the tests required at each stage of production.

Assay	Host strain	MCB	WCB	EOPCs*
Identity and purity				
Viability	+	+	+	+
Bacterial strain characterisation	+	+	–	+
Genotyping / phenotyping	+	+	–	+
Presence of the plasmid				
– Sequence of the DNA plasmid	–	+	–	+
– Copy number	–	+	+	+
– Restriction map	–	+	+	+
– Percentage of cells retaining the plasmid	–	+	+	+
Adventitious agents				
Purity by plating	+	+	+	+
Presence of bacteriophage	+	+	–	+

* EOPCs are cells with a passage number at least equivalent to that used for production. The analysis has to be done once to validate each new WCB, except for purity, which has to be tested for each fermentation.

IDENTITY AND PURITY TESTING

Viability. The number of viable cells is determined by plating a diluted aliquot of bacterial cells on an appropriate medium and counting individual colonies.

Biochemical and physiological bacterial strain characterisation. Depending on the bacterial strain used for production, relevant biochemical and physiological characterisation is performed to confirm cell identity at the species level.

Genotyping / phenotyping. The genotype of bacterial cells is verified by determination of the suitable specific phenotypic markers or by appropriate genetic analysis.

Presence of the plasmid

Sequencing. The whole nucleotide sequence of the plasmid is verified.

Copy number. The plasmid DNA is isolated and purified from a known number of bacteria and the copy number determined by a suitable method such as quantitative PCR (*2.6.21*).

Restriction map. Restriction endonuclease digestion is performed with sufficient resolution to verify that the structure of the plasmid is unaltered in bacterial cells.

Percentage of cells retaining the plasmid. Bacterial elements present in the plasmid, such as selectable genetic markers, are used to define the percentage of bacteria retaining the plasmid.

ADVENTITIOUS AGENTS AND ENDOGENOUS VIRUSES

Purity by plating. Bacterial cells are streaked out onto suitable media and incubated in the required conditions in order to detect potential bacterial contaminants. In order to test for inhibition of the growth of contaminating organisms, additional tests in the presence of a definite amount of relevant positive control bacteria are carried out. A suitable number of colonies is examined; no contamination is detected.

Presence of bacteriophage. Bacterial cells are plated and incubated in a medium allowing proliferation of bacteriophages, to test for bacteriophage presence. The test is validated by the use of a reference bacteriophage strain and permissive cells as positive controls. A suitable number of colonies is examined; no contamination is detected.

ADENOVIRUS VECTORS FOR HUMAN USE

DEFINITION

Adenovirus vectors for human use are freeze-dried or liquid preparations of recombinant adenoviruses, genetically modified to transfer genetic material to human somatic cells *in vivo* or *ex vivo*.

PRODUCTION

VECTOR CONSTRUCTION

There are different approaches for the design and construction of an adenovirus vector. The purpose of clinical use determines which approach is optimal. A method is chosen that minimises the risk of generating replication-competent adenovirus vectors or that effectively eliminates helper viruses that might be used during production.

VECTOR PRODUCTION

The production method shall have been shown to yield a vector of consistent quality. Unless otherwise justified and authorised, the vector in the final product shall have undergone no more passages from the master seed lot than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

The genetic and phenotypic stability of the vector at or beyond the maximum passage level used for production is assessed by suitable methods.

SUBSTRATE FOR VECTOR PROPAGATION

The vector is propagated in continuous cell lines (*5.2.3*) based on a cell bank system. The occurrence of replication-competent adenoviruses may be significant when large regions of homology exist between the viral genome and the genome of the complementation cells. This occurrence may be minimised by minimising the homology between both genomes. The use of cells with no sequence homology with the vector is recommended for production.

VECTOR SEED LOT

Production of the vector is based on a seed-lot system.

The strain of adenovirus used is identified by historical records that include information on its origin and its subsequent manipulation, notably deleted or modified regions. A detailed description of the genetic insert(s) and the flanking control regions is established, including the nucleotide sequence. The method by which the genetic insert is introduced into the vector is documented.

Only a seed lot that complies with the following requirements may be used for vector production.

Identification. The vector is identified in the master seed lot and each working seed lot by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Genetic and phenotypic characterisation. The following tests are carried out.

- The entire genome of the vector is sequenced at a passage level comparable to a production batch and the analytically determined sequence is compared to the theoretical sequence based on vector construction and available databases.
- Restriction enzyme analysis is performed on the vector DNA of the master seed lot, each working seed lot and a production batch. The viral DNA is extracted, purified and digested with sufficient resolution. The digested fragments are separated by gel electrophoresis or capillary electrophoresis and the observed restriction pattern is compared to the theoretical restriction pattern based on vector construction.

– A suitable number of isolated sub-clones are tested for expression of the genetic insert product(s) and biological activity at a passage level comparable to a production batch. Sub-clones giving lower levels of expression or biological activity need further characterisation.

Vector concentration. The titre of infectious vector or the concentration of vector particles in the master seed lot and each working seed lot are determined.

Extraneous agents (*2.6.16*). The master seed lot and each working seed lot comply with the tests for extraneous agents.

Replication-competent adenoviruses. Replication-competent adenoviruses are generated by homologous recombination between the recombinant viral DNA and the adenovirus sequences integrated into the genome of the complementation cells.

Detection of replication-competent adenoviruses is performed by a suitable method approved by the competent authority. It is generally performed by an infectivity assay on sensitive detector cell lines, which are not able to complement for the genes deleted from the vector. Other indicators of viral replication may be used as appropriate.

When replication-competent adenoviruses are not supposed to be present in the test sample, considering vector construction and cell lines used, at least 2, but preferably 3 or 4 successive passages are performed on the detector cell line, where applicable. Detection of a cytopathic effect at the end of the passages reveals the presence of replication-competent adenoviruses in the preparation. Positive controls are included in each assay to monitor its sensitivity.

When replication-competent adenoviruses are expected to be present in the test sample, plaque-assays or limit dilution assays on a detector cell line may be performed.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done in an area with a suitable containment level where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration, but it is preferable to have a substrate free from antibiotics during production. Unless otherwise justified and authorised, at no stage during production is penicillin or streptomycin used. A portion of the production cell cultures is set aside as uninfected cell cultures (control cells).

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

Identification. The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Vector concentration. The titre of infectious vector and the concentration of vector particles in single harvests are determined.

Extraneous agents (*2.6.16*). The single harvest complies with the tests for extraneous agents.

Control cells. Control cells comply with a test for identification (*5.2.3*) and a test for extraneous agents (*2.6.16*).

PURIFIED HARVEST

Several single harvests may be pooled before the purification process. The purification process is validated to demonstrate the satisfactory removal of impurities.

Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

Identification. The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Genomic integrity. Genomic integrity of the vector is verified by suitable methods such as restriction enzyme analysis.

Vector concentration. The titre of infectious vector and the concentration of vector particles in purified harvests are determined.

Residual host-cell protein. The concentration of residual host-cell protein is determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

Residual host-cell DNA. The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative polymerase chain reaction (PCR) is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual reagents. Where reagents are used during the production process, tests for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

Residual antibiotics. Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from general method *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

FINAL BULK

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*). It complies with the test for sterility.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for bovine serum albumin (when bovine serum is used to manufacture the vector) and replication-competent adenoviruses have been carried out with satisfactory results on the final bulk, they may be omitted on the final lot.

IDENTIFICATION

The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

TESTS

Osmolality (*2.2.35*): within the limits approved for the particular preparation.

pH (*2.2.3*): within the limits approved for the particular preparation.

Extractable volume (*2.9.17*). It complies with the test for extractable volume.

Residual moisture (*2.5.12*): within the limits approved for the particular freeze-dried preparation.

Bovine serum albumin: not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*), where bovine serum has been used during production.

Replication-competent adenovirus concentration: within the limits approved for the particular preparation.

Vector aggregates. Vector aggregates are determined by suitable methods (for example, light scattering).

Sterility (*2.6.1*). It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*): less than the limit approved for the particular preparation.

Thermal stability. Maintain samples of the vector final lot at a temperature and for a length of time that are adapted and authorised for the particular preparation. Determine the total infectious vector concentration after heating, as described below under Assay. Determine in parallel the vector concentration of a non-heated sample. The estimation of the difference between the total vector concentration without heating and after heating is within the limits approved for the particular preparation.

ASSAY

Vector particle concentration. Physical titration is performed by a suitable technique (for example, liquid chromatography, absorbance measurement or NAT (*2.6.21*)). Use an appropriate vector reference standard to validate each assay.

The vector particle concentration of the preparation to be examined is not less than the concentration stated on the label.

Infectious vector titre. Titrate the preparation to be examined by inoculation into cell cultures. Titrate an appropriate vector reference standard to validate each assay.

The assay is invalid if:

- the confidence interval ($P = 0.95$) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

Ratio of vector particle concentration to infectious vector titre: within the limits approved for the particular preparation.

Expression of the genetic insert product. The expression of the genetic insert product(s) is determined wherever possible, following inoculation of cell cultures with the particular preparation at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

Biological activity. Unless otherwise justified and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:

- the content of active substance;
- the recommended human dose, expressed in vector particle concentration;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

POXVIRUS VECTORS FOR HUMAN USE

DEFINITION

Poxvirus vectors for human use are freeze-dried or liquid preparations of recombinant poxviruses, genetically modified to transfer genetic material to human somatic cells *in vivo* or *ex vivo*.

PRODUCTION

VECTOR CONSTRUCTION

The general design of a poxvirus vector is currently as follows: the genetic insert is inserted downstream of a poxvirus promoter. This expression cassette is inserted into the poxvirus genome in such a manner that it interrupts a viral gene non-essential for replication or is positioned between 2 virus open reading frames.

In most strategies used so far for the construction of the vector, the expression cassette is first inserted within the target site of a virus DNA fragment cloned into a bacterial plasmid. The plasmid is then introduced into host cells, cultured *in vitro*, which are simultaneously infected with the parental poxvirus. DNA recombination occurs within the infected cells, between homologous sequences in the viral genome and viral sequences in the plasmid so as to transfer the genetic insert into the targeted site of the viral genome. The correct targeting of the inserted DNA is checked by restriction-enzyme mapping, NAT (*2.6.21*) and sequencing. Successive plaque-cloning steps are performed to purify the recombinant poxvirus from the mixture of parental and recombinant poxviruses. A variety of methods (for example, foreign marker genes, DNA hybridisation, immunological detection, phenotypic changes in the virus) are employed to facilitate recognition and/or selection of the recombinant poxvirus from the background of parental virus. Where foreign marker genes have been transiently employed, they are removed by appropriate methods from the final recombinant poxvirus.

An alternative strategy for creating poxvirus vectors begins with the *in vitro* construction of a full-length virus genome harbouring the expression cassette within a chosen target site. This recombinant genome is then introduced into host cells simultaneously infected with a helper poxvirus that is unable to multiply. The helper virus may be a poxvirus of the same species whose ability to multiply has been inactivated, or another poxvirus species that does not multiply in the host cells.

The construction of non-replicative poxvirus vectors relies on specific host cell lines or primary cells that are naturally permissive, or on host cell lines that have been modified to express an essential poxvirus gene. These cells fulfill the general requirements for the production of medicinal products (*5.2.3*) and do not allow the generation of replicative vectors.

VECTOR PRODUCTION

The production method shall have been shown to yield a vector of consistent quality. Unless otherwise justified and authorised, the vector in the final product shall have undergone no more passages from the master seed lot than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

The genetic and phenotypic stability of the vector at or beyond the maximum passage level used for production is assessed by suitable methods.

SUBSTRATE FOR VECTOR PROPAGATION

The vector is propagated under aseptic conditions in human diploid cells (*5.2.3*), in continuous cell lines (*5.2.3*) or in cultures of chick-embryo cells derived from a chicken flock free from specified pathogens (*5.2.2*). When the vector is propagated in a continuous cell line or in human diploid cells, a cell-bank system is established.

VECTOR SEED LOT

Production of the vector is based on a seed-lot system.

The strain of poxvirus used is identified by historical records that include information on its origin and its subsequent manipulation, notably deleted or modified regions. A detailed description of the genetic insert(s) and the flanking control regions is established, including the nucleotide sequence. The method by which the genetic insert is introduced into the vector is documented.

Only a seed lot that complies with the following requirements may be used for vector production.

Identification. The vector is identified in the master seed lot and each working seed lot by immunochemical methods (*2.7.1*) or NAT (*2.6.21*).

Genetic and phenotypic characterisation. The following tests are carried out.

- The entire genome of the vector is sequenced at a passage level comparable to a production batch and the analytically determined sequence is compared to the theoretical sequence based on vector construction and available databases.

- Restriction enzyme analysis is performed on the vector DNA of the master seed lot, each working seed lot and a production batch. The viral DNA is extracted, purified and digested with sufficient resolution. The digested fragments are separated by gel electrophoresis or capillary electrophoresis and the observed restriction pattern is compared to the theoretical restriction pattern based on vector construction.
- A suitable number of isolated sub-clones are tested for expression of the genetic insert product(s) and biological activity at a passage level comparable to a production batch. Sub-clones giving lower levels of expression or biological activity need further characterisation.
- The host range is verified by determining the replication properties of the vector and comparing them with that of the parental virus, at a passage level comparable to a production batch.

Infectious vector titre. The titre of infectious vector in the master seed lot and each working seed lot is determined.

Extraneous agents (*2.6.16*). The master seed lot and each working seed lot comply with the tests for extraneous agents, except where cytopathic strains cannot be neutralised and the vector causes interference. Where a test cannot be performed, carry out a suitable validated alternative.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area with a suitable containment level where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration, but it is preferable to have a substrate free from antibiotics during production. Unless otherwise justified and authorised, at no stage during production is penicillin or streptomycin used. A portion of the production cell culture is set aside as uninfected cell cultures (control cells).

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

Identification. The vector is identified by immunochemical methods (*2.7.1*) or NAT (*2.6.21*).

Infectious vector titre. The titre of infectious vector in single harvests is determined.

Extraneous agents (*2.6.16*). The single harvest complies with the tests for extraneous agents, except where cytopathic strains cannot be neutralised and the vector causes interference. Where a test cannot be performed, carry out a suitable validated alternative.

Control cells. If human diploid cells or a continuous cell line are used for production, the control cells comply with a test for identification (*5.2.3*). They comply with the tests for extraneous agents (*2.6.16*).

PURIFIED HARVEST

Processing is carried out under aseptic conditions. Several single harvests may be pooled before the purification process. The harvest is first clarified to remove cells and then, where applicable, purified by validated methods.

Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

Identification. The vector is identified by immunochemical methods (*2.7.1*) or NAT (*2.6.21*).

Genomic integrity. Genomic integrity of the vector is verified by suitable methods such as restriction enzyme analysis.

Infectious vector titre. The titre of infectious vector in purified harvests is determined.

Ratio of infectious vector titre to total protein concentration. The total protein concentration is determined by a suitable method (*2.5.33*). The ratio between infectious vector titre and total protein concentration is calculated.

Residual host-cell protein. The concentration of residual host-cell protein is determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

Residual host-cell DNA. The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual reagents. Where reagents are used during the production process, tests for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

Residual antibiotics. Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from general method *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

FINAL BULK

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*). It complies with the test for sterility.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the test for bovine serum albumin (when bovine serum is used to manufacture the vector) has been carried out with satisfactory results on the final bulk, it may be omitted on the final lot.

IDENTIFICATION

The vector is identified by immunochemical methods (*2.7.1*) or NAT (*2.6.21*).

TESTS

Osmolality (*2.2.35*): within the limits approved for the particular preparation.

pH (*2.2.3*): within the limits approved for the particular preparation.

Extractable volume (*2.9.17*). It complies with the test for extractable volume.

Residual moisture (*2.5.12*): within the limits approved for the particular freeze-dried preparation.

Bovine serum albumin: not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*), where bovine serum has been used during production.

Sterility (*2.6.1*). It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*): less than the limit approved for the particular preparation.

Thermal stability. Maintain samples of the vector final lot at a temperature and for a length of time that are adapted and authorised for the particular preparation. Determine the total infectious vector concentration after heating, as described below under Assay. Determine in parallel the vector concentration of a non-heated sample. The estimation of the difference between the total vector concentration without heating and after heating is within the limits approved for the particular preparation.

ASSAY

Infectious vector titre. Titrate at least 3 vials of the preparation to be examined by inoculation into cell cultures. Titrate a vial of an appropriate vector reference standard to validate each assay.

The vector titre of the preparation to be examined is not less than the minimum titre stated on the label.

The assay is invalid if:

- the confidence interval ($P = 0.95$) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

Expression of the genetic insert product. The expression of the genetic insert product(s) is determined, wherever possible, following inoculation of cell cultures with the particular preparation at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

Biological activity. Unless otherwise justifed and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:

- the minimum vector titre per human dose;
- the recommended human dose;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

RETROVIRIDAE-DERIVED VECTORS FOR HUMAN USE

DEFINITION

Retroviridae-derived vectors for human use are liquid or freeze-dried preparations of recombinant retroviruses, lentiviruses or spumaviruses, genetically modified to render them replication-incompetent, which are used to transfer genetic material to human somatic cells *in vivo* or *ex vivo*. This section applies to non-replicative vectors.

PRODUCTION

VECTOR CONSTRUCTION

A typical vector is composed of:

- the minimal genome from parental viruses containing the structural genetic elements shown to be indispensable for vector production;
- the required regulatory genetic elements for expression of the genetic insert (for example, long terminal repeats (LTRs));
- the genetic insert.

The vector construction is designed to prevent the generation of replication-competent viruses.

VECTOR PRODUCTION

The production method shall have been shown to yield a vector of consistent quality. Unless otherwise justified and authorised, the packaging or producer cells shall have undergone no more cell doublings from the master cell bank (MCB) than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy. The genetic and phenotypic stability of the packaging or producer cells at or beyond the maximum number of cell doublings used for production is assessed by suitable methods.

Vectors are produced in continuous cell lines (*5.2.3*) using a cell-bank system. Production may involve either stably or transiently transfected cells.

DEFINITIONS

Packaging cells: a source cell line stably transfected with plasmids containing the viral genes necessary for production of empty vector particles: *gag*, *pol*, *env*.

Producer cells: contain the viral genes and expression cassette necessary for vector production.

- In stable production systems, the producer cells are generated by stable transfection of the packaging cell line by a transfer plasmid containing the sequence of interest.
- In transient production systems, the producer cells are generated at the time of manufacture by simultaneous transfection of the source cell line with both the viral genes and the transgene expression plasmid, or by transient transfection of the packaging cell line by a transfer plasmid containing the sequence of interest.

PRODUCTION INTERMEDIATES

Packaging cells

Copy number. The genomic DNA is isolated and purified from a known number of cells and the *gag*, *pol* and *env* genes copy number is determined by a suitable method such as quantitative PCR (*2.6.21*).

Sequence integrity of the viral genes. Complete nucleotide sequencing of the inserted viral genes and their regulatory elements is performed.

Genetic stability. Genetic stability of the packaging cells is verified at or beyond the maximum number of cell doublings used for production.

Plasmids

Production of the vector requires the use of plasmid intermediates. For each plasmid DNA used during production, a complete description is established, including identification, source, means of isolation and nucleotide sequence. The source and function of component parts of these plasmids, such as the origin of replication, viral and eukaryotic promoters and genes encoding selection markers, are documented.

Production of plasmid intermediates is based on a bacterial cell-bank system. The MCB complies with the requirements of the section Bacterial cells used for the manufacture of plasmid vectors for human use. Plasmids are purified by suitable techniques.

Only plasmid batches that comply with the following requirements may be used for the production of the vector.

Identification. Plasmids are identified by restriction enzyme analysis, sequencing or NAT (*2.6.21*).

Genomic integrity. Genomic integrity of the plasmid is verified by suitable methods such as restriction enzyme analysis of the viral genes, the genetic insert and their respective regulation elements.

Plasmid DNA. The following indications are given as examples.

DNA concentrations greater than 500 ng/mL may be determined using absorbance measurement at 260 nm. A 50 µg/mL double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/mL are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

Residual host-cell DNA. The content of residual host-cell DNA is determined using a suitable method, unless the production process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Bacterial endotoxins (*2.6.14*): less than the limit approved for the particular preparation.

Sterility (*2.6.1*). It complies with the test for sterility.

Producer cells used in a stable production system

Copy number. The copy number of the integrated viral genes and expression cassette is determined by a suitable method.

Genetic stability. Genetic stability of the producer cells at or beyond the maximum number of cell doublings used for production is confirmed.

Sequence integrity of the viral genes and expression cassette. Complete nucleotide sequencing of the inserted viral genes, the expression cassette and their respective regulation elements (for example, LTRs, promoters, psi sequence, polyadenylation signal) is performed.

Replication-competent viruses. The detection of replication-competent viruses is performed by suitable methods. Detection may be based on a co-cultivation for several cell doublings of the producer cells with a permissive cell line, followed by detection (either by observation of a cytopathic or haemadsorbing effect on indicator cells like PG4 S+L-, by detection using indicator cell lines by NAT (*2.6.21*) or by marker-rescue assay). Positive controls are included in each assay to monitor its sensitivity. No replication competent viruses are found.

PRODUCTION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done in an area with a suitable containment level where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media must be qualified. It is preferable to have a substrate free from antibiotics during production. Unless otherwise justified and authorised, at no stage during production is penicillin or streptomycin used.

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

Identification. The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Vector concentration. The titre of infectious vector and/or the concentration of vector particles in single harvests is determined.

Extraneous agents. Each single harvest complies with the tests for extraneous agents (*2.6.16*).

Control cells. Where a transient production process is used, control cells comply with a test for identification (*5.2.3*) and a test for extraneous agents (*2.6.16*).

PURIFIED HARVEST

Several single harvests may be pooled before purification. Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

Identification. The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Genomic integrity. Genomic integrity of the vector is verified by a suitable method.

Vector concentration. The infectious particle titre is determined by a suitable method, for example infection of permissive cells followed by quantitative NAT (for example, quantitative PCR), Southern blot or protein expression. For lentivirus vectors, the physical titre is measured, for example by ELISA (p24).

Replication-competent viruses. Detection of replication-competent viruses is performed by suitable methods. It is generally performed by amplification on permissive cells followed by NAT (*2.6.21*), by detection of a viral antigen (for example, p24 by ELISA) or by marker-rescue assay. Positive controls are included in each assay to monitor its sensitivity.

Detection of replication-competent viruses is performed on the purified harvest or on the final lot. No replication-competent viruses are found.

Residual host-cell protein. The concentration of residual host-cell protein is determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

Residual host-cell DNA. The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual reagents. Where reagents are used during production, tests for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

Residual antibiotics. Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from general method *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

Residual plasmids. Where a transient production process is used, the concentration of residual contaminating plasmids must be quantified.

FINAL BULK

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*). It complies with the test for sterility.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for bovine serum albumin (when bovine serum is used to manufacture the vector) and replication-competent viruses have been carried out with satisfactory results on the purified harvest, they may be omitted on the final lot.

IDENTIFICATION

Retroviridae-derived vectors are identified by NAT (*2.6.21*), immunochemical methods (*2.7.1*) or restriction enzyme analysis.

TESTS

Osmolality (*2.2.35*): within the limits approved for the particular preparation.

pH (*2.2.3*): within the limits approved for the particular preparation.

Extractable volume (*2.9.17*). It complies with the test for extractable volume.

Residual moisture (*2.5.12*): within the limits approved for the particular freeze-dried preparation.

Bovine serum albumin: where bovine serum has been used during production, not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*).

Replication-competent viruses. Detection of replication-competent viruses is performed by suitable methods. It is generally performed by amplification on permissive cells followed by NAT (*2.6.21*), detection of a viral antigen (for example, p24 by ELISA) or marker-rescue assay. Positive controls are included in each assay to monitor its sensitivity.

Detection of replication-competent viruses is performed on the purified harvest or on the final lot. No replication-competent viruses are found.

Sterility (*2.6.1*). It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*): less than the limit approved for the particular preparation.

ASSAY

Vector-particle concentration. Physical titration is performed by a suitable technique (for example, immunochemical methods (*2.7.1*) or NAT (*2.6.21*)). Use an appropriate vector reference standard to validate each assay.

Infectious vector titre. Titrate the preparation to be examined by inoculation into cell cultures. Titrate an appropriate vector reference standard to validate each assay.

The infectious vector titre of the preparation to be examined is not less than the minimum titre stated on the label.

The assay is invalid if:

- the confidence interval ($P = 0.95$) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

Ratio of vector-particle concentration to infectious vector titre: within the limits approved for the particular product, where applicable.

Expression of the genetic insert product. The expression of the genetic insert product(s) is determined wherever possible, following inoculation of cell cultures with the product at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

Biological activity. Unless otherwise justified and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:

- the minimum vector titre per human dose;
- the recommended human dose;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

ADENO-ASSOCIATED-VIRUS VECTORS FOR HUMAN USE

DEFINITION

Adeno-associated-virus (AAV) vectors for human use are freeze-dried or liquid preparations of recombinant AAV (rAAV), genetically modified to transfer genetic material to human somatic cells *in vivo* or *ex vivo*.

PRODUCTION

VECTOR CONSTRUCTION

rAAV vectors are developed by replacement of the *rep* and *cap* genes with the genetic insert of interest. The inverted terminal repeat (ITR) sequences are retained in the rAAV vector since these are the only AAV sequences absolutely required in *cis* to function as the origin of replication. The *rep* and *cap* genes are required in *trans* and function for replication and packaging respectively. In summary, the rAAV vector contains the ITRs and the genetic insert.

Wild-type AAV normally replicate only in the presence of helper functions, provided by a coinfecting adenovirus or herpes virus. Therefore, there are different approaches to the manufacture of an AAV vector. The manufacturing strategy chosen is designed to minimise the risk for the generation of replication-competent AAV vectors and effectively eliminate helper viruses that might be used during production.

VECTOR PRODUCTION

The production method shall have been shown to yield a vector of consistent quality and stability.

To produce AAV vectors, several strategies are currently used, for example:

- transient co-transfection of a cell line with plasmids containing the ITRs and the genetic insert, *rep* and *cap* genes and helper functions;
- infection with a replication-deficient helper virus of a producer cell line harbouring *rep* and *cap* genes, the ITRs and the genetic insert;
- infection of a permissive cell line with 1 or several production viruses encoding *rep* and/or *cap* and/or the genetic insert and the ITRs, and that may or may not provide helper functions (helper viruses and baculoviruses, respectively).

Depending on the strategy used to produce AAV vectors, different production intermediates are required (plasmids, viruses used for production, packaging cells).

The occurrence of replication-competent AAV may be significant when regions of homology exist between the genomes of the production intermediates and the rAAV vector. This occurrence may be minimised by reducing the homology between these genomes to a minimum. The use of production intermediates with no sequence homology is recommended for production.

The genetic and phenotypic stability of the vector at or beyond the maximum number of passage levels used for production is assessed by suitable methods.

PRODUCTION INTERMEDIATES

Viruses used for production and the rAAV vector are produced in continuous cell lines (*5.2.3*) using a seed lot and a cell-bank system.

Packaging and producer cells

Copy number. The genomic DNA is isolated and purified from a known number of cells and the copy number of the inserted viral genes and of the expression cassette is determined by a suitable method such as quantitative PCR (*2.6.21*).

Sequence integrity of the viral genes and expression cassette. Complete nucleotide sequencing of the inserted viral genes, of their regulatory elements and where applicable, of the expression cassette is performed.

Genetic stability. Genetic stability of the cells is verified at or beyond the maximum number of cell doublings used for production.

Wild-type AAV. The absence of wild-type AAV is verified using NAT (*2.6.21*).

Plasmids

Production of the AAV vector by transient co-transfection requires the use of plasmid intermediates. For each plasmid DNA used during production, a complete description is established, including identification, source, means of isolation and nucleotide sequence. The source and function of component parts of these plasmids, such as the origin of replication, viral and eukaryotic promoters and genes encoding selection markers, are documented.

Production of plasmid intermediates is based on a bacterial cell-bank system. The master cell bank complies with the requirements of the section Bacterial cells used for the manufacture of plasmid vectors for human use. Plasmids are purified by suitable techniques.

Only plasmid batches that comply with the following requirements may be used for the production of the AAV vector.

Identification. Plasmids are identified by restriction enzyme analysis, sequencing or NAT (*2.6.21*).

Genomic integrity. Genomic integrity of the plasmid is verified by suitable methods such as restriction enzyme analysis of the region corresponding to *rep*, *cap* and the expression cassette.

Plasmid DNA. The following indications are given as examples.

DNA concentrations greater than 500 ng/mL may be determined using absorbance measurement at 260 nm. A 50 µg/mL double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/mL are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

Residual host-cell DNA. The content of residual host-cell DNA is determined using a suitable method, unless the production process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Bacterial endotoxins (*2.6.14*) : less than the limit approved for the particular preparation.

Sterility (*2.6.1*). It complies with the test for sterility.

Viruses used for production

Their production is based on a seed lot and a cell-bank system or, where applicable (for example, for baculoviruses), on a transient system. The strain of virus used is identified by historical records that include information on its origin and its subsequent manipulation, notably deleted or modified regions. The nucleotide sequence of the viruses is documented.

Only a virus used for production that complies with the following requirements may be used.

Identification. Viruses used for production are identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Genomic integrity. Genomic integrity of the virus used for production is verified by suitable methods such as restriction enzyme analysis. Where viruses are modified to express *rep* or *cap* genes or the expression cassette, genomic integrity is assessed by sequencing or by quantitative PCR of these regions.

Genetic stability. Where a stable production system is used, genetic stability is verified at or beyond the maximum number of cell doublings used for production.

Virus titration. The infectious titre is determined by a suitable assay.

Wild-type AAV. Where applicable, the absence of wild-type AAV in helper virus seed lots is verified using NAT (*2.6.21*).

Replication-competent viruses. Detection of replication-competent viruses is performed by suitable methods. No replication-competent viruses are found.

Extraneous agents (*2.6.16*). It complies with the test for extraneous agents. In addition, detection of potential contamination with specific insect viruses is required where applicable.

PRODUCTION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done in an area with a suitable classified space with appropriate containment level where no other cells, viruses or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production, and at no stage during production is penicillin or streptomycin used. A portion of the production cell cultures is set aside as uninfected cell cultures (control cells).

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

Identification. The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Vector concentration. The titre of infectious vector and the concentration of vector particles in single harvests are determined.

Extraneous agents (*2.6.16*). The single harvest complies with the tests for extraneous agents.

Control cells. Control cells comply with a test for identification (*5.2.3*) and a test for extraneous agents (*2.6.16*) and specific insect viruses, where insect cell lines are used for production.

PURIFIED HARVEST

Several single harvests may be pooled before the purification process. The purification process is validated to demonstrate satisfactory removal of impurities.

Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

Identification. The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Genetic characterisation. The following tests are carried out.

- The entire genome of the vector is sequenced for a suitable number of production runs at the level of the purified harvest or final bulk and the analytically determined sequence is compared to the theoretical sequence based on vector construction and available databases.
- Genomic integrity is checked on the vector DNA. PCR analysis may be used.

Vector concentration. The titre of infectious vector and the concentration of vector particles are determined.

Residual viruses used for production. Residual viruses used for production are assessed by plaque assays or tissue culture infective dose 50 ($TCID_{50}$) on permissive cell lines or by quantitative PCR, according to the production system used.

Residual proteins. The concentrations of residual host-cell and/or viral proteins are determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

Residual DNA. The content of residual producer-cell DNA and of residual DNA from intermediates such as plasmids and production viruses where applicable, is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual reagents. Where reagents are used during production, tests for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

Residual antibiotics. Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from general

method *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

FINAL BULK

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*). It complies with the test for sterility.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for bovine serum albumin (when bovine serum is used to manufacture the vector), replication-competent AAV and residual viruses used for production have been carried out with satisfactory results on the final bulk, they may be omitted on the final lot.

IDENTIFICATION

The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

TESTS

Osmolality (*2.2.35*): within the limits approved for the particular preparation.

pH (*2.2.3*): within the limits approved for the particular preparation.

Extractable volume (*2.9.17*). It complies with the test for extractable volume.

Residual moisture (*2.5.12*): within the limits approved for the particular freeze-dried product.

Bovine serum albumin: where bovine serum has been used during production, not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*).

Replication-competent AAV concentration: within the limits approved by the competent authority.

Detection of replication-competent AAV is performed by a replication assay on a permissive cell line previously infected with a helper virus and analysis of the replicative forms by Southern blot on low-molecular-weight DNA, or by detection of the *rep* gene by quantitative PCR.

Vector aggregates. Vector aggregates are determined by suitable methods (for example, light scattering).

Sterility (*2.6.1*). It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*): less than the limit approved for the particular preparation.

ASSAY

Vector-particle concentration. Vector-particle concentration is determined using a suitable method such as quantitative PCR by comparison with a standard curve obtained using the recombinant AAV plasmid or an AAV reference standard. This concentration is within the limits approved for the particular product.

Infectious vector titre. Titrate the preparation to be examined by inoculation into cell cultures. Titrate an appropriate vector reference standard to validate each assay.

The infectious vector titre of the preparation to be examined is not less than the minimum amount stated on the label.

The assay is invalid if:

- the confidence interval ($P = 0.95$) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

Ratio of vector-particle concentration to infectious vector titre: within the limits approved for the particular product.

Expression of the genetic insert product. The expression of the genetic insert product is determined wherever possible, following inoculation of cell cultures with the product at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

Biological activity. Unless otherwise justified and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:

- the content of active substance;
- the recommended human dose;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

METHODS OF ANALYSIS

TEXT N°	TITLE	PRODUCT TYPE(S)
2.6.1	Sterility	All vaccines[1].
5.1.6	Alternative methods for control of microbiological quality	All vaccines[2].
2.6.2	Mycobacteria	Vaccines using cell cultures or primary avian tissues for production.
2.6.7	Mycoplasmas	Vaccines using cell cultures or primary avian tissues for production.
5.1.3	Efficacy of antimicrobial preservation	Multi-dose vaccines containing a preservative.
2.6.14	Bacterial endotoxins	All vaccines[1].
5.1.10	Guidelines for using the test for bacterial endotoxins	All vaccines[1].
2.7.2	Microbiological assay of antibiotics	Where antibiotics are used during the production process[3], the residual antibiotic concentration may be determined by a microbiological assay adapted from chapter 2.7.2 or by other suitable methods (e.g. liquid chromatography).
2.6.34	Host-cell protein assays	For recombinant-protein based vaccines and purified viral vectored vaccines.
2.5.33	Total protein	Certain live attenuated viral vaccines and recombinant viral vectored vaccines that are less amenable to purification.

1 Unless otherwise justified and authorised, as described in the General Monograph Vaccines for human use (0153).

2 A comprehensive validation package, including the demonstration of equivalence with the compendial test, is a prerequisite when opting to use alternative microbiological methods for sterility.

3 It is preferable to have a production free from antibiotics. Unless otherwise justified, at no stage during production is penicillin or streptomycin used.

TEXT N°	TITLE	PRODUCT TYPE(S)
2.6.35	Quantification and characterisation of residual host-cell DNA	Vaccines produced in continuous cell lines[4].
2.2.29	Liquid chromatography	When the method is selected.
2.6.21	Nucleic acid amplification techniques	When the method is selected.
2.7.1	Immunochemical methods	When the method is selected.
2.7.24	Flow cytometry	When the method is selected.
2.2.1	Clarity and degree of opalescence of liquids	Vaccines in liquid forms, lyophilised vaccines after reconstitution.
2.2.2	Degree of coloration of liquids	Vaccines in liquid forms, lyophilised vaccines after reconstitution.
2.2.3	Potentiometric determination of pH	Vaccines in liquid forms, lyophilised vaccines after reconstitution.
2.2.35	Osmolality	Vaccines for parenteral administration.
2.5.12	Water: semi-micro determination	Lyophilised vaccines.
2.9.17	Test for extractable volume of parenteral preparations	Vaccines for parenteral administration.
2.9.20	Particulate contamination: visible particles	Vaccines in liquid forms, lyophilised vaccines after reconstitution.

4 See also General Chapter *5.2.3. Cell substrates for the production of vaccines for human use*

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corrected 7.7

2.6.1. STERILITY[(1)]

The test is applied to substances, preparations or articles which, according to the Pharmacopoeia, are required to be sterile. However, a satisfactory result only indicates that no contaminating micro-organism has been found in the sample examined in the conditions of the test.

PRECAUTIONS AGAINST MICROBIAL CONTAMINATION

The test for sterility is carried out under aseptic conditions. In order to achieve such conditions, the test environment has to be adapted to the way in which the sterility test is performed. The precautions taken to avoid contamination are such that they do not affect any micro-organisms which are to be revealed in the test. The working conditions in which the tests are performed are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.

CULTURE MEDIA AND INCUBATION TEMPERATURES

Media for the test may be prepared as described below, or equivalent commercial media may be used provided that they comply with the growth promotion test.

The following culture media have been found to be suitable for the test for sterility. Fluid thioglycollate medium is primarily intended for the culture of anaerobic bacteria; however, it will also detect aerobic bacteria. Soya-bean casein digest medium is suitable for the culture of both fungi and aerobic bacteria.

Fluid thioglycollate medium

L-Cystine	0.5 g
Agar	0.75 g
Sodium chloride	2.5 g
Glucose monohydrate/Glucose	5.5 g/5.0 g
Yeast extract (water-soluble)	5.0 g
Pancreatic digest of casein	15.0 g
Sodium thioglycollate or	0.5 g
Thioglycollic acid	0.3 mL
Resazurin sodium solution (1 g/L of resazurin sodium), freshly prepared	1.0 mL
Water R	1000 mL

pH after sterilisation 7.1 ± 0.2

Mix the L-cystine, agar, sodium chloride, glucose, water-soluble yeast extract and pancreatic digest of casein with the *water R* and heat until solution is effected. Dissolve the sodium thioglycollate or thioglycollic acid in the solution and, if necessary, add *1 M sodium hydroxide* so that, after sterilisation, the solution will have a pH of 7.1 ± 0.2. If filtration is necessary, heat the solution again without boiling and filter while hot through moistened filter paper. Add the resazurin sodium solution, mix and place the medium in suitable vessels which provide a ratio of surface to depth of medium such that not more than the upper half of the medium has undergone a colour change indicative of oxygen uptake at the end of the incubation period. Sterilise using a validated process. If the medium is stored, store at a temperature between 2 °C and 25 °C in a sterile, airtight container. If more than the upper one-third of the medium has acquired a pink colour, the medium may be restored once by heating the containers in a water-bath or in free-flowing steam until the pink colour disappears and cooling quickly, taking care to prevent the introduction of non-sterile air into the container. Do not use the medium for a longer storage period than has been validated.

Fluid thioglycollate medium is to be incubated at 30-35 °C.

For products containing a mercurial preservative that cannot be tested by the membrane-filtration method, fluid thioglycollate medium incubated at 20-25 °C may be used instead of soya-bean casein digest medium provided that it has been validated as described in growth promotion test.

Where prescribed or justified and authorised, the following alternative thioglycollate medium may be used. Prepare a mixture having the same composition as that of the fluid thioglycollate medium, but omitting the agar and the resazurin sodium solution, sterilise as directed above. The pH after sterilisation is 7.1 ± 0.2. Heat in a water-bath prior to use and incubate at 30-35 °C under anaerobic conditions.

Soya-bean casein digest medium

Pancreatic digest of casein	17.0 g
Papaic digest of soya-bean meal	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Glucose monohydrate/Glucose	2.5 g/2.3 g
Water R	1000 mL

pH after sterilisation 7.3 ± 0.2

Dissolve the solids in *water R*, warming slightly to effect solution. Cool the solution to room temperature. Add *1 M sodium hydroxide*, if necessary, so that after sterilisation the solution will have a pH of 7.3 ± 0.2. Filter, if necessary, to clarify, distribute into suitable vessels and sterilise using a validated process. Store at a temperature between 2 °C and 25 °C in a sterile well-closed container, unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated.

Soya-bean casein digest medium is to be incubated at 20-25 °C.

The media used comply with the following tests, carried out before or in parallel with the test on the product to be examined.

Sterility. Incubate portions of the media for 14 days. No growth of micro-organisms occurs.

Growth promotion test of aerobes, anaerobes and fungi. Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Suitable strains of micro-organisms are indicated in Table 2.6.1.-1.

Inoculate portions of fluid thioglycollate medium with a small number (not more than 100 CFU) of the following micro-organisms, using a separate portion of medium for each of the following species of micro-organism: *Clostridium sporogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*. Inoculate portions of soya-bean casein digest medium with a small number (not more than 100 CFU) of the following micro-organisms, using a separate portion of medium for each of the following species of micro-organism: *Aspergillus brasiliensis*, *Bacillus subtilis*, *Candida albicans*. Incubate for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi.

Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot.

The media are suitable if a clearly visible growth of the micro-organisms occurs.

(1) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

Table 2.6.1.-1. – *Strains of the test micro-organisms suitable for use in the growth promotion test and the method suitability test*

Aerobic bacteria	
Staphylococcus aureus	ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518, NBRC 13276
Bacillus subtilis	ATCC 6633, CIP 52.62, NCIMB 8054, NBRC 3134
Pseudomonas aeruginosa	ATCC 9027, NCIMB 8626, CIP 82.118, NBRC 13275
Anaerobic bacterium	
Clostridium sporogenes	ATCC 19404, CIP 79.3, NCTC 532, ATCC 11437, NBRC 14293
Fungi	
Candida albicans	ATCC 10231, IP 48.72, NCPF 3179, NBRC 1594
Aspergillus brasiliensis	ATCC 16404, IP 1431.83, IMI 149007, NBRC 9455

METHOD SUITABILITY TEST

Carry out a test as described below under Test for sterility of the product to be examined using exactly the same methods except for the following modifications.

Membrane filtration. After transferring the contents of the container or containers to be tested to the membrane add an inoculum of a small number of viable micro-organisms (not more than 100 CFU) to the final portion of sterile diluent used to rinse the filter.

Direct inoculation. After transferring the content of the container or containers to be tested (for catgut and other surgical sutures for veterinary use: strands) to the culture medium add an inoculum of a small number of viable micro-organisms (not more than 100 CFU) to the medium.

In both cases use the same micro-organisms as those described above under Growth promotion test of aerobes, anaerobes and fungi. Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

If clearly visible growth of micro-organisms is obtained after the incubation, visually comparable to that in the control vessel without product, either the product possesses no antimicrobial activity under the conditions of the test or such activity has been satisfactorily eliminated. The test for sterility may then be carried out without further modification.

If clearly visible growth is not obtained in the presence of the product to be tested, visually comparable to that in the control vessels without product, the product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. Modify the conditions in order to eliminate the antimicrobial activity and repeat the method suitability test.

This method suitability test is performed:

a) when the test for sterility has to be carried out on a new product;

b) whenever there is a change in the experimental conditions of the test.

The method suitability test may be performed simultaneously with the test for sterility of the product to be examined.

TEST FOR STERILITY OF THE PRODUCT TO BE EXAMINED

The test may be carried out using the technique of membrane filtration or by direct inoculation of the culture media with the product to be examined. Appropriate negative controls are included. The technique of membrane filtration is used whenever the nature of the product permits, that is, for filterable aqueous preparations, for alcoholic or oily preparations and for preparations miscible with or soluble in aqueous or oily solvents provided these solvents do not have an antimicrobial effect in the conditions of the test.

Membrane filtration. Use membrane filters having a nominal pore size not greater than 0.45 µm whose effectiveness to retain micro-organisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters, for example, for strongly alcoholic solutions. Specially adapted filters may be needed for certain products, e.g. for antibiotics.

The technique described below assumes that membranes about 50 mm in diameter will be used. If filters of a different diameter are used the volumes of the dilutions and the washings should be adjusted accordingly. The filtration apparatus and membrane are sterilised by appropriate means. The apparatus is designed so that the solution to be examined can be introduced and filtered under aseptic conditions; it permits the aseptic removal of the membrane for transfer to the medium or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

Aqueous solutions. If appropriate, transfer a small quantity of a suitable, sterile diluent such as a 1 g/L neutral solution of meat or casein peptone pH 7.1 ± 0.2 onto the membrane in the apparatus and filter. The diluent may contain suitable neutralising substances and/or appropriate inactivating substances for example in the case of antibiotics.

Transfer the contents of the container or containers to be tested to the membrane or membranes, if necessary after diluting to the volume used in the method suitability test with the chosen sterile diluent but in any case using not less than the quantities of the product to be examined prescribed in Table 2.6.1.-2. Filter immediately. If the product has antimicrobial properties, wash the membrane not less than 3 times by filtering through it each time the volume of the chosen sterile diluent used in the method suitability test. Do not exceed a washing cycle of 5 times 100 mL per filter, even if during the method suitability test it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. Transfer the whole membrane to the culture medium or cut it aseptically into 2 equal parts and transfer one half to each of 2 suitable media. Use the same volume of each medium as in the method suitability test. Alternatively, transfer the medium onto the membrane in the apparatus. Incubate the media for not less than 14 days.

Soluble solids. Use for each medium not less than the quantity prescribed in Table 2.6.1.-2 of the product dissolved in a suitable solvent such as the solvent provided with the preparation, water for injections, saline or a 1 g/L neutral solution of meat or casein peptone and proceed with the test as described above for aqueous solutions using a membrane appropriate to the chosen solvent.

Oils and oily solutions. Use for each medium not less than the quantity of the product prescribed in Table 2.6.1.-2. Oils and oily solutions of sufficiently low viscosity may be filtered without dilution through a dry membrane. Viscous oils may be diluted as necessary with a suitable sterile diluent such as isopropyl myristate shown not to have antimicrobial activity in the conditions of the test. Allow the oil to penetrate the membrane by its own weight then filter, applying the pressure or suction gradually. Wash the membrane at least 3 times by filtering through it each time about 100 mL of a suitable sterile solution such as 1 g/L neutral meat or casein peptone containing a suitable emulsifying agent at a concentration

Table 2.6.1.-2. – *Minimum quantity to be used for each medium*

Quantity per container	Minimum quantity to be used for each medium unless otherwise justified and authorised
Liquids	
– less than 1 mL	The whole contents of each container
– 1-40 mL	Half the contents of each container but not less than 1 mL
– greater than 40 mL and not greater than 100 mL	20 mL
– greater than 100 mL	10 per cent of the contents of the container but not less than 20 mL
Antibiotic liquids	1 mL
Insoluble preparations, creams and ointments to be suspended or emulsified	Use the contents of each container to provide not less than 200 mg
Solids	
– less than 50 mg	The whole contents of each container
– 50 mg or more but less than 300 mg	Half the contents of each container but not less than 50 mg
– 300 mg to 5 g	150 mg
– greater than 5 g	500 mg
Catgut and other surgical sutures for veterinary use	3 sections of a strand (each 30 cm long)

shown to be appropriate in the method suitability test, for example polysorbate 80 at a concentration of 10 g/L. Transfer the membrane or membranes to the culture medium or media or vice versa as described above for aqueous solutions, and incubate at the same temperatures and for the same times.

Ointments and creams. Use for each medium not less than the quantities of the product prescribed in Table 2.6.1.-2. Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1 per cent in isopropyl myristate as described above, by heating, if necessary, to not more than 40 °C. In exceptional cases it may be necessary to heat to not more than 44 °C. Filter as rapidly as possible and proceed as described above for oils and oily solutions.

Direct inoculation of the culture medium. Transfer the quantity of the preparation to be examined prescribed in Table 2.6.1.-2 directly into the culture medium so that the volume of the product is not more than 10 per cent of the volume of the medium, unless otherwise prescribed.

If the product to be examined has antimicrobial activity, carry out the test after neutralising this with a suitable neutralising substance or by dilution in a sufficient quantity of culture medium. When it is necessary to use a large volume of the product it may be preferable to use a concentrated culture medium prepared in such a way that it takes account of the subsequent dilution. Where appropriate, the concentrated medium may be added directly to the product in its container.

Oily liquids. Use media to which have been added a suitable emulsifying agent at a concentration shown to be appropriate in the method suitability test, for example polysorbate 80 at a concentration of 10 g/L.

Ointments and creams. Prepare by diluting to about 1 in 10 by emulsifying with the chosen emulsifying agent in a suitable sterile diluent such as a 1 g/L neutral solution of meat or casein peptone. Transfer the diluted product to a medium not containing an emulsifying agent.

Incubate the inoculated media for not less than 14 days. Observe the cultures several times during the incubation period. Shake cultures containing oily products gently each day. However when fluid thioglycollate medium is used for the detection of anaerobic micro-organisms keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

Catgut and other surgical sutures for veterinary use. Use for each medium not less than the quantities of the product prescribed in Table 2.6.1.-2. Open the sealed package using aseptic precautions and remove 3 sections of the strand for each culture medium. Carry out the test on 3 sections, each 30 cm long, cut off from the beginning, the centre and the end of the strand. Use whole strands from freshly opened cassette packs. Transfer each section of the strand to the selected medium. Use sufficient medium to cover adequately the material to be tested (20 mL to 150 mL).

OBSERVATION AND INTERPRETATION OF RESULTS

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 mL) of the medium to fresh vessels of the same medium and then incubate the original and transfer vessels for not less than 4 days.

If no evidence of microbial growth is found, the product to be examined complies with the test for sterility. If evidence of microbial growth is found the product to be examined does not comply with the test for sterility, unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product to be examined. The test may be considered invalid only if one or more of the following conditions are fulfilled:

a) the data of the microbiological monitoring of the sterility testing facility show a fault;

b) a review of the testing procedure used during the test in question reveals a fault;

c) microbial growth is found in the negative controls;

d) after determination of the identity of the micro-organisms isolated from the test, the growth of this species or these species may be ascribed unequivocally to faults with respect to the material and/or the technique used in conducting the sterility test procedure.

If the test is declared to be invalid it is repeated with the same number of units as in the original test.

If no evidence of microbial growth is found in the repeat test the product examined complies with the test for sterility. If microbial growth is found in the repeat test the product examined does not comply with the test for sterility.

Table 2.6.1.-3. – *Minimum number of items to be tested*

Number of items in the batch*	Minimum number of items to be tested for each medium, unless otherwise justified and authorised**
Parenteral preparations	
– Not more than 100 containers	10 per cent or 4 containers, whichever is the greater
– More than 100 but not more than 500 containers	10 containers
– More than 500 containers	2 per cent or 20 containers (10 containers for large-volume parenterals) whichever is less
Ophthalmic and other non-injectable preparations	
– Not more than 200 containers	5 per cent or 2 containers, whichever is the greater
– More than 200 containers	10 containers
– If the product is presented in the form of single-dose containers, apply the scheme shown above for preparations for parenteral administration	
Catgut and other surgical sutures for veterinary use	2 per cent or 5 packages whichever is the greater, up to a maximum total of 20 packages
Bulk solid products	
– Up to 4 containers	Each container
– More than 4 containers but not more than 50 containers	20 per cent or 4 containers, whichever is the greater
– More than 50 containers	2 per cent or 10 containers, whichever is the greater

* If the batch size is not known, use the maximum number of items prescribed.

**If the contents of one container are enough to inoculate the 2 media, this column gives the number of containers needed for both the media together.

APPLICATION OF THE TEST TO PARENTERAL PREPARATIONS, OPHTHALMIC AND OTHER NON-INJECTABLE PREPARATIONS REQUIRED TO COMPLY WITH THE TEST FOR STERILITY

When using the technique of membrane filtration, use, whenever possible, the whole contents of the container, but not less than the quantities indicated in Table 2.6.1.-2, diluting where necessary to about 100 mL with a suitable sterile solution, such as 1 g/L neutral meat or casein peptone.

When using the technique of direct inoculation of media, use the quantities shown in Table 2.6.1.-2, unless otherwise justified and authorised. The tests for bacterial and fungal sterility are carried out on the same sample of the product to be examined. When the volume or the quantity in a single container is insufficient to carry out the tests, the contents of 2 or more containers are used to inoculate the different media.

MINIMUM NUMBER OF ITEMS TO BE TESTED

The minimum number of items to be tested in relation to the size of the batch is given in Table 2.6.1.-3.

Guidelines on the test for sterility are given in general chapter 5.1.9.

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5.1.6. ALTERNATIVE METHODS FOR CONTROL OF MICROBIOLOGICAL QUALITY

The following chapter is published for information.

1. GENERAL INTRODUCTION

The objective of this chapter is to facilitate the implementation and use of alternative microbiological methods where this can lead to efficient microbiological control and improved assurance for the quality of pharmaceutical products.

The microbiological methods described in the European Pharmacopoeia have been used for over a century and these methods for detecting, enumerating and identifying micro-organisms still serve microbiologists well. Over the years, these methods have been invaluable for the production of microbiologically safe pharmaceutical products. However, these microbiological methods are slow, and in the case of sterility tests, results are not available before an incubation period of 14 days. Consequently, the results from these methods seldom enable proactive corrective action to be taken.

Alternative methods for the control of microbiological quality have shown potential for real-time or near real-time results with the possibility of earlier corrective action. These new methods, if validated and adapted for routine use, can also offer significant improvements in the quality of testing.

Alternative methods may be used for in-process samples of pharmaceutical products, particularly for the application of Process Analytical Technology (PAT), for environmental monitoring and for industrial utilities (e.g. production and distribution of water, steam etc.), thereby contributing to the quality control of these products.

In this chapter, alternative microbiological methods for pharmaceutical application are described. For each method, the basic principle is stated and the advantages and disadvantages of the method are discussed along with any critical aspects to be considered. Potential uses that may be envisaged based on the principles of the method concerned are given, but it is not intended to suggest that such applications have been realised or that the list provided is exhaustive.

It is not the intention of this chapter to recommend one method over another, nor is it the intention to provide an exclusive or exhaustive list of alternative methods that can be used for pharmaceutical microbiological control. The information herein may be used, however, in the process of choosing an alternative microbiological method as a supplement or as an alternative to pharmacopoeial microbiological methods and to give guidance on validation of the chosen method. If a suitable method is described in the Pharmacopoeia, this method is the reference method. In this rapidly developing field, other methods are likely to appear and the guidance offered herein may be equally applicable in these cases.

There are 3 major types of determination specific to microbiological tests:

- qualitative tests for the presence or absence of micro-organisms;
- quantitative tests for enumeration of micro-organisms;
- identification tests.

1-1. *QUALITATIVE TESTS FOR THE PRESENCE OR ABSENCE OF MICRO-ORGANISMS*

In conventional microbiological analysis, this type of test is characterised by the use of turbidity or other growth-related changes in a culture medium as evidence of the presence of viable micro-organisms in the test sample. The most common example of this test is the test for sterility (*2.6.1*). Other examples include those tests designed to evaluate the presence or absence of a particular type of viable micro-organism in a sample. The conventional sterility test may be replaced by, for example, tests based on bioluminescence or solid phase cytometry, gas detection or autofluorescence. Nucleic acid amplification techniques (NAT) (*2.6.21*) may also be used for the detection of mycoplasmas (*2.6.7*).

1-2. *QUANTITATIVE TESTS FOR ENUMERATION OF MICRO-ORGANISMS*

Membrane filtration and plate count methods are conventional methods used to estimate the number of viable micro-organisms present in a sample. The Most Probable Number (MPN) method is another example of such methods and was developed as a means of estimating the number of viable micro-organisms present in a sample not amenable to direct plating. Examples of alternative methods for enumeration include autofluorescence, flow cytometry, direct epifluorescent filter technique (DEFT) and solid phase cytometry.

1-3. *IDENTIFICATION TESTS*

Biochemical and morphological characterisation of an unknown micro-organism is the classical approach to identification. Recently developed methods have streamlined and automated aspects of this identification, especially in the areas of data handling, analysis and storage. Several alternative approaches that have been integrated into these methods include biochemical reactions, carbon substrate utilisation, characterisation of fatty acid composition, mass spectroscopy and Raman spectroscopy, restriction endonuclease banding patterns and the use of genome sequencing methods such as 16S rRNA gene sequence analysis for prokaryotes.

Traditional biochemical and phenotypic techniques have been shown to be less accurate and precise than genotypic methods.

Pure cultures are required for a precise identification and such cultures must be fresh and cultivated in appropriate media.

Databases are part of the systems and are included in the primary validation. As identification methods depend on the use of databases, the extent of coverage of the database with respect to the range of micro-organisms of interest must be taken into account during validation. Appropriate software allows customisation of the database, thereby allowing the user to add micro-organisms not previously included. This possibility must be considered during the validation.

2. GENERAL PRINCIPLES OF ALTERNATIVE METHODS

Alternative microbiological methods employ direct and indirect methods of detection; in some instances amplification of the signal is achieved by enrichment methods. In recognition of these differences, and for convenience within this chapter, alternative methods for the control of microbiological quality are divided into 3 categories:

- growth-based methods, where a detectable signal is usually achieved by a period of culture;
- direct measurement, where individual cells are differentiated and/or imaged;
- cell component analysis, where the expression of specific cell components offers an indirect measure of microbial presence and identification of micro-organisms.

In some instances, these distinctions are artificial, but enable a working classification to be created.

2-1. *GROWTH-BASED METHODS*

2-1-1. **General critical aspects of methods based on early detection of growth**

Such methods are critically dependent on microbial growth in order to provide an indication of the presence and/or number of micro-organisms. For the typically low levels of microbial contamination seen in pharmaceutical products, detection may take 24 h or longer. Increased sensitivity

can be achieved with filtered products. In this case, after filtration, the membrane filter is incubated in or on the medium and the result is expressed as presence or absence in the quantity corresponding to the filtered volume. These systems, if they use an incubation step in liquid media, do not offer quantitative information, but a presence/absence determination in the quantity analysed. Analysis of more than one sample quantity may offer a semi-quantitative estimation (limit test). The major benefit of early detection methods compared to classical methods is often the capacity to simultaneously process a large number of samples and the potential to obtain a result in a shorter time.

The methods described below can be used for quantitative, semi-quantitative or qualitative analyses. They are also non-destructive, therefore subsequent identification of the micro-organism is possible.

2-1-2. **Electrochemical methods**

Principles of measurement. Micro-organisms multiplying and metabolising in appropriate growth media produce highly charged ionic metabolites from weakly charged organic nutrients leading to the modification of electrical properties in such media. These changes in impedance (measured by conductance or capacitance) are monitored with electrodes included in the culture vessels and in contact with the culture medium. The measurable end-point is the time taken to detect a predetermined impedance change; for particular types of micro-organisms, the detection time is inversely proportional to the initial inoculum size. For yeasts and moulds, which only produce small changes in electrical impedance, an indirect measurement of conductance can be used. Direct measurement of capacitance can also be carried out.

Critical aspects. There is no direct relationship between the original microbial level and the detectable end-point.

Potential uses. Microbiological assay of antibiotics, efficacy of antimicrobial preservation and presence/absence testing.

2-1-3. **Measurement of consumption or production of gas**

Principles of measurement. Appropriate growth media is utilised by actively multiplying and metabolising micro-organisms, leading to the production of metabolites or the elimination of specific nutrients. These methods detect microbial growth either by changes in the electrical properties of a sensor in response to a change in gas composition or by colorimetric changes of a sensor in response to physico-chemical changes in the growth medium in contact with that sensor. The systems are based on non-destructive techniques which enable subsequent identification or strain typing of the micro-organisms. Bacteria and/or fungi may be grown in closed containers and continuous monitoring can be performed using automated instruments that measure gas evolution (e.g. CO_2) or consumption (e.g. O_2) as surrogate markers of microbial growth. Furthermore, the production of metabolites or elimination of nutrients can lead to changes in pH or redox potential. All of these changes can be measured either directly or indirectly as changes in colorimetric markers in the growth medium.

Critical aspects. There is no direct relationship between the original microbial level and the detectable end-point. The incubation temperature, the physiological state and type of micro-organism, the initial load and the algorithm for data processing can significantly affect the results or the time to detection.

Potential uses. Presence/absence testing of filterable or non-filterable samples (e.g. final drug products, in-process control samples, media fill or container closure integrity testing).

2-1-4. **Bioluminescence**

Principles of measurement. Adenosine triphosphate (ATP) is a well-documented marker of cell viability. In this method, ATP first needs to be released from the micro-organisms using an appropriate extractant, followed by an assay using the luciferin/luciferase enzyme system, which emits light in proportion to the ATP present. The signal-to-noise ratio can be increased by addition of ADP and converting this ADP into released ATP.

Qualitative method: micro-organisms are cultivated in liquid medium. The emitted light is measured with a bioluminometer and is expressed in relative light units (RLU) (e.g. bioluminescence in a tube or a well of a microtitre plate). The RLU obtained from the sample is compared with a pre-determined threshold value. The result is positive if the RLU obtained with the analysed sample exceeds the threshold value.

Quantitative method: micro-organisms are captured on a membrane and cultivated by incubation on agar medium. Using a charge coupled device (CCD) camera, the ATP released from microcolonies can be detected by light emission and a quantitative determination is possible.

Critical aspects. If the sample has a high level of bacterial contamination, the detection is rapid. For low levels of contamination, it is necessary to increase the number of micro-organisms using an incubation step in culture media (liquid or solid). The yield of ATP varies from one micro-organism to another and can depend on several factors including the species, the growth phase of the cell, the nutritional status, the cellular stress or the cellular age. Additional factors such as turbidity, sample colour or product matrix effects can also influence bioluminescence measurements. Extraction of ATP is generally a destructive process which should be considered with respect to any subsequent need for identification of detected micro-organisms.

Potential uses. Presence/absence testing of filterable or non-filterable samples (e.g. final drug products, in-process control samples, media fill), total aerobic microbial count (TAMC), environmental and water monitoring, testing for efficacy of antimicrobial preservation.

2-1-5. **Turbidimetry**

Principles of measurement. Microbial growth leads to detectable changes in medium opacity, which can be accurately quantified by optical density measurements at a specified wavelength. In its simplest form, such measurements are performed using a standard spectrophotometer, generally over a wavelength range of 420-615 nm. Alternative automated systems employ microtitre plate readers offering a continuous readout with early detection of optical density change.

Critical aspects. Attempts have been made to extrapolate the initial microbial contamination from the time to detection, but this is limited to healthy micro-organisms with reproducible growth characteristics.

Potential uses. By means of calibration graphs, determination of the inoculum size of microbial suspensions for use in pharmacopoeial tests. In automated mode, microbiological assay of antibiotics and testing for efficacy of antimicrobial preservation.

2-1-6. **Growth detection using selective and/or indicative media**

Principles of measurement. The ability to detect the presence of specific enzymes using suitable chromogenic substrates has led to the development of a large number of methods for the identification of micro-organisms employing either manual or automated techniques. The incorporation of such substrates into a selective or non-selective primary isolation medium can eliminate the need for further subculture and biochemical testing for the identification of certain micro-organisms. Consequently, chromogenic liquid or solid culture media are designed to reveal specific enzymatic activities for detection and differentiation of micro-organisms. In these particular media, defined substrates are introduced into the formulation and are metabolised by the specific cell enzyme of a given bacterium or fungus during growth. These substrates, which

are linked to coloured indicators, are chosen according to the diagnostic enzymatic activity sought. Furthermore, chromogenic broth can be used for early or improved detection of contamination (e.g. in media fill or broth-based detection methods).

The use of innovative media presents several advantages, namely improved discrimination of colonies in a mixed culture, ease of use and ease of interpretation. In addition, response times are shorter as the growth and identification of the micro-organism are simultaneous.

Critical aspects. Validation of the media must be undertaken carefully to ensure a combination of specificity, selectivity and robustness. The quality of the signal is based not only on the careful choice of the enzymes or indicators used as the basis of detection (as these enzymes may be present in different micro-organism genera), but also on the physico-chemical characteristics of the medium, e.g. pH.

Potential uses. Detection of specified micro-organisms and qualitative testing (e.g. media fill and container closure integrity testing) and quantitative testing (e.g. water testing).

2-2. *DIRECT MEASUREMENT*

2-2-1. **Solid phase cytometry**

Principles of measurement. Micro-organisms are stained for viability by exposure to a conjugated, initially non-fluorogenic, fluorophore. An intact cellular membrane is required to retain and accumulate the fluorophore within the cytoplasm. Inside metabolically-active microbial cells, the conjugate is enzymatically cleaved and the fluorescent derivative is released intracellularly. Micro-organisms are collected on a membrane filter either before or after viability staining.

Membrane surfaces retaining vital-stained cells are then scanned by a laser beam and epifluorescent excitation allows the detection of single, viable fluorescent micro-organisms. Appropriate software allows differentiation of viable micro-organisms from autofluorescent particles. The high sensitivity and rapidity of the method permit detection of microbial contaminants within a few hours. Total cell counts (viable and non-viable) can be obtained using fluorescent staining.

Critical aspects. Metabolically active, fastidious and viable non-culturable micro-organisms can all be detected. This may result in reappraisal of the microbial limits established for the samples under evaluation. Spores require initiation of germination to enable detection. Single cell detection may be achievable, but identification of isolates might not be possible. False positives may occur due to autofluorescent particles that can be difficult to differentiate from micro-organisms. Signal discrimination and enhancement can be aided by microcolony growth.

Potential uses. Rapid and sensitive method for the non-specific evaluation of microbial contamination.

2-2-2. **Flow cytometry**

Principles of measurement. Fluorophore-labelled micro-organisms can be detected in suspension as they pass through a flow cytometer. Viable micro-organisms can be differentiated from non-viable particles by use of a viability-indicating fluorophore (see 2-2-1). The cell suspension stream is dispersed into a narrow channel and exposed to a laser which excites the fluorophore. Micro-organisms and particles are then counted in different channels depending on whether or not they contain a fluorescent cell.

Critical aspects. Direct flow cytometry may be applied to the microbiological analysis of both filterable and non-filterable materials, and after possible enrichment in the case of the low contamination levels. It gives near real-time detection, but is not as sensitive as solid phase cytometry. To increase sensitivity for use in the pharmaceutical field, it is often necessary to add an incubation step in culture media, in which case the method becomes a combination of a growth-based method and a direct detection method. Particle size and number may have a significant effect on performance, and samples may require serial dilution. With the exception of filterability, similar considerations to those in solid phase cytometry apply. Clumping of bacteria can be a problem (e.g. *Staphylococcus aureus*).

Potential uses. In contrast to solid phase cytometry, this method offers the potential to detect and enumerate microbial contamination in materials containing particulate matter and if the material cannot be filtered. If a pre-incubation step is needed, the method becomes a qualitative determination.

2-2-3. **Direct epifluorescent filtration technique (DEFT)**

Principles of measurement. This technique may be considered a forerunner of solid phase cytometry. Micro-organisms, concentrated by filtration of the sample, are stained with a fluorescent dye (formerly acridine orange and now more commonly 4′,6-diamidino-2-phenylindole (DAPI)), that can be detected by epifluorescent illumination. Fluorescent vital staining techniques, as employed in solid phase cytometry (see 2-2-1), are amenable to DEFT, and fluorescent redox dyes such as 5-cyano-2,3-ditolyltetrazolium chloride (CTC) can be used to highlight respiring cells. Coupled with microscopy, the method allows rapid detection of micro-organisms with an absolute sensitivity that is dependent on the volume of product filtered and the number of fields of view examined. Semi-automated auto-focusing systems coupled to image analysis have served to improve the utility of this method. A modification of the principle involves sampling using an adhesive sheet (which permits collection of cells from surfaces), subsequent staining on the sheet itself, followed by direct observation using an epifluorescence microscope.

Critical aspects. The distribution of micro-organisms on the membrane affects method robustness. The intensity of fluorescence can be influenced by the staining process and the metabolic status of the micro-organisms. Fluorescence is not necessarily an indicator of viability. A brief period of culture on the filter surface prior to staining allows microcolony formation; these microcolonies stain readily, can be easily enumerated and are demonstrable evidence of viability.

Potential uses. DEFT is generally limited to low viscosity fluids, although pre-dilution or pre-filtration has occasionally been applied to viscous or particulate products. Monitoring of microbial contamination has been successfully applied to aqueous pharmaceuticals.

2-2-4. **Autofluorescence**

Principles of measurement. The presence of endogenous autofluorescent molecules and metabolites (e.g. NADPH, flavoproteins) within micro-organisms allows the early detection and quantitative enumeration of microcolonies or single cells. For direct measurements, the laser-induced autofluorescence of a single micro-organism is captured by a detector, while for growth-based systems, automated sequential imaging of the membrane surface on agar medium over the incubation period is employed and image overlay allows differentiation of growing microcolonies from fluorescent particulates. The emitted light is detected by a CCD camera. Non-destructive detection allows identification of contaminants at the end of the incubation period.

Critical aspects. For a non-growth based measurement, viable, but non-culturable, micro-organisms might be detected. It may be difficult to distinguish between culturable micro-organisms, viable but non-culturable micro-organisms and/or other particles.

Potential uses. Environmental monitoring, filterable in-process samples, water testing and product release for both sterile and non-sterile applications.

2-3. *CELL COMPONENT ANALYSIS*

2-3-1. **Phenotypic techniques**

2-3-1-1. *Immunological methods*

Principles of measurement. Antibody-antigen reactions can be employed to detect unique cellular determinants of specific micro-organisms. These reactions can be linked to agglutination phenomena and colorimetric or fluorimetric end-points, which offer both quantitative and qualitative detection. Enzyme-linked immunosorbent assays (ELISA) offer simple solid-phase methodologies.

Critical aspects. Immunological detection methods depend on the unique expression of specific identifiers, but do not necessarily demonstrate the presence of viable micro-organisms.

Potential uses. Detection and identification of specified micro-organisms.

2-3-1-2. *Fatty acid profiles*

Principles of measurement. The fatty acid composition of micro-organisms is stable, well conserved and shows a high degree of homogeneity within different taxonomic groups. The isolate is grown on a standard medium and harvested. The fatty acids are saponified, methylated and extracted, and the occurrence and amount of the resulting fatty acid methyl esters are measured using high-resolution gas chromatography. The fatty acid composition of an unknown isolate is compared with a database of known isolates for a possible match and identification.

Critical aspects. The use of fatty acid profiles for microbial identification requires a high degree of standardisation. It is critical for the fatty acid composition of microbial cells that isolates are grown using standardised media and standard incubation conditions. Standard conditions for operation of the gas chromatograph must also be employed, with frequent runs of calibration standards and known isolates being very important.

Potential uses. Identification or characterisation of environmental and product microbial contamination (for contaminant tracing and detection of specified micro-organisms).

2-3-1-3. *Fourier transform infrared (FTIR) spectroscopy*

Principles of measurement. A Fourier transformation of the infrared spectrum of whole micro-organisms gives a stable, recognisable pattern typical of the taxonomic groups of micro-organisms. The analysis of the FTIR pattern can be performed with commercially available instruments. The isolate is grown on a standard medium and harvested. Cell mass is transferred to a carrier, and the infrared spectrum is recorded. The Fourier transformation is calculated and the pattern is compared with a database of known isolates for a possible match and identification.

Critical aspects. The use of FTIR patterns for microbial identification requires a high degree of standardisation. It is critical for the FTIR pattern of microbial cells that isolates are grown using standardised media and standard incubation conditions. The cells must be in the same state of the growth cycle when analysed, and particular attention must be paid to this in the validation process.

Potential uses. Identification or characterisation of environmental and product microbial contamination (for contaminant tracing and detection of specified micro-organisms).

2-3-1-4. *Mass spectrometry*

Principles of measurement. Ionised particles released by exposing microbial isolates to a laser in a vacuum can be analysed by mass spectrometry, providing characteristic spectra. Similarly, intact microbial cells, when subject to intense ionisation under matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry, release a distinctive pattern of charged species. Such spectra can be compared with known profiles.

Critical aspects. The isolates must be cultured under standardised conditions prior to analysis.

Potential uses. Identification or characterisation of environmental and product microbial contaminants (for contaminant tracing and detection of specified micro-organisms).

2-3-1-5. *Biochemical assays based on physiological reactions*

Principles of measurement. Systems capable of performing biochemical assays based on physiological reactions are used for the identification of micro-organisms. In the presence of a pure colony, the five basic steps for these assays are preparation, inoculation, incubation, readings and interpretation. These steps are usually preceded by a description of the colony morphology, a differentiation test (e.g. Gram stain), a description of the cellular morphology and/or other early biochemical differentiation tests (e.g. oxidase, catalase, coagulase) in order to determine the appropriate testing protocol.

The Gram stain is often a key characteristic upon which further testing is based. Alternatives to the traditional staining method include the potassium hydroxide (KOH) string test, the aminopeptidase test, a fluorescent staining method and a limulus amoebocyte lysate (LAL) based assay. Test kits are available for the latter 3 methods. The fluorescent staining method requires a fluorescence microscope or a flow cytometer.

Microbial cell suspensions are tested using biochemical (assimilation or susceptibility) test kits (plates or strips). Anaerobic and aerobic micro-organisms develop characteristic reactions to selected biochemical substances. They are also known to utilise specific carbon, nitrogen, phosphorus and sulfur sources or to be inhibited by a specific concentration of an antimicrobial agent. The results are based on measurable changes (e.g. turbidity, chromogenic or fluorogenic reaction) due to the growth or inhibition of the micro-organism under investigation. Comparison of the metabolic and/or antimicrobial resistance profile with a database allows for identification of the culture. These methods can be performed manually or by semi- or fully automated instruments. Complementary tests can be performed in cases of poor discrimination. Subcultures can help in cases of indeterminate results.

Critical aspects. A fresh physiological culture is required. The performance of the system is also dependent on the selected phenotypic parameters, which must be stable, significant and in sufficient number.

Potential uses. Identification or characterisation of environmental and product microbial contamination (for contaminant tracing and detection of specified micro-organisms).

2-3-2. **Genotypic techniques**

Identification and detection of micro-organisms as well as characterisation of strains belonging to the same species may be achieved by direct detection of nucleotide target sequences that are unique for a particular microbial species or microbial group, and are targets of the genotypic (DNA or RNA-based) detection techniques. These detection techniques may be separated into 3 broad categories: direct hybridisation, nucleic acid amplification and genetic fingerprinting.

2-3-2-1. *Direct hybridisation*

General principles of measurement. DNA probes are short, labelled, single-strand segments of DNA that hybridise with a complementary region of microbial DNA or RNA. The probe or target DNA is usually labelled with either radioactive, fluorescent or chromogenic molecules in order to provide a hybridisation signal. Hybridisation assays include fluorescence *in situ* hybridisation (FISH) and microarray-based techniques.

General critical aspects. Hybridisation generally requires a large amount of the target DNA for analysis, which may result in lower detection sensitivity. The availability of suitable probes may be limited.

Potential uses. Due to the high specificity of the sequence-based hybridisation reaction, this method may be used for both detection and identification of micro-organisms.

2-3-2-2. *Nucleic acid amplification techniques (NAT)*

General principles of measurement. NAT relies on the reiteration of the DNA polymerisation process, leading to an exponential increase of a specific nucleic acid fragment. The polymerase chain reaction (PCR) is the most widely used method for target DNA amplification. In this cyclic process, a specific DNA fragment is copied by a thermostable DNA polymerase enzyme in the presence of nucleotides and oligonucleotide primers, previously designed to flank the target sequence and to hybridise with it (see also general chapter *2.6.21*). After PCR, the amplified nucleic acid targets can be analysed using several methods of post-amplification analysis: fragment size analysis in gel electrophoresis, DNA sequencing or specific detection by hybridisation with a fluorescent-labelled probe. Real-time PCR eliminates the need for further post-amplification processing and offers the additional advantage that the likelihood of cross-contamination is minimised. An important advantage of real-time PCR is the ability to quantify the starting amount of the DNA target sequence in the original sample, in contrast to conventional PCR techniques, which are based on end-point detection. Since the amount of PCR product detected at the beginning of the exponential phase of the amplification reaction correlates with the initial starting amount of the DNA target, modern real-time PCR techniques have been developed to measure this exponential phase of the reaction. Automated real-time PCR systems are commercially available. For identification of species, either species-specific probes or primers can be used.

RNA can also be amplified by both conventional and real-time PCR after transcription into cDNA using a reverse transcriptase enzyme. This technique is known as reverse transcriptase PCR (RT-PCR) and it enables detection and identification of RNA viruses or viable organisms. Alternatively, specific RNA-based amplification techniques, for example nucleic acid sequence-based amplification or transcription-mediated amplification, are available. Both techniques produce RNA amplicons, in contrast to PCR which only produces DNA amplicons, even when starting from an RNA target.

Types of target to be amplified. Regardless of the type of NAT used, the specificity of the test is determined by the target DNA sequence under evaluation. For identification/characterisation purposes, the 16S or 23S ribosomal RNA genes may be used as targets. The 16S rRNA gene is an evolutionary-conserved gene present in all bacterial species, and is a broad range target as it is a universal marker for bacterial detection. The 23S rRNA gene is not widely used as a single target, but the 16S-23S rRNA transcribed intergenic spacer regions can be employed to distinguish between certain closely related species and/or to identify subtypes. Alternative broad-range targets include the *groEL* and *tuf* genes. Apart from broad-range targets, species-specific sequences can be used as targets for micro-organism identification. Depending on the species, either specific surface antigens, virulence factors or genes which code toxins may be amplified to detect and identify micro-organisms.

General critical aspects:

- the target and the primers chosen must be specific for a particular micro-organism or group of micro-organisms;
- the sensitivity of the methods is highly dependent on the efficiency of the lysis protocol and how successfully the DNA targets can be purified and concentrated in the sample;
- the presence of inhibitors of the enzymatic process results in false negative reactions;
- the procedures are prone to cross-contamination from background DNA resulting in false positive results.

Depending on the aim, a choice must be made between amplification of either a DNA or an RNA target, as this target choice affects the correlation with viability. DNA targets are generally more widely used for identification purposes, but the use of DNA as a marker has the disadvantage that dead micro-organisms can also be detected. As mRNA is rapidly degraded in dead cells, it is considered a marker for viability. Furthermore, mRNA is the obligatory target for the identification of RNA viruses.

Critical aspects of (semi-) quantitative detection by real-time PCR. Quantification of the target requires generation of appropriate standards and the use of standardised procedures.

Critical aspects of RT-PCR. RNA is less stable compared to DNA, so it requires more attention during processing. Depending on the quality of the RNA isolation, the efficiency of the cDNA synthesis can vary. RT-PCR can be used to specifically detect RNA if DNA contamination of the RNA sample is low.

Critical aspects of using the 16S or 23S rRNA gene as a target for species identification. 16S rRNA gene sequencing is a valuable method for identification of bacteria provided that appropriate universal primers from databases are selected. Its discriminatory power depends on the variability and the length of the 16S rRNA gene within a certain species. Regarding the use of assays targeting the 16S-23S rRNA intergenic spacer regions, the choice of appropriate species-specific primers/probes is of critical importance due to the potential polymorphism of such regions.

Potential uses of NAT. Due to the high sensitivity and specificity of amplification techniques, they are suitable for both detection and identification of micro-organisms. Real-time PCR is needed for quantitative or semi-quantitative analysis of the target. Besides quantitative determinations, the real-time PCR technique allows simultaneous detection of multiple targets in a single sample, as long as appropriate primers and probes that allow for multiplexing are employed. The sequencing of different genes (e.g.16S rDNA, 23S rDNA, rpoB, Gyr) is best applied to the identification of micro-organisms.

2-3-2-3. *Genetic fingerprinting*

Principles of measurement. Genetic fingerprinting is the identification of a strain on the basis of its DNA profile (or RNA for RNA viruses). Individual DNA profiles may be different due to genetic diversity between strains of the same species, and the aim of the fingerprinting methods is to discriminate between these strains. The classical genetic fingerprinting technique characterises micro-organisms using restriction fragments of chromosomal DNA from bacterial and fungal genomes.

Different strains from the same species may exhibit different patterns and these differences are referred to as restriction fragment length polymorphisms (RFLPs). As cutting the chromosomal DNA with restriction enzymes generates too many fragment bands to be efficiently and accurately compared, several modifications of the conventional RFLP-based method have been developed. Examples of the kind of technologies used are ribotyping, pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). Several other fingerprinting methods use PCR to selectively amplify defined subsets of DNA restriction fragments from the entire genome, for example random amplified polymorphic DNA (RAPD) and variable number tandem repeats (VNTR).

Critical aspects. All fingerprinting techniques require that the micro-organism is present as a pure culture. Depending on the method, a preliminary enrichment cultivation step may be necessary if a defined quantity or a specific DNA

preparation is required for the test, e.g. AFLP and PFGE. The discriminatory power, the reproducibility, the expertise needed and the labour-burden vary among techniques. The major criticism of conventional RFLP analysis is the complexity of the banding patterns. The discriminatory power of ribotyping (based on patterns of rRNA genes) is less than that of PFGE (based on patterns of the whole genomic DNA) or some PCR-based methods, but it has the advantage that it can be a highly automated system. Although PFGE is one of the most highly discriminatory fingerprinting methods, it is time-consuming and technically demanding in the laboratory as it is not automated. It also requires the use of standardised protocols. AFLP has high reproducibility, but requires technical expertise and the interpretation of results may need automated computer analysis. The reproducibility of RAPD may be poor, so it must be performed in a standardised way.

Potential uses. Genetic fingerprinting methods are mainly used for strain discrimination (characterisation below species level). They are a powerful tool for investigating and tracing the source and the spread of microbial contamination.

3. VALIDATION OF ALTERNATIVE MICROBIOLOGICAL METHODS

3-1. *INTRODUCTION*

Validation, whilst subject to a variety of context-specific definitions, can be generally defined as a method to establish documented evidence that a process will consistently achieve its intended goal. Therefore, to validate an alternative microbiological method, it is essential to understand and define what the procedure is intended to achieve.

Typically, pharmaceutical microbiological methods use specific characteristics of micro-organisms as indicators or detection principles in order to determine microbiological quality. The information generally sought is presence/absence, number, viability and/or identity of micro-organisms in a given product or environment. Any given method will usually provide an indirect and conditional measure of microbiological quality. For example, the total number and viability of micro-organisms can be indicated by the number of colonies appearing under a certain set of conditions of sample preparation, cultivation and incubation; reproduction in classical microbiology is hence taken as the general indicator for viability. There are other parameters, however, that can be used as a viability measure, such as the level of ATP or the accumulation or metabolism of substrates in living cells. The results from different viability-indicating methods may not always be identical; micro-organisms may not be able to reproduce on a given medium, but may still accumulate and metabolise a substrate. Conversely, micro-organisms may be unable, at a given state of damage, to accumulate a substrate, but may still be able to recover and reproduce.

Similar considerations arise with the multiplicity of methods used for identification of micro-organisms. Therefore, while characterisation of the pattern of metabolic activity is frequently used for species identification, alternative methods also exist. Again, the outcomes obtained may not be fully consistent for the different identification methods, as one answer may be appropriate for the construction of a correct phylogenetic correlation tree, while another may be more useful in the context of pathogenicity or other property of the differentiated micro-organisms.

3-2. *VALIDATION PROCESS*

Two levels of validation must be envisaged for the application of alternative microbiological methods, namely primary validation and validation for the intended use. The supplier of the alternative technology typically performs primary validation of a method, whereas validation for the intended use, which is a verification of the suitability or applicability of the method in a given situation, must be seen as the responsibility of the user.

Where specific equipment is critical for the application of a method, the equipment, including computer hardware and software, must be fully qualified.

3-2-1. **Description of the technique**

In order to characterise a specific microbiological method, the principle of detection must be clearly described by the supplier. Through primary validation, the method must be fully detailed with respect to the conditions required for application, the materials and equipment needed and the expected signal. The user shall critically review the available information.

3-2-2. **Risk-benefit analysis**

For validation of specific alternative microbiological methods, it is critical that the purpose of the quality assurance procedure is precisely outlined, as this defines the type and depth of information needed. The information obtained by, and the limitations of, the pharmacopoeial method and the alternative method must be considered and compared in a risk-benefit analysis.

The risk level in adopting an alternative method varies depending on the technology considered, the methodology it replaces, the nature of the measurements taken (qualitative, quantitative or identification), the particular product or process attribute being evaluated, the location of the measurement in the manufacturing process chain and various other factors.

Risk analysis tools may be utilised in order to determine which alternative method is to be implemented, to assist in the justification of its implementation or to better understand the impact of implementation on production and/or product quality. An alternative method can be justified for use if the information obtained gives a scientifically sound measure of microbiological quality, and if the limitations of the method are not more severe than those of the pharmacopoeial method.

3-2-3. **Primary validation**

The supplier, using a panel of test micro-organisms appropriate for the intended use, must characterise the principle of detection. Depending on the type of alternative method, relevant validation criteria shall be selected from those listed below:

- prerequisite treatment of sample or micro-organisms;
- type of response;
- specificity;
- detection limit;
- quantitation limit;
- range;
- linearity;
- accuracy and precision;
- robustness of the method in a model system.

3-2-4. **Validation for the intended use**

Validation for the intended use should encompass the entire process, from the decision to change any aspects of a microbiological testing programme to on-going routine use. It should consist of the following phases:

- user requirement specification (URS);
- design qualification (DQ);
- installation qualification (IQ);
- operational qualification (OQ);
- performance qualification (PQ).

The supplier and user have different tasks to perform with regard to the validation and implementation of an alternative method. These tasks are summarised in Table 5.1.6.-1.

Table 5.1.6.-1 – *Tasks to be undertaken during the validation process*

Activity	Normally carried out by	
	Supplier	User
Primary validation	+	-(1)
URS (instrument, application)	-	+
Description of the technique	+	-(2)
Risk benefit analysis	-(3)	+
Design qualification (DQ)	-	+
Installation qualification (IQ)	-(4)	+
Operational qualification (OQ)	-(4)	+
Performance qualification (PQ):		
- verification of primary validation data given by the supplier;	-	+
- verification for the intended use (e.g. sterility testing, TAMC/TYMC, …);	-	+
- method suitability test	-	+

(1) The user performs primary validation if they employ the alternative method for a use other than that defined by the supplier.

(2) The user shall critically review information provided by the supplier.

(3) As part of commercialisation, the supplier may list advantages of the alternative method over pharmacopoeial techniques.

(4) IQ/OQ for complex equipment, IQ/OQ is often outsourced to supplier.

3-2-4-1. *User requirement specification (URS)*

The URS describes the functions that the method must be capable of performing and will form the basis of the method selection process. It is an essential document, as acceptance testing will be based on the requirements detailed therein. It is important to consider data management capabilities at this stage, particularly within a regulatory context. The URS shall at least address the following items:

- application of the instrument:
  - the type of analysis to be performed (e.g. quantitative, semi-quantitative, qualitative or identification).
- detection limit or quantitation limit (sensitivity):
  - the detection limit may be linked to time to detection (TTD);
  - the required level of sensitivity, which will depend on the current specification, the dilution regime and the test sample size for the existing test method under replacement.
- specificity:
  - the ability of the alternative test method to selectively detect the micro-organisms or classes of micro-organisms; this should be based on historical data generated from the pharmacopoeial test method and complemented by information from the supplier of the alternative method;
  - the ability to detect only the required viable micro-organisms;
  - for identification methods, the extent of coverage of the database with respect to the range of micro-organisms of interest.
- number and type of samples:
  - the nature of samples to be tested and the manufacturing output per batch or work-shift.
- time to detection (TTD) or time to result (TTR):
  - the TTD or TTR is an important attribute for alternative microbiological methods; for monitoring purposes, a relatively short TTD (e.g. a few hours) allows corrective actions to be taken at an early stage; for quality control purposes, a short TTD may be less critical.
- data management capabilities:
  - the new instrumentation may need to have laboratory information management system (LIMS) interface capability and external server compatibility, and the data management tools should be defined; evidence of software validation and functional testing reports will be required to support each part of the software and firmware functions.

3-2-4-2. *Design qualification (DQ)*

The DQ provides documented evidence that the design of any associated equipment is suitable for correct performance of the method. Most alternative method systems are based on commercial off-the-shelf equipment. The DQ is most suitably performed, therefore, by the instrument developer/manufacturer. Nevertheless, the user shall verify that the equipment meets the specifications laid down in the URS for the intended application.

3-2-4-3. *Installation qualification (IQ)*

The IQ provides documented evidence that the equipment has been provided and installed in accordance with its specifications.

3-2-4-4. *Operational qualification (OQ)*

The OQ provides documented evidence that the installed equipment operates within predetermined limits when used in accordance with its operational procedures.

3-2-4-5. *Performance qualification (PQ)*

The PQ provides documented evidence that the method, with the equipment installed and operated according to operational procedures, consistently performs in accordance with predetermined criteria and thereby yields correct results for the method. This is typically done with a panel of micro-organisms (e.g. pharmacopoeial test strains, in-house isolates or stressed/slow-growing micro-organisms). This assures that the conditions employed by the user laboratory make it possible to satisfy the criteria described by the supplier of the method in the model system used for the primary validation.

Verification of primary validation data given by the supplier (see 3-2-3). The method is verified using the panel of test micro-organisms given by the corresponding pharmacopoeial chapter. The alternative method must be applied according to the specified procedure of the supplier, without the samples to be analysed under the responsibility of the user, and must be shown to give comparable results as characterised in the model system used by the supplier.

Verification for the intended use (e.g. sterility testing, total aerobic microbial count (TAMC)/total combined yeasts/moulds count (TYMC), etc). The following points, where applicable, should be addressed:

- compatibility of the response with the sample preparation that the user normally performs for product testing (method suitability testing);
- limit and range of detection of the method with regard to sample size and sample availability;
- specificity of the response with regard to the influence of the product ingredients;
- linearity of the response with regard to the types of samples to be analysed;
- accuracy and precision of the response with regard to the types of samples to be analysed.

Acceptance criteria for the method will need to be defined as a function of the application and the validation data.

3-3. *TYPES OF MICROBIOLOGICAL TESTS*

Validation of a microbiological method is the process whereby it is experimentally established by the user that the performance characteristics of the method meet the requirements of the intended application. As microbiological tests have 3 basic applications (qualitative, quantitative and identification), 3 separate sets of validation criteria are required. These criteria are described below and summarised in Table 5.1.6.-2.

Table 5.1.6.-2 – *Validation criteria for qualitative, quantitative and identification tests*

Criterion	Qualitative test	Quantitative test	Identification test
Accuracy	+(1)	+	+
Precision	-	+	-
Specificity	+	+	+
Detection limit	+	-(2)	-
Quantitation limit	-	+	-
Linearity	-	+	-
Range	-	+	-
Robustness	+	+	+
Suitability testing	+	+	-
Equivalence testing	+	+	-

(1) Performing an accuracy test of the alternate method with respect to the pharmacopoeial method can be used instead of the validation of the limit of detection test.

(2) May be needed in some cases.

3-3-1. **Validation of alternative qualitative tests for the presence or absence of micro-organisms**

3-3-1-1. *Specificity*

The specificity of an alternative qualitative method is its ability to detect only the required micro-organisms, i.e. does not generate false positive results. This can be demonstrated using a panel of appropriate micro-organisms. Where relevant for the purpose of the test, mixtures of micro-organisms are used during validation. For qualitative methods that rely on growth to demonstrate presence or absence of micro-organisms, specificity is adequately addressed by demonstrating the growth promotion properties of the media. For those methods that do not require growth as an indicator of microbial presence, the specificity assures that extraneous matter in the test system does not interfere with the test.

3-3-1-2. *Detection Limit*

The detection limit of an alternative qualitative method is the lowest number of micro-organisms in a sample that can be detected under the stated analytical conditions. A microbiological limit test determines the presence or absence of micro-organisms in a defined quantity of the sample under test. Due to the nature of microbiological tests, the detection limit reflects the number of micro-organisms present in the original sample before any dilution or incubation steps. The detection limit of the alternative method must not be a number greater than that of the pharmacopoeial method.

It is essential that the detection limit is determined using a sufficient number of replicates and a number of independent determinations.

3-3-1-3. *Robustness*

The robustness of an alternative qualitative method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters (e.g. incubation period or incubation temperature range). Robustness is a validation parameter best suited to determination by the supplier of the method. Nevertheless, if the user modifies critical parameters, any effect on robustness must be evaluated. Robustness of a qualitative method is judged by its ability to detect the test micro-organisms after deliberate variations to the method parameters.

3-3-1-4. *Suitability testing*

The alternative method must be applied according to the specified procedure and with the samples to be analysed under the responsibility of the user. It must be shown that the test sample does not interfere with the system's detection capacity or microbial recovery. Specific points to be addressed are:

- the ability of the test to detect micro-organisms in the presence of the sample matrix;
- verifying if the sample matrix interferes with the alternative system (e.g. background signal or inhibiting chemical reactions).

Acceptance criteria for the method in routine use will need to be defined as a function of the application and the validation data.

3-3-1-5. *Equivalence testing*

Equivalence testing of 2 qualitative methods can be conducted directly on the validation parameters. This approach requires an adequate comparison experiment at low levels of inoculation (e.g. less than 5 CFU) with sufficient numbers of replicates for relevant strains of test micro-organisms. Alternatively, and in some cases additionally, equivalence testing can be carried out by the parallel testing of a predefined number of samples or for a predefined period of time. This parallel testing can be justified based on a risk assessment. The alternative method must enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

3-3-2. **Validation of alternative quantitative tests for enumeration of micro-organisms**

3-3-2-1. *Accuracy*

The accuracy of an alternative quantitative method is the closeness of the test results obtained by the alternative method to those obtained by the pharmacopoeial method. Accuracy must be demonstrated across the practical range of the test. It is usually expressed as the percentage recovery of micro-organisms by the alternative method compared to the percentage recovery using the pharmacopoeial method, taking into account statistical analysis.

Accuracy may be shown by preparing and testing a suspension of micro-organisms at the upper end of the test range and serially diluting to the lower end of the test range. For example, if the alternative method is meant to replace the pharmacopoeial plate count method for viable counts, then a reasonable range might be 10^0-10^6 CFU/mL. If instead, it is a replacement for the MPN method, a much narrower range may be used. At least 1 suspension for each test micro-organism dilution must be analysed.

The alternative method should be shown to recover at least as many micro-organisms as the pharmacopoeial method using appropriate statistical analysis.

The protocol used to check the linearity of the method (see 3-3-2-5) may also be used to check the accuracy. The suspensions of micro-organisms prepared for the alternative method are counted at the same time using the pharmacopoeial method.

3-3-2-2. *Precision*

The precision of an alternative quantitative method is the degree of agreement between individual test results when the procedure is applied repeatedly to multiple samplings of homogeneous suspensions of micro-organisms under the prescribed conditions. Precision should be split into repeatability and intermediate precision under normal or routine operating conditions. Repeatability (also referred to as within-run variability) refers to the use of the microbiological method with the same sample (replicate) in the same laboratory over a short period of time with the same analyst and the same equipment. It gives the minimum variability

of the method. Intermediate precision (includes run-to-run variability and within-run variability) refers to the use of the microbiological method applied to different sample preparations of the product under test in the same laboratory with different analysts, equipment and/or on different days. It gives the maximum variability of the method. The precision of a microbiological method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation). At least 1 suspension in the middle of the test range is analysed. The number of replicates is chosen so that the entire test can be carried out during the same working session, i.e. under the same operating conditions and without any change in the suspension of micro-organisms. For intermediate precision, other working sessions are then carried out under conditions of maximum variability (different reagents, operators and/or days, etc.). The variance in the results observed in each of the working sessions is calculated. If the variances are homogeneous, the variance of the repeatability can be calculated. The inter-group variance of the results is also calculated and the resultant variance of the intermediate precision is given as the sum of the variance of the repeatability and the inter-group variance. The coefficient of variation is then calculated. Alternative methods must demonstrate precision comparable to that of the pharmacopoeial methods.

3-3-2-3. *Specificity*

The specificity of an alternative quantitative method is its ability to quantify only the required micro-organisms, i.e. does not generate false positive results. This may be demonstrated using a panel of appropriate micro-organisms. Where relevant for the purpose of the test, mixtures of micro-organisms are used during validation. For those methods that do not require growth as an indicator of microbial presence, the specificity assures that extraneous matter in the test system does not interfere with the test.

3-3-2-4. *Quantitation limit*

The quantitation limit of an alternative quantitative method is the lowest number of CFUs in a sample which can be quantitatively determined with suitable precision and accuracy. It is essential that the quantitation limit is determined from a number of replicates. The results of the linearity and accuracy studies can also be used. In this case, the lowest concentration in the linear range is considered to be the quantitation limit of the method. The quantitation limit of the alternative method must not be greater than that of the pharmacopoeial method.

3-3-2-5. *Linearity*

The linearity of an alternative quantitative method is its ability (within a given range) to produce results that are proportional to the concentration of micro-organisms present in the sample. The linearity must be determined over a reasonable range (e.g. 10^0-10^6 CFU/mL) so as to correspond to the purpose of the alternative method. One approach would be to select different concentrations of each test micro-organism and test several replicates. For each concentration, an appropriate number of replicates is chosen to confirm linearity. The number of replicates is chosen so that the entire test can be carried out during the same working session. After checking the homogeneity of the variances of the results obtained for each concentration, the regression line is calculated. Linearity is demonstrated if the estimated slope is significant and if the test for deviation from linearity is non-significant (see general chapter *5.3*).

3-3-2-6. *Range*

The range of an alternative quantitative method is the interval between the upper and lower levels of micro-organisms as determined from the related studies of precision, accuracy and linearity using the specified method; it is dependent on the intended application.

3-3-2-7. *Robustness*

The robustness of an alternative quantitative method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters (e.g. incubation period or incubation temperature range). Robustness is a validation parameter best suited to determination by the supplier of the method. Nevertheless, if the user modifies critical parameters, the effects on robustness must be evaluated. Robustness of an alternative quantitative method is judged by its ability to accurately enumerate the test micro-organisms after deliberate variations to the method parameters.

3-3-2-8. *Suitability testing*

The alternative method must be applied according to the specified procedure and with the samples to be analysed under the responsibility of the user. It must be shown that the test sample does not interfere with the system's enumeration capacity or microbial recovery. Specific points to be addressed are:

- the ability of the test to detect micro-organisms in the presence of the sample matrix;
- verifying if the sample matrix interferes with the alternative system (e.g. background signal or inhibiting chemical reactions).

Acceptance criteria for the method are defined as a function of the application and of the validation data.

3-3-2-9. *Equivalence testing*

Equivalence testing of 2 quantitative methods can be conducted directly on the validation parameters. This approach requires an adequate comparison experiment at low levels of inoculation (e.g. less than 5 CFU) with sufficient numbers of replicates for relevant strains of test micro-organisms. Alternatively, and in some cases additionally, equivalence testing can be carried out by the parallel testing of a predefined number of samples or for a predefined period of time. This parallel testing can be justified based on a risk assessment.

If the result of the alternative method can be expressed as the number of CFUs per weight or per volume, statistical analysis of the results shall demonstrate that the results of the alternative method enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

If the result of the alternative method cannot be expressed as the number of CFUs, equivalence testing is performed using suitable parameters, followed by statistical analysis to demonstrate that the results of the alternative method enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

3-3-3. **Validation of alternative identification tests**

There is a large body of evidence that different methods vary considerably in their ability to identify micro-organisms. It must be accepted that a method of identification needs to be internally consistent, but may differ from others in its identification of micro-organisms.

3-3-3-1. *Accuracy*

The accuracy of an alternative identification method is its ability to identify the desired micro-organism to the required taxonomic level. It must be demonstrated using well-characterised reference micro-organisms, e.g. type strains. Accuracy of the identification method is usually expressed as the number of correct identifications divided by the total number of identifications.

3-3-3-2. *Specificity*

The specificity of an alternative identification method is its ability to discriminate micro-organisms actually present from interfering factors that cause false identification results. Such factors include chemical substances and

mixtures of micro-organisms, which cause the test to identify micro-organisms not actually present in the sample material (e.g. the presence of mixtures of DNA material from 2 micro-organisms in a sequencing test leading to the false identification of a third micro-organism).

3-3-3-3. *Robustness*

The robustness of an alternative identification method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters (e.g. incubation period or incubation temperature range). Robustness is a validation parameter best suited to determination by the supplier of the method. Nevertheless, if the user modifies critical parameters, the effects on robustness have to be evaluated. Robustness of an identification method is judged by its ability to correctly identify the test micro-organisms after deliberate variations to the method parameters.

2.6.2. MYCOBACTERIA

If the sample to be examined may be contaminated by micro-organisms other than mycobacteria, treat it with a suitable decontamination solution, such as acetylcysteine-sodium hydroxide solution or sodium laurilsulfate solution.

01/2008:20602 Inoculate 0.2 mL of the sample in triplicate onto each of 2 suitable solid media (Löwenstein-Jensen medium and Middlebrook 7H10 medium are considered suitable). Inoculate 0.5 mL in triplicate into a suitable liquid medium. Incubate all media at 37 °C for 56 days.

Establish the fertility of the media in the presence of the preparation to be examined by inoculation of a suitable strain of a *Mycobacterium* sp. such as BCG and if necessary use a suitable neutralising substance.

If contaminating micro-organisms develop during the first 8 days of incubation, repeat the test and carry out at the same time a bacteriological sterility test.

If at the end of the incubation time no growth of mycobacteria occurs in any of the test media, the preparation complies with the test.

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corrected 6.1

2.6.7. MYCOPLASMAS

Where the test for mycoplasmas is prescribed for a master cell bank, for a working cell bank, for a virus seed lot or for control cells, both the culture method and the indicator cell culture method are used. Where the test for mycoplasmas is prescribed for a virus harvest, for a bulk vaccine or for the final lot (batch), the culture method is used. The indicator cell culture method may also be used, where necessary, for screening of media.

Nucleic acid amplification techniques (NAT) may be used as an alternative to one or both of the other methods after suitable validation.

CULTURE METHOD

CHOICE OF CULTURE MEDIA

The test is carried out using a sufficient number of both solid and liquid media to ensure growth in the chosen incubation conditions of small numbers of mycoplasmas that may be present in the product to be examined. Liquid media must contain phenol red. The range of media chosen is shown to have satisfactory nutritive properties for at least the micro-organisms shown below. The nutritive properties of each new batch of medium are verified for the appropriate micro-organisms in the list. When testing for mycoplasmas in the product to be examined, at least 1 of the following species will be included as a positive control:

- *Acholeplasma laidlawii* (vaccines for human and veterinary use where an antibiotic has been used during production);
- *Mycoplasma gallisepticum* (where avian material has been used during production or where the vaccine is intended for use in poultry);
- *Mycoplasma hyorhinis* (non-avian veterinary vaccines);
- *Mycoplasma orale* (vaccines for human and veterinary use);
- *Mycoplasma pneumoniae* (vaccines for human use) or other suitable species of D-glucose fermenter such as *Mycoplasma fermentans*;
- *Mycoplasma synoviae* (where avian material has been used during production or where the vaccine is intended for use in poultry).

The test strains are field isolates having undergone a limited number of subcultures (not more than 15), and are stored frozen or freeze-dried. After cloning, the strains are identified as being of the required species by comparison with type cultures, for example:

A. laidlawii	NCTC 10116	CIP 75.27	ATCC 23206
M. gallisepticum	NCTC 10115	CIP 104967	ATCC 19610
M. fermentans	NCTC 10117	CIP 105680	ATCC 19989
M. hyorhinis	NCTC 10130	CIP 104968	ATCC 17981
M. orale	NCTC 10112	CIP 104969	ATCC 23714
M. pneumoniae	NCTC 10119	CIP 103766	ATCC 15531
M. synoviae	NCTC 10124	CIP 104970	ATCC 25204

Acholeplasma laidlawii BRP, *Mycoplasma fermentans BRP*, *Mycoplasma hyorhinis BRP*, *Mycoplasma orale BRP* and *Mycoplasma synoviae BRP* are suitable for use as low-passage reference strains.

INCUBATION CONDITIONS

Incubate liquid media in tightly stoppered containers at 35-38 °C. Incubate solid media in microaerophilic conditions (nitrogen containing 5-10 per cent of carbon dioxide and sufficient humidity to prevent desiccation of the agar surface) at 35-38 °C.

NUTRITIVE PROPERTIES

Carry out the test for nutritive properties for each new batch of medium. Inoculate the chosen media with the appropriate test micro-organisms; use not more than 100 CFU per 60 mm diameter plate containing 9 mL of solid medium and per 100 mL container of liquid medium; use a separate plate and container for each species of micro-organism. Incubate the media and make subcultures from 0.2 mL of liquid medium to solid medium at the specified intervals (see below under Test for mycoplasmas in the product to be examined). The solid medium complies with the test if adequate growth is found for each test micro-organism (growth obtained does not differ by a factor greater than 5 from the value calculated with respect to the inoculum). The liquid medium complies with the test if growth on agar plates subcultured from the broth is found for at least 1 subculture for each test micro-organism.

INHIBITORY SUBSTANCES

The test for inhibitory substances is carried out once for a given product and is repeated whenever there is a change in production method that may affect the detection of mycoplasmas.

To demonstrate absence of inhibitory substances, carry out the test for nutritive properties in the presence and absence of the product to be examined. If growth of a test micro-organism occurs more than 1 subculture sooner in the absence of the product to be examined than in its presence, or if plates directly inoculated with the product to be examined have fewer than 1/5 of the number of colonies of those inoculated without the product to be examined, inhibitory substances are present and they must be neutralised or their effect otherwise countered, for example by passage in substrates not containing inhibitors or dilution in a larger volume of medium before the test. If dilution is used, larger medium volumes may be used or the inoculum volume may be divided among several 100 mL flasks. The effectiveness of the neutralisation or other process is checked by repeating the test for inhibitory substances after neutralisation.

TEST FOR MYCOPLASMAS IN THE PRODUCT TO BE EXAMINED

Inoculate 10 mL of the product to be examined per 100 mL of each liquid medium. If it has been found that a significant pH change occurs upon the addition of the product to be examined, the liquid medium is restored to its original pH value by the addition of a solution of either sodium hydroxide or hydrochloric acid. Inoculate 0.2 mL of the product to be examined on each plate of each solid medium. Incubate liquid media for 20-21 days. Incubate solid media for not less than 14 days, except those corresponding to the 20-21 day subculture, which are incubated for 7 days. At the same time incubate an uninoculated 100 mL portion of each liquid medium and agar plates, as a negative control. On days 2-4 after inoculation, subculture each liquid medium by inoculating 0.2 mL on at least 1 plate of each solid medium. Repeat the procedure between the 6th and 8th days, again between the 13th and 15th days and again between the 19th and 21st days of the test. Observe the liquid media every 2 or 3 days and if a colour change occurs, subculture. If a liquid medium shows bacterial or fungal contamination, the test is invalid. The test is valid if at least 1 plate per medium and per inoculation day can be read. Include in the test positive controls prepared by inoculation of not more than 100 CFU of at least 1 test micro-organism on agar medium or into broth medium. Where the test for mycoplasmas is carried out regularly and where possible, it is recommended

to use the test micro-organisms in regular rotation. The test micro-organisms used are those listed under Choice of culture media.

INTERPRETATION OF RESULTS

At the end of the prescribed incubation period, examine all inoculated solid media microscopically for the presence of mycoplasma colonies. The product complies with the test if growth of typical mycoplasma colonies has not occurred. The product does not comply with the test if growth of typical mycoplasma colonies has occurred on any of the solid media. The test is invalid if 1 or more of the positive controls do not show growth of mycoplasmas on at least 1 subculture plate. The test is invalid if 1 or more of the negative controls show growth of mycoplasmas. If suspect colonies are observed, a suitable validated method may be used to determine whether they are due to mycoplasmas.

The following section is published for information.

RECOMMENDED MEDIA FOR THE CULTURE METHOD

The following media are recommended. Other media may be used, provided that their ability to sustain the growth of mycoplasmas has been demonstrated on each batch in the presence and absence of the product to be examined.

HAYFLICK MEDIA (RECOMMENDED FOR THE GENERAL DETECTION OF MYCOPLASMAS)

Liquid medium

Beef heart infusion broth (1)	90.0 mL
Horse serum (unheated)	20.0 mL
Yeast extract (250 g/L)	10.0 mL
Phenol red (0.6 g/L solution)	5.0 mL
Penicillin (20 000 IU/mL)	0.25 mL
Deoxyribonucleic acid (2 g/L solution)	1.2 mL

Adjust to pH 7.8.

Solid medium

Prepare as described above replacing beef heart infusion broth by beef heart infusion agar containing 15 g/L of agar.

FREY MEDIA (RECOMMENDED FOR THE DETECTION OF M. SYNOVIAE)

Liquid medium

Beef heart infusion broth (1)	90.0 mL
Essential vitamins (2)	0.025 mL
Glucose monohydrate (500 g/L solution)	2.0 mL
Swine serum (inactivated at 56 °C for 30 min)	12.0 mL
β-Nicotinamide adenine dinucleotide (10 g/L solution)	1.0 mL
Cysteine hydrochloride (10 g/L solution)	1.0 mL
Phenol red (0.6 g/L solution)	5.0 mL
Penicillin (20 000 IU/mL)	0.25 mL

Mix the solutions of β-nicotinamide adenine dinucleotide and cysteine hydrochloride and after 10 min add to the other ingredients. Adjust to pH 7.8.

Solid medium

Beef heart infusion broth (1)	90.0 mL
Agar, purified (3)	1.4 g

Adjust to pH 7.8, sterilise by autoclaving then add:

Essential vitamins (2)	0.025 mL
Glucose monohydrate (500 g/L solution)	2.0 mL
Swine serum (unheated)	12.0 mL
β-Nicotinamide adenine dinucleotide (10 g/L solution)	1.0 mL
Cysteine hydrochloride (10 g/L solution)	1.0 mL
Phenol red (0.6 g/L solution)	5.0 mL
Penicillin (20 000 IU/mL)	0.25 mL

FRIIS MEDIA (RECOMMENDED FOR THE DETECTION OF NON-AVIAN MYCOPLASMAS)

Liquid medium

Hanks' balanced salt solution (modified) (4)	800 mL
Distilled water	67 mL
Brain heart infusion (5)	135 mL
PPLO Broth (6)	248 mL
Yeast extract (170 g/L)	60 mL
Bacitracin	250 mg
Meticillin	250 mg
Phenol red (5 g/L)	4.5 mL
Horse serum	165 mL
Swine serum	165 mL

Adjust to pH 7.40-7.45.

Solid medium

Hanks' balanced salt solution (modified) (4)	200 mL
DEAE-dextran	200 mg
Agar, purified (3)	15.65 g

Mix well and sterilise by autoclaving. Cool to 100 °C. Add to 1740 mL of liquid medium as described above.

(1) *Beef heart infusion broth*

Beef heart (for preparation of the infusion)	500 g
Peptone	10 g
Sodium chloride	5 g
Distilled water	to 1000 mL

Sterilise by autoclaving.

(2) *Essential vitamins*

Biotin	100 mg
Calcium pantothenate	100 mg
Choline chloride	100 mg
Folic acid	100 mg
i-Inositol	200 mg
Nicotinamide	100 mg
Pyridoxal hydrochloride	100 mg
Riboflavine	10 mg
Thiamine hydrochloride	100 mg
Distilled water	to 1000 mL

(3) *Agar, purified*

A highly refined agar for use in microbiology and immunology, prepared by an ion-exchange procedure that results in a product having superior purity, clarity and gel strength. It contains about:

Water	12.2 per cent
Ash	1.5 per cent
Acid-insoluble ash	0.2 per cent
Chlorine	0
Phosphate (calculated as P_2O_5)	0.3 per cent
Total nitrogen	0.3 per cent

Copper	8 ppm
Iron	170 ppm
Calcium	0.28 per cent
Magnesium	0.32 per cent

(4) *Hanks' balanced salt solution (modified)*

Sodium chloride	6.4 g
Potassium chloride	0.32 g
Magnesium sulfate heptahydrate	0.08 g
Magnesium chloride hexahydrate	0.08 g
Calcium chloride, anhydrous	0.112 g
Disodium hydrogen phosphate dihydrate	0.0596 g
Potassium dihydrogen phosphate, anhydrous	0.048 g
Distilled water	to 800 mL

(5) *Brain heart infusion*

Calf-brain infusion	200 g
Beef-heart infusion	250 g
Proteose peptone	10 g
Glucose monohydrate	2 g
Sodium chloride	5 g
Disodium hydrogen phosphate, anhydrous	2.5 g
Distilled water	to 1000 mL

(6) *PPLO broth*

Beef-heart infusion	50 g
Peptone	10 g
Sodium chloride	5 g
Distilled water	to 1000 mL

INDICATOR CELL CULTURE METHOD

Cell cultures are stained with a fluorescent dye that binds to DNA. Mycoplasmas are detected by their characteristic particulate or filamentous pattern of fluorescence on the cell surface and, if contamination is heavy, in surrounding areas. Mitochondria in the cytoplasm may be stained but are readily distinguished from mycoplasmas.

If for viral suspensions the interpretation of results is affected by marked cytopathic effects, the virus may be neutralised using a specific antiserum that has no inhibitory effects on mycoplasmas or a cell culture substrate that does not allow growth of the virus may be used. To demonstrate the absence of inhibitory effects of serum, carry out the positive control tests in the presence and absence of the antiserum.

VERIFICATION OF THE SUBSTRATE

Use Vero cells or another cell culture (for example, the production cell line) that is equivalent in effectiveness for detecting mycoplasmas. Test the effectiveness of the cells to be used by applying the procedure shown below and inoculating not more than 100 CFU or CFU-like micro-organisms of suitable reference strains of *M. hyorhinis* and *M. orale*. The following strains have been found to be suitable:

M. hyorhinis			ATCC 29052
M. orale	NCTC 10112	CIP 104969	ATCC 23714

The cells are suitable if both reference strains are detected.

The indicator cells must be subcultured without an antibiotic before use in the test.

TEST METHOD

1. Seed the indicator cell culture at a suitable density (for example, 2×10^4 to 2×10^5 cells/mL, 4×10^3 to 2.5×10^4 cells/cm^2) that will yield confluence after 3 days of growth. Inoculate 1 mL of the product to be examined into the cell culture vessel and incubate at 35-38 °C.

2. After at least 3 days of incubation, when the cells have grown to confluence, make a subculture on cover slips in suitable containers or on some other surface (for example, chambered slides) suitable for the test procedure. Seed the cells at low density so that they reach 50 per cent confluence after 3-5 days of incubation. Complete confluence impairs visualisation of mycoplasmas after staining and must be avoided.

3. Remove the medium and rinse the indicator cells with *phosphate buffered saline pH 7.4 R*, then add a suitable fixing solution (a freshly prepared mixture of 1 volume of *glacial acetic acid R* and 3 volumes of *methanol R* is suitable when *bisbenzimide R* is used for staining).

4. Remove the fixing solution and wash the cells with sterile *water R*. Dry the slides completely if they are to be stained more than 1 h later (particular care is needed for staining of slides after drying owing to artefacts that may be produced).

5. Add a suitable DNA stain and allow to stand for a suitable time (*bisbenzimide working solution R* and a standing time of 10 min are suitable).

6. Remove the stain and rinse the monolayer with *water R*.

7. Mount each coverslip, where applicable (a mixture of equal volumes of *glycerol R* and *phosphate-citrate buffer solution pH 5.5 R* is suitable for mounting). Examine by fluorescence (for bisbenzimide stain a 330 nm/380 nm excitation filter and an LP 440 nm barrier filter are suitable) at 400 × magnification or greater.

8. Compare the microscopic appearance of the test cultures with that of the negative and positive controls, examining for extranuclear fluorescence. Mycoplasmas produce pinpoints or filaments over the indicator cell cytoplasm. They may also produce pinpoints and filaments in the intercellular spaces. Multiple microscopic fields are examined according to the protocol established during validation.

INTERPRETATION OF RESULTS

The product to be examined complies with the test if fluorescence typical of mycoplasmas is not present. The test is invalid if the positive controls do not show fluorescence typical of mycoplasmas. The test is invalid if the negative controls show fluorescence typical of mycoplasmas.

NUCLEIC ACID AMPLIFICATION TECHNIQUES (NAT)

NAT (*2.6.21*) may be used for detection of mycoplasmas by amplification of nucleic acids extracted from a test sample with specific primers that reveal the presence of the target nucleic acid. NAT indicate the presence of a particular nucleic acid sequence and not necessarily the presence of viable mycoplasmas. A number of different techniques are available. This general chapter does not prescribe a particular method for the test. The procedure applied must be validated as described, taking account of the guidelines presented at the end of this section. Where a commercial kit is used, certain elements of the validation may be carried out by the manufacturer and information provided to the user but it must be remembered that full information on the primers may not be available and that production of the kit may be modified or discontinued.

NAT are applied where prescribed in a monograph. They may also be used instead of the culture method and the indicator cell culture method after suitable validation.

Direct NAT can be applied in the presence of cytotoxic material and where a rapid method is needed.

Cell-culture enrichment followed by NAT: the test sample and a suitable cell substrate (as described under the indicator cell-culture method) are cultured together for a suitable period; the nucleic acids are then extracted from cells and supernatant and used for detection by NAT.

VALIDATION

Reference standards are required at various stages during validation and for use as controls during routine application of the test. The reference standards may be mycoplasmas or nucleic acids.

For validation of the limit of detection, the following species represent an optimal selection in terms of the frequency of occurrence as contaminants and phylogenetic relationships:

- *A. laidlawii*;
- *M. fermentans*;
- *M. hyorhinis* (where cell-culture enrichment is used, a fastidious strain such as ATCC 29052 is included);
- *M. orale*;
- *M. pneumoniae* or *M. gallisepticum*;
- *M. synoviae* (where there is use of or exposure to avian material during production);
- *Mycoplasma arginini;*
- *Spiroplasma citri* (where there is use of or exposure to insect or plant material during production).

Demonstration of specificity requires the use of a suitable range of bacterial species other than mycoplasmas. Bacterial genera with close phylogenetic relation to mycoplasmas are most appropriate for this validation; these include *Clostridium, Lactobacillus* and *Streptococcus*.

Comparability studies for use of NAT as an alternative method. For each mycoplasma test species:

- as an alternative to the culture method: the NAT test system must be shown to detect 10 CFU/mL;
- as an alternative to the indicator cell culture method: the NAT test system must be shown to detect 100 CFU/mL;

or an equivalent limit of detection in terms of the number of copies of mycoplasma nucleic acid in the test sample (using suitable reference standards of mycoplasma nucleic acid).

CONTROLS

Internal controls. Internal controls are necessary for routine verification of absence of inhibition. The internal control may contain the primer binding-site, or some other suitable sequence may be used. It is preferably added to the test material before isolating the nucleic acid and therefore acts as an overall control (extraction, reverse transcription, amplification, detection).

External controls. The external positive control contains a defined number of target-sequence copies or CFUs from 1 or more suitable species of mycoplasma chosen from those used during validation of the test conditions. 1 of the positive controls is set close to the positive cut-off point to demonstrate that the expected sensitivity is achieved. The external negative control contains no target sequence but does not necessarily represent the same matrix as the test article.

INTERPRETATION OF RESULTS

The primers used may also amplify non-mycoplasmal bacterial nucleic acid, leading to false positive results. Procedures are established at the time of validation for dealing with confirmation of positive results, where necessary.

The following section is published for information.

Validation of nucleic acid amplification techniques (NAT) for the detection of mycoplasmas: guidelines

1. *SCOPE*

Nucleic acid amplification techniques (NAT) are either qualitative or quantitative tests for the presence of nucleic acid. For the detection of mycoplasma contamination of various samples such as vaccines and cell substrates, qualitative tests are adequate and may be considered to be limit tests. These guidelines describe methods to validate qualitative nucleic acid amplification analytical procedures for assessing mycoplasma contamination. They may also be applicable for real-time NAT used as limit tests for the control of contaminants.

The 2 characteristics regarded as the most important for validation of the analytical procedure are the specificity and the detection limit. In addition, the robustness of the analytical procedure should be evaluated.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part or all of the analytical procedure, documented validation points already covered by the kit manufacturer can replace validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. detection limit, robustness, cross-detection of other classes of bacteria).

NAT may be used as:

- a complementary test (for example, for cytotoxic viral suspensions) or for in-process control purposes;
- an alternative method to replace an official method (indicator cell culture method or culture method).

These guidelines will thus separate these 2 objectives by presenting first a guideline for the validation of the NAT themselves, and second, a guideline for a comparability study between NAT and official methods.

2. *GUIDELINE FOR MYCOPLASMA NAT VALIDATION*

3 parameters should be evaluated: specificity, detection limit and robustness.

2-1. **Specificity**. Specificity is the ability to unequivocally assess target nucleic acid in the presence of components that may be expected to be present.

The specificity of NAT is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and detection steps).

The ability of the NAT to detect a large panel of mycoplasma species will depend on the choice of primers, probes and method parameters. This ability should be demonstrated using characterised reference panels (e.g. reference strains provided by the EDQM). Since NAT systems are usually based on a mix of primers, the theoretical analysis of primers and probes by comparison with databases is not recommended, because interpretation of the results may be quite complex and may not reflect the experimental results.

Moreover, as it is likely that the primers will detect other bacterial species, the potential cross-detection should be documented in the validation study. Bacterial genera such as gram-positive bacteria with close phylogenetic relation to mycoplasmas are most appropriate for this validation; these include *Clostridium*, *Lactobacillus* and *Streptococcus*. However, this is not an exhaustive list and species to be tested will depend on the theoretical ability (based on primers/probes sequences) of the NAT system to detect such other species.

Based on the results from this validation of the specificity, if a gap in the specificity of the method is identified (such as detection of non-mycoplasmal bacterial nucleic acid), an appropriate strategy must be proposed in the validation study to allow interpretation of positive results on a routine basis. For example, a second test may be performed using an alternative method without this specificity gap or using an official method.

2-2. **Detection limit**. The detection limit of an individual analytical procedure is the lowest amount of target nucleic acid in a sample that can be detected but not necessarily quantitated as an exact value.

For establishment of the detection limit, a positive cut-off point should be determined for the nucleic acid amplification analytical procedure. The positive cut-off point (as defined in general chapter *2.6.21*) is the minimum number of target sequence copies per volume of sample that can be detected in 95 per cent of test runs. This positive cut-off point is influenced by the distribution of mycoplasmal genomes in the individual samples being tested and by factors such as enzyme efficiency, and can result in different 95 per cent cut-off values for individual analytical test runs.

To determine the positive cut-off point, a dilution series of characterised and calibrated (either in CFUs or nucleic acid copies) in-house working strains or EDQM standards should be tested on different days to examine variation between test runs.

For validation of the limit of detection, the following species represent an optimal selection in terms of the frequency of occurrence as contaminants and phylogenetic relationships:

- *A. laidlawii*;
- *M. fermentans*;
- *M. hyorhinis*;
- *M. orale*;
- *M. pneumoniae* or *M. gallisepticum*;
- *M. synoviae* (where there is use of or exposure to avian material during production);
- *M. arginini*;
- *S. citri* (where there is use of or exposure to insect or plant material during production).

For each strain, at least 3 independent 10-fold dilution series should be tested, with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution, to enable a statistical analysis of the results.

For example, a laboratory may test 3 dilution series on different days with 8 replicates for each dilution, 4 dilution series on different days with 6 replicates for each dilution, or 6 dilution series on different days with 4 replicates for each dilution. In order to keep the number of dilutions at a manageable level, a preliminary test should be performed to obtain a preliminary value for the positive cut-off point (i.e. the highest dilution giving a positive signal). The range of dilutions can then be chosen around the predetermined preliminary cut-off point. The concentration of mycoplasmas (CFUs or copies) that can be detected in 95 per cent of test runs can then be calculated using an appropriate statistical evaluation.

These results may also serve to evaluate the variability of the analytical procedure.

2-3. **Robustness**. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. $MgCl_2$, primers or deoxyribonucleotides) are tested. Modifications of extraction kits or extraction procedures as well as different thermal cycler types may also be evaluated.

Finally, robustness of the method can be evaluated through collaborative studies.

3. *GUIDELINE FOR COMPARABILITY STUDY*

NAT may be used instead of official methods (indicator cell culture method and/or culture method). In this case a comparability study should be carried out. This comparability study should include mainly a comparison of the respective detection limits of the alternative method and official methods. However, specificity (mycoplasma panel detected, putative false positive results) should also be considered. For the detection limit, acceptability criteria are defined as follows:

- if the alternative method is proposed to replace the culture method, the NAT system must be shown to detect 10 CFU/mL for each mycoplasma test species described in paragraph 2-2;
- if the alternative method is proposed to replace the indicator cell culture method, the NAT system must be shown to detect 100 CFU/mL for each mycoplasma test species described in paragraph 2-2.

For both cases, suitable standards calibrated for the number of nucleic acid copies and the number of CFUs may be used for establishing that these acceptability criteria are reached. The relation between CFUs and nucleic acid copies for the reference preparations should be previously established to compare the performance of the alternative NAT method with the performance of the official methods.

1 of the following 2 strategies can be used to perform this comparability study:

- perform the NAT alternative method in parallel with the official method(s) to evaluate simultaneously the detection limit of both methods using the same samples of calibrated strains;
- compare the performance of the NAT alternative method using previously obtained data from official method validation. In this case, calibration of standards used for both validations as well as their stabilities should be documented carefully.

Comparability study reports should describe all the validation elements described in section 2 (specificity, limit of detection and variability, as well as robustness) in order to assess all the advantages and/or disadvantages of the alternative NAT method compared to official methods.

01/2011:50103

5.1.3. EFFICACY OF ANTIMICROBIAL PRESERVATION

If a pharmaceutical preparation does not itself have adequate antimicrobial activity, antimicrobial preservatives may be added, particularly to aqueous preparations, to prevent proliferation or to limit microbial contamination which, during normal conditions of storage and use, particularly for multidose containers, could occur in a product and present a hazard to the patient from infection and spoilage of the preparation. Antimicrobial preservatives must not be used as a substitute for good manufacturing practice.

The efficacy of an antimicrobial preservative may be enhanced or diminished by the active constituent of the preparation or by the formulation in which it is incorporated or by the container and closure used. The antimicrobial activity of the preparation in its final container is investigated over the period of validity to ensure that such activity has not been impaired by storage. Such investigations may be carried out on samples removed from the final container immediately prior to testing.

During development of a pharmaceutical preparation, it shall be demonstrated that the antimicrobial activity of the preparation as such or, if necessary, with the addition of a suitable preservative or preservatives provides adequate protection from adverse effects that may arise from microbial contamination or proliferation during storage and use of the preparation.

The efficacy of the antimicrobial activity may be demonstrated by the test described below. The test is not intended to be used for routine control purposes.

TEST FOR EFFICACY OF ANTIMICROBIAL PRESERVATION

The test consists of challenging the preparation, wherever possible in its final container, with a prescribed inoculum of suitable micro-organisms, storing the inoculated preparation at a prescribed temperature, withdrawing samples from the container at specified intervals of time and counting the organisms in the samples so removed.

The preservative properties of the preparation are adequate if, in the conditions of the test, there is a significant fall or no increase, as appropriate, in the number of micro-organisms in the inoculated preparation after the times and at the temperatures prescribed. The acceptance criteria, in terms of decrease in the number of micro-organisms with time, vary for different types of preparations according to the degree of protection intended (see Tables 5.1.3.-1/2/3).

Test micro-organisms

Pseudomonas aeruginosa	ATCC 9027; NCIMB 8626; CIP 82.118.
Staphylococcus aureus	ATCC 6538; NCTC 10788; NCIMB 9518; CIP 4.83.
Candida albicans	ATCC 10231; NCPF 3179; IP 48.72.
Aspergillus brasiliensis	ATCC 16404; IMI 149007; IP 1431.83.

Single-strain challenges are used and the designated micro-organisms are supplemented, where appropriate, by other strains or species that may represent likely contaminants to the preparation. It is recommended, for example, that *Escherichia coli* (ATCC 8739; NCIMB 8545; CIP 53.126) is used for all oral preparations and *Zygosaccharomyces rouxii* (NCYC 381; IP 2021.92) for oral preparations containing a high concentration of sugar.

Preparation of inoculum

Preparatory to the test, inoculate the surface of casein soya bean digest agar (*2.6.12*) for bacteria or Sabouraud-dextrose agar without the addition of antibiotics (*2.6.12*) for fungi, with the recently grown stock culture of each of the specified micro-organisms. Incubate the bacterial cultures at 30-35 °C for 18-24 h, the culture of *C. albicans* at 20-25 °C for 48 h, and the culture of *A. brasiliensis* at 20-25 °C for 1 week or until good sporulation is obtained. Subcultures may be needed after revival before the micro-organism is in its optimal state, but it is recommended that their number be kept to a minimum.

To harvest the bacterial and *C. albicans* cultures, use a sterile suspending fluid, containing 9 g/L of *sodium chloride R*, for dispersal and transfer of the surface growth into a suitable vessel. Add sufficient suspending fluid to reduce the microbial count to about 10^8 micro-organisms per millilitre. To harvest the *A. brasiliensis* culture, use a sterile suspending fluid containing 9 g/L of *sodium chloride R* and 0.5 g/L of *polysorbate 80 R* and adjust the spore count to about 10^8 per millilitre by adding the same solution.

Remove immediately a suitable sample from each suspension and determine the number of colony-forming units per millilitre in each suspension by plate count or membrane filtration (*2.6.12*). This value serves to determine the inoculum and the baseline to use in the test. The suspensions shall be used immediately.

METHOD

To count the viable micro-organisms in the inoculated products, use the agar medium used for the initial cultivation of the respective micro-organisms.

Inoculate a series of containers of the product to be examined, each with a suspension of one of the test organisms to give an inoculum of 10^5 to 10^6 micro-organisms per millilitre or per gram of the preparation. The volume of the suspension of inoculum does not exceed 1 per cent of the volume of the product. Mix thoroughly to ensure homogeneous distribution.

Maintain the inoculated product at 20-25 °C, protected from light. Remove a suitable sample from each container, typically 1 mL or 1 g, at zero hour and at appropriate intervals according to the type of the product and determine the number of viable micro-organisms by plate count or membrane filtration (*2.6.12*). Ensure that any residual antimicrobial activity of the product is eliminated by dilution, by filtration or by the use of a specific inactivator. When dilution procedures are used, due allowance is made for the reduced sensitivity in the recovery of small numbers of viable micro-organisms. When a specific inactivator is used, the ability of the system to support the growth of the test organisms is confirmed by the use of appropriate controls.

The procedure is validated to verify its ability to demonstrate the required reduction in count of viable micro-organisms.

ACCEPTANCE CRITERIA

The criteria for evaluation of antimicrobial activity are given in Tables 5.1.3.-1/2/3 in terms of the $\log_{10}$ reduction in the number of viable micro-organisms against the value obtained for the inoculum.

Table 5.1.3.-1. - *Parenteral preparations, eye preparations, intrauterine preparations and intramammary preparations*

		$\log_{10}$ reduction				
		6 h	24 h	7 d	14 d	28 d
Bacteria	A	2	3	-	-	NR
	B	-	1	3	-	NI
Fungi	A	-	-	2	-	NI
	B	-	-	-	1	NI

NR: no recovery.

NI: no increase in number of viable micro-organisms compared to the previous reading.

The A criteria express the recommended efficacy to be achieved. In justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions, the B criteria must be satisfied.

Table 5.1.3.-2. - *Ear preparations, nasal preparations, preparations for cutaneous application and preparations for inhalation*

		Log_{10} reduction			
		2 d	**7 d**	**14 d**	**28 d**
Bacteria	A	2	3	-	NI
	B	-	-	3	NI
Fungi	A	-	-	2	NI
	B	-	-	1	NI

NI: no increase in number of viable micro-organisms compared to the previous reading.

The A criteria express the recommended efficacy to be achieved. In justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions, the B criteria must be satisfied.

Table 5.1.3.-3. - *Oral preparations, oromucosal preparations and rectal preparations*

	Log_{10} reduction	
	14 d	**28 d**
Bacteria	3	NI
Fungi	1	NI

NI: no increase in number of viable micro-organisms compared to the previous reading.

The above criteria express the recommended efficacy to be achieved.

01/2018:20614

2.6.14. BACTERIAL ENDOTOXINS[(1)]

The test for bacterial endotoxins (BET) is used to detect or quantify endotoxins from gram-negative bacteria using amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). There are 3 techniques for this test: the gel-clot technique, which is based on gel formation; the turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and the chromogenic technique, based on the development of colour after cleavage of a synthetic peptide-chromogen complex.

The following 6 methods are described in the present chapter:

Method A. Gel-clot method: limit test

Method B. Gel-clot method: quantitative test

Method C. Turbidimetric kinetic method

Method D. Chromogenic kinetic method

Method E. Chromogenic end-point method

Method F. Turbidimetric end-point method

Proceed by any of the 6 methods for the test. In the event of doubt or dispute, the final decision is made based upon method A unless otherwise indicated in the monograph.

The test is carried out in a manner that avoids endotoxin contamination.

1. APPARATUS

Depyrogenate all glassware and other heat-stable apparatus in a hot-air oven using a validated process. A commonly used minimum time and temperature is 30 min at 250 °C. If employing plastic apparatus, such as microtitre plates and pipette tips for automatic pipetters, use apparatus shown to be free of detectable endotoxin and which does not interfere in the test.

NOTE: in this chapter, the term 'tube' includes all types of receptacles, for example microtitre plate wells.

2. REAGENTS, TEST SOLUTIONS

(1) Amoebocyte lysate

Amoebocyte lysate is a lyophilised product obtained from amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). This reagent refers only to a product manufactured in accordance with the regulations of the competent authority.

NOTE: amoebocyte lysate reacts with some β-glucans in addition to endotoxins. Amoebocyte lysate preparations which do not react with glucans are available; they are prepared by removing from amoebocyte lysate the G factor, which reacts with glucans, or by inhibiting the G factor reacting system of amoebocyte lysate. These preparations may be used for endotoxin testing in the presence of glucans.

(2) Lysate solution

Dissolve amoebocyte lysate in water for BET or in a buffer, as recommended by the lysate manufacturer, by gentle stirring. Store the reconstituted lysate, refrigerated or frozen, as indicated by the manufacturer.

(3) Water for BET (water for bacterial endotoxins test)

Water for injections R or water produced by other procedures that shows no reaction with the lysate employed at the detection limit of the reagent.

3. PREPARATION OF THE STANDARD ENDOTOXIN STOCK SOLUTION

The standard endotoxin stock solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard, for example *endotoxin standard BRP*.

Endotoxin is expressed in International Units (IU). The equivalence in IU of the International Standard is stated by the World Health Organization.

NOTE: one International Unit (IU) of endotoxin is equal to one Endotoxin Unit (E.U.).

Follow the specifications in the package leaflet and on the label for preparation and storage of the standard endotoxin stock solution.

4. PREPARATION OF THE STANDARD ENDOTOXIN SOLUTIONS

After vigorously mixing the standard endotoxin stock solution, prepare appropriate serial dilutions of this solution using water for BET.

Use the solutions as soon as possible to avoid loss of activity by adsorption.

5. PREPARATION OF THE TEST SOLUTIONS

Prepare the test solutions by dissolving or diluting active substances or medicinal products using water for BET. Some substances or preparations may be more appropriately dissolved or diluted in other aqueous solutions. If necessary, adjust the pH of the test solution (or dilution thereof) so that the pH of the mixture of the lysate and test solution falls within the pH range specified by the lysate manufacturer, usually 6.0 to 8.0. The pH may be adjusted by the use of acid, base or a suitable buffer, as recommended by the lysate manufacturer. Acids and bases may be prepared from concentrates or solids with water for BET in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

6. DETERMINATION OF THE MAXIMUM VALID DILUTION

The Maximum Valid Dilution (MVD) is the maximum allowable dilution of a sample at which the endotoxin limit can be determined. Determine the MVD using the following formulae:

$$\mathrm{MVD} = \frac{\text{endotoxin limit} \times \text{concentration of test solution}}{\lambda}$$

Endotoxin limit: the endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass,

M = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

The endotoxin limit for active substances administered parenterally is specified in units such as IU/mL, IU/mg, IU/Unit of biological activity, etc., in monographs.

(1) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

Concentration of test solution:

- mg/mL if the endotoxin limit is specified by mass (IU/mg),
- Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit),
- mL/mL if the endotoxin limit is specified by volume (IU/mL).

λ = the labelled lysate sensitivity in the gel-clot technique (IU/mL) or the lowest concentration used in the standard curve of the turbidimetric or chromogenic techniques.

7. GEL-CLOT TECHNIQUE (METHODS A AND B)

The gel-clot technique allows detection or quantification of endotoxins and is based on clotting of the lysate in the presence of endotoxins. The minimum concentration of endotoxins required to cause the lysate to clot under standard conditions is the labelled lysate sensitivity. To ensure both the precision and validity of the test, confirm the labelled lysate sensitivity and perform the test for interfering factors as described under 1. Preparatory testing.

1. PREPARATORY TESTING

(i) Confirmation of the labelled lysate sensitivity

Confirm in 4 replicates the labelled sensitivity λ, expressed in IU/mL, of the lysate solution prior to use in the test. Confirmation of the lysate sensitivity is carried out when a new lot of lysate is used or when there is any change in the test conditions which may affect the outcome of the test.

Prepare standard solutions of at least 4 concentrations equivalent to 2λ, λ, 0.5λ and 0.25λ by diluting the standard endotoxin stock solution with water for BET.

Mix a volume of the lysate solution with an equal volume of 1 of the standard solutions (such as 0.1 mL aliquots) in each tube. When single test vials or ampoules containing lyophilised lysate are employed, add solutions of standards directly to the vial or ampoule. Incubate the reaction mixture for a constant period according to the recommendations of the lysate manufacturer (usually at 37 ± 1 °C for 60 ± 2 min), avoiding vibration. Test the integrity of the gel: for tubes, take each tube in turn directly from the incubator and invert it through approximately 180° in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if an intact gel is not formed.

The test is considered valid when the lowest concentration of the standard solutions shows a negative result in all replicate tests.

The end-point is the lowest concentration in the series of decreasing concentrations of standard endotoxin that clots the lysate. Determine the geometric mean end-point concentration by calculting the mean of the logarithms of the end-point concentrations of the 4 dilution series, take the antilogarithm of this value, as indicated by the following expression:

$$\text{Geometric mean end-point concentration} = \text{antilog}\frac{\sum e}{f}$$

$\sum e$ = sum of the $\log_{10}$ end-point concentrations of the dilution series used,

f = number of replicates.

The geometric mean end-point concentration is the measured sensitivity of the lysate solution (IU/mL). If this is not less than 0.5λ and not more than 2λ, the labelled sensitivity is confirmed and is used in the tests performed with this lysate.

(ii) Test for interfering factors

Prepare solutions A, B, C and D as shown in Table 2.6.14.-1, and use the test solutions at a dilution less than the MVD, not containing any detectable endotoxins, operating as described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

The geometric mean end-point concentrations of solutions B and C are determined using the expression described in 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

The test for interfering factors must be repeated when any changes are made to the experimental conditions that are likely to influence the result of the test.

The test is considered valid when all replicates of solutions A and D show no reaction and the result of solution C confirms the labelled lysate sensitivity.

If the sensitivity of the lysate determined with solution B is not less than 0.5λ and not greater than 2λ, the test solution does not contain interfering factors under the experimental conditions used. Otherwise, the test solution interferes with the test.

If the preparation being examined interferes with the test at a dilution less than the MVD, repeat the test for interfering factors using a greater dilution, not exceeding the MVD. The use of a more sensitive lysate permits a greater dilution of the preparation being examined and this may contribute to the elimination of interference.

Interference may be overcome by suitable validated treatment, such as filtration, neutralisation, dialysis or heat treatment. To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment.

Table 2.6.14.-1

Solution	Endotoxin concentration/Solution to which endotoxin is added	Diluent	Dilution factor	Endotoxin concentration	Number of replicates
A	None/Test solution	-	-	-	4
B	2λ/Test solution	Test solution	1	2λ	4
			2	1λ	4
			4	0.5λ	4
			8	0.25λ	4
C	2λ/Water for BET	Water for BET	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D	None/Water for BET	-	-	-	2

Solution A = solution of the preparation being examined that is free of detectable endotoxins.
Solution B = test for interference.
Solution C = control of the labelled lysate sensitivity.
Solution D = negative control (water for BET).

2. LIMIT TEST (METHOD A)

(i) Procedure

Prepare solutions A, B, C and D as shown in Table 2.6.14.-2, and perform the test on these solutions following the procedure described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

Table 2.6.14.-2

Solution	Endotoxin concentration/Solution to which endotoxin is added	Number of replicates
A	None/Diluted test solution	2
B	2λ/Diluted test solution	2
C	2λ/Water for BET	2
D	None/Water for BET	2

Prepare solution A and solution B (positive product control) using a dilution not greater than the MVD and treatments as described in 1. Preparatory testing, (ii) Test for interfering factors. Solutions B and C (positive controls) contain the standard endotoxin at a concentration corresponding to twice the labelled lysate sensitivity. Solution D (negative control) consists of water for BET.

(ii) Interpretation

The test is considered valid when both replicates of solution B and C are positive and those of solution D are negative.

When a negative result is found for both replicates of solution A, the preparation being examined complies with the test.

When a positive result is found for both replicates of solution A, the preparation being examined does not comply with the test.

When a positive result is found for one replicate of solution A and a negative result is found for the other, repeat the test. In the repeat test, the preparation being examined complies with the test if a negative result is found for both replicates of solution A. The preparation does not comply with the test if a positive result is found for one or both replicates of solution A.

However, if the preparation does not comply with the test at a dilution less than the MVD, the test may be repeated using a greater dilution, not exceeding the MVD.

3. QUANTITATIVE TEST (METHOD B)

(i) Procedure

The test quantifies bacterial endotoxins in the test solution by titration to an end-point. Prepare solutions A, B, C and D as shown in Table 2.6.14.-3, and test these solutions according to the procedure described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

(ii) Calculation and interpretation

The test is considered valid when the following 3 conditions are met:

(a) both replicates of solution D (negative control) are negative,

(b) both replicates of solution B (positive product control) are positive,

(c) the geometric mean end-point concentration of solution C is in the range of 0.5λ to 2λ.

To determine the endotoxin concentration of solution A, calculate the end-point concentration for each replicate, by multiplying each end-point dilution factor by λ.

The endotoxin concentration in the test solution is the end-point concentration of the replicates. If the test is conducted with a diluted test solution, calculate the concentration of endotoxin in the original solution by multiplying the result by the dilution factor.

If none of the dilutions of the test solution is positive in a valid test, report the endotoxin concentration as less than λ (or, if a diluted sample was tested, report as less than the lowest dilution factor of the sample × λ). If all dilutions are positive, the endotoxin concentration is reported as equal to or greater than the largest dilution factor multiplied by λ (e.g. in Table 2.6.14.-3, the initial dilution factor × 8 × λ).

The preparation being examined meets the requirements of the test if the endotoxin concentration in both replicates is less than that specified in the monograph.

Table 2.6.14.-3

Solution	Endotoxin concentration/Solution to which endotoxin is added	Diluent	Dilution factor	Endotoxin concentration	Number of replicates
A	None/Test solution	Water for BET	1	-	2
			2	-	2
			4	-	2
			8	-	2
B	2λ/Test solution		1	2λ	2
C	2λ/Water for BET	Water for BET	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D	None/Water for BET	-	-	-	2

Solution A = test solution at the dilution, not exceeding the MVD, with which the test for interfering factors was carried out. Subsequent dilution of the test solution must not exceed the MVD. Use water for BET to make a dilution series of 4 tubes containing the test solution at concentrations of 1, 1/2, 1/4 and 1/8, relative to the dilution used in the test for interfering factors. Other dilutions up to the MVD may be used as appropriate.

Solution B = solution A containing standard endotoxin at a concentration of 2λ (positive product control).

Solution C = a dilution series of 4 tubes of water for BET containing the standard endotoxin at concentrations of 2λ, λ, 0.5λ and 0.25λ.

Solution D = water for BET (negative control).

8. PHOTOMETRIC QUANTITATIVE TECHNIQUES (METHODS C, D, E AND F)

1. TURBIDIMETRIC TECHNIQUE (METHODS C AND F)

This technique is a photometric test to measure the increase in turbidity. Based on the test principle employed, this technique may be classified as being either the end-point-turbidimetric test or the kinetic-turbidimetric test.

The end-point-turbidimetric test (Method F) is based on the quantitative relationship between the endotoxin concentration and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period.

The kinetic-turbidimetric test (Method C) is a method to measure either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance or transmission, or the rate of turbidity development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (usually 37 ± 1 °C).

2. CHROMOGENIC TECHNIQUE (METHODS D AND E)

This technique is used to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the lysate. Depending on the test principle employed, this technique may be classified as being either the end-point-chromogenic test or the kinetic-chromogenic test.

The end-point-chromogenic test (Method E) is based on the quantitative relationship between the endotoxin concentration and the quantity of chromophore released at the end of an incubation period.

The kinetic-chromogenic test (Method D) measures either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance, or the rate of colour development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (usually 37 ± 1 °C).

3. PREPARATORY TESTING

To assure the precision or validity of the turbidimetric and chromogenic techniques, preparatory tests are conducted to show that the criteria for the standard curve are satisfied and that the test solution does not interfere with the test.

Validation of the test method is required when any changes are made to the experimental conditions that are likely to influence the result of the test.

(i) Assurance of criteria for the standard curve

The test must be carried out for each lot of lysate reagent.

Using the standard endotoxin solution, prepare at least 3 endotoxin concentrations within the range indicated by the lysate manufacturer to generate the standard curve. Perform the test using at least 3 replicates of each standard endotoxin solution as recommended by the lysate manufacturer (volume ratios, incubation time, temperature, pH, etc.).

If the desired range is greater than 2 $\log_{10}$ in the kinetic methods, additional standards must be included to bracket each $\log_{10}$ increase in the range of the standard curve.

The absolute value of the correlation coefficient, $|r|$, must be greater than or equal to 0.980, for the range of endotoxin concentrations set up.

(ii) Test for interfering factors

Select an endotoxin concentration at or near the middle of the endotoxin standard curve.

Prepare solutions A, B, C and D as shown in Table 2.6.14.-4. Perform the test on at least 2 replicates of these solutions as recommended by the lysate manufacturer (volume of test solution and lysate solution, volume ratio of test solution to lysate solution, incubation time, etc.).

Table 2.6.14.-4

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of replicates
A	None	Test solution	Not less than 2
B	Middle concentration of the standard curve	Test solution	Not less than 2
C	At least 3 concentrations (lowest concentration is designated λ)	Water for BET	Each concentration not less than 2
D	None	Water for BET	Not less than 2

Solution A = test solution, that may be diluted not to exceed the MVD.

Solution B = preparation to be examined at the same dilution as solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve.

Solution C = standard endotoxin solution at the concentrations used in the validation of the method as described under 3. Preparatory testing, (i) Assurance of criteria for the standard curve (positive controls).

Solution D = water for BET (negative control).

The test is considered valid when the following conditions are met:

- the absolute value of the correlation coefficient of the standard curve generated using solution C is greater than or equal to 0.980;
- the result with solution D does not exceed the limit of the blank value required in the description of the lysate reagent employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution (if any) (solution A, Table 2.6.14.-4) from that in the solution containing the added endotoxin (solution B, Table 2.6.14.-4).

The test solution is considered free of interfering factors if under the conditions of the test, the measured concentration of the endotoxin added to the test solution is within 50-200 per cent of the known added endotoxin concentration, after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is out of the specified range, the test solution is considered to contain interfering factors. Repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the test solution or diluted test solution not to exceed the MVD may be eliminated by suitable validated treatment, such as filtration, neutralisation, dialysis or heat treatment. To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment.

4. TEST

(i) Procedure

Follow the procedure described in 3. Preparatory testing, (ii) Test for interfering factors.

(ii) Calculation

Calculate the endotoxin concentration of each replicate of solution A using the standard curve generated by the positive control solution C.

The test is considered valid when the following 3 requirements are met:

(1) the results obtained with solution C comply with the requirements for validation defined under 3. Preparatory testing, (i) Assurance of criteria for the standard curve,

(2) the endotoxin recovery, calculated from the endotoxin concentration found in solution B after subtracting the endotoxin concentration found in solution A, is within the range of 50-200 per cent,

(3) the result obtained with solution D (negative control) does not exceed the limit of the blank value required in the description of the lysate employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

(iii) Interpretation

The preparation being examined complies with the test if the mean endotoxin concentration of the replicates of solution A, after correction for dilution and concentration, is less than the endotoxin limit for the product.

Guidelines on the test for bacterial endotoxins are given in general chapter 5.1.10.

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5.1.10. GUIDELINES FOR USING THE TEST FOR BACTERIAL ENDOTOXINS

1. INTRODUCTION

Endotoxins from gram-negative bacteria are the most common cause of toxic reactions resulting from contamination of pharmaceutical products with pyrogens; their common pyrogenic activity is much higher than that of other known pyrogenic substances. These endotoxins are lipopolysaccharides. Although there are a small number of pyrogens that possess a different structure, the conclusion is generally justified that the absence of bacterial endotoxins in a substance or product implies the absence of pyrogenic components, provided the presence of non-endotoxin pyrogenic substances can be ruled out. The monocyte-activation test (*2.6.30*) is a suitable method to use to rule out the presence of non-endotoxin pyrogens in substances or products.

The presence of endotoxins in a substance or product may be masked by factors interfering with the reaction between the endotoxins, the test reagents and the amoebocyte lysate. Also, the ability to detect endotoxins may be affected by storage conditions or storage time. Hence, the analyst who wishes to implement a test for bacterial endotoxins or to replace the pyrogen test by a test for bacterial endotoxins has to demonstrate that a valid test can be carried out on the substance or product concerned; this may entail a procedure for removing interference.

As indicated in general chapter *2.6.14. Bacterial endotoxins*, information must be available on the following 2 aspects before a test on a sample can be regarded as valid.

- The suitability of the material to be used for the test has to be established. The absence of endotoxins in the water for BET (water for bacterial endotoxins test) and in the other reagents and consumables must be assured and the sensitivity of the amoebocyte lysate must be checked to confirm the sensitivity declared by the manufacturer.
- As the substance or product to be examined may interfere with the test, the sensitivity of the amoebocyte lysate is determined in the presence and in the absence of the substance or product to be examined. There must be no difference between the 2 sensitivity values.

General chapter *2.6.14. Bacterial endotoxins* indicates methods for removing interfering factors; in the case of interference, another test must be carried out after such a method has been applied to check whether the interference has indeed been neutralised or removed.

This general chapter explains the reasons for the requirements in the test for bacterial endotoxins, then deals with reading and interpretation of the results.

Replacement of the rabbit pyrogen test required in a pharmacopoeial monograph by an amoebocyte lysate test, or by other methods such as the monocyte-activation test or a test using recombinant factor C reagent as a replacement for the amoebocyte lysate, constitutes the use of an alternative method of analysis and hence requires demonstration that the method is appropriate for the given substance or product and gives a result consistent with that obtained with the prescribed method as described in the General Notices (see also section 13).

The prescribed method for bacterial endotoxins may be stated in the monograph on a given substance or product. The use of a method other than the method prescribed in the monograph is considered as the use of an alternative method. Where no method is stated, any of methods A to F of general chapter *2.6.14. Bacterial endotoxins* can be used.

2. METHOD AND ACCEPTANCE CRITERIA

2-1. *METHODS AND PRECAUTIONS TO BE TAKEN*

The addition of endotoxins to amoebocyte lysate may result in turbidity, precipitation or gelation (gel-clot); initially only the gel-clot method was used in the Pharmacopoeia as an evaluation criterion in the test for bacterial endotoxins. The advantage was the simplicity of basing the decision to pass or fail the substance or product to be examined on the absence or presence of a gel-clot, visible with the naked eye. The quantitative methods C, D, E and F were developed later: they require more instrumentation, but they are easier to automate for the regular testing of large numbers of samples of the same substance or product.

Endotoxins may be adsorbed onto the surface of tubes or pipettes made from certain plastics or types of glass. Interference may appear due to the release of substances from plastic materials. Hence, the materials used must be checked.

2-2. *ENDOTOXIN LIMIT CONCENTRATION*

The decision to use the test for bacterial endotoxins as a limit test implies firstly that an endotoxin limit concentration must be defined for the substance or product to be examined, and secondly that the objective of the test is to know whether the endotoxin concentration in the sample to be examined is below or above this limit. The quantitative methods C, D, E and F make it possible to determine the endotoxin concentration in the sample to be examined, but for compliance with the Pharmacopoeia and in routine quality control the final question is whether or not this concentration exceeds a defined limit.

The dose of the substance or product to be examined must be taken into account in setting the endotoxin limit concentration: the limit is set so as to ensure that, as long as the endotoxin concentration in the substance or product remains below this limit, even the maximal dose administered by the intended route per hour does not contain sufficient endotoxin to cause a toxic reaction.

When the endotoxin concentration in the substance or product exactly equals the endotoxin limit concentration, gelation will occur, as is the case when the endotoxin concentration is much higher, and the substance or product will fail the test, because the all-or-none character of the test makes it impossible to differentiate between a concentration exactly equal to the endotoxin limit concentration and one that is higher. It is only when no gelation occurs that the analyst may conclude that the endotoxin concentration is below the endotoxin limit.

For substances or products in the solid state, this endotoxin limit concentration per mass unit or per International Unit (IU) of substance or product has to be converted into a concentration of endotoxin per millilitre of solution to be examined, as the test can only be carried out on a solution. The case of substances or products that already exist in the liquid state (such as infusion fluids) is discussed below.

2-3. *CALCULATION OF THE ENDOTOXIN LIMIT*

The endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass;

M = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered per hour.

The endotoxin limit depends on the product and its route of administration and may be stated in the monograph. Values for K are suggested in Table 5.1.10.-1.

For other routes, the acceptance criterion for bacterial endotoxins is generally determined on the basis of results obtained during the development of the preparation.

Table 5.1.10.-1

Route of administration	*K*
Intravenous	5.0 IU of endotoxin per kilogram of body mass
Intravenous for radiopharmaceuticals	2.5 IU of endotoxin per kilogram of body mass
Intrathecal	0.2 IU of endotoxin per kilogram of body mass
Parenteral formulations administered per square metre of body surface	100 IU of endotoxin/m^2

2-4. *CONSIDERATIONS WHEN ESTABLISHING AN ENDOTOXIN LIMIT FOR A SPECIFIC SUBSTANCE OR PRODUCT*

The endotoxin limit for a substance or product is established with consideration of the following aspects.

Calculated endotoxin limit. The endotoxin limit is calculated as described in section 2-3. This represents a safety limit not to be exceeded if the product is to be administered to humans.

Limit prescribed in an individual substance monograph. The limit stated in an individual substance monograph frequently reflects what is achievable in a controlled production environment. The limit prescribed in a monograph can therefore be lower than the calculated endotoxin limit. However a manufacturer may specify a limit that is more stringent than that stated in the monograph.

Process capability. The capability of the process to reduce or remove bacterial endotoxins during manufacture might result in lower endotoxin limits for specific processes.

Additional safety requirements. Precautions are taken in consideration of patient population (such as paediatric use, malnourished or cachectic patients, etc.), specific local requirements (e.g. countries might wish to operate with a lower average body weight of 60 kg instead of 70 kg frequently employed in Europe) or any additional safety margins requested by the competent authority.

Formulation of the product. The limit must take into consideration any theoretical bacterial endotoxin load introduced by any other components used for reconstitution and/or dilution of the product (e.g. water for injections) or introduced by starting materials and/or raw materials.

2-5. *MAXIMUM VALID DILUTION*

Which dilution of the substance or product is to be used in the test to obtain maximal assurance that a negative result means that the endotoxin concentration of the substance or product is less than the endotoxin limit and that a positive result means that the lysate detected an endotoxin concentration equal to or greater than the endotoxin limit? This dilution depends on the endotoxin limit and on the sensitivity of the lysate; it is called the maximum valid dilution (MVD) and its value may be calculated using the following expression:

$$\frac{\textit{endotoxin limit} \times \textit{concentration of test solution}}{\lambda}$$

Concentration of test solution:

- in mg/mL if the endotoxin limit is specified by mass (IU/mg);
- in Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit);
- in mL/mL if the endotoxin limit is specified by volume (IU/mL).

λ = the labelled lysate sensitivity in the gel-clot technique (IU/mL) or the lowest concentration used in the standard curve of the turbidimetric or chromogenic techniques.

When the value of the MVD is not a whole number, a convenient whole number smaller than the MVD may be used for routine purposes (which means preparing a solution of the substance or product that is less diluted than the MVD indicates). In this case, a negative result indicates that the endotoxin concentration of the substance or product lies below the limit value. However, when the endotoxin concentration of the substance or product in such a test is less than the endotoxin limit but high enough to make the reaction with the lysate result in a clot, the test may be positive under these conditions. Hence, when a test with this 'convenient' dilution factor is positive, the substance or product is diluted to the MVD and the test is repeated. In any case of doubt or dispute, the MVD must be used.

This stresses the importance of the confirmation of the sensitivity of the lysate.

Example

A 50 mg/mL solution of phenytoin sodium (intended for intravenous injection) has to be tested. Determine the MVD, given the following variables:

M = maximum human dose = 15 mg per kilogram of body mass;

c = 50 mg/mL;

K = 5 IU of endotoxin per kilogram of body mass;

λ = 0.4 IU of endotoxin per millilitre.

$$MVD = \frac{5 \times 50}{15} \times \frac{1}{0.4} = 41.67$$

For routine tests on this product, it may be expedient to dilute 1 mL of the solution to be examined to 20 mL (MVD/2 rounded to the next lower whole number). However, if this test result is positive the analyst will have to dilute 1 mL to 41.67 mL and repeat the test. A dilution to 41.67 mL is also necessary when the test is performed to settle a dispute.

3. RISK ASSESSMENT

As stated in section 1 of this general chapter, the conclusion is generally justified that the absence of bacterial endotoxins in a substance or product implies the absence of pyrogenic components, provided the presence of non-endotoxin pyrogenic substances can be ruled out. To rule out the presence of non-endotoxin pyrogens in substances or products, the use of the monocyte-activation test (*2.6.30*) is recommended at release or during development of the production process; if any changes are made to the production process that could influence the quality of the product regarding pyrogenicity, the monocyte-activation test is repeated. Examples of such changes include the use of different raw materials, a different production site and different process parameters.

The decision to use the test for bacterial endotoxins as the sole pyrogenicity test is to be made after careful evaluation of the risk of the substance or product containing non-endotoxin pyrogens. The risk assessment is made with consideration given to any factor that could result in the inclusion of pyrogens not detected by the test for bacterial endotoxins. The items below constitute a non-exhaustive list of factors to be considered in the risk assessment.

Production process (chemical synthesis, fermentation, biotechnological method). For products of fermentation, the expression system is to be considered (prokaryotic, eukaryotic) and, for a prokaryotic expression system, whether gram-positive or gram-negative bacteria are used. Also, the culture media components are examined with consideration given to their origin (synthetic, animal, plant).

Bioburden. The potential presence of gram-positive bacteria and fungi as contaminants of the active substance, excipients or starting materials and raw materials used in the production of the medicinal product, and the origin of the raw materials

(synthetic, animal, plant) have to be taken into consideration. The quality of the water plays an important role on the overall evaluation.

Capability of the downstream process. It must be verified whether bacterial endotoxin removal steps are part of the downstream process.

Safety. The target population and the route of administration (e.g. intravenous, intrathecal) have to be taken into account in the risk assessment.

Stability of the detectable endotoxins. It has to be considered that the ability to detect endotoxins can be affected by interaction with certain components, storage conditions or storage time, temperature and handling of the test sample. Procedures that demonstrate stability of the detectable endotoxin content have to be established for storing, handling and mixing of samples.

4. REFERENCE MATERIAL

Endotoxin standard BRP is intended for use as the reference preparation. It has been assayed against the WHO International Standard for Endotoxin and its potency is expressed in International Units of endotoxin per vial. The International Unit of endotoxin is defined as the specific activity of a defined mass of the International Standard.

For routine purposes, another preparation of endotoxin may be used, provided it has been assayed against the International Standard for Endotoxin or the BRP and its potency is expressed in International Units of endotoxin.

NOTE: 1 International Unit (IU) of endotoxin is equal to 1 Endotoxin Unit (EU).

5. WATER FOR BET

Water for BET is sterile water that is free of detectable levels of endotoxin. Usually it is commercially available and certified.

General chapter *2.6.14. Bacterial endotoxins* indicates that methods other than triple distillation may be used to prepare water for BET. Reverse osmosis has been used with good results; some analysts may prefer to distil the water more than 3 times. Whatever method is used, the resultant product must be free of detectable bacterial endotoxins.

6. pH OF THE MIXTURE

In the test for bacterial endotoxins, optimum gel-clot occurs for a mixture at pH 6.0-8.0. However, the addition of the lysate to the sample may result in a lowering of the pH.

7. VALIDATION OF THE LYSATE

It is important to follow the manufacturer's instructions for the preparation of the solutions of the lysate.

The positive end-point dilution factors in gel-clot methods A and B are converted to logarithms. The reason is that if the frequency distribution of these logarithmic values is plotted, it usually approaches a normal distribution curve much more closely than the frequency distribution of the dilution factors themselves; in fact it is so similar that it is acceptable to use the normal frequency distribution as a mathematical model and to calculate confidence limits with Student's *t*-test.

8. PRELIMINARY TEST FOR INTERFERING FACTORS

Some substances or products cannot be tested directly for the presence of bacterial endotoxins because they are not miscible with the reagents, they cannot be adjusted to pH 6.0-8.0 or they inhibit or activate enzymatic reaction (such as β-D-glucans).

Therefore a preliminary test is required to check for the presence of interfering factors; when these are found the analyst must demonstrate that the procedure to remove them has been effective and that by applying this procedure, any bacterial endotoxins present have not been removed.

The object of the preliminary test is to test the null hypothesis that the sensitivity of the lysate in the presence of the substance or product to be examined does not differ significantly from the sensitivity of the lysate in the absence of the product. A simple criterion is used in methods A and B: the null hypothesis is accepted when the sensitivity of the lysate in the presence of the product is at least 0.5 times and not more than twice the sensitivity of the lysate by itself.

The test for interfering factors in gel-clot methods A and B requires the use of a sample of the substance or product in which no endotoxins are detectable. This presents a theoretical problem when an entirely new product has to be tested. Hence, a different approach was designed for quantitative methods C, D, E and F.

Note that methods D and E, which use a chromogenic peptide, require reagents that are absent in methods A, B, C and F, and hence compliance of methods A, B, C or F with the requirements for interfering factors cannot be extrapolated to method D or method E without further testing.

9. REMOVAL OF INTERFERING FACTORS

The procedures to remove interfering factors must not increase or decrease (for example, by adsorption) the amount of endotoxin in the substance or product to be examined. The correct way of checking this is to apply the procedures to a spiked sample of the substance or product to be examined, that is, a sample to which a known amount of endotoxin has been added, and then to measure the recovery of the endotoxin after the removal process has been conducted.

Methods C and D. If the nature of the product to be examined results in an interference that cannot be removed by classical methods (e.g. dilution or centrifugation), it may be possible to determine the standard curve in the same type of substance or product freed from endotoxins by appropriate treatment or by dilution of the substance or product. The endotoxins test is then carried out by comparison with this standard curve.

Ultrafiltration with cellulose triacetate asymmetric membrane filters has been found to be suitable in most cases. The filters must be properly validated, because under some circumstances cellulose derivatives (β-D-glucans) can cause false positive results.

Another option to remove interfering factors is a 2-step procedure in which 1) endotoxin within the interfering sample is fixed on a solid phase, and 2) after removal of the interfering substance (e.g. by washing) the endotoxin is detected unimpaired under suitable testing conditions.

10. THE PURPOSE OF THE CONTROLS

The purpose of the control made up with water for BET and the reference preparation of endotoxin at twice the concentration of the labelled lysate sensitivity is to verify the activity of the lysate at the time and under the conditions of the test (for method A and B). The purpose of the negative control is to verify the absence of a detectable concentration of endotoxin in the water for BET.

The positive control, which contains the product to be examined at the concentration used in the test, is intended to show the absence of inhibiting factors at the time and under the conditions of the test.

11. READING AND INTERPRETATION OF RESULTS

Minute amounts of bacterial endotoxin in the water for BET, or in any other reagent or material to which the lysate is exposed during the test, may escape detection as long as they do not reach the sensitivity limit of the lysate. However, they may raise the amount of bacterial endotoxin in the solution containing the substance or product to be examined to just above the sensitivity limit and cause a positive reaction.

The risk of this happening may be reduced by testing the water for BET and the other reagents and materials with the most sensitive lysate available, or at least one that is more sensitive than the one used in the test on the product. Even then, the risk of such a 'false positive result' cannot be ruled out completely.

12. IMPLEMENTATION OF METHODS DESCRIBED IN THE PH. EUR.

As stated in the General Notices, the test methods given in monographs and general chapters have been validated in accordance with accepted scientific practice and current recommendations on analytical validation. The methods described in general chapters *2.6.14. Bacterial endotoxins*, *2.6.30. Monocyte-activation test* and *2.6.32. Test for bacterial endotoxins using recombinant factor C* therefore do not have to be re-validated *per se*, other than in consideration of their use for a specific substance or product in a specific analytical environment.

The procedure and the materials and reagents used in the method must be validated as described for the test concerned.

The absence of interfering factors (and, if necessary, the procedure for removing them) is verified on samples of at least 3 production batches.

As stated in general chapter *2.6.30. Monocyte-activation test*, the monocyte-activation test is primarily intended as a replacement of the rabbit pyrogen test. Guidelines on which methods to use (A, B or C) and on how to validate the monocyte-activation test are described in general chapter *2.6.30. Monocyte-activation test*.

13. REPLACEMENT OF A METHOD PRESCRIBED IN A MONOGRAPH

13-1. *REPLACEMENT BY ANOTHER METHOD DESCRIBED IN THE PH. EUR.*

Replacement of a method prescribed in a monograph by another method described in the Ph. Eur. is to be regarded as the use of an alternative method in the replacement of a pharmacopoeial test, as described in the General Notices. The analyst has to demonstrate that a valid test can be carried out on the substance or product concerned.

The alternative method does not have to be re-validated *per se*, other than in consideration of its use for a specific substance or product in a specific analytical environment and of its equivalence to the prescribed method.

13-2. *REPLACEMENT BY A METHOD NOT DESCRIBED IN THE PH. EUR.*

Replacement of a method prescribed in a monograph by a method not described in the Ph. Eur. is to be regarded as the use of an alternative method in the replacement of a pharmacopoeial test, as described in the General Notices.

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2.7.2. MICROBIOLOGICAL ASSAY OF ANTIBIOTICS

The potency of an antibiotic is estimated by comparing the inhibition of growth of sensitive micro-organisms produced by known concentrations of the antibiotic to be examined and a reference substance.

The reference substances used in the assays are substances whose activity has been precisely determined with reference to the corresponding international standard or international reference preparation.

The assay must be designed in a way that will permit examination of the validity of the mathematical model on which the potency equation is based. If a parallel-line model is chosen, the 2 log dose-response (or transformed response) lines of the preparation to be examined and the reference preparation must be parallel; they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a given probability, usually $P = 0.05$. Other mathematical models, such as the slope ratio model, may be used provided that proof of validity is demonstrated.

Unless otherwise stated in the monograph, the confidence limits ($P = 0.95$) of the assay for potency are not less than 95 per cent and not more than 105 per cent of the estimated potency.

Carry out the assay by method A or method B.

A. DIFFUSION METHOD

Liquefy a medium suitable for the conditions of the assay and inoculate it at a suitable temperature, for example 48 °C to 50 °C for vegetative forms, with a known quantity of a suspension of micro-organisms sensitive to the antibiotic to be examined, such that clearly defined zones of inhibition of suitable diameter are produced with the concentrations of the antibiotic used for the assay. Immediately pour into Petri dishes or large rectangular dishes a quantity of the inoculated medium to form a uniform layer 2-5 mm thick. Alternatively, the medium may consist of 2 layers, only the upper layer being inoculated.

Store the dishes so that no appreciable growth or death of the micro-organisms occurs before the dishes are used and so that the surface of the medium is dry at the time of use.

Using the solvent and the buffer solution indicated in Table 2.7.2.-1, prepare solutions of the reference substance and of the antibiotic to be examined having known concentrations and presumed to be of equal activity. Apply the solutions to the surface of the medium, for example, in sterile cylinders of porcelain, stainless steel or other suitable material, or in cavities prepared in the agar. The same volume of solution must be added to each cylinder or cavity. Alternatively, use sterile absorbent paper discs of suitable quality; impregnate the discs with the solutions of the reference substance or the solutions of the antibiotic to be examined and place on the surface of the agar.

In order to assess the validity of the assay, use not fewer than 3 doses of the reference substance and 3 doses of the antibiotic to be examined having the same presumed activity as the doses of the reference substance. It is preferable to use a series of doses in geometric progression. In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay as described above must be applied.

Arrange the solutions on each Petri dish or on each rectangular dish according to a statistically suitable design, except for small Petri dishes that cannot accommodate more than 6 solutions, arrange the solutions of the antibiotic to be examined and the solutions of the reference substance in an alternate manner to avoid interaction of the more concentrated solutions.

Incubate at a suitable temperature for about 18 h. A period of diffusion prior to incubation, usually 1-4 h, at room temperature or at about 4 °C, as appropriate, may be used to minimise the effects of the variation in time between the application of the solutions and to improve the regression slope.

Measure the diameters to the nearest 0.1 mm or the areas of the circular inhibition zones to the nearest 0.01 mm^2 and calculate the potency using appropriate statistical methods.

Use in each assay the number of replications per dose sufficient to ensure the required accuracy and precision. The assay may be repeated and the results combined statistically to obtain the required accuracy and precision and to ascertain whether the potency of the antibiotic to be examined is not less than the minimum required.

B. TURBIDIMETRIC METHOD

Inoculate a suitable medium with a suspension of the chosen micro-organism having a sensitivity to the antibiotic to be examined such that a sufficiently large inhibition of microbial growth occurs in the conditions of the test. Use a known quantity of the suspension chosen so as to obtain a readily measurable opacity after an incubation period of about 4 h.

Use the inoculated medium immediately after its preparation.

Using the solvent and the buffer solution indicated in Table 2.7.2.-2 prepare solutions of the reference substance and of the antibiotic to be examined having known concentrations presumed to be of equal activity.

In order that the validity of the assay may be assessed, use not fewer than 3 doses of the reference substance and 3 doses of the antibiotic to be examined having the same presumed activity as the doses of the reference substance. It is preferable to use a series of doses in geometric progression. In order to obtain the required linearity, it may be necessary to select from a large number 3 consecutive doses, using corresponding doses for the reference substance and the antibiotic to be examined.

Distribute an equal volume of each of the solutions into identical test-tubes and add to each tube an equal volume of inoculated medium (for example, 1 mL of the solution and 9 mL of the medium). For the assay of tyrothricin add 0.1 mL of the solution to 9.9 mL of inoculated medium.

Prepare at the same time 2 control tubes without antibiotic, both containing the inoculated medium and to one of which is added immediately 0.5 mL of *formaldehyde R*. These tubes are used to set the optical apparatus used to measure the growth.

Place all the tubes, randomly distributed or in a Latin square or randomised block arrangement, in a water-bath or other suitable apparatus fitted with a means of bringing all the tubes rapidly to the appropriate incubation temperature and maintain them at that temperature for 3-4 h, taking precautions to ensure uniformity of temperature and identical incubation time.

After incubation, stop the growth of the micro-organisms by adding 0.5 mL of *formaldehyde R* to each tube or by heat treatment and measure the opacity to 3 significant figures using suitable optical apparatus. Alternatively use a method which allows the opacity of each tube to be measured after exactly the same period of incubation.

Table 2.7.2.-1. – *Diffusion assay*

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Amphotericin B	*Amphotericin B for microbiological assay CRS*	*Dimethyl sulfoxide R*	pH 10.5 (0.2 M)	*Saccharomyces cerevisiae* ATCC 9763 IP 1432-83	F - pH 6.1	35-37 °C
Bacitracin zinc	*Bacitracin zinc CRS*	*0.01 M hydrochloric acid*	pH 7.0 (0.05 M)	*Micrococcus luteus* NCTC 7743 CIP 53.160 ATCC 10240	A - pH 7.0	35-39 °C
Bleomycin sulfate	*Bleomycin sulfate CRS*	*Water R*	pH 6.8 (0.1 M)	*Mycobacterium smegmatis* ATCC 607	G - pH 7.0	35-37 °C
Colistimethate sodium	*Colistimethate sodium CRS*	*Water R*	pH 6.0 (0.05 M)	*Bordetella bronchiseptica* NCTC 8344 CIP 53.157 ATCC 4617	B - pH 7.3	35-39 °C
				Escherichia coli NCIMB 8879 CIP 54.127 ATCC 10536	B - pH 7.3	35-39 °C
Colistin sulfate	*Colistin sulfate for microbiological assay CRS*	*Water R*	pH 6.0 (0.05 M)	*Bordetella bronchiseptica* NCTC 8344 CIP 53.157 ATCC 4617	B - pH 7.3	35-39 °C
				Escherichia coli NCIMB 8879 CIP 54.127 ATCC 10536	B - pH 7.3	35-39 °C
Framycetin sulfate	*Framycetin sulfate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	E - pH 7.9	30-37 °C
				Bacillus pumilus NCTC 8241 CIP 76.18	E - pH 7.9	30-37 °C
Gentamicin sulfate	*Gentamicin sulfate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus pumilus* NCTC 8241 CIP 76.18	A - pH 7.9	35-39 °C
				Staphylococcus epidermidis NCIMB 8853 CIP 68.21 ATCC 12228	A - pH 7.9	35-39 °C
Josamycin	*Josamycin CRS*	*Methanol R* (see the monograph)	pH 5.6	*Bacillus subtilis* CIP 52.62 ATCC 6633 NCTC 10400	A - pH 6.6	35-37 °C
Josamycin propionate	*Josamycin propionate CRS*	*Methanol R* (see the monograph)	pH 5.6	*Bacillus subtilis* CIP 52.62 ATCC 6633 NCTC 10400	A - pH 6.6	35-37 °C
Kanamycin monosulfate Kanamycin acid sulfate	*Kanamycin monosulfate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9	30-37 °C
				Staphylococcus aureus NCTC 7447 CIP 53.156 ATCC 6538 P	A - pH 7.9	35-39 °C

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Neomycin sulfate	*Neomycin sulfate for microbiological assay CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus pumilus* NCTC 8241 CIP 76.18	E - pH 7.9	30-37 °C
				Bacillus subtilis NCTC 10400 CIP 52.62 ATCC 6633	E - pH 7.9	30-37 °C
Netilmicin sulfate	*Netilmicin sulfate CRS*	*Water R*	pH 8.0 ± 0.1	*Staphylococcus aureus* ATCC 6538 P CIP 53.156	A - pH 7.9	32-35 °C
Nystatin	*Nystatin CRS*	*Dimethylformamide R*	pH 6.0 (0.05 M) containing 5 per cent *V/V* of *dimethylformamide R*	*Candida tropicalis* CIP 1433-83 NCYC 1393	F - pH 6.0	30-37 °C
				Saccharomyces cerevisiae NCYC 87 CIP 1432-83 ATCC 9763	F - pH 6.0	30-32 °C
Polymyxin B sulfate	*Polymyxin B sulfate for microbiological assay CRS*	*Water R*	pH 6.0 (0.05 M)	*Bordetella bronchiseptica* NCTC 8344 CIP 53.157 ATCC 4617	B - pH 7.3	35-39 °C
Rifamycin sodium	*Rifamycin sodium CRS*	*Methanol R*	pH 7.0 (0.05 M)	*Micrococcus luteus* NCTC 8340 CIP 53.45 ATCC 9341	A - pH 6.6	35-39 °C
Spiramycin	*Spiramycin CRS*	*Methanol R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9	30-32 °C
Streptomycin sulfate	*Streptomycin sulfate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 8236 CIP 1.83	A - pH 7.9	30-37 °C
				Bacillus subtilis NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9	30-37 °C
Teicoplanin	*Teicoplanin CRS*	pH 6.0 (0.05 M)	pH 6.0 (0.05 M)	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	H - pH 7.8-8.0	35-37 °C
Tylosin for veterinary use Tylosin phosphate for veterinary use Tylosin tartrate for veterinary use	*Tylosin CRS*	2.5 per cent *V/V* solution of *methanol R* in *0.1 M phosphate buffer solution pH 7.0 R*	A mixture of 40 volumes of *methanol R* and 60 volumes of *0.1 M phosphate buffer solution pH 8.0 R*	*Micrococcus luteus* NCTC 8340 CIP 53.45 ATCC 9341	A - pH 8.0	32-35 °C
Vancomycin hydrochloride	*Vancomycin hydrochloride CRS*	*Water R*	pH 8.0	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	A - pH 8.0	37-39 °C

Calculate the potency using appropriate statistical methods.

Linearity of the dose-response relationship, transformed or untransformed, is often obtained only over a very limited range. It is this range which must be used in calculating the activity and it must include at least 3 consecutive doses in order to permit linearity to be verified. In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay must be applied.

Use in each assay the number of replications per dose sufficient to ensure the required accuracy and precision. The assay may be repeated and the results combined statistically to obtain the required accuracy and precision and to ascertain whether the potency of the antibiotic to be examined is not less than the minimum required.

Table 2.7.2.-2. – *Turbidimetric assay*

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Colistimethate sodium	*Colistimethate sodium CRS*	*Water R*	pH 7.0	*Escherichia coli* NCIMB 8666 CIP 2.83 ATCC 9637	C - pH 7.0	35-37 °C
Colistin sulfate	*Colistin sulfate for microbiological assay CRS*	*Water R*	pH 7.0	*Escherichia coli* NCIMB 8666 CIP 2.83 ATCC 9637	C - pH 7.0	35-37 °C
Framycetin sulfate	*Framycetin sulfate CRS*	*Water R*	pH 8.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Gentamicin sulfate	*Gentamicin sulfate CRS*	*Water R*	pH 7.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Gramicidin	*Gramicidin CRS*	*Methanol R*	pH 7.0*	*Enterococcus hirae* CIP 58.55 ATCC 10541 *Staphylococcus aureus* ATCC 6538 P	C - pH 7.0	35-37 °C
	*Addition of a detergent may be necessary to avoid adsorption on the material during the dilutions, for example 0.1 mg/mL of *polysorbate 80 R*					
Josamycin	*Josamycin CRS*	*Methanol R* (see the monograph)	pH 5.6	*Staphylococcus aureus* CIP 53.156 ATCC 6538 P NCTC 7447	C - pH 8.0	35-37 °C
Josamycin propionate	*Josamycin propionate CRS*	*Methanol R* (see the monograph)	pH 5.6	*Staphylococcus aureus* CIP 53.156 ATCC 6538 P NCTC 7447	C - pH 8.0	35-37 °C
Kanamycin monosulfate Kanamycin acid sulfate	*Kanamycin monosulfate CRS*	*Water R*	pH 8.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Neomycin sulfate	*Neomycin sulfate for microbiological assay CRS*	*Water R*	pH 8.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Rifamycin sodium	*Rifamycin sodium CRS*	*Methanol R*	pH 7.0	*Escherichia coli* NCIMB 8879 CIP 54.127 ATCC 10536	C - pH 7.0	35-37 °C
Spiramycin	*Spiramycin CRS*	*Methanol R*	pH 7.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Streptomycin sulfate	*Streptomycin sulfate CRS*	*Water R*	pH 8.0	*Klebsiella pneumoniae* NCTC 7427 CIP 53.153 ATCC 10031	C - pH 7.0	35-37 °C

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Tylosin for veterinary use Tylosin tartrate for veterinary use	*Tylosin CRS*	2.5 per cent *V/V* solution of *methanol R* in *0.1 M phosphate buffer solution pH 7.0 R*	pH 7.0	*Staphylococcus aureus* NCTC 6571 ATCC 9144 CIP 53.154	C - pH 7.0	37 °C
Tyrothricin	*Gramicidin CRS*	*Alcohol R*	*Alcohol R*	*Enterococcus hirae* ATCC 10541	C - pH 7.0	37 °C
Vancomycin hydrochloride	*Vancomycin hydrochloride CRS*	*Water R*	pH 8.0	*Staphylococcus aureus* CIP 53.156 ATCC 6538 P	C - pH 7.0	37-39 °C

The following section is published for information.

Recommended micro-organisms

The following text details the recommended micro-organisms and the conditions of use. Other micro-organisms may be used provided that they are shown to be sensitive to the antibiotic to be examined and are used in appropriate media and appropriate conditions of temperature and pH. The concentrations of the solutions used should be chosen so as to ensure that a linear relationship exists between the logarithm of the dose and the response in the conditions of the test.

Preparation of inocula. *Bacillus cereus* var. *mycoides*; *Bacillus subtilis*; *Bacillus pumilus*. Spore suspensions of the organisms to be used as inocula are prepared as follows.

Grow the organism at 35-37 °C for 7 days on the surface of a suitable medium to which has been added 0.001 g/L of *manganese sulfate R*. Using sterile *water R*, wash off the growth, which consists mainly of spores. Heat the suspension at 70 °C for 30 min and dilute to give an appropriate concentration of spores, usually 10×10^6 to 100×10^6 per millilitre. The spore suspensions may be stored for long periods at a temperature not exceeding 4 °C.

Alternatively, spore suspensions may be prepared by cultivating the organisms in medium C at 26 °C for 4-6 days, then adding, aseptically, sufficient *manganese sulfate R* to give a concentration of 0.001 g/L and incubating for a further 48 h. Examine the suspension microscopically to ensure that adequate spore formation has taken place (about 80 per cent) and centrifuge. Re-suspend the sediment in sterile *water R* to give a concentration of 10×10^6 to 100×10^6 spores per millilitre, and then heat to 70 °C for 30 min. Store the suspension at a temperature not exceeding 4 °C.

Bordetella bronchiseptica. Grow the test organism on medium B at 35-37 °C for 16-18 h. Wash off the bacterial growth with sterile *water R* and dilute to a suitable opacity.

Staphylococcus aureus; *Klebsiella pneumoniae*; *Escherichia coli*; *Micrococcus luteus*; *Staphylococcus epidermidis*. Prepare as described above for *B. bronchiseptica* but using medium A and adjusting the opacity to one which has been shown to produce a satisfactory dose-response relationship in the turbidimetric assay, or to produce clearly defined zones of inhibition of convenient diameter in the diffusion assay, as appropriate.

Saccharomyces cerevisiae; *Candida tropicalis*. Grow the test organism on medium F at 30-37 °C for 24 h. Wash off the growth with a sterile 9 g/L solution of *sodium chloride R*. Dilute to a suitable opacity with the same solution.

Buffer solutions. Buffer solutions having a pH between 5.8 and 8.0 are prepared by mixing 50.0 mL of *0.2 M potassium dihydrogen phosphate R* with the quantity of *0.2 M sodium hydroxide* indicated in Table 2.7.2.-3. Dilute with freshly prepared *distilled water R* to produce 200.0 mL.

Table 2.7.2.-3

pH	*0.2 M Sodium hydroxide* (mL)
5.8	3.72
6.0	5.70
6.2	8.60
6.4	12.60
6.6	17.80
6.8	23.65
7.0	29.63
7.2	35.00
7.4	39.50
7.6	42.80
7.8	45.20
8.0	46.80

These buffer solutions are used for all microbiological assays shown in Table 2.7.2.-1 with the exception of bleomycin sulfate and amphotericin B.

For bleomycin sulfate, prepare the buffer solution pH 6.8 as follows: dissolve 6.4 g of *potassium dihydrogen phosphate R* and 18.9 g of *disodium hydrogen phosphate dodecahydrate R* in *water R* and dilute to 1000 mL with *water R*.

For amphotericin B, prepare the 0.2 M phosphate buffer solution pH 10.5 as follows: dissolve 35 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*, add 20 mL of *1 M sodium hydroxide* and dilute to 1000.0 mL with *water R*.

Culture media. The following media or equivalent media may be used.

Medium A

Peptone	6 g
Pancreatic digest of casein	4 g
Beef extract	1.5 g
Yeast extract	3 g
Glucose monohydrate	1 g
Agar	15 g
Water	to 1000 mL

Medium B

Pancreatic digest of casein	17 g
Papaic digest of soya bean	3 g
Sodium chloride	5 g

Dipotassium hydrogen phosphate	2.5 g
Glucose monohydrate	2.5 g
Agar	15 g
Polysorbate 80	10 g
Water	to 1000 mL

The polysorbate 80 is added to the hot solution of the other ingredients after boiling, and immediately before adjusting to volume.

Medium C

Peptone	6 g
Beef extract	1.5 g
Yeast extract	3 g
Sodium chloride	3.5 g
Glucose monohydrate	1 g
Dipotassium hydrogen phosphate	3.68 g
Potassium dihydrogen phosphate	1.32 g
Water	to 1000 mL

Medium D

Heart extract	1.5 g
Yeast extract	1.5 g
Peptone-casein	5 g
Glucose monohydrate	1 g
Sodium chloride	3.5 g
Dipotassium hydrogen phosphate	3.68 g
Potassium dihydrogen phosphate	1.32 g
Potassium nitrate	2 g
Water	to 1000 mL

Medium E

Peptone	5 g
Meat extract	3 g
Disodium hydrogen phosphate,12H_2O	26.9 g
Agar	10 g
Water	to 1000 mL

The disodium hydrogen phosphate is added as a sterile solution after sterilisation of the medium.

Medium F

Peptone	9.4 g
Yeast extract	4.7 g
Beef extract	2.4 g
Sodium chloride	10.0 g
Glucose monohydrate	10.0 g
Agar	23.5 g
Water	to 1000 mL

Medium G

Glycerol	10 g
Peptone	10 g
Meat extract	10 g
Sodium chloride	3 g
Agar	15 g
Water	to 1000 mL

pH 7.0 ± 0.1 after sterilisation.

Medium H

Peptone	5.0 g
Agar	15.0 g
Beef extract powder	3.0 g
Water	to 1000 mL

pH 7.8 - 8.0 adjusted with *0.1 M sodium hydroxide*.

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2.6.34. HOST-CELL PROTEIN ASSAYS

This general chapter provides guidance for the development and validation of host-cell protein (HCP) assays used to test products obtained by recombinant DNA technology. It does not exclude the use of alternative approaches that are acceptable to the competent authority.

INTRODUCTION

Host-cell proteins (HCPs) are process-related impurities derived from the host organism used for the production of a medicinal product by recombinant DNA technology. In order to mitigate their potential adverse effects (e.g. immunogenicity), HCP content is expected to be reduced to the lowest possible level.

HCP clearance during the purification process must be assessed and the HCP content determined using an HCP assay that has been evaluated and validated for a given product.

The HCP acceptance limit, typically expressed in nanograms of HCP per milligram of active substance (ppm), must be justified with regard to the HCP clearance capacity of the purification process and with regard to the potential impact of residual HCP on patients, taking into account the worst-case quantity of HCP that could be administered with the product.

HCPs are generally measured using an immuno-based assay containing, as reagents, the HCP antigen preparation (hereinafter 'the HCP antigens') or HCP reference standard and the corresponding polyclonal antibodies (antisera). Antisera must cover a broad spectrum of HCPs representative of the product concerned.

Sandwich-type enzyme-linked immunosorbent assays (ELISA) are the most commonly employed assays to assess quantitatively the level of HCPs. It should be noted that HCP content measured by ELISA does not represent absolute HCP mass content. The sensitivity is the result of the observed cumulative responses of many individual HCPs in comparison to the response of an HCP reference standard. The use of orthogonal analytical methods (e.g. electrophoresis, HPLC, Western blot, mass spectrometry) to characterise the various HCPs in the product is recommended to support the development and selection of the assay.

ASSAY SELECTION

Several types of assay are available, with selection taking into account several factors, including the stage of development of the product, the nature of the host cell and the protein immunogenicity, the expression mode, the manufacturing process, and prior knowledge. When selecting and developing the assay, its life cycle (e.g. reagent supply, consistency, assay validation, process change) must also be considered.

TYPES OF ASSAY

Process-specific assays

Process-specific HCP assays (also called product-specific HCP assays) are developed and validated taking into account the specificity of the production process, and using the same host organism expressing the recombinant product.

The HCP antigens are derived from a mock run of the active substance manufacturing process (or a process representative of it) up to a step capable of generating a broad spectrum of HCPs in sufficient quantities.

The antisera raised must cover a broad range of HCPs, in order to detect as many different HCPs as possible and also to accommodate process variations.

Platform assays

Platform assays are developed by individual manufacturers and customised for the processes and host organism used by the manufacturer for production. The same sets of reference standards and reagents may be used to monitor HCPs in several products manufactured in the same host organism, provided that upstream processes (and downstream, if relevant) are sufficiently similar for these products. The suitability of the antiserum should be evaluated as described above for process-specific assays.

Generic assays

Commercially available HCP test kits are commonly referred to as generic HCP assays. They are intended to work broadly across similar expression hosts. Detailed information on the preparation of the reagents may not be disclosed by the vendor. For instance, the HCP antigens may be derived from a combination of strains of an expression host species, and the process(es) used may not mimic the process applied for the product of interest. The suitability of the antiserum should be evaluated as described above for process-specific assays.

CRITERIA FOR ASSAY SELECTION

In view of the potential safety issues associated with residual HCPs in the active substance, a risk assessment is performed to support the choice between a generic, a platform or a process-specific strategy, taking into account the stage of development of the product.

For early development a generic assay or a platform assay may be used. For later development phases, process-specific assays must be considered, as they are generally regarded as superior, especially when compared to generic assays. This is because process-specific assays are more likely to show immunoreactivity against representative HCPs.

Platform or generic assays may be used, provided that the assay is appropriately characterised and validated against process-specific HCPs.

PRODUCTION AND TESTING OF THE HCP ANTIGEN PREPARATION

The HCP reagents (HCP antigens and anti-HCP antibodies) are produced in such a way as to facilitate replication of the production when a replenishment for the HCP assay is needed.

The HCP antigens are used to generate the polyclonal antibody reagent for the HCP immunoassay by immunising one or more suitable animal species. In addition, they serve as the HCP reference standard in the HCP immunoassay.

As far as possible, the HCP antigens must cover the relevant HCP population expected to be derived from the manufacturing process of the protein of interest.

The HCP population must also be broad enough to cover worst-case purification scenarios and to provide robustness against potential manufacturing process changes during the life cycle of the product.

PROCESS-SPECIFIC ASSAYS

Null cell line

Development of a process-specific assay involves the selection of a null cell line that does not contain the expression gene for the product of interest and is derived from the same cell line that has been used to establish the production cell line. This null cell line may be non-transfected or mock-transfected. A mock-transfected cell line is created by transfecting the parental cell line with a blank plasmid, i.e. the plasmid used to create the production cell line, but missing the gene coding for the protein of interest.

Mock production process

Upstream

The antigens produced for process-specific assays are obtained by a mock production process that mimics the intended manufacturing process, using the null cell line and, as far as possible, the same operating conditions.

As for any mock production, the process used represents an approximation of the intended manufacturing process and leads to differences (e.g. different scale, operating parameters, product interaction). However, the impact of those differences needs to be considered carefully because they may affect the composition of the HCP population.

For example, a mock fermentation of an inclusion body manufacturing process may not deliver the desired inclusion bodies if the product is not present. Therefore, depending on the null cell line used (e.g. mock-transfected or not), the antigens may need to be isolated differently compared to the intended manufacturing process.

In some situations, operating parameters for the mock production may be adjusted to cover worst-case scenarios (e.g. to deliver antigens covering a broad spectrum of different HCP species). For example, the antigen-containing cell culture supernatant may be harvested beyond the minimum level of cell viability in order to include more cytosolic proteins, which are released by additional cell lysis.

Downstream

The HCP antigens derived from the upstream process are usually only minimally processed (filtration, concentration), in order to obtain a representative spectrum of HCPs. Further purification is generally not recommended as there will be a risk of losing HCP species.

However, in cases where the antigens are not representative (e.g. resulting in low coverage), mixing of mock materials from different processing steps can be considered. Enrichment may also be achieved by pooling materials from mock fermentation or purification runs using different operating conditions, or from selective purification steps (e.g. to reduce large amounts of the few immunodominant HCPs).

Cross-contamination with the protein of interest

The HCP antigens must be produced in a manner that avoids contamination with even minute traces of the product in order to avoid cross-reactivity with the polyclonal antibodies.

To achieve this goal, dedicated or single-use equipment is used as much as possible. Where multi-purpose equipment is used, it must be cleaned appropriately. In addition, the risk of contamination when filling or handling the antigens in the laboratory environment must also be considered.

Characterisation and testing

Before using the HCP antigens for immunisation, the protein content is assessed (total protein assay) and the absence of the protein of interest verified.

Comparison of the HCP population with the mock and the intended production process is performed, typically by SDS-PAGE and/or two-dimensional (2D) electrophoresis with a high sensitivity stain. The aim of this comparison is to show that the HCP antigens resulting from the mock production process contain most of the representative HCP species of the intended manufacturing process. Where necessary, complementary information may be gathered by orthogonal methods, e.g. mass spectrometry.

PLATFORM ASSAYS

Null cell line

Development of a platform assay involves a null cell line that does not contain the expression gene for the product of interest, and uses the same host species. This null cell line may be non-transfected or mock-transfected, and may be used for the production of HCP antigens for products from a given company's manufacturing platform.

Mock production process

Upstream

The HCP antigens produced for platform assays are obtained by a mock production process that mimics the platform upstream process that is used for several products, and typically uses the same media components. As for any mock production, the process used represents an approximation of the intended manufacturing process, which may impact the composition of the HCP population (see process-specific assays).

Downstream

As for other assays, the HCP antigens derived from the upstream process are, in general, only minimally processed (e.g. no or limited number of purification steps) to obtain a broad spectrum of HCPs, although mixing and pooling strategies may also be used to widen the spectrum of HCP species.

Characterisation and testing

As for process-specific assays, both the protein content and the absence of the protein of interest are tested. Comparison of the HCP population with the mock and the intended production process is performed.

GENERIC ASSAYS

Generic assays are commercially available and are developed by the vendor.

Detailed information on the preparation of the reagents may not be disclosed by the vendor. For instance, the null cell line may be derived from a combination of strains of an expression host species, and the process(es) used may not mimic the process applied for the product of interest.

Nevertheless, the generic assay must be selected with consideration given to the intended manufacturing process (e.g. appropriate host cell line), and be appropriately validated for the product of interest and phase of development. As a consequence, if generic assays are used in later stages of development or during commercial manufacturing, it is recommended to validate the assay and control lot-to-lot reagent consistency using either appropriate upstream fractions from the production process or a mock preparation generated using a null cell line.

PRODUCTION AND CHARACTERISATION OF THE ANTI-HCP ANTIBODY REAGENT

PROCESS-SPECIFIC AND PLATFORM ASSAYS

Immunisation

One of the challenges of the immunisation step is to generate polyclonal antibodies that are highly specific and sensitive for each of the antigenic proteins in the complex mixture of HCPs used as an immunogen. An animal's immune response must be stimulated against both the stronger and the weaker antigens.

An animal host that yields a sufficient quantity and diversity of HCP-specific immunoglobulin G (IgG) is selected.

Where both the polyclonal capture and the polyclonal detection antibodies are from the same source, it can be assumed that they recognise different epitopes on the same HCP in the assay. Alternatively, polyclonal anti-HCP antibodies from different animal species may be used. Using several animals for a given species may reduce the impact of individual variations in immune competence and provide additional response diversity, resulting in maximised antibody coverage against the HCP antigens.

An immune response to a limited number of HCP antigens may be obtained rapidly, particularly when adjuvants are used to boost the immune response. However, in complex mixtures, differential enhancement of the immune response towards weaker antigens or those at lower concentrations may be necessary.

It usually takes several immunisations to reach a maximum immunological response and, depending on the frequency of immunisation, the process can take 3-6 months to complete.

The immune response against the HCPs for a given immunisation scheme has to be monitored by determining the antibody titre using, for example, an ELISA, and by comparing the results of 1D or 2D electrophoresis after protein staining and a Western blot, where the polyclonal anti-HCP antibodies

are used as primary antibody. In practice, some minor proteins that elicit a strong immune response may not be visible in the protein-stained gel, and some poorly antigenic proteins that are detectable by protein staining may not elicit a detectable immune response. To achieve sample-dilution linearity in complex multi-analyte immunoassays, it is essential that the immune reagent simultaneously and specifically recognises as many individual analytes as possible in an assay sample and that it is present in stoichiometric excess. For this purpose, a series of sample dilutions from different process steps may be tested by ELISA using purified anti-HCP antibodies from bleedings that have shown suitable coverage by Western blot.

Finally, based on the results of the tests described above, antisera from different animals are pooled, retested and purified.

Purification and preparation

The HCP antibodies must be purified before an assay can be developed.

Typically, this is achieved by protein A- or protein G-chromatography and/or HCP antigen affinity chromatography. In the case of HCP antigen affinity chromatography, the antigens used for immunisation are immobilised on column chromatography media and the specific antibodies are captured by applying the antisera onto the column.

Additional purification to remove potential aggregates might be required by gel permeation chromatography.

For the ELISA, a part of the purified anti-HCP antibodies is conjugated to a detection label (e.g. biotin or horseradish peroxidase).

The purified anti-HCP antibodies and the sera must be stored at a temperature that ensures their stability.

Characterisation and testing

The suitability of the derived HCP assay reagent is assessed by demonstrating the coverage of the HCPs representative of the manufacturing process by the anti-HCP antibodies.

For this purpose, 2D electrophoresis of the HCP antigens is performed. The protein pattern of the immunostain is compared with the protein pattern of the total stain. The anti-HCP antibodies must recognise a broad range of HCPs over the full range of charge and molecular size. Other methods using native conditions may be considered.

GENERIC ASSAYS

Immunisation, purification and preparation of the anti-HCP antibody reagent are carried out by the vendor and details may not be available.

Characterisation and testing of anti-HCP antibodies are performed as for other assay types. Typically, there is limited control over lot-to-lot reagent consistency. Appropriate comparative lot testing is therefore required.

VALIDATION OF THE HCP ASSAY

The HCP ELISA is developed to detect and quantify a heterogeneous mixture of antigens at varying concentrations, and with a reagent containing antibodies that are not represented at a one-to-one ratio. The section below is intended to target the specifics in development and validation of this type of ELISA.

HCP assays such as ELISA are validated with regard to accuracy, specificity, precision, quantitation and detection limits, linearity, range and robustness.

During the life cycle of the product, a full or partial revalidation of the assay may be required, for example when implementing a manufacturing process change that may impact the suitability of the HCP reagent.

Accuracy

Accuracy is demonstrated by spike/recovery analysis of the HCP reference standard in a relevant background matrix (e.g. the active substance or a sample from a relevant purification step).

Specificity

Specificity is demonstrated by the absence of interference from the matrix background (including the active substance). For instance, data from the accuracy study can be used to assess specificity.

Precision

As for any other quantitative assay, repeatability, intermediate precision and reproducibility are appropriately demonstrated.

Quantitation and detection limits

Sensitivity is usually in the ppm range and is normally described through the quantitation limit (QL). QL is typically determined by HCP spike recovery studies in the active substance or an appropriate sample matrix, and is calculated from the minimal spike providing a response with predefined accuracy and precision from replicate analyses.

Detection limit (DL) is often not determined (optional validation parameter).

Linearity

The linearity of the HCP assay is demonstrated using dilution series of the HCP standard and spike/recovery experiments (accuracy study).

Additionally, due to the nature of HCP assays, the multiple HCP analytes and polyclonal anti-HCP antibodies, sample-dilution non-linearity may be observed, i.e. back-calculated results increase with increasing dilutions of samples, which in most cases is related to the excess of one or more individual HCPs in the sample when compared to the available antibodies in the HCP immunoassay. As a consequence, dilution linearity must be properly assessed for the relevant process steps by comparison of target versus measured HCP concentrations at varying sample dilutions. Dilution linearity is demonstrated if the acceptance criteria for assay variation are met for different sample dilutions. Studies demonstrating dilution linearity can be carried out either during method development or at the latest during method validation.

If a sample shows dilution non-linearity, multiple sample dilutions are prepared beyond the range where non-linear behaviour is observed.

The final HCP value is typically reported as the average HCP concentration obtained for a minimum of 2 dilutions within the linear dilution range. If justified, 1 dilution may be sufficient.

Range

The range of the assay is typically defined by the HCP concentrations for which a suitable level of precision, accuracy and linearity has been demonstrated.

Robustness

The evaluation of robustness is considered during the development phase.

CHANGE OF HCP ASSAY AND/OR REAGENT

The quantities of antigens and antibodies must be large enough to supply the HCP assay for several years. Therefore, the supply, quality and consistency of reagents must be appropriately managed throughout the life cycle of the assay.

For generic HCP assays, in order to ensure the consistency and quality of the reagents, recharacterisation or revalidation of the assay may be required for each new batch of reagent, as their quality may change from one batch to another.

For process-specific and for platform assays, there are generally 2 situations where new HCP assay reagents may be required:

- the HCP reference standard and/or antibody are depleted; antibody may then be purified from a frozen serum stock or a new immunisation is required;
- a manufacturing process change can impact the HCP composition for the purification intermediates or the final product; the assay reagent may not be properly suited to detect and quantify the modified HCPs; a manufacturing process change can therefore render the reagents unsuitable for assay use.

Newly prepared reagents must be thoroughly characterised (e.g. by 2D-SDS-PAGE/Western blot for coverage, 2D-SDS-PAGE/differential gel electrophoresis (DIGE)/identification by MS). Afterwards, the validation status of the assay using the new reagents must be assessed. It is recommended to perform these experiments side-by-side with the currently used reagents.

Table 2.6.34.-1 outlines a recommendation for reagent characterisation and assessment of immunoassay validation, as a consequence of a depletion of assay reagents or a process change.

Table 2.6.34.-1

	Depleted reagents		Process change
	HCP reference standard	**Anti-HCP antibody**	
Reagent characterisation	The protein concentration of the new reference standard is determined preferably using the same method as for the current reference standard to ensure that the protein concentrations are comparable. Using suitable methods (e.g. 1D-/2D-PAGE, 2D-DIGE), the similarity in protein composition between the new and current HCP reference standards is assessed.	Total protein concentration of the new antibody is determined. The final assay concentration must be titrated for the new lot in order to achieve a similar standard curve as for the current lot. For detection antibodies, the detection label: protein stoichiometry is controlled and ensured to be similar to the current antibody lot. Immunoreactivity of the new antibody is compared qualitatively (by visual comparison) or semi-quantitatively (coverage determination) against the current lot by suitable methods (e.g. 1D or 2D Western blot). Due to the variability of the method, it is particularly advisable to perform this characterisation side-by-side with the current antibody lot.	The effects of process changes that have the potential to impact the HCP composition are analysed by suitable methods (e.g. 1D-/2D-PAGE, Western blot, HCP assay). If the process change does not lead to a relevant change in HCP composition, the current HCP reagents are also suitable for the new process. If the process change does lead to a relevant change in HCP composition, but the suitability of the current HCP reagents was demonstrated, the current HCP reagents are also suitable for the new process. If the process change does lead to a relevant change in HCP composition, but the current HCP reagents were shown to be unsuitable for the new process, a new assay must to be developed including a mock fermentation according to the new process and a new immunisation.
Testing of reagents in HCP assays	The new HCP reference standard is quantitatively tested against the current reference standard for spike recovery at different concentrations covering the validated assay range. Standard curves obtained with the new versus the current reference standard are assessed for similarity.	Standard curves obtained when using new versus current antibody lots are compared. A bridging study is performed with testing of relevant process samples (e.g. purification steps from harvest to the final active substance). In a side-by-side experiment, new antibodies must detect HCP levels at different process steps equally or with an improved quantitation limit.	A mock run harvest from the new process is tested for spike recovery using the current HCP assay. Relevant process samples (e.g. purification steps from harvest to the final active substance) from the new and the previous process are tested side-by-side.

	Depleted reagents		Process change
	HCP reference standard	**Anti-HCP antibody**	
Assessment of validation status	If reagent characterisation and ELISA testing demonstrate suitability of the new HCP reference standard, the current reference standard can be replaced. No revalidation of the test method is required. If the new HCP reference standard differs significantly in protein composition and/or assay performance from the current reference standard, revalidation is required.	If reagent characterisation and ELISA testing demonstrate that the new antibody is suitable, the current antibody can be replaced. No revalidation of the test method is required. If the new antibody differs significantly in Western blot immunoreactivity or immunoassay sensitivity and/or assay performance compared to the current antibody, revalidation is required.	If, for the new process, the antibody shows similar or higher immunoreactivity compared to the previous process, and the HCP assay shows adequate recovery from the mock harvest of the new process and also similar or higher sensitivity for samples from the relevant process steps, then the current assay and reagents are considered suitable for the new process. No revalidation of the test method is required. If reagents appear suitable to detect HCP from the new process, but the ELISA indicates significant differences in spike recovery of the mock sample or of HCP levels at relevant process steps, then revalidation is required. The process change might also impact dilution linearity of test samples from certain process steps; if these steps are essential for the HCP control strategy, revalidation or even generation of new antibody reagents might be required. In case of a major change in HCP composition with the new process that leads to either a mismatch in protein composition compared to the current assay standard, reduced immunoreactivity of the antibody, or significantly decreased immunoassay sensitivity, then new assay reagents are prepared and the HCP assay is validated with the new reagents.
PAGE: polyacrylamide gel electrophoresis DIGE: differential gel electrophoresis			

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corrected 6.0

2.5.33. TOTAL PROTEIN

Many of the assay methods described in this chapter can be performed using kits from commercial sources.

METHOD 1

Protein in solution absorbs ultraviolet light at a wavelength of 280 nm, due to the presence of aromatic amino acids, mainly tyrosine and tryptophan, in the protein structure. This property can be used for assay purposes. If the buffer used to dissolve the protein has a high absorbance relative to that of water, an interfering substance is present. This interference may be obviated by using the buffer as compensation liquid but if the interfering substance produces a high absorbance, the results may nevertheless be compromised. At low concentrations, protein adsorbed onto the cell may significantly reduce the content in solution. This can be prevented by preparing samples at higher concentration or by using a non-ionic detergent in the preparation.

Test solution. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a protein concentration between 0.2 mg/mL and 2 mg/mL.

Reference solution. Prepare a solution of a suitable reference substance for the protein to be determined, in the same buffer and at the same protein concentration as the test solution.

Procedure. Keep the test solution, the reference solution and the compensation liquid at the same temperature during the performance of this test. Determine the absorbances (*2.2.25*) of the test solution and the reference solution in quartz cells at 280 nm, using the prescribed buffer as the compensation liquid. The response must be linear in the range of protein concentrations to be assayed to obtain accurate results.

Light scattering. The accuracy of the determination of protein can be diminished by the scattering of light by the test sample. If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250 nm to 300 nm), scattering of the light beam results in an apparent increase in absorbance of the test sample. To calculate the absorbance at 280 nm due to light scattering, determine the absorbances of the test solution at wavelengths of 320 nm, 325 nm, 330 nm, 335 nm, 340 nm, 345 nm and 350 nm. Plot the logarithm of the observed absorbance against the logarithm of the wavelength and determine the standard curve best fitting the plotted points by linear regression. Extrapolate the curve to determine the logarithm of the absorbance at 280 nm. The antilogarithm of this value is the absorbance attributed to light scattering. Correct the observed values by subtracting the absorbance attributed to light scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a 0.2 µm filter that does not adsorb protein or clarification by centrifugation may be performed to reduce the effect of light scattering, especially if the solution is noticeably turbid.

Calculations. Use corrected values for the calculations. Calculate the concentration of protein in the test solution (C_U) from the following equation:

$$C_U = C_S(A_U / A_S)$$

where C_S is the concentration of protein in the reference solution and A_U and A_S are the corrected absorbances of the test solution and the reference solution, respectively.

METHOD 2

This method (commonly referred to as the Lowry assay) is based on the reduction by protein of the phosphomolybdotungstic mixed acid chromogen in the phosphomolybdotungstic reagent, which results in an absorbance maximum at 750 nm. The phosphomolybdotungstic reagent reacts primarily with tyrosine residues in the protein. Colour development reaches a maximum in 20 min to 30 min at room temperature, after which there is a gradual loss of colour. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test sample may be used. Most interfering substances cause a lower colour yield; however, some detergents cause a slight increase in colour. A high salt concentration may cause a precipitate to form. Because different protein species may give different colour response intensities, the reference substance and test protein must be the same. Where separation of interfering substances from the protein in the test sample is necessary, proceed as directed below for interfering substances prior to preparation of the test solution. The effect of interfering substances may be minimised by dilution, provided the concentration of the test protein remains sufficient for accurate measurement.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the standard curve. A suitable buffer will produce a solution of pH 10.0 to 10.5.

Reference solutions. Dissolve the reference substance for the protein to be determined in the prescribed buffer. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 5 µg/mL and 100 µg/mL.

Blank. Use the buffer used to prepare the test solution and the reference solutions.

Copper sulfate reagent. Dissolve 100 mg of *copper sulfate pentahydrate R* and 0.2 g of *sodium tartrate R* in *distilled water R* and dilute to 50 mL with the same solvent. Dissolve 10 g of *anhydrous sodium carbonate R* in *distilled water R* and dilute to 50 mL with the same solvent. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Use within 24 h.

Alkaline copper reagent. Mix 1 volume of copper sulfate reagent, 2 volumes of a 50 g/L solution of *sodium dodecyl sulfate R* and 1 volume of a 32 g/L solution of *sodium hydroxide R*. Store at room temperature and use within 2 weeks.

Diluted phosphomolybdotungstic reagent. Mix 5 mL of *phosphomolybdotungstic reagent R* with 55 mL of *distilled water R*. Store in an amber bottle, at room temperature.

Procedure. To 1.0 mL of each reference solution, of the test solution and of the blank, add 1.0 mL of alkaline copper reagent and mix. Allow to stand for 10 min. Add 0.5 mL of the diluted phosphomolybdotungstic reagent, mix and allow to stand at room temperature for 30 min. Determine the absorbances (*2.2.25*) of the solutions at 750 nm, using the solution from the blank as compensation liquid.

Calculations. The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against the protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

Interfering substances. In the following procedure, deoxycholate-trichloroacetic acid is added to a test sample to remove interfering substances by precipitation of proteins before determination; this technique can also be used to concentrate proteins from a dilute solution.

Add 0.1 mL of a 1.5 g/L solution of *sodium deoxycholate R* to 1 mL of a solution of the substance to be examined. Mix using a vortex mixer and allow to stand at room temperature for 10 min. Add 0.1 mL of a 720 g/L solution of *trichloroacetic acid R* and mix using a vortex mixer. Centrifuge at 3000 *g* for 30 min, decant the liquid and remove any residual liquid with a pipette. Redissolve the protein pellet in 1 mL of alkaline copper reagent.

METHOD 3

This method (commonly referred to as the Bradford assay) is based on the absorption shift from 470 nm to 595 nm observed when the acid blue 90 dye binds to protein. The acid blue 90 dye binds most readily to arginine and lysine residues in the protein which can lead to variation in the response of the assay to different proteins. The protein used as reference substance must therefore be the same as the protein to be determined. There are relatively few interfering substances, but it is preferable to avoid detergents and ampholytes in the test sample. Highly alkaline samples may interfere with the acidic reagent.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the standard curve.

Reference solutions. Dissolve the reference substance for the protein to be determined in the prescribed buffer. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 0.1 mg/mL and 1 mg/mL.

Blank. Use the buffer used to prepare the test solution and the reference solutions.

Acid blue 90 reagent. Dissolve 0.10 g of *acid blue 90 R* in 50 mL of *alcohol R*. Add 100 mL of *phosphoric acid R*, dilute to 1000 mL with *distilled water R* and mix. Filter the solution and store in an amber bottle at room temperature. Slow precipitation of the dye occurs during storage. Filter the reagent before using.

Procedure. Add 5 mL of acid blue 90 reagent to 0.100 mL of each reference solution, of the test solution and of the blank. Mix by inversion. Avoid foaming, which will lead to poor reproducibility. Determine the absorbances (*2.2.25*) of the standard solutions and of the test solution at 595 nm, using the blank as compensation liquid. Do not use quartz (silica) spectrophotometer cells because the dye binds to this material.

Calculations. The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

METHOD 4

This method (commonly referred to as the bicinchoninic acid or BCA assay) is based on reduction of the cupric (Cu^{2+}) ion to cuprous (Cu^{1+}) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. Few substances interfere with the reaction. When interfering substances are present their effect may be minimised by dilution, provided that the concentration of the protein to be determined remains sufficient for accurate measurement. Alternatively, the protein precipitation procedure given in Method 2 may be used to remove interfering substances. Because different protein species may give different colour response intensities, the reference protein and protein to be determined must be the same.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the concentrations of the reference solutions.

Reference solutions. Dissolve the reference substance for the protein to be determined in the prescribed buffer. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 10 µg/mL and 1200 µg/mL.

Blank. Use the buffer used to prepare the test solution and the reference solutions.

BCA reagent. Dissolve 10 g of *disodium bicinchoninate R*, 20 g of *sodium carbonate monohydrate R*, 1.6 g of *sodium tartrate R*, 4 g of *sodium hydroxide R*, and 9.5 g of *sodium hydrogen carbonate R* in *distilled water R*. Adjust, if necessary, to pH 11.25 with a solution of *sodium hydroxide R* or a solution of *sodium hydrogen carbonate R*. Dilute to 1000 mL with *distilled water R* and mix.

Copper-BCA reagent. Mix 1 mL of a 40 g/L solution of *copper sulfate pentahydrate R* and 50 mL of BCA reagent.

Procedure. Mix 0.1 mL of each reference solution, of the test solution and of the blank with 2 mL of the copper-BCA reagent. Incubate the solutions at 37 °C for 30 min, note the time and allow the mixtures to cool to room temperature. Within 60 min of the end of incubation, determine the absorbances (*2.2.25*) of the reference solutions and of the test solution in quartz cells at 562 nm, using the blank as compensation liquid. After the solutions have cooled to room temperature, the colour intensity continues to increase gradually.

Calculations. The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

METHOD 5

This method (commonly referred to as the biuret assay) is based on the interaction of cupric (Cu^{2+}) ion with protein in alkaline solution and resultant development of absorbance at 545 nm. This test shows minimal difference between equivalent IgG and albumin samples. Addition of the sodium hydroxide and the biuret reagent as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the biuret reagent will give IgG samples a higher response than albumin samples. The trichloroacetic acid method used to minimise the effects of interfering substances also can be used to determine the protein content in test samples at concentrations below 500 µg/mL.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in a 9 g/L solution of *sodium chloride R* to obtain a solution having a concentration within the range of the concentrations of the reference solutions.

Reference solutions. Dissolve the reference substance for the protein to be determined in a 9 g/L solution of *sodium chloride R*. Dilute portions of this solution with a 9 g/L solution of *sodium chloride R* to obtain not fewer than three reference solutions having protein concentrations evenly spaced over a suitable range situated between 0.5 mg/mL and 10 mg/mL.

Blank. Use a 9 g/L solution of *sodium chloride R*.

Biuret reagent. Dissolve 3.46 g of *copper sulfate pentahydrate R* in 10 mL of hot *distilled water R*, and allow to cool (Solution A). Dissolve 34.6 g of *sodium citrate R* and 20.0 g of *anhydrous sodium carbonate R* in 80 mL of hot *distilled water R*, and allow to cool (Solution B). Mix solutions A and B and dilute to 200 mL with *distilled water R*. Use within 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

Procedure. To one volume of the test solution add an equal volume of a 60 g/L solution of *sodium hydroxide R* and mix. Immediately add biuret reagent equivalent to 0.4 volumes of the test solution and mix rapidly. Allow to stand at a temperature between 15 °C and 25 °C for not less than 15 min. Within 90 min of addition of the biuret reagent, determine the absorbances (*2.2.25*) of the reference solutions and of the test solution at the maximum at 545 nm, using the blank as compensation liquid. Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.

Calculations. The relationship of absorbance to protein concentration is approximately linear within the indicated range of protein concentrations for the reference solutions. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. Calculate the correlation coefficient for the standard curve. A suitable system is one that yields a line having a correlation coefficient not less than 0.99. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

Interfering substances. To minimise the effect of interfering substances, the protein can be precipitated from the test sample as follows: add 0.1 volumes of a 500 g/L solution of *trichloroacetic acid R* to 1 volume of a solution of the test sample, withdraw the supernatant layer and dissolve the precipitate in a small volume of *0.5 M sodium hydroxide*. Use the solution obtained to prepare the test solution.

METHOD 6

This fluorimetric method is based on the derivatisation of the protein with *o*-phthalaldehyde, which reacts with the primary amines of the protein (*N*-terminal amino acid and the ε-amino group of lysine residues). The sensitivity of the assay can be increased by hydrolysing the protein before adding *o*-phthalaldehyde. Hydrolysis makes the α-amino group of the constituent amino acids available for reaction with the phthalaldehyde reagent. The method requires very small quantities of the protein. Primary amines, such as tris(hydroxymethyl)aminomethane and amino acid buffers, react with phthalaldehyde and must be avoided or removed. Ammonia at high concentrations reacts with phthalaldehyde. The fluorescence obtained when amine reacts with phthalaldehyde can be unstable. The use of automated procedures to standardise this procedure may improve the accuracy and precision of the test.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in a 9 g/L solution of *sodium chloride R* to obtain a solution having a concentration within the range of the concentrations of the reference solutions. Adjust the solution to pH 8 to 10.5 before addition of the phthalaldehyde reagent.

Reference solutions. Dissolve the reference substance for the protein to be determined in a 9 g/L solution of *sodium chloride R*. Dilute portions of this solution with a 9 g/L solution of *sodium chloride R* to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 10 µg/mL and 200 µg/mL. Adjust the solutions to pH 8 to 10.5 before addition of the phthalaldehyde reagent.

Blank solution. Use a 9 g/L solution of *sodium chloride R*.

Borate buffer solution. Dissolve 61.83 g of *boric acid R* in *distilled water R* and adjust to pH 10.4 with a solution of *potassium hydroxide R*. Dilute to 1000 mL with *distilled water R* and mix.

Phthalaldehyde stock solution. Dissolve 1.20 g of *phthalaldehyde R* in 1.5 mL of *methanol R*, add 100 mL of borate buffer solution and mix. Add 0.6 mL of a 300 g/L solution of *macrogol 23 lauryl ether R* and mix. Store at room temperature and use within 3 weeks.

Phthalaldehyde reagent. To 5 mL of phthalaldehyde stock solution add 15 µL of *2-mercaptoethanol R*. Prepare at least 30 min before use. Use within 24 h.

Procedure. Mix 10 µL of the test solution and of each of the reference solutions with 0.1 mL of phthalaldehyde reagent and allow to stand at room temperature for 15 min. Add 3 mL of *0.5 M sodium hydroxide* and mix. Determine the fluorescent intensities (*2.2.21*) of solutions from the reference solutions and from the test solution at an excitation wavelength of 340 nm and an emission wavelength between 440 and 455 nm. Measure the fluorescent intensity of a given sample only once, since irradiation decreases the fluorescence intensity.

Calculations. The relationship of fluorescence to protein concentration is linear. Plot the fluorescent intensities of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the fluorescent intensity of the test solution, determine the concentration of protein in the test solution.

METHOD 7

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test sample can affect the determination of protein by this method. Nitrogen analysis techniques destroy the test sample during the analysis but are not limited to protein presentation in an aqueous environment.

Procedure A. Proceed as prescribed for the determination of nitrogen by sulfuric acid digestion (*2.5.9*) or use commercial instrumentation for Kjeldahl nitrogen assay.

Procedure B. Commercial instrumentation is available for nitrogen analysis. Most nitrogen analysis instruments use pyrolysis (i.e. combustion of the sample in oxygen at temperatures approaching 1000 °C), which produces nitric oxide (NO) and other oxides of nitrogen (NO_x) from the nitrogen present in the substance to be examined. Some instruments convert the nitric oxides to nitrogen gas, which is quantified using a thermal-conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O_3) to produce excited nitrogen dioxide (NO_2*), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference material that is relatively pure and is similar in composition to the test proteins is used to optimise the injection and pyrolysis parameters and to evaluate consistency in the analysis.

Calculations. The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with a suitable reference substance.

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2.6.35. QUANTIFICATION AND CHARACTERISATION OF RESIDUAL HOST-CELL DNA

This general chapter describes analytical methods that may be used to measure the content and to characterise the size of residual host-cell DNA in biological products produced in cell substrates. It does not exclude the use of alternative approaches that are acceptable to the competent authority.

INTRODUCTION

Several sensitive analytical methods exist for the quantification of residual host-cell DNA, including real-time quantitative PCR (qPCR) (Method A) and an immunoenzymatic method (Method B).

qPCR may also be used to assess the residual host-cell DNA size distribution, as a characterisation test depending on the nature of the cell substrate (e.g. continuous cell lines) and on the amount of residual host-cell DNA.

A suitable method is selected depending on the nature of the biological product to be tested and taking into account the characteristics and limitations of each method as summarised in Table 2.6.35.-1.

Table 2.6.35.-1 - *Comparison of the characteristics of qPCR and immunoenzymatic methods*

Characteristics	qPCR (Method A)	Immunoenzymatic method (Method B)
Can be used to assess DNA size distribution	Yes	No
Limit of quantification (*may vary depending on the matrix, method and interfering substances*)	0.01-10 pg/mL	2-10 pg/mL
Specificity (total DNA versus specific DNA)	Specific DNA	Total DNA
Interfering substances	Proteins	Detergents/proteins/solvents/RNA
Limitations	Fragments smaller than the PCR product can not be detected and quantified.	Not applicable for DNA-based products. The DNA content is underestimated for fragments below 1000 base pairs (bp). Detectability depends on DNA size. Undetectable short DNA fragments (below 80 nucleotides) can interfere with the assay through reagent consumption. Products must be free of bacterial DNA. Narrow quantification range: 5-150 pg/well.

SAMPLE PREPARATION

The concentration of residual host-cell DNA may vary depending on the type of biological product and the DNA clearance capacity of the manufacturing process.

Depending on the sample matrix, pretreatment of the sample may be necessary to ensure appropriate recovery of the residual host-cell DNA.

When analysing highly purified protein samples such as recombinant proteins or monoclonal antibodies, simple digestion with proteinase or affinity chromatography may be sufficient to recover the residual host-cell DNA. For more complex matrices such as viral vaccines and viral vectors, an additional virus lysis step may be required to release the residual host-cell DNA from the viral particles.

The immunoenzymatic method is especially sensitive to interference from proteins. This can be avoided by performing an initial pretreatment step, which may include digestion with proteinase K and sodium dodecyl sulfate (SDS). This step may suffice to recover the residual host-cell DNA. However, in other cases, the residual host-cell DNA may be bound to the sample components, and/or soluble interfering substances may be present, in which case it may be necessary to extract the residual host-cell DNA from the sample.

The DNA can be extracted using protocols which have demonstrated a satisfactory recovery rate during spiking experiments. Several suitable methods exist, including DNA precipitation or DNA-specific binding to a matrix (e.g. magnetic beads or silica columns). Commercial kits can be used to extract residual host-cell DNA samples. Some of these kits use a chaotrope (sodium iodide) and a detergent (sodium lauroylsarcosinate) to disrupt the association between the residual host-cell DNA and the sample components. The residual host-cell DNA in the sample is then recovered by co-precipitation with a carrier molecule such as glycogen in the presence of ethanol or 2-propanol. Several independent extraction procedures may be required, depending on the reproducibility of the spike recovery. Negative controls must be included in each extraction procedure. In some cases, dilution of the samples may be recommended to reduce the matrix effect. A correction factor may also be applied to take into account the recovery rate of the spike.

METHOD A – REAL-TIME QUANTITATIVE PCR (qPCR)

This method can be used to quantify a cellular DNA target sequence from a variety of samples. For the quantification of residual host-cell DNA, qPCR targeting either a stable sequence within a highly conserved host-cell region or targeting repetitive elements to enhance the sensitivity of the test can be used. When repetitive elements are targeted, it may be difficult to eliminate potential background noise due to environmental DNA (e.g. when using Alu human sequences). The specificity of the qPCR method must be established during the validation studies by demonstrating the absence of cross-reactivity with unrelated sequences.

Alternatively, digital PCR methods may be used.

qPCR amplification

The detection and quantification of residual host-cell DNA by qPCR may involve the use of either a non-specific fluorescent dye that intercalates with any double-stranded DNA, or sequence-specific DNA probes. The qPCR principle described in general chapter *2.6.21. Nucleic acid amplification techniques* applies.

The number of cycles required for the fluorescent measurement to exceed a threshold value (Ct or Cp) correlates to the starting amount of residual host-cell DNA in the sample.

If several extractions are performed, the resulting samples must be analysed at an appropriate dilution.

PCR negative controls are used.

A standard curve is plotted using serial dilutions of host-cell genomic DNA in order to allow residual host-cell DNA levels in biological products to be determined based on their Ct or Cp values. The use of carefully characterised representative

genomic DNA extracted from the cells used for the production of the biological product is recommended for the preparation of the standard.

The same methodology is applied for residual host-cell DNA size evaluation. At least 2 sets of primers can be designed to amplify overlapping fragments of different sizes in the target sequence.

Suitability criteria

Control samples. In order to control the risk of contamination and to ensure adequate sensitivity, each PCR assay includes the following controls:

- *a negative control for qPCR* and *a negative control for extraction*, composed of a sample of a suitable matrix already proven to be free of the target sequence(s);
- *a positive control for qPCR*, which contains a defined number of target sequence copies or a defined DNA concentration which is determined individually for each assay system;
- *a control for extraction*, typically an internal control added to the test material as a defined concentration or number of target-sequence copies. In this case, the amplicons must be clearly discernible and may be detected in a separate qPCR. Alternatively, an external control consisting of test sample spiked with a well-characterised level of genomic DNA may be used.

Extraction recovery must fall within predetermined values based on the performance of the assay as demonstrated during assay validation.

Genomic DNA standard curve. The standard curve is linear over the chosen range.

The coefficient of determination R^2 associated with the standard curve must be greater or equal to 0.98. The PCR efficiency falls within pre-established limits.

The coefficient of variation for the different extracts or replicates is not higher than a predefined criterion.

Calculation

If several extractions are performed, each extracted sample is analysed individually. The residual host-cell DNA content is calculated from the genomic standard curve by averaging the values obtained for the different extractions or replicates. A correction factor may also be applied to take into account the recovery rate for total DNA quantification in the samples.

For the characterisation of residual host-cell DNA size, the distribution of the overlapping fragments of different sizes is calculated as the ratio of the number of copies for each amplicon size to the number of copies of the smallest amplicon size.

METHOD B – IMMUNOENZYMATIC METHOD

The immunoenzymatic method is a non-specific technique for the quantification of residual host-cell DNA (regardless of its origin). It is therefore also a total DNA assay, and consequently, it is not only critical to avoid contamination through environmental DNA, but all materials and reagents used must be DNA-free. The samples to be tested must be free of microbial contamination and all samples, controls and standards must be processed under controlled conditions until the denaturation step.

By design, the method detects single-stranded DNA.

Principle

This total DNA assay consists of 4 steps:

- *denaturation and formation of complexes*, where the DNA is denatured into single-stranded DNA by heating the sample. The denatured DNA is mixed with a single reagent that contains a single-stranded DNA-binding protein conjugated to streptavidin and a monoclonal anti-DNA antibody conjugated to urease. The DNA-binding protein and the monoclonal antibody are specific for single-stranded DNA but are not sequence-specific. The liquid phase, in the presence of streptavidin, facilitates the formation of a complex with the single-stranded DNA from the sample.
- *filtration*, where the complex is filtered through a biotinylated nitrocellulose membrane. The biotin in the membrane captures the complexes by binding to streptavidin. The membrane is washed to remove any unbound reagents. Non-specific binding is avoided by the use of an albumin-coated nitrocellulose membrane.
- *detection*, where the membrane is placed in a reader, which contains a urea solution that reacts with the urease in the DNA complex and produces ammonia. The associated change in pH is measured by a potentiometric sensor in µV/s and is directly proportional to the amount of DNA in the sample.
- *analysis*, where the raw data from the sample and from the standard curve are analysed using appropriate software to determine the residual host-cell DNA content in the sample.

All samples and negative controls are tested spiked and unspiked. The spike solution (1000 pg/mL) is prepared by dilution of a concentrated standard (calf thymus DNA) at 5000 pg/mL.

Suitability criteria

Control samples

- the amount of DNA in the positive control falls within the range indicated on the batch certificate provided by the supplier;
- spike recovery in the negative control is between 80 per cent and 120 per cent.

Samples

- when several replicates are analysed, the coefficient of variation for the different replicates is not higher than a predefined criterion;
- spike recovery is between 80 per cent and 120 per cent.

Calculation

The content of residual host-cell DNA is calculated in picograms per millilitre using the following expression:

$$\frac{ID \times (C - A)}{V}$$

ID = ratio for dilution and sampling;

C = raw mean value (in picograms per tube) for the test tubes containing the diluted sample;

A = raw mean value (in picograms per tube) for the test tubes containing the negative control;

V = volume in the test tube, in millilitres (usually 0.5 mL per tube).

Where necessary, this result may be corrected by the extraction recovery (e.g. mean recovery for a given product).

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2.2.29. LIQUID CHROMATOGRAPHY

PRINCIPLE

Liquid chromatography (LC) is a method of chromatographic separation based on the difference in the distribution of species between 2 non-miscible phases, in which the mobile phase is a liquid which percolates through a stationary phase contained in a column.

LC is mainly based on mechanisms of adsorption, mass distribution, ion exchange, size exclusion or stereochemical interaction.

Unless otherwise specified, all the information below is valid for both standard LC and LC using reduced particle-size columns (e.g. sub-2.0 µm).

The latter requires instrumentation that is able to withstand higher pressures (typically up to 100 MPa, i.e. about 15 000 psi), generates lower extra-column band broadening, provides improved gradient mixing and allows a higher sampling rate in the detection system.

EQUIPMENT

The equipment typically consists of:

- a pumping system;
- an injector;
- a chromatographic column (a column temperature controller may be used);
- 1 or more detectors;
- a data acquisition system.

The mobile phase is supplied from 1 or more reservoirs and is pumped to the injector, then through the column, usually at a constant rate, and then through the detector(s).

PUMPING SYSTEMS

LC pumping systems deliver the mobile phase at a controlled flow rate. Pressure fluctuations are to be minimised, for example by passing the pressurised solvent through a pulse-dampening device. Tubing and connections are capable of withstanding the pressures developed by the pumping system. LC pumps may be fitted with a facility for 'bleeding' the system of entrapped air bubbles.

Microprocessor-controlled pumping systems are capable of accurately delivering a mobile phase of either constant (isocratic elution) or varying composition (gradient elution), according to a defined programme. In the case of gradient elution, pumping systems which deliver solvent(s) from several reservoirs are available and solvent mixing can be achieved on either the low or high-pressure side of the pump(s).

INJECTORS

The sample solution is introduced into the flowing mobile phase at or near the head of the column using an injection system which can operate at high pressure. Fixed-loop and variable volume devices operated manually or by an autosampler are used. Partial filling of loops during manual injection may adversely affect injection volume precision.

STATIONARY PHASES

There are many types of stationary phases employed in LC, including:

- silica or alumina, commonly used in normal-phase LC (polar stationary phase and non-polar mobile phase), where the separation is based on differences in adsorption on the stationary phase and/or mass distribution between the mobile phase and the stationary phase (partition chromatography);
- a variety of chemically modified supports prepared from polymers, silica or porous graphite, used in normal-phase and reversed-phase LC (non-polar stationary phase and polar mobile phase), where the separation is based principally on partition of the molecules;
- resins or polymers with acidic or basic groups, used in ion-exchange chromatography, where separation is based on competition between the ions to be separated and those in the mobile phase;
- porous silica or polymers, used in size-exclusion chromatography (*2.2.30*), where separation is based on differences between the volumes of the molecules, corresponding to steric exclusion;
- specially modified stationary phases, e.g. cellulose or amylose derivatives, proteins or peptides, cyclodextrins etc., for the separation of enantiomers (chiral chromatography).

Most separations are based on reversed-phase LC utilising chemically modified silica as the stationary phase. The surface of the support, i.e. the silanol groups of silica, is reacted with various silane reagents to produce covalently bound silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.

Unless otherwise stated by the manufacturer, silica-based reversed-phase columns are considered to be stable in mobile phases having an apparent pH in the range 2.0 to 8.0. Columns containing porous graphite or particles of polymeric materials such as styrene-divinylbenzene copolymer are stable over a wider pH range.

Analysis using normal-phase LC with unmodified silica or polar chemically modified silica (e.g. cyanopropyl or diol) as the stationary phase, with a non-polar mobile phase is applicable in certain cases.

For analytical separations, the particle size of the most commonly used stationary phases varies between 2 and 10 µm. The particles may be spherical or irregular, and of varying porosity and specific surface area. These properties contribute to the chromatographic behaviour of a particular stationary phase. In the case of reversed phases, the nature of the stationary phase, the extent of bonding, e.g. expressed as the carbon loading, and whether the stationary phase is end-capped (i.e. part of the residual silanol groups are silylated) are additional determining factors. Tailing of peaks, particularly of basic substances, can occur when residual silanol groups are present.

In addition to porous particles, superficially porous or monolithic materials may be used.

Unless otherwise prescribed in the monograph, columns made of stainless steel of varying length and internal diameter (Ø) are used for analytical chromatography. Columns with internal diameters of less than 2 mm are often referred to as microbore columns.

The temperature of the mobile phase and the column must be kept constant during the analysis. A column temperature may be specified in the monograph for optimal performance but most separations are performed at 20-25 °C.

MOBILE PHASES

For normal-phase LC, low-polarity organic solvents are generally employed. The residual water content of the solvents used in the mobile phase is to be strictly controlled to obtain reproducible results.

In reversed-phase LC, aqueous mobile phases, usually with organic solvents and/or modifiers, are employed.

The components of the mobile phase are usually filtered to remove particles greater than 0.45 µm in size (or greater than 0.2 µm when the stationary phase is made of sub-2.0 µm particles, and when special detectors, e.g. light scattering detectors, are used). Multicomponent mobile phases are prepared by measuring the required volumes (unless masses

are specified) of the individual components, followed by mixing. Alternatively, the solvents may be delivered by individual pumps controlled by proportioning valves, by which mixing is performed according to the desired proportion. Solvents are normally degassed before pumping by sparging with helium, sonication and/or using in-line membrane/vacuum modules to avoid the creation of gas bubbles in the detector cell.

Solvents for the preparation of the mobile phase are normally free of stabilisers and, if an ultraviolet detector is employed, are transparent at the wavelength of detection. Solvents and other components employed are to be of appropriate quality. In particular, *water for chromatography R* is used for the preparation of mobile phases when water, or an aqueous solution, is 1 of the components. Any necessary adjustments of the pH are made to the aqueous component of the mobile phase and not the mixture. If buffer solutions or saline solutions are used, adequate rinsing of the system is carried out with a mixture of water and a small proportion of the organic part of the mobile phase (5 per cent *V/V*) to prevent crystallisation of salts after completion of the analysis.

Mobile phases may contain other components, for example a counter-ion for ion-pair chromatography or a chiral selector for chiral chromatography using an achiral stationary phase.

DETECTORS

Ultraviolet/visible (UV/Vis) spectrophotometers (including diode array detectors) (*2.2.25*), are the most commonly employed detectors. Fluorescence spectrophotometers, differential refractometers (RI), electrochemical detectors (ECD), light scattering detectors, charged aerosol detectors (CAD), mass spectrometers (MS) (*2.2.43*), radioactivity detectors, multi-angle light scattering (MALS) detectors or other detectors may be used.

PROCEDURE

Equilibrate the column with the prescribed mobile phase and flow rate, at 20-25 °C or at the temperature specified in the monograph, until a stable baseline is achieved. Prepare the solution(s) of the substance to be examined and the reference solution(s) required. The solutions must be free from solid particles.

Criteria for assessing the suitability of the system are described in general chapter *2.2.46. Chromatographic separation techniques*. The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

07/2014:20621

2.6.21. NUCLEIC ACID AMPLIFICATION TECHNIQUES

1. INTRODUCTION

Nucleic acid amplification techniques are based on 2 different approaches:

1. amplification of a target nucleic acid sequence using, for example, polymerase chain reaction (PCR), ligase chain reaction (LCR), or isothermal ribonucleic acid (RNA) amplification;
2. amplification of a hybridisation signal using, for example, for deoxyribonucleic acid (DNA), the branched DNA (bDNA) method; in this case signal amplification is achieved without subjecting the nucleic acid to repetitive cycles of amplification.

In this general chapter, the PCR method is described as the reference technique. Alternative methods may be used, if they comply with the quality requirements described below.

2. SCOPE

This section establishes the requirements for sample preparation, *in vitro* amplification of DNA sequences and detection of the specific PCR product. With the aid of PCR, defined DNA sequences can be detected. RNA sequences can also be detected following reverse transcription of the RNA to complementary DNA (cDNA) and subsequent amplification.

3. PRINCIPLE OF THE METHOD

PCR is a procedure that allows specific *in vitro* amplification of segments of DNA or of RNA after reverse transcription into cDNA.

Following denaturation of double-stranded DNA into single-stranded DNA, 2 synthetic oligonucleotide primers of opposite polarity anneal to their respective complementary sequences in the DNA to be amplified. The short double-stranded regions that form as a result of specific base pairing between the primers and the complementary DNA sequence border the DNA segment to be amplified, and serve as starting positions for *in vitro* DNA synthesis by means of a heat-stable DNA polymerase.

Amplification of the DNA occurs in cycles consisting of:

- heat denaturation of the nucleic acid (target sequence) into 2 single strands;
- specific annealing of the primers to the target sequence under suitable reaction conditions;
- extension of the primers, which are bound to both single strands, by DNA polymerase at a suitable temperature (DNA synthesis).

Repeated cycles of heat denaturation, primer annealing and DNA synthesis results in an exponential amplification of the DNA segment limited by the primers.

The specific PCR product known as an amplicon can be detected by a variety of methods of appropriate specificity and sensitivity.

Multiplex PCR assays use several primer pairs designed for simultaneous amplification of different targets in one reaction.

4. TEST MATERIAL

Because of the high sensitivity of PCR, the samples must be protected against external contamination with target sequences. Sampling, storage and transport of the test material are performed under conditions that minimise degradation of the target sequence. In the case of RNA target sequences, special precautions are necessary since RNA is highly sensitive to degradation by ribonucleases. Care must be taken since some added reagents, such as anticoagulants or preservatives, may interfere with the test procedure.

5. TEST METHOD

5.1. Prevention of contamination

The risk of contamination requires a strict segregation of the areas depending on the material handled and the technology used. Points to consider include movement of personnel, gowning, material flow and air supply and decontamination procedures.

The system should be sub-divided into compartments such as:

- master-mix area (area where exclusively template-free material is handled, e.g. primers, buffers, etc.);
- pre-PCR (area where reagents, samples and controls are handled);
- PCR amplification (amplified material is handled in a closed system);
- post-PCR detection (the only area where the amplified material is handled in an open system).

If closed systems are used, the strict segregation of areas is not required.

5.2. Sample preparation

When preparing samples, the target sequence to be amplified needs to be efficiently extracted or liberated from the test material in a reproducible manner and in such a way that amplification under the selected reaction conditions is possible. A variety of physico-chemical extraction procedures and/or enrichment procedures may be employed.

Additives present in test material may interfere with PCR. The procedures described under 7.3.2. must be used as a control for the presence of inhibitors originating from the test material.

In the case of RNA-templates, care must be taken to avoid ribonuclease activity.

5.3. Amplification

PCR amplification of the target sequence is conducted under defined cycling conditions (temperature profile for denaturation of double-stranded DNA, annealing and extension of primers; incubation times at selected temperatures; ramp rates). These depend on various parameters such as:

- the length and base composition of primer and target sequences;
- the type of DNA polymerase, buffer composition and reaction volume used for the amplification;
- the type of thermocycler used and the thermal conductivity rate between the apparatus, reaction tube and reaction fluid.

5.4. Detection

The amplicon generated by PCR may be identified by size, sequence, chemical modification or a combination of these parameters. Detection and characterisation by size may be achieved by gel electrophoresis (using agarose or polyacrylamide slab gels or capillary electrophoresis) or column chromatography (for example, liquid chromatography). Detection and characterisation by sequence composition may be achieved by the specific hybridisation of probes having a sequence complementary to the target sequence or by cleavage of the amplified material reflecting target-specific restriction-enzyme sites. Detection and characterisation by chemical modification may be achieved by, for example, incorporation of a fluorophore into the amplicons and subsequent detection of fluorescence following excitation.

Detection of amplicons may also be achieved by using probes labelled to permit a subsequent chemiluminescent, radioisotopic or immuno-enzyme-coupled detection.

6. EVALUATION AND INTERPRETATION OF RESULTS

A valid result is obtained within a test only if the positive control(s) is unambiguously positive and the negative control(s) is unambiguously negative. Due to the very high sensitivity of the PCR method and the inherent risk of contamination, it is necessary to confirm positive results by repeating the complete test procedure in duplicate, where possible on a new aliquot of the sample. The sample is considered positive if at least one of the repeat tests gives a positive result. As soon as a measurable target threshold is defined, a quantitative test system is required.

7. QUALITY ASSURANCE

7.1. Validation of the PCR assay system

The validation programme must include validation of instrumentation and the PCR method employed. Reference should be made to the ICH guideline Q2(R1) Validation of Analytical Procedures: Text and Methodology.

Appropriate official working reference preparations or in-house reference preparations calibrated against International Standards for the target sequences are used for validation of a PCR test, when available.

7.1.1. Determination of the positive cut-off point

During validation of qualitative tests, the positive cut-off point must be determined. The positive cut-off point is defined as the minimum number of target sequences per volume sample that can be detected in 95 per cent of test runs. The positive cut-off point depends on interrelated factors such as the volume of the sample extracted and the efficacy of the extraction methodology, the transcription of the target RNA into cDNA, the amplification process and the detection.

To define the detection limit of the assay system, reference must be made to the positive cut-off point for each target sequence and the test performance above and below the positive cut-off point.

7.1.2. Quantitative assay systems

For a quantitative assay, the following parameters are determined during validation: accuracy, precision, specificity, quantitation limit, linearity, range and robustness.

7.2. Quality control of reagents

All reagents crucial for the methodology used have to be controlled prior to use in routine applications. Their acceptance/withdrawal is based on pre-defined quality criteria.

Primers are a crucial component of the PCR assay and as such their design, their purity and the validation of their use in a PCR assay require careful attention. Primers may be modified (for example, by conjugation with a fluorophore or antigen) in order to permit a specific method of detection of the amplicon, provided such modifications do not inhibit accurate and efficient amplification of the target sequence.

7.3. Run controls

7.3.1. External controls

In order to minimise the risk of contamination and to ensure adequate sensitivity, the following external controls are included in each PCR assay:

- positive control: this contains a defined number of target-sequence copies, the number being close to the positive cut-off value, and determined individually for each assay system and indicated as a multiple of the positive cut-off value of the assay system;
- negative control: a sample of a suitable matrix already proven to be free of the target sequences.

7.3.2. Internal control

Internal controls are defined nucleic acid sequences containing, unless otherwise prescribed, the primer binding sites. Internal controls must be amplified with defined efficacy, and the amplicons must be clearly discernible. Internal controls must be of the same type of nucleic acid (DNA/RNA) as the material to be tested. The internal control is preferably added to the test material before isolating the nucleic acid and therefore acts as an overall control (extraction, reverse transcription, amplification, detection).

7.3.3. Threshold control

The threshold control for quantitative assays is a test sample with the analyte at a concentration that is defined as the threshold not to be exceeded. It contains the analyte suitably calibrated in International Units and is analysed in parallel in each run of a quantitative assay.

7.4. External quality assessment

Participation in external quality assessment programmes is an important PCR quality assurance procedure for each laboratory and each operator.

The following sections are published for information.

Validation of nucleic acid amplification techniques (NAT) for the detection of hepatitis C virus (HCV) RNA in plasma pools: guidelines

1. SCOPE

The majority of nucleic acid amplification analytical procedures are qualitative (quantal) tests for the presence of nucleic acid with some quantitative tests (either in-house or commercial) being available. For the detection of HCV RNA contamination of plasma pools, qualitative tests are adequate and may be considered to be a limit test for the control of impurities as described in the *Pharmeuropa* Technical Guide for the elaboration of monographs, December 1999, Chapter III 'Validation of analytical procedures'. These guidelines describe methods to validate only qualitative nucleic acid amplification analytical procedures for assessing HCV RNA contamination of plasma pools. Therefore, the 2 characteristics regarded as the most important for validation of the analytical procedure are the specificity and the detection limit. In addition, the robustness of the analytical procedure should be evaluated.

However, this document may also be used as a basis for the validation of nucleic acid amplification in general.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part or all of the analytical procedure, documented validation points already covered by the kit manufacturer can substitute for the validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. detection limit, robustness, cross-contamination).

2. SPECIFICITY

Specificity is the ability to assess unequivocally nucleic acid in the presence of components that may be expected to be present.

The specificity of nucleic acid amplification analytical procedures is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and the detection steps).

When designing primers and probes, the specificity of the primers and probes to detect only HCV RNA should be investigated by comparing the chosen sequences with sequences in published data banks. For HCV, primers (and probes) will normally be chosen from areas of the 5' non-coding region of the HCV genome which are highly conserved for all genotypes.

The amplified product should be unequivocally identified by using one of a number of methods such as amplification with nested primers, restriction enzyme analysis, sequencing, or hybridisation with a specific probe.

In order to validate the specificity of the analytical procedure, at least 100 HCV RNA-negative plasma pools should be tested and shown to be non-reactive. Suitable samples of non-reactive pools are available from the European Directorate for the Quality of Medicines & HealthCare (EDQM).

The ability of the analytical procedure to detect all HCV genotypes will again depend on the choice of primers, probes and method parameters. This ability should be demonstrated using characterised reference panels. However, in view of the difficulty in obtaining samples of some genotypes (e.g. genotype 6), the most prevalent genotypes (e.g. genotypes 1 and 3 in Europe) should be detected at a suitable level.

3. DETECTION LIMIT

The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample that can be detected but not necessarily quantitated as an exact value.

The nucleic acid amplification analytical procedure used for the detection of HCV RNA in plasma pools usually yields qualitative results. The number of possible results is limited to 2: either positive or negative. Although the determination of the detection limit is recommended, for practical purposes, a positive cut-off point should be determined for the nucleic acid amplification analytical procedure. The positive cut-off point (as defined in the general chapter *2.6.21*) is the minimum number of target sequences per volume sample that can be detected in 95 per cent of test runs. This positive cut-off point is influenced by the distribution of viral genomes in the individual samples being tested and by factors such as enzyme efficiency, and can result in different 95 per cent cut-off values for individual analytical test runs.

In order to determine the positive cut-off point, a dilution series of a working reagent or of the *hepatitis C virus BRP*, which has been calibrated against the WHO HCV International Standard, should be tested on different days to examine variation between test runs. At least 3 independent dilution series should be tested with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution, to enable a statistical analysis of the results.

For example, a laboratory could test 3 dilution series on different days with 8 replicates for each dilution, 4 dilution series on different days with 6 replicates for each dilution, or 6 dilution series on different days with 4 replicates for each dilution. In order to keep the number of dilutions at a manageable level, a preliminary test (using, for example, $\log_{10}$ dilutions of the plasma pool sample) should be carried out in order to obtain a preliminary value for the positive cut-off point (i.e. the highest dilution giving a positive signal). The range of dilutions can then be chosen around the predetermined preliminary cut-off point (using, for example, a dilution factor of 0.5 $\log_{10}$ or less and a negative plasma pool for the dilution matrix). The concentration of HCV RNA that can be detected in 95 per cent of test runs can then be calculated using an appropriate statistical evaluation.

These results may also serve to demonstrate the intra-assay variation and the day-to-day variation of the analytical procedure.

4. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. $MgCl_2$, primers or dNTP) are tested. To demonstrate robustness, at least 20 HCV RNA negative plasma pools (selected at random) spiked with HCV RNA to a final concentration of 3 times the previously determined 95 per cent cut-off value should be tested and found positive.

Problems with robustness may also arise with methods that use an initial ultracentrifugation step prior to extraction of the viral RNA. Therefore, to test the robustness of such methods, at least 20 plasma pools containing varying levels of HCV RNA, but lacking HCV-specific antibodies, should be tested and found positive.

Cross-contamination prevention should be demonstrated by the accurate detection of a panel of at least 20 samples consisting of alternate samples of negative plasma pools and negative plasma pools spiked with high concentrations of HCV (at least 10^2 times the 95 per cent cut-off value or at least 10^4 IU/mL).

5. QUALITY ASSURANCE

For biological tests such as NAT, specific problems may arise that influence both the validation and the interpretation of results. The test procedures must be described precisely in the form of standard operating procedures (SOPs). These should cover:

- the mode of sampling (type of container, etc.);
- the preparation of mini-pools (where appropriate);
- the conditions of storage before analysis;
- the exact description of the test conditions, including precautions taken to prevent cross-contamination or destruction of the viral RNA, reagents and reference preparations used;
- the exact description of the apparatus used;
- the detailed formulae for calculation of results, including statistical evaluation.

The use of a suitable run control (for example, an appropriate dilution of *hepatitis C virus BRP* or plasma spiked with an HCV sample calibrated against the WHO HCV International Standard) can be considered a satisfactory system-suitability check and ensures that the reliability of the analytical procedure is maintained whenever used.

Technical qualification. An appropriate installation and operation qualification programme should be implemented for each critical piece of the equipment used. For confirmation of analytical procedure performance after a change of critical equipment (e.g. thermocyclers), the change should be documented by conducting a parallel test on 8 samples of a plasma pool that is spiked with HCV RNA to a final concentration of 3 times the previously determined 95 per cent cut-off value. All results should be positive.

Operator qualification. An appropriate qualification programme should be implemented for each operator involved in the testing.

Validation of nucleic acid amplification techniques (NAT) for the quantification of B19 virus (B19V) DNA in plasma pools: guidelines

1. SCOPE

The European Pharmacopoeia requires that plasma pools used for manufacture of certain products are tested for the presence of B19 virus (B19V) DNA with a threshold concentration that must not be exceeded. In order to comply with these requirements, quantitative NAT tests are preferred. The characteristics regarded as the most important for validation of the quantitative NAT procedure are accuracy, precision, specificity, quantitation limit, linearity and range. In addition, the robustness of the analytical procedure should be evaluated.

This guideline describes methods to validate NAT analytical procedures for assessing B19V DNA contamination of plasma pools based on the ICH guidelines. However, this document may also be used as a basis for the validation of quantitative NAT in general.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part or all of the analytical procedure, documented validation points already covered by the kit manufacturer can substitute for the validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. precision, accuracy, range, robustness).

2. ACCURACY

Accuracy expresses the closeness of agreement between the value that is accepted as either a conventional true value or an accepted reference value and the value found. The accuracy of an assay is dependent on the calibration of the assay and on the variance of the different assay steps. Though it is recommended to establish the accuracy across the specified range of the analytical procedure, the most important assessment of accuracy is in the range of the threshold concentration. In the case of B19V NAT assays for investigation of plasma pools it is recommended to assess the accuracy of the calibrated assay by assaying at least 5 concentrations (dilution factor of 0.5 $\log_{10}$) of *B19 virus DNA for NAT testing BRP* or another material, suitably calibrated in International Units against the actual WHO B19 DNA International Standard, covering the range of the currently recommended threshold concentration of 10.0 IU/µL B19V DNA (e.g. 10^5 IU/mL, $10^{4.5}$ IU/mL, 10^4 IU/mL, $10^{3.5}$ IU/mL and 10^3 IU/mL), with at least 3 replicates for each dilution. Accuracy should be reported for the different concentrations in terms of percentage determined compared with the known amount of B19V DNA. It should reflect the level of technology of the respective assays, which should also be defined, for example in collaborative studies.

3. PRECISION

Precision expresses the closeness of agreement between a series of measurements, obtained from multiple sampling of the same homogenous sample. The precision is defined at 3 levels:

- repeatability expresses the precision under the same operating conditions over a short interval of time (intra-assay precision); it is assessed by using 1 assay and testing 3 replicates of appropriate dilutions of a B19V DNA-positive sample suitably calibrated in International Units and covering the whole quantitative range of the assay; the coefficient of variation for the individual samples is calculated (intra-assay variability);
- intermediate precision expresses the intra-laboratory variations (inter-assay precision); it is established by assaying replicates (as routinely used for the assay) of appropriate dilutions of a B19V DNA-positive sample suitably calibrated in International Units covering the whole quantitative range of the assay under different circumstances (e.g. different days, different analysts, different equipment, different reagents); the coefficient of variation for the individual samples is calculated;
- reproducibility expresses the precision between different laboratories (inter-laboratory precision); it is assessed by participation in quantitative collaborative studies on B19V DNA-NAT assays, e.g. under the Proficiency Testing Scheme (PTS), including the comparative analysis of the obtained quantitative results, where appropriate.

4. SPECIFICITY

Specificity expresses the ability to assess unequivocally nucleic acid in the presence of components that may be expected to be present. The specificity of NAT analytical procedures is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and the detection steps).

When designing primers and probes, the specificity of the primers and probes to detect only human B19V DNA should be investigated by comparing the chosen sequences with sequences in published data banks. There should be no major homology found with sequences unrelated to B19V.

The amplified product should be unequivocally identified by using one of a number of methods such as amplification with nested primers, restriction enzyme analysis, sequencing, or hybridisation with a specific probe.

In order to examine the specificity of the analytical procedure, at least 20 B19V DNA-negative plasma pools should be tested and shown to be non-reactive.

Parvovirus B19 genotypes. The International Committee on Taxonomy of Viruses (ICTV) has classified representatives of the 3 genotypes as strains of human parvovirus B19. Genotype 1 represents prototype B19V, genotype 2 represents viral sequences like A6, and genotype 3 represents V9-like sequences. By performing sequence alignment with respective B19V genotype sequences available from nucleic acid sequence databases, primers and probes should be designed to detect and quantify consistently the different human parvovirus B19 genotypes. Reference materials should be used to check the approach chosen. Since biological reference preparations reflecting some genotypes might be difficult to obtain, respective plasmid preparations or synthetic nucleic acids may also serve as a characterised target sequence source. However, those cannot be used to validate the extraction procedure.

5. QUANTITATION LIMIT

The quantitation limit is the lowest amount of nucleic acid in a sample that can be determined quantitatively with suitable precision and accuracy. The quantitation limit of the B19V NAT assay is assessed during the repeatability and intermediate-precision studies by limiting dilution analysis. The lowest concentration of target nucleic acids that is quantitated with suitable precision and accuracy is defined.

6. LINEARITY

The linearity of an assay is its ability to obtain test results that are directly proportional to the concentration of the nucleic acid. The linearity of the B19V NAT assay is assessed during the repeatability and intermediate-precision studies by testing replicates of diluted samples with the concentrations covering the whole quantitative range. The interval between the upper and the lower concentration of the target nucleic acid where test results are directly proportional to the concentrations is defined.

7. RANGE

The range of an assay is the interval between the upper and the lower concentration of nucleic acid in the sample for which it has been demonstrated that the procedure has a suitable level of precision, accuracy and linearity. The range of the B19V NAT assay is assessed during the repeatability and intermediate-precision studies by testing replicates of diluted samples. The interval between the upper and the lower concentration that can be expressed with an acceptable degree of accuracy and precision is defined.

8. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The evaluation of robustness should be considered during the development phase. It should

show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. Nonetheless, the robustness of NAT can be demonstrated during the development of the method when small variations in the concentrations of reagents, for example $MgCl_2$, primers or dNTP, are tested. To demonstrate robustness, at least 20 B19V DNA-negative plasma pool samples spiked with B19V DNA at the threshold concentration should be tested and found to have acceptable quantitative values.

Cross-contamination prevention should be demonstrated by the accurate detection of a panel of at least 20 samples consisting of alternate samples of plasma pools without B19V DNA or with levels below the threshold concentration (10 samples) and plasma pools spiked with high concentrations of B19V DNA (at least 10^2 times the threshold level, 10 samples).

9. QUALITY ASSURANCE

For biological tests such as NAT, specific problems may arise that may influence both the validation and the interpretation of results. The test procedures must be described precisely in the form of standard operating procedures (SOPs). These should cover:

- the mode of sampling (type of container, etc.);
- the preparation of mini-pools by manufacturers (where appropriate);
- the conditions of storage before analysis;
- the exact description of the test conditions including precautions taken to prevent cross-contamination or destruction of the viral nucleic acids, reagents and reference preparations used;
- the exact description of the apparatus used;
- the detailed formulae for calculation of results, including statistical evaluation.

The inclusion of an appropriate threshold control (for example, plasma spiked with a B19V DNA sample suitably calibrated in International Units, such as *B19 virus DNA for NAT testing BRP*) is considered to be a satisfactory system-suitability check and ensures that the reliability of the analytical procedure is maintained whenever used.

Technical qualification. An appropriate installation and operation qualification programme should be implemented for each critical piece of the equipment used. For confirmation of analytical procedure performance after a change of critical equipment (e.g. thermocyclers), the change should be documented by conducting a parallel test on 8 samples of a plasma pool that is spiked with a concentration of B19V DNA around the threshold concentration. All results should be acceptable and reflect the features of the assay as determined during the validation phase.

Operator qualification. An appropriate qualification programme should be implemented for each operator involved in the testing.

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2.7.1. IMMUNOCHEMICAL METHODS

Immunochemical methods are based on the selective, reversible and non-covalent binding of antigens by antibodies. These methods are employed to detect or quantify either antigens or antibodies. The formation of an antigen-antibody complex may be detected, and the amount of complex formed may be measured by a variety of techniques. The provisions of this general method apply to immunochemical methods using labelled or unlabelled reagents, as appropriate.

The results of immunochemical methods depend on the experimental conditions and the nature and quality of the reagents used. It is essential to standardise the components of an immunoassay and to use, wherever available, international reference preparations for immunoassays.

The reagents necessary for many immunochemical methods are available as commercial assay kits, that is, a set including reagents (particularly the antigen or the antibody) and materials intended for the *in vitro* estimation of a specified substance as well as instructions for their proper use. The kits are used in accordance with the manufacturers' instructions; it is important to ascertain that the kits are suitable for the analysis of the substance to be examined, with particular reference to selectivity and sensitivity. Guidance concerning immunoassay kits is provided by the World Health Organization, Technical Report Series 658 (1981).

METHODS IN WHICH A LABELLED ANTIGEN OR A LABELLED ANTIBODY IS USED

Methods using labelled substances may employ suitable labels such as enzymes, fluorophores, luminophores and radioisotopes. Where the label is a radioisotope, the method is described as a "radio-immunoassay". The recommendations for the measurement of radioactivity given in the monograph on *Radiopharmaceutical Preparations (0125)* are applicable to immunoassays involving radioisotopes. All work with radioactive materials must be carried out in conformity with national legislation and internationally accepted codes of practice for protection against radiation hazards.

METHODS IN WHICH AN UNLABELLED ANTIGEN OR ANTIBODY IS USED

Immunoprecipitation methods

Immunoprecipitation methods include flocculation and precipitation reactions. When a solution of an antigen is mixed with its corresponding antibody under suitable conditions, the reactants form flocculating or precipitating aggregates. The ratio of the reactants which gives the shortest flocculation time or the most marked precipitation is called the optimal ratio, and is usually produced by equivalent amounts of antigen and antibody. Immunoprecipitation can be assessed visually or by light-scattering techniques (nephelometric or turbidimetric assay). An increase in sensitivity can be obtained by using antigen- or antibody-coated particles (e.g. latex) as reactants.

In flocculation methods, stepwise dilutions of one of the reactants is usually used whereas, in immunodiffusion (ID) methods, the dilution is obtained by diffusion in a gel medium: concentration gradients of one or both of the reactants are obtained, thus creating zones in the gel medium where the ratio of the reactants favours precipitation. While flocculation methods are performed in tubes, immunodiffusion methods may be performed using different supports such as tubes, plates, slides, cells or chambers.

Where the immunoprecipitating system consists of one antigen combining with its corresponding antibody, the system is referred to as *simple*; when it involves related but not serologically identical reactants, the system is *complex* and where several serologically unrelated reactants are involved, the system is *multiple*.

In *simple diffusion methods*, a concentration gradient is established for only one of the reactants diffusing from an external source into the gel medium containing the corresponding reactant at a comparatively low concentration.

Single radial immunodiffusion (SRID) is a simple quantitative immunodiffusion technique. When the equilibrium between the external and the internal reactant has been established, the circular precipitation area, originating from the site of the external reactant, is directly proportional to the amount of the antigen applied and inversely proportional to the concentration of the antibody in the gel.

In *double diffusion methods*, concentration gradients are established for both reactants. Both antigen and antibody diffuse from separate sites into an initially immunologically neutral gel.

Comparative double diffusion methods are used for qualitatively comparing various antigens versus a suitable antibody or vice versa. The comparison is based on the presence or absence of interaction between the precipitation patterns. Reactions of identity, non-identity or partial identity of antigens/antibodies can be distinguished.

Immunoelectrophoretic methods

Immunoelectrophoresis (IE) is a qualitative technique combining 2 methods: gel electrophoresis followed by immunodiffusion.

Crossed immunoelectrophoresis is a modification of the IE method. It is suitable both for qualitative and quantitative analysis. The first part of the procedure is an ordinary gel electrophoresis, after which a longitudinal gel strip, containing the separated fractions to be determined, is cut out and transferred to another plate. The electrophoresis in the second direction is carried out perpendicular to the previous electrophoretic run in a gel containing a comparatively low concentration of antibodies corresponding to the antigens. For a given antibody concentration and gel thickness, the relationship between the area of the respective precipitation peaks and the amount of the corresponding antigen is linear.

Electroimmunoassay, often referred to as *rocket immuno-electrophoresis* is a rapid quantitative method for determining antigens with a charge differing from that of the antibodies or vice versa. The electrophoresis of the antigen to be determined is carried out in a gel containing a comparatively lower concentration of the corresponding antibody. The test material and dilutions of a standard antigen used for calibration are introduced into different wells in the gel. During electrophoresis, migrating peak-shaped precipitation zones originating from the wells are developed. The front of the precipitate becomes stationary when the antigen is no longer in excess. For a given antibody concentration, the relationship between the distance travelled by the precipitate and the amount of antigen applied is linear.

Counter-immunoelectrophoresis is a rapid quantitative method allowing concentration gradients of external antigen and external antibody to be established in an electric field depending on the different charges. Dilutions of a standard for calibration and dilutions of the test material are introduced into a row of wells in a gel and a fixed amount of the corresponding reactant is introduced into an opposite row of wells. The titre of the test material may be determined as the highest dilution showing a precipitation line.

A number of modifications of crossed immunoelectrophoresis and electroimmunoassay methods exist.

Other techniques combine separation of antigens by molecular size and serological properties.

Visualisation and characterisation of immunoprecipitation lines

These may be performed by selective or non-selective stains, by fluorescence, by enzyme or isotope labelling or other relevant techniques. Selective staining methods are usually performed for characterisation of non-protein substances in the precipitates.

In translucent gels such as agar or agarose, the precipitation line becomes clearly visible in the gel, provided that the concentration of each of the reactants is appropriate.

VALIDATION OF THE METHOD

Validation criteria

A quantitative immunochemical method is not valid unless:

1) The antibody or antigen does not significantly discriminate between the test and standard. For a labelled reactant, the corresponding reactant does not significantly discriminate between the labelled and unlabelled compound,

2) The method is not affected by the assay matrix, that is, any component of the test sample or its excipients, which can vary between samples. These may include high concentrations of other proteins, salts, preservatives or contaminating proteolytic activity,

3) The limit of quantitation is below the acceptance criteria stated in the individual monograph,

4) The precision of the assay is such that the variance of the results meets the requirements stated in the individual monographs,

5) The order in which the assay is performed does not give rise to systematic errors.

Validation methods

In order to verify these criteria, the validation design includes the following elements:

1) The assay is performed at least in triplicate,

2) The assay includes at least 3 different dilutions of the standard preparation and 3 dilutions of sample preparations of presumed activity similar to the standard preparation,

3) The assay layout is randomised,

4) If the test sample is presented in serum or formulated with other components, the standard is likewise prepared,

5) The test includes the measurement of non-specific binding of the labelled reactant,

6) For displacement immunoassay:

(a) maximum binding (zero displacement) is determined,

(b) dilutions cover the complete response range from values close to non-specific binding to maximum binding, preferably for both standard and test preparations.

STATISTICAL CALCULATION

To analyse the results, response curves for test and standard may be analysed by the methods described in *5.3. Statistical Analysis of Results of Biological Assays and Tests*.

Significant non-parallelism indicates that the antibody or antigen discriminates between test and standard, and the results are not valid.

In displacement immunoassays, the values for non-specific binding and maximum displacement at high test or standard concentration must not be significantly different. Differences may indicate effects due to the matrix, either inhibition of binding or degradation of tracer.

01/2008:20724

2.7.24. FLOW CYTOMETRY

Flow cytometry consists of a multiparametric analysis of optical properties of individual particles in a fluidic system.

Cells or particles in suspension are individually distributed into a linear array (stream), which flows through a detection device. Solid tissues have to be reduced to a single-cell suspension to be analysed.

The spectrum of parameters measurable by flow cytometry includes volume and morphological complexity of cells or cell-like structures, cell pigments, DNA content, RNA content, proteins, cell surface markers, intracellular markers, enzymatic activity, pH, membrane and fluidity.

It is possible to collect 2 morphological parameters plus 1 or more fluorescence signals per single cell. The multiparametric analysis allows the definition of cell populations by their phenotype.

APPARATUS

Focusing, magnifying, and choice of light source are optimised to allow the automatic detection and measurement of morphological differences and staining patterns. Flow cytofluorimetric analysis meets the following criteria:

- choice of light source depending on the parameters to be analysed;
- adjustment of instrument settings depending on the cell type to be analysed (for example, cell cultures, leucocytes, platelets, bacteria, spermatozoa, yeast) and the analysis to be performed (for example, phenotyping, cell cycle, apoptosis, cytokines, membrane fluidity, fluorescent protein).

Flow cytometry is characterised by the automated quantification of set parameters for a high number of single cells during each analysis session. For example, 100 000 particles or more (practically unlimited) are analysed one after the other, typically in about 1 min. The detection limit is as low as 100 fluorescent molecules per cell.

A flow cytometer apparatus has 5 main components:

- a fluidic system and a flow cell;
- a light source;
- a detection and Analogue to Digital Conversion (ADC) system;
- an amplification system;
- a computer provided with software for analysis of the signals.

FLUIDIC SYSTEM AND FLOW CELL

The single cell is exposed to the light source and detected in the flow cell. The fluidic system carries the suspended cells individually from the sample tube to the laser intercept point. To achieve this, the sample stream is drawn out to a very thin fluid thread by a sheath fluid in the flow cell (hydrodynamic focusing). The light beam is focused in an elliptical shape, by 2 confocal lenses, into the flow cell channel through which the cells pass. The flow rate must be constant during routine cell surface marker analysis and must ensure a suitable distance between the cells to allow counting.

LIGHT SOURCES

Commonly used light sources are:

- lamps (mercury, xenon);
- high power water-cooled lasers (argon, krypton, dye laser);
- low power air-cooled lasers (argon (488 nm), red helium-neon (633 nm), green helium-neon, helium-cadmium (UV));
- diode lasers (blue, green, red, violet).

SIGNAL DETECTION

When a particle passes across the light beam, it scatters some of the light in all directions. Fluorescent dyes, when added to the particle, give off their own light (fluorescence), which is also radiated in all directions. 2 types of signals may thereby be generated:

- scatter of light;
- fluorescence emission.

The instrument's light detectors collect some of this scattered and fluorescent light and produce electronic signals proportional to the amount of light collected.

Scatter. 2 parameters of light scattering are measured:

- the amount scattered mainly forward (forward scatter (FS))
- the amount scattered at 90° from the direction of the light beam (side scatter (SS)).

Forward scatter correlates with the cell volume while side scatter is influenced by parameters such as the shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness, and correlates with the morphological complexity of the cell, so that the higher the SS intensity, the higher the cell complexity. As a function of the morphological characteristics of cells, scatter signals will always be generated during a flow analysis; they are defined as intrinsic parameters.

Fluorescence. Depending on the type and number of light sources, when a cell passes through the sensing area, it will emit fluorescent light. Fluorescence signals are generated from fluorescent dyes naturally present in the cells (for example, co-enzymes, chlorophyll, seaweed pigments) and/or from fluorescent probes taken up by the cells when stained for the analysis of specific characteristics (for example, fluorescent antibodies, nucleic acid dyes, pH probes, calcium probes, fluorescent proteins). Nowadays, there is a large number and a wide range of different types of fluorescent probes available. The optical filters must be adapted to the fluorochromes used and changed if necessary. Each fluorescent probe is characterised by its excitation spectrum and its emission spectrum. They are chosen depending on the nature of the excitation source and the detection system, and according to the specific purpose of the analysis.

SIGNAL MANAGEMENT AND ANALOGUE TO DIGITAL CONVERSION

Scatter and fluorescence signals emitted by cells when passing across the laser beam are sorted and addressed to their detectors using optical filters. The detectors are transducers (photomultiplier tubes (PMTs)) that convert light signals radiated from the cells into voltage pulses.

The process of counting each pulse in the appropriate channel is known as Analogue to Digital Conversion (ADC). The process is finally shown as a frequency histogram.

Amplification. Voltage pulses need to be amplified for optimal visualisation. The amplification process accentuates the differences between cell signals, and consequently increases the resolution among cell populations of different characteristics (for example, the differentiation of viable from non-viable cells, or non-specific fluorescence from antigen-specific fluorescence after staining with a fluorescent monoclonal antibody).

There are 2 methods of amplification: linear or logarithmic; the choice between the 2 types is made for every single signal according to the morphological characteristics of the cells and the staining reagents used (for example, fluorescent monoclonal antibodies, nucleic acid dyes).

Linear amplification, which enhances the differences among strong pulses, is used with those parameters that generate high intensity signals, for example:

- cell scatters;
- fluorescence from nucleic acid dyes for cell cycle studies.

Logarithmic amplification, in contrast, is for weak pulses and parameters or analysis conditions that may generate both weak and strong pulses, for example:

- cell antigens;
- scatter from platelets, bacteria, yeast;
- fluorescence from nucleic acid dyes for apoptosis studies.

Compensation of fluorescence signals. Each fluorescent dye has an absorption wavelength spectrum and a higher emission wavelength spectrum. When using 2 or more fluorescent probes simultaneously for staining cells (for example, 4-antigen immunophenotyping), the fluorochromes emission spectra may overlap. As a consequence, each fluorescence detector will sense its own specific fluorescent light and a variable quantity of light emitted by the other fluorescent probes. This results in signal over-evaluation and poor separation of the cell populations.

The solution is in the use of an electronic matrix that allows the selective subtraction of the interfering signals from each fluorescence signal after detector sensing (fluorescence compensation).

Fluorescence compensation requires the use of fluorescence calibrators, preferably positive cell samples stained with the fluorochromes of interest, combined in a manner equivalent to that for the antibody used for the analysis.

SIGNAL PLOTTING AND DISPLAY

After amplification and compensation, the signals are plotted in 2 or 3 dimensions. Histograms show the signal intensities versus the cell counts for a given parameter. Cytograms, in which each dot represents a cell, result from the combination of 2 signal intensities (dual-parameter dot plots). The type and number of plots and signal combinations are chosen on the basis of the specimens and dyes used. When analysing acquired data, the flow cytometry software can also generate other kinds of graphs (such as overlays, surface plots, tomograms, contour plots, density plots, overlay plots). Statistical data such as mean fluorescent intensities (and their shifts in time or their dependence on cell function) can also be used.

DATA ANALYSIS

Different kinds of cell populations may be present inside the cell suspensions to be analysed, some of which are unwanted (such as dead cells, debris or macro-aggregates), or simply not relevant for the analysis (for example, granulocytes when studying lymphocytes). This depends on the cell sample type (whole blood, bone marrow, cell cultures, biological fluids, cell suspensions from solid tissues) and on the handling procedures (for example, staining methods, lysis, fixation, cryopreservation, thawing, paraffin-embedded tissue preparation).

As a consequence, not all the signals generated during a flow cytometry analysis belong to the cells to be studied. 2 strategies are adopted to exclude unwanted and irrelevant cell signals.

The 1st is used during data acquisition. It is a noise threshold, applied to 1 (or more) significant parameter(s), set to acquire only the cells with signal intensities higher than the pre-defined discrimination value for that parameter. Due to its characteristics of a strong signal with a low grade of interference, forward scatter is the parameter most often used as discriminator.

The 2nd, applied during data analysis, consists of the use of gating regions to restrict the analysis only to signals from those populations that satisfy given morphological and expression profile characteristics. 2 types of logical gating are commonly used. The 1st is the morphological gate. The cell populations are identified using their morphological signals (FS and SS). A region gate is drawn around the population of interest (for example, lymphocytes, viable cells) then the fluorescence plots are gated into the selected region. The 2nd is the fluorescence-based gate. The cell population of interest is identified on the basis of the expression intensity of an antigen or a dye, then a gate region is drawn around it. Afterwards the fluorescence plots are gated into the selected region.

The analysis software allows the creation of multiple gate regions, using a sequential logic order. This feature is especially useful when studying rare cell populations or for sorting purposes.

CONTROLS

Internal control. The system's optical alignment must be validated before analysis using adapted fluorospheres and the optimum fluidic stability is checked. The data obtained are reported and allow the periodical review of control values against the mean performance value. A positive control is highly desirable to prove that the test antibody is functional and to allow the proper setting of the flow cytometer. The positive control must include samples known to be positive for the marker of interest.

External control. To ensure reliability in the data obtained or to check inter-laboratory reproducibility, participation in a proficiency testing study is recommended.

07/2017:20201

2.2.1. CLARITY AND DEGREE OF OPALESCENCE OF LIQUIDS

Opalescence is the effect of light being absorbed or scattered by submicroscopic particles or optical density inhomogeneities. The absence of any particles or inhomogeneities in a solution results in a clear solution.

A liquid is considered *clear* if its clarity is the same as that of *water R* or of the solvent used, or if its opalescence is not more pronounced than that of reference suspension I (see Table 2.2.1.-1), when examined under the conditions described below.

Requirements in monographs are expressed in terms of the visual method by comparing with the defined reference suspensions (see Table 2.2.1.-1). However, instrumental methods may also be used for determining compliance with monograph requirements once the suitability of the instrument has been established as described below and calibration with reference suspensions I-IV and with *water R* or the solvent used has been performed.

VISUAL METHOD

Using identical test-tubes of colourless, transparent, neutral glass with a flat base and an internal diameter of 15-25 mm, compare the liquid to be examined with a reference suspension freshly prepared as described below. Ensure that the depths of the layers in the 2 test-tubes are the same (about 40 mm).

Compare the liquids in diffused daylight 5 min after preparation of the reference suspension, viewing vertically against a black background.

System suitability. The diffusion of light must be such that reference suspension I can readily be distinguished from *water R*, and that reference suspension II can readily be distinguished from reference suspension I (see Table 2.2.1.-1).

INSTRUMENTAL METHOD

The instrumental assessment of clarity and opalescence provides a more discriminatory test that does not depend on the visual acuity of the analyst. Numerical results are more useful for process control and quality monitoring, especially in stability studies. For example, previous numerical data on stability can be extrapolated to determine whether a given batch of a preparation will exceed shelf-life limits prior to the expiry date.

TURBIDIMETRY AND NEPHELOMETRY

When a suspension is viewed at right angles to the direction of the incident light, the system appears opalescent due to the scattering of light by the particles of the suspension (Tyndall effect). A certain portion of the light beam entering a turbid liquid is transmitted, another portion is absorbed and the remaining portion is scattered by the suspended particles. The light-scattering effect of suspended particles can be measured either indirectly by observation of the transmitted light (turbidimetry) or directly by measuring the scattered light (nephelometry). Turbidimetry and nephelometry are more reliable in low turbidity ranges, where there is a linear relationship between turbidity values and detector signals. As the degree of turbidity increases, not all the particles are exposed to the incident light and the scattered or the transmitted radiation of other particles is hindered on its way to the detector.

For quantitative measurements, the construction of calibration curves is essential. Linearity must be based on at least 4 levels of concentrations. Reference suspensions must show a sufficiently stable degree of turbidity and must be produced under well-defined conditions.

MEASUREMENTS IN RATIO MODE

The determination of opalescence of coloured liquids is done using instruments with ratio mode, since colour provides a negative interference, attenuating both incident and scattered light and lowering the turbidity value. The effect is so great, even for moderately coloured samples, that conventional nephelometers cannot be used.

In turbidimetry or nephelometry with ratio mode, the ratio of the transmission measurement to the 90° scattered light measurement is determined. This procedure compensates for the light that is diminished by the colour of the sample. Instruments with ratio mode use as light source a tungsten lamp with spectral sensitivity at about 550 nm operating at a filament colour temperature of 2700 K. Other suitable light sources may also be used. Silicon photodiodes and photomultipliers are commonly used as detectors and record changes in light scattered or transmitted by the sample. The light scattered at 90 ± 2.5° is measured by the primary detector. Other detectors measure back and forward scatter (reflected light) as well as transmitted light. The results are obtained by calculating the ratio of the 90° scattered light measured to the sum of the components of forward scattered and transmitted light values.

The instruments used are calibrated against standards of known turbidity and are capable of automatic measurement of turbidity. The test results are obtained directly from the instrument and compared to the specifications in the individual monograph.

Alternatively, the influence of the colour of the sample may also be eliminated by using an infrared light-emitting diode (IR LED) having an emission maximum at 860 nm with a 60 nm spectral bandwidth as the light source of the instrument.

INSTRUMENT REQUIREMENTS

Instruments complying with the following characteristics and verified using reference suspensions as described below may be used instead of visual examination for determination of compliance with monograph requirements.

- *Measuring unit*: NTU (nephelometric turbidity units). NTU is based on the turbidity of a primary standard of formazin. FTU (formazin turbidity units) or FNU (formazin nephelometric units) are also used, and are equivalent to NTU in regions of low turbidity (up to 40 NTU). These units are used in all 3 instrumental methods (nephelometry, turbidimetry and in ratio mode).
- *Measuring range*: 0.01-1100 NTU.
- *Resolution*: 0.01 NTU within the range 0-9.99 NTU; 0.1 NTU within the range 10.0-99.9 NTU; and 1 NTU for the range > 100 NTU.
- *Accuracy*: ± (10 per cent of reading + 0.01 NTU) within the range 0-20 NTU; ± 7.5 per cent within the range 20-1100 NTU.
- *Repeatability*: ± 0.05 NTU within the range 0-20 NTU; ± 2 per cent of the reading within the range 20-1100 NTU.

Instruments with measuring range or resolution, accuracy and repeatability capabilities other than those mentioned above may be used provided they are sufficiently validated and are capable for the intended use.

CONTROL OF INSTRUMENT PERFORMANCE

- *Calibration*: performed with at least 4 reference suspensions of formazin covering the measuring range of interest. Reference suspensions described in this chapter or suitable reference standards calibrated against the primary reference suspensions may be used.
- *Stray light*: < 0.15 NTU within the range 0-10 NTU; < 0.5 NTU within the range 10-1100 NTU. Stray light is defined as that light that reaches the nephelometric detector without being a result of scatter from the sample. Stray light is always a positive interference and is a significant source

of error in low-range turbidity measurements. Sources of stray light include: imperfections in and scratches on sample cells, internal reflections of the optical system, contamination of the optics or sample cell chamber with dust, and electronic noise. Instrument design can also affect stray light. The influence of stray light becomes negligible in ratio mode measurements.

The test methodology for the specific substance/product to be analysed must also be verified to demonstrate its analytical capability. The instrument and methodology shall be consistent with the attributes of the substance to be examined.

Measurements of standards and samples should be carried out under the same temperature conditions, preferably between 20 °C and 25 °C.

REFERENCE SUSPENSIONS

Formazin has several desirable characteristics that make it an excellent turbidity standard. It can be reproducibly prepared from assayed raw materials. The physical characteristics make it a desirable light-scatter calibration standard. The formazin polymer consists of chains of different lengths, which fold into random configurations. This results in a wide variety of particle shapes and sizes, which allows the analysis of different particle sizes and shapes that are found in real samples. Stabilised formazin suspensions that can be used to prepare stable, diluted turbidity standards are commercially available and may be used after comparison with the standards prepared as described.

All steps of the preparation of reference suspensions as described below are carried out at 25 ± 3 °C.

Hydrazine sulfate solution. Dissolve 1.0 g of *hydrazine sulfate R* in *water R* and dilute to 100.0 mL with the same solvent. Allow to stand for 4-6 h.

Primary opalescent suspension (formazin suspension). In a 100 mL ground-glass-stoppered flask, dissolve 2.5 g of *hexamethylenetetramine R* in 25.0 mL of *water R*. Add 25.0 mL of the hydrazine sulfate solution. Mix and allow to stand for 24 h. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be mixed thoroughly before use.

Standard of opalescence. Dilute 15.0 mL of the primary opalescent suspension to 1000.0 mL with *water R*. This suspension is freshly prepared and may be stored for up to 24 h.

Reference suspensions. Prepare the reference suspensions according to Table 2.2.1.-1. Mix and shake before use.

Table 2.2.1.-1

	I	II	III	IV
Standard of opalescence	5.0 mL	10.0 mL	30.0 mL	50.0 mL
Water R	95.0 mL	90.0 mL	70.0 mL	50.0 mL

Measurements of reference suspensions I-IV in ratio mode show a linear relationship between the concentrations and measured NTU values (see Table 2.2.1.-2).

Table 2.2.1.-2

Formazin suspensions	Opalescent values (NTU)
Reference suspension I	3
Reference suspension II	6
Reference suspension III	18
Reference suspension IV	30
Standard of opalescence	60
Primary opalescent suspension	4000

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2.2.2. DEGREE OF COLORATION OF LIQUIDS[(1)]

◊A solution is *colourless* if it has the appearance of *water R* or the solvent used for the preparation of the solution to be examined, or is not more intensely coloured than reference solution B_9.

Report the results together with the method used (method I, method II or method III).

VISUAL METHODS

The examination of the degree of coloration of liquids in the range brown-yellow-red is carried out using one of the 2 methods below, as prescribed in the monograph.

METHOD I

Using identical tubes of colourless, transparent, neutral glass with an external diameter of 12 mm, compare 2.0 mL of the liquid to be examined with 2.0 mL of *water R* of the solvent used for the preparation of the solution to be examined, or the reference solution (see Tables of reference solutions) prescribed in the monograph. Compare the colours in diffuse daylight, viewing horizontally against a white background.

METHOD II

Using identical tubes of colourless, transparent, neutral glass with a flat base and an internal diameter of 15-25 mm, compare the liquid to be examined with *water R*, with the solvent used for the preparation of the solution to be examined, or with the reference solution (see Tables of reference solutions) prescribed in the monograph, the depth of the layer being 40 mm. Compare the colours in diffuse daylight, viewing vertically against a white background.

PREPARATION OF REFERENCE SOLUTIONS

Primary solutions

Yellow solution. Dissolve 46 g of *ferric chloride R* in about 900 mL of a mixture of 25 mL of *hydrochloric acid R* and 975 mL of *water R* and dilute to 1000.0 mL with the same mixture. Titrate and adjust the solution to contain 45.0 mg of $FeCl_3,6H_2O$ per millilitre by adding the same acidic mixture. Protect the solution from light.

Titration. In a 250 mL conical flask fitted with a ground-glass stopper, introduce 10.0 mL of the solution, 15 mL of *water R*, 5 mL of *hydrochloric acid R* and 4 g of *potassium iodide R*, close the flask, allow to stand in the dark for 15 min and add 100 mL of *water R*. Titrate the iodine released with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 27.03 mg of $FeCl_3,6H_2O$.

Red solution. Dissolve 60 g of *cobalt chloride R* in about 900 mL of a mixture of 25 mL of *hydrochloric acid R* and 975 mL of *water R* and dilute to 1000.0 mL with the same mixture. Titrate and adjust the solution to contain 59.5 mg of $CoCl_2,6H_2O$ per millilitre by adding the same acidic mixture.

Titration. In a 250 mL conical flask fitted with a ground-glass stopper, introduce 5.0 mL of the solution, 5 mL of *dilute hydrogen peroxide solution R* and 10 mL of a 300 g/L solution of *sodium hydroxide R*. Boil gently for 10 min, allow to cool and add 60 mL of *dilute sulfuric acid R* and 2 g of *potassium iodide R*. Close the flask and dissolve the precipitate by shaking gently. Titrate the iodine released with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator. The end-point is reached when the solution turns pink.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 23.79 mg of $CoCl_2,6H_2O$.

Blue primary solution. Dissolve 63 g of *copper sulfate pentahydrate R* in about 900 mL of a mixture of 25 mL of *hydrochloric acid R* and 975 mL of *water R* and dilute to 1000.0 mL with the same mixture. Titrate and adjust the solution to contain 62.4 mg of $CuSO_4,5H_2O$ per millilitre by adding the same acidic mixture.

Titration. Into a 250 mL conical flask fitted with a ground-glass stopper, introduce 10.0 mL of the solution, 50 mL of *water R*, 12 mL of *dilute acetic acid R* and 3 g of *potassium iodide R*. Titrate the iodine released with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator. The end-point is reached when the solution shows a slight pale brown colour.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 24.97 mg of $CuSO_4,5H_2O$.

Standard solutions

Using the 3 primary solutions, prepare the 5 standard solutions as follows.

Table 2.2.2.-1

Standard solution	Volumes in millilitres			
	Yellow solution	Red solution	Blue solution	Hydrochloric acid (10 g/L HCl)
B (brown)	3.0	3.0	2.4	1.6
BY (brownish-yellow)	2.4	1.0	0.4	6.2
Y (yellow)	2.4	0.6	0.0	7.0
GY (greenish-yellow)	9.6	0.2	0.2	0.0
R (red)	1.0	2.0	0.0	7.0

Reference solutions for Methods I and II

Using the 5 standard solutions, prepare the following reference solutions.

Table 2.2.2.-2. - *Reference solutions B*

Reference solution	Volumes in millilitres	
	Standard solution B	Hydrochloric acid (10 g/L HCl)
B_1	75.0	25.0
B_2	50.0	50.0
B_3	37.5	62.5
B_4	25.0	75.0
B_5	12.5	87.5
B_6	5.0	95.0
B_7	2.5	97.5
B_8	1.5	98.5
B_9	1.0	99.0

(1) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

Table 2.2.2.-3. - *Reference solutions BY*

Reference solution	Volumes in millilitres	
	Standard solution BY	Hydrochloric acid (10 g/L HCl)
BY_1	100.0	0.0
BY_2	75.0	25.0
BY_3	50.0	50.0
BY_4	25.0	75.0
BY_5	12.5	87.5
BY_6	5.0	95.0
BY_7	2.5	97.5

Table 2.2.2.-4. - *Reference solutions Y*

Reference solution	Volumes in millilitres	
	Standard solution Y	Hydrochloric acid (10 g/L HCl)
Y_1	100.0	0.0
Y_2	75.0	25.0
Y_3	50.0	50.0
Y_4	25.0	75.0
Y_5	12.5	87.5
Y_6	5.0	95.0
Y_7	2.5	97.5

Table 2.2.2.-5. - *Reference solutions GY*

Reference solution	Volumes in millilitres	
	Standard solution GY	Hydrochloric acid (10 g/L HCl)
GY_1	25.0	75.0
GY_2	15.0	85.0
GY_3	8.5	91.5
GY_4	5.0	95.0
GY_5	3.0	97.0
GY_6	1.5	98.5
GY_7	0.75	99.25

Table 2.2.2.-6. - *Reference solutions R*

Reference solution	Volumes in millilitres	
	Standard solution R	Hydrochloric acid (10 g/L HCl)
R_1	100.0	0.0
R_2	75.0	25.0
R_3	50.0	50.0
R_4	37.5	62.5
R_5	25.0	75.0
R_6	12.5	87.5
R_7	5.0	95.0

Storage

For Method I, the reference solutions may be stored in sealed tubes of colourless, transparent, neutral glass of 12 mm external diameter, protected from light.

For Method II, prepare the reference solutions immediately before use from the standard solutions.◊

INSTRUMENTAL METHOD – METHOD III

PRINCIPLE

The observed colour of an object depends primarily on its light-absorbing characteristics. However, a variety of conditions such as light-source differences, spectral energy of the illuminant, visual sensitivity of the observer, size differences, background differences and directional differences affect the perception of colour. Hue, lightness (or brightness) and saturation are 3 attributes of the colour. Instrumental measurement under defined conditions allows numerical expression of a colour. The base of any instrumental measurement of colour is that the human eye has been shown to detect colour via 3 types of receptors.

Instrumental methods for measurement of colour provide more objective data than the subjective viewing of colours by a small number of individuals. With adequate maintenance and calibration, instrumental methods can provide accurate, precise and consistent measurements of colour that do not drift with time. Through extensive colour-matching experiments with human subjects having normal colour vision, distribution coefficients (weighting factors) have been established for each wavelength in the visible spectrum, giving the relative amount of stimulation of each receptor type caused by the light of that wavelength.

The International Commission on Illumination (CIE) has developed models taking into account the light source and the angle at which the observer is looking at the target (field of view). In a visual test for coloration of solutions, there are requirements that lead to the use of a 2° angle and diffuse daylight (illuminant C). The mean sensitivity of the human eye is represented by the distribution coefficients $\bar{x}_\lambda$, $\bar{y}_\lambda$ and $\bar{z}_\lambda$ (Figure 2.2.2.-1).

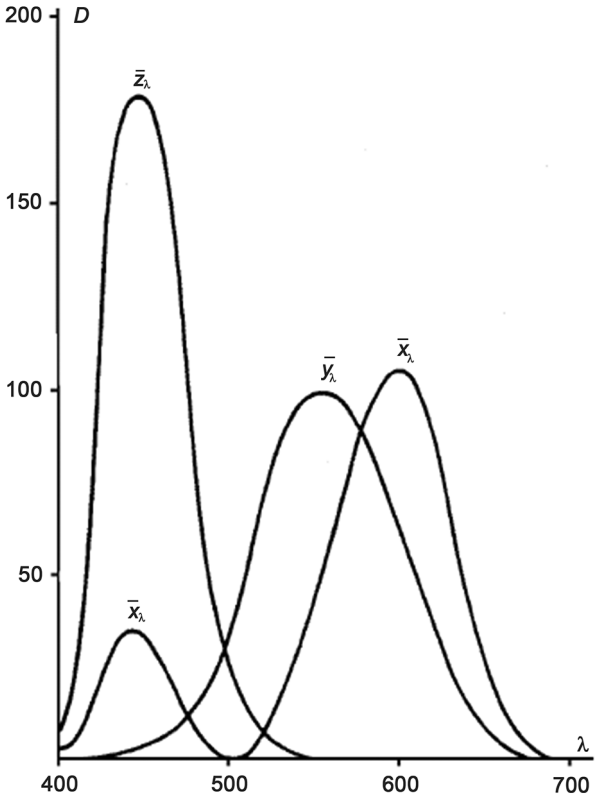

Figure 2.2.2.-1. – *Mean sensitivity of the human eye represented by distribution coefficients, CIE 2° Standard Observer (D = distribution coefficient; λ = wavelength in nanometres)*

For any colour, the amount of stimulation of each receptor type is defined by the set of tristimulus values (*XYZ*).

The relationship between the distribution coefficients and the tristimulus values (X, Y and Z) is given by the following equations, expressed in terms of integrals:

$$X = k\int_0^\infty f_\lambda \bar{x}_\lambda S_\lambda d\lambda$$

$$Y = k\int_0^\infty f_\lambda \bar{y}_\lambda S_\lambda d\lambda$$

$$Z = k\int_0^\infty f_\lambda \bar{z}_\lambda S_\lambda d\lambda$$

$$k = 100 / \int_0^\infty \bar{y}_\lambda S_\lambda d\lambda$$

k = normalising constant characterising the stimulation of one receptor type and the used illumination;

S_λ = relative spectral power distribution of the illuminant;

$\bar{x}_\lambda$, $\bar{y}_\lambda$ and $\bar{z}_\lambda$ = colour matching distribution coefficients for CIE 2° Standard Observer;

f_λ = spectral transmittance T_λ of the material;

λ = wavelength, in nanometres.

In practical calculations of tristimulus values, the integration is approximated by a summation, as follows:

$$X = k\sum_\lambda T_\lambda \bar{x}_\lambda S_\lambda \Delta\lambda$$

$$Y = k\sum_\lambda T_\lambda \bar{y}_\lambda S_\lambda \Delta\lambda$$

$$Z = k\sum_\lambda T_\lambda \bar{z}_\lambda S_\lambda \Delta\lambda$$

$$k = \frac{100}{\sum_\lambda S_\lambda \bar{y}_\lambda \Delta\lambda}$$

The tristimulus values can be used to calculate the CIE *Lab* colour space co-ordinates: L^**(lightness or brightness)*, a^**(red-green)* and b^**(yellow-blue)*; these are defined by:

$$L^* = 116f(Y / Y_n) - 16$$

$$a^* = 500[f(X / X_n) - f(Y / Y_n)]$$

$$b^* = 200[f(Y / Y_n) - f(Z / Z_n)]$$

where X_n, Y_n and Z_n are the tristimulus values of *water R* and

$f(X / X_n) = (X / X_n)^{1/3}$ if $X / X_n > (6 / 29)^3$,

otherwise $f(X / X_n) = 841 / 108(X / X_n) + 4 / 29$;

$f(Y / Y_n) = (Y / Y_n)^{1/3}$ if $(Y / Y_n) > (6 / 29)^3$,

otherwise $f(Y / Y_n) = 841 / 108(Y / Y_n) + 4 / 29$;

$f(Z / Z_n) = (Z / Z_n)^{1/3}$ if $(Z / Z_n) > (6 / 29)^3$,

otherwise $f(Z / Z_n) = 841 / 108(Z / Z_n) + 4 / 29$.

In the spectrophotometric method, transmittance values are obtained at discrete wavelengths throughout the visible spectrum. These values are then used to calculate the tristimulus values through the use of weighting factors $\bar{x}_\lambda$, $\bar{y}_\lambda$, and $\bar{z}_\lambda$ for a 2° Standard Observer and CIE standard illuminant C (see the current International Commission on Illumination publication, CIE).

SPECTROPHOTOMETRIC METHOD

Using a suitable spectrophotometer according to the manufacturer's instructions, determine the transmittance (T) at least over the range 400-700 nm, at intervals of not greater than 10 nm. Express the result as a percentage. Calculate the tristimulus values X, Y, and Z and the colour co-ordinates L^*, a^* and b^*.

DETERMINATION OF COLORATION

Calibrate the instrument according to the manufacturer's recommendations. Carry out system performance tests prior to each measurement or at regular intervals, depending on the use of the apparatus. For this purpose, use certified reference materials within the measurement range.

Operate the apparatus according to the manufacturer's instructions and test the sample solution and reference solution(s) under the same conditions (e.g. path length of the cuvette, temperature).

For transmittance measurements, use *water R* as the standard, assigning it a transmittance of 100.0 per cent at all wavelengths in the visible spectrum. Then the weighting factors $\bar{x}_\lambda$, $\bar{y}_\lambda$ and $\bar{z}_\lambda$ for CIE standard illuminant C are used to calculate the tristimulus values corresponding to colour co-ordinates $L^* = 100$, $a^* = 0$ and $b^* = 0$.

Reference measurements can be made using the colour co-ordinates of *water R* or freshly prepared pharmacopoeial reference solutions, or using the respective colour co-ordinates stored in the instrument manufacturer's database, provided the latter have been obtained under the same testing conditions.

If the test solution is turbid or hazy, it is filtered or centrifuged. If the test solution is not filtered or centrifuged, any haziness or turbidity is reported with the results. Air bubbles are to be avoided or, where applicable, removed.

The instrumental method is used to compare 2 solutions in terms of their colour or colour difference, or a deviation from a defined colour. Calculate the colour difference (ΔE^*_{tr}) between the test solution (t) and a reference solution (r) using the following equation:

$$\Delta E^*_{tr} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differences in colour co-ordinates.

The CIE *LCh* colour co-ordinates may be used instead of the CIE *Lab* colour co-ordinates.

Assessment of location within the $L^*a^*b^*$ colour space. Instruments may provide information on the actual location of the test solution within the $L^*a^*b^*$ colour space. Using appropriate algorithms, correspondence to pharmacopoeial reference solutions (such as 'test solution equals reference solution XY', 'test solution close to reference solution XY' or 'test solution between reference solutions XY and XZ') can be obtained.

07/2016:20203

2.2.3. POTENTIOMETRIC DETERMINATION OF pH

The pH of an aqueous solution is defined as the negative logarithm of the activity of its hydrogen ions, expressed conventionally as the hydrogen ion concentration of the solution. For practical purposes, its definition is an experimental one. The pH of a solution to be examined is related to that of a reference solution (pH_s) by the following equation:

$$pH = pH_s - \frac{E - E_s}{k}$$

in which E is the potential, expressed in volts, of the cell containing the solution to be examined and E_s is the potential, expressed in volts, of the cell containing the solution of known pH (pH_s), k is the change in potential per unit change in pH, expressed in volts and calculated from the Nernst equation.

Table 2.2.3.-1. – *Values of k at different temperatures*

Temperature (°C)	*k* (V)
15	0.0572
20	0.0582
25	0.0592
30	0.0601
35	0.0611

The potentiometric determination of pH is made by measuring the potential difference between 2 appropriate electrodes immersed in the solution to be examined; 1 of these electrodes is sensitive to hydrogen ions (usually a glass electrode) and the other is the reference electrode (e.g. a silver-silver chloride electrode). They are often combined as 1 compact electrode, together with a temperature probe.

Apparatus. The measuring apparatus is usually a voltmeter with an input resistance at least 100 times that of the electrodes used. It is normally graduated in pH units and has a sensitivity such that discrimination of at least 0.05 pH unit or at least 0.003 V may be achieved.

Recent pH meters are microprocessor-controlled and are operated using the manufacturer's firmware or software, according to given instructions.

Management of electrodes. The electrodes are stored appropriately and according to the manufacturer's recommendations (e.g. in an electrolyte solution or a suitable storage solution). Before measurement, the electrodes are visually checked. Refillable electrodes are checked for the absence of air bubbles in the glass bulb and to ensure that the inner electrolyte solution level is satisfactory. The filling orifice has to remain open during the measurement. It is also recommended that the diaphragm of the reference electrode is checked. Before first use, or if the electrode has been stored out of electrolyte solution, it is usually necessary to condition it, according to the recommendations of the manufacturer. If pH stabilisation is too slow (i.e. a long response time), or a zero point shift, reduced slope or any other difficulties in calibration are observed, the electrode will probably need to be cleaned or replaced. The cleaning is performed depending on the type of sample and as prescribed in the manufacturer's manual. Regular cleaning is recommended.

Calibration and measurement conditions. Unless otherwise prescribed in the monograph, all measurements are carried out at the same temperature as that used for calibration (± 2.5 °C), usually between 20 °C and 25 °C. Table 2.2.3.-2 shows the variation of pH with respect to temperature of a number of reference buffer solutions used for calibration. Follow the manufacturer's instructions for temperature correction.

The calibration consists of the determination of the slope (e.g. 95-105 per cent) and the offset of the measuring system. Most commercial pH meters offer a "self test" or "start-up test" where, for example, the slope and asymmetry potential are tested and compared to the manufacturer's specifications. The apparatus is calibrated using at least 2 buffer solutions chosen so that the expected pH value of the solution to be examined lies between the pH values of the buffer solutions. The range must be at least 2 pH units. The pH of another buffer solution of intermediate pH, read from the scale, must not differ by more than 0.05 pH units from the value corresponding to that solution.

Reference buffer solutions are preferably commercially available certified reference materials. Alternatively, buffer solutions can be prepared in-house according to Table 2.2.3.-2. These solutions must be traceable to primary standards. Calibration has to be performed on a regular basis, preferably each day of use or before each series of measurements.

Immerse the electrodes in the solution to be examined and take the reading in the same conditions as those applied for the reference buffer solutions.

If suspensions, emulsions or solutions of non-aqueous or partially non-aqueous character are measured on a system calibrated as described above, the pH reading can only be considered to be an approximation of the true value. Suitable electrodes have to be used for pH measurements of such mixtures.

Table 2.2.3.-2. – *pH of reference buffer solutions at various temperatures*

Temperature (°C)	Potassium tetraoxalate 0.05 M	Potassium hydrogen tartrate saturated at 25 °C	Potassium dihydrogen citrate 0.05 M	Potassium hydrogen phthalate 0.05 M	Potassium dihydrogen phosphate 0.025 M + Disodium hydrogen phosphate 0.025 M	Potassium dihydrogen phosphate 0.0087 M + Disodium hydrogen phosphate 0.0303 M	Disodium tetraborate 0.01 M	Sodium carbonate 0.025 M + Sodium bicarbonate 0.025 M	Calcium hydroxide, saturated at 25°C
	$C_4H_3KO_8,2H_2O$	$C_4H_5KO_6$	$C_6H_7KO_7$	$C_8H_5KO_4$	KH_2PO_4+ Na_2HPO_4	KH_2PO_4+ Na_2HPO_4	$Na_2B_4O_7$, $10H_2O$	Na_2CO_3+ $NaHCO_3$	$Ca(OH)_2$
15	1.67		3.80	4.00	6.90	7.45	9.28	10.12	12.81
20	1.68		3.79	4.00	6.88	7.43	9.23	10.06	12.63
25	1.68	3.56	3.78	4.01	6.87	7.41	9.18	10.01	12.45
30	1.68	3.55	3.77	4.02	6.85	7.40	9.14	9.97	12.29

Temperature (°C)	Potassium tetraoxalate 0.05 M	Potassium hydrogen tartrate saturated at 25 °C	Potassium dihydrogen citrate 0.05 M	Potassium hydrogen phthalate 0.05 M	Potassium dihydrogen phosphate 0.025 M + Disodium hydrogen phosphate 0.025 M	Potassium dihydrogen phosphate 0.0087 M + Disodium hydrogen phosphate 0.0303 M	Disodium tetraborate 0.01 M	Sodium carbonate 0.025 M + Sodium bicarbonate 0.025 M	Calcium hydroxide, saturated at 25°C
	$C_4H_3KO_8,2H_2O$	$C_4H_5KO_6$	$C_6H_7KO_7$	$C_8H_5KO_4$	KH_2PO_4+ Na_2HPO_4	KH_2PO_4+ Na_2HPO_4	$Na_2B_4O_7$, $10H_2O$	Na_2CO_3+ $NaHCO_3$	$Ca(OH)_2$
35	1.69	3.55	3.76	4.02	6.84	7.39	9.10	9.93	12.13
$\frac{\Delta pH^{(1)}}{\Delta t}$	+ 0.001	− 0.0014	− 0.0022	+ 0.0012	− 0.0028	− 0.0028	− 0.0082	− 0.0096	− 0.034

(1) pH variation per degree Celsius.

PREPARATION OF REFERENCE BUFFER SOLUTIONS

Potassium tetraoxalate 0.05 M. Dissolve 12.61 g of $C_4H_3KO_8,2H_2O$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Potassium hydrogen tartrate, saturated at 25 °C. Shake an excess of $C_4H_5KO_6$ vigorously with *carbon dioxide-free water R* at 25 °C. Filter or decant. Prepare immediately before use.

Potassium dihydrogen citrate 0.05 M. Dissolve 11.41 g of $C_6H_7KO_7$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent. Prepare immediately before use.

Potassium hydrogen phthalate 0.05 M. Dissolve 10.13 g of $C_8H_5KO_4$, previously dried for 1 h at 110 ± 2 °C, in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Potassium dihydrogen phosphate 0.025 M + Disodium hydrogen phosphate 0.025 M. Dissolve 3.39 g of KH_2PO_4 and 3.53 g of Na_2HPO_4, both previously dried for 2 h at 120 ± 2 °C, in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Potassium dihydrogen phosphate 0.0087 M + Disodium hydrogen phosphate 0.0303 M. Dissolve 1.18 g of KH_2PO_4 and 4.30 g of Na_2HPO_4, both previously dried for 2 h at 120 ± 2 °C, in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Disodium tetraborate 0.01 M. Dissolve 3.80 g of $Na_2B_4O_7,10H_2O$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent. Store protected from atmospheric carbon dioxide.

Sodium carbonate 0.025 M + Sodium hydrogen carbonate 0.025 M. Dissolve 2.64 g of Na_2CO_3 and 2.09 g of $NaHCO_3$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent. Store protected from atmospheric carbon dioxide.

Calcium hydroxide, saturated at 25 °C. Shake an excess of *calcium hydroxide R* with *carbon dioxide-free water R* and decant at 25 °C. Store protected from atmospheric carbon dioxide.

STORAGE OF BUFFER SOLUTIONS

Store buffer solutions in suitable chemically-resistant, airtight containers, such as type I glass bottles or plastic containers suitable for aqueous solutions.

07/2019:20235

2.2.35. OSMOLALITY

PRINCIPLE

GENERAL

Osmolality is a measure of the total number of chemical entities per kilogram of solvent, and thus provides an indication of the osmotic pressure of the solution. Osmolality is dependent on the molal concentration of the solute(s) in the solution, on their dissociation and on the deviation of the solution from ideal behaviour (Raoult's law).

The unit of osmolality is the osmole per kilogram (osmol/kg), but the submultiple milliosmole per kilogram (mosmol/kg) is more commonly used.

The osmolality (ξ_m) of a solution containing *i* solutes is given by the expression:

$$\xi_m = \sum v_i m_i \Phi_{m,i}$$

v_i = number of entities formed by the dissociation of one molecule of the i^{th} solute; if the solute is non-ionic (non-dissociating), v_i equals 1,

m_i = molality of the i^{th} solute in the solution, in moles per kilogram of solvent,

$\Phi_{m,i}$ = molal osmotic coefficient, a dimensionless factor.

The molal osmotic coefficient is a measure of the deviation of the solution from ideal behaviour. For an ideal solution, osmolality equals molality (Φ=1).

In the case of a real, non-ideal solution, the molal osmotic coefficient is influenced by the interactions occurring amongst the components (i.e. molecules, ions, solvent) of the solution. The more complex the composition of the solution, the harder it becomes to determine Φ.

For this reason, the measurement of a colligative property such as the freezing-point depression is used as a practical means of determining osmolality by obtaining an overall measure of the contribution of the various solutes present in a solution.

PRINCIPLE OF MEASUREMENT

Unless otherwise prescribed, osmolality is determined by measuring the freezing-point depression (ΔT_f) of a solution. The relationship between osmolality and freezing-point depression is given by the expression:

$$\Delta T_f = k_f \xi_m$$

where k_f is the molal cryoscopic constant, which is solvent-dependent. For water, the value of k_f is 1.86 K/osmol (i.e. adding 1 mol of a non-dissociating solute to 1 kg of water results in a decrease in freezing-point of 1.86 K).

EQUIPMENT

An osmometer for freezing-point depression measurement typically consists of:

- an appropriate sample container;
- a means of cooling the sample;
- a temperature-sensitive resistor (thermistor), with an appropriate current or potential difference measurement device that can indicate a temperature depression or give osmolality values directly;
- a means of mixing the sample and/or inducing solidification when supercooling occurs.

PROCEDURE

CALIBRATION

Prepare reference solutions as specified in Table 2.2.35.-1, as necessary, using dried *sodium chloride R*. Commercially available certified solutions for osmometer calibration, with osmolalities equal or similar to those listed in Table 2.2.35.-1, may be used. Calibrate the equipment according to the manufacturer's instructions using *water R* to determine the zero value and at least 2 of the reference solutions listed in Table 2.2.35.-1. Confirm the calibration using at least one additional reference solution with a known osmolality (see Table 2.2.35.-1). Select a reference solution preferably with an osmolality within ± 50 mosmol/kg of the expected value for the solution to be examined or close to the centre of the expected osmolality range of the solutions to be examined. It is recommended that the reading is within ± 4 mosmol/kg of the osmolality of the chosen reference solution.

Table 2.2.35.-1. – *Reference solutions for osmometer calibration*

Mass of *sodium chloride R* in *water R* (g/kg)	Osmolality, ξ_m (mosmol/kg)	Freezing-point depression, ΔT_f (K)
3.087	100	0.186
6.260	200	0.372
9.463	300	0.558
12.684	400	0.744
15.916	500	0.930
19.147	600	1.116
22.380	700	1.302

METHOD

Rinse the sample container with the solution to be examined before each measurement. Programme the device inducing solidification to start at a defined temperature below the expected freezing-point of the solution to be examined. Introduce an appropriate volume of the solution to be examined into the sample container according to the manufacturer's instructions, and start the cooling system. The equipment indicates when equilibrium has been reached.

Perform the test under the conditions (cooling temperature and volume) used to calibrate the equipment. Depending on the type of equipment, the osmolality can be read directly or can be calculated from the measured freezing-point depression.

The test is not valid unless the measured osmolality of the solution to be examined lies within the calibrated osmolality range.

04/2018:20512

2.5.12. WATER: SEMI-MICRO DETERMINATION

The semi-micro determination of water is based upon the quantitative reaction of water with sulfur dioxide and iodine in a suitable anhydrous medium in the presence of a base with sufficient buffering capacity.

APPARATUS

The apparatus consists of a titration vessel with:

- 2 identical platinum electrodes;
- tight inlets for introduction of solvent and titrant;
- an inlet for introduction of air via a desiccant;
- a sample inlet fitted with a stopper or, for liquids, a septum.

Inlet systems for introduction of dry nitrogen or for aspiration of solvents may also be fitted.

The titration is carried out according to the instrument supplier's instructions. Care is taken throughout the determination to avoid exposure of reagents and solvents to atmospheric moisture. The end-point is determined using 2 identical indicator electrodes connected to an electrical source that maintains between the electrodes either a constant current (*2.2.65. Voltametric titration*) or a constant voltage (*2.2.19. Amperometric titration*). Where direct titration is used (method A), addition of titrant causes either a decrease in voltage where constant current is maintained or an increase in current where constant voltage is maintained, until the end-point is reached. Instruments with automatic end-point detection are commonly used. Instrument qualification is carried out according to established quality system procedures, for example using a suitable certified reference material (*sodium aminosalicylate dihydrate for equipment qualification CRS* may be used).

STANDARDISATION

To the titration vessel, add *methanol R*, dried if necessary, or the solvent recommended by the supplier of the titrant. Where applicable for the apparatus used, eliminate residual water from the measurement cell or carry out a pre-titration. Introduce a suitable amount of water in an appropriate form (*water R* or a certified reference material) and carry out the titration, stirring for the necessary time. The water equivalent is not less than 80 per cent of that indicated by the supplier. Standardise the titrant before the first use and at suitable intervals thereafter.

Unless otherwise prescribed, use Method A.

METHOD A

Introduce into the titration vessel *methanol R*, or the solvent indicated in the monograph or recommended by the supplier of the titrant. Where applicable for the apparatus used, eliminate residual water from the measurement cell or carry out a pre-titration. Introduce the substance to be examined rapidly and carry out the titration, stirring for the necessary extraction time.

METHOD B

Introduce into the titration vessel *methanol R*, or the solvent indicated in the monograph or recommended by the supplier of the titrant. Where applicable for the apparatus used, eliminate residual water from the measurement cell or carry out a pre-titration. Introduce the substance to be examined rapidly and in a suitable state of division. Add an accurately measured volume of the titrant, sufficient to give an excess of about 1 mL or the prescribed volume. Allow to stand protected from light for 1 min or the prescribed time, with stirring. Titrate the excess of reagent using *methanol R* or the prescribed solvent, containing an accurately known quantity of water.

SUITABILITY

The accuracy of the determination with the chosen titrant must be verified for each combination of substance, titrant and solvent to be examined. The following procedure, given as an example, is suitable for samples containing 2.5-25 mg of water.

The water content of the substance to be examined is determined using the reagent/solvent system chosen. Thereafter, in the same titration vessel, sequential known amounts of water, corresponding to about 50-100 per cent of the amount found in the substance to be examined, are added in an appropriate form (at least 5 additions) and the water content is determined after each addition. Calculate the percentage recovery (r) after each addition using the following expression:

$$r = 100\frac{W_2}{W_1}$$

W_1 = amount of water added, in milligrams;

W_2 = amount of water found, in milligrams.

Calculate the mean percentage recovery ($\bar{r}$). The reagent/solvent system is considered to be acceptable if $\bar{r}$ is between 97.5 per cent and 102.5 per cent.

Calculate the regression line. The x-axis represents the cumulative water added whereas the y-axis represents the sum of the initial water content determined for the substance (M) and the cumulative water determined after each addition. Calculate the slope (b), the intercept with the y-axis (a) and the intercept of the extrapolated calibration line with the x-axis (d).

Calculate the percentage errors (e_1 and e_2) using the following expressions:

$$e_1 = 100\frac{a - M}{M}$$

$$e_2 = 100\frac{|d| - M}{M}$$

a = the y-axis intercept, in milligrams of water;

d = the x-axis intercept, in milligrams of water;

M = water content of the substance, in milligrams of water.

The reagent/solvent system is considered to be acceptable if:

- $|e_1|$ and $|e_2|$ are not greater than 2.5 per cent;
- b is between 0.975 and 1.025.

04/2010:20917

2.9.17. TEST FOR EXTRACTABLE VOLUME OF PARENTERAL PREPARATIONS[1]

Suspensions and emulsions are shaken before withdrawal of the contents and before the determination of the density. Oily and viscous preparations may be warmed according to the instructions on the label, if necessary, and thoroughly shaken immediately before removing the contents. The contents are then cooled to 20-25 °C before measuring the volume.

SINGLE-DOSE CONTAINERS

Select 1 container if the nominal volume is 10 mL or more, 3 containers if the nominal volume is more than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. Take up individually the total contents of each container selected into a dry syringe of a capacity not exceeding 3 times the volume to be measured, and fitted with a 21-gauge needle not less than 2.5 cm in length. Expel any air bubbles from the syringe and needle, then discharge the contents of the syringe without emptying the needle into a standardised dry cylinder (graduated to contain rather than to deliver the designated volumes) of such size that the volume to be measured occupies at least 40 per cent of its graduated volume. Alternatively, the volume of the contents in millilitres may be calculated as the mass in grams divided by the density.

For containers with a nominal volume of 2 mL or less, the contents of a sufficient number of containers may be pooled to obtain the volume required for the measurement provided that a separate, dry syringe assembly is used for each container.

The contents of containers holding 10 mL or more may be determined by opening them and emptying the contents directly into the graduated cylinder or tared beaker.

The volume is not less than the nominal volume in case of containers examined individually, or, in case of containers with a nominal volume of 2 mL or less, is not less than the sum of the nominal volumes of the containers taken collectively.

MULTIDOSE CONTAINERS

For injections in multidose containers labelled to yield a specific number of doses of a stated volume, select one container and proceed as directed for single-dose containers using the same number of separate syringe assemblies as the number of doses specified.

The volume is such that each syringe delivers not less than the stated dose.

CARTRIDGES AND PREFILLED SYRINGES

Select 1 container if the nominal volume is 10 mL or more, 3 containers if the nominal volume is more than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. If necessary, fit the containers with the accessories required for their use (needle, piston, syringe) and transfer the entire contents of each container without emptying the needle into a dry tared beaker by slowly and constantly depressing the piston. Determine the volume in millilitres calculated as the mass in grams divided by the density.

The volume measured for each of the containers is not less than the nominal volume.

PARENTERAL INFUSIONS

Select one container. Transfer the contents into a dry measuring cylinder of such a capacity that the volume to be determined occupies at least 40 per cent of the nominal volume of the cylinder. Measure the volume transferred.

The volume is not less than the nominal volume.

(1) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

01/2020:20920

2.9.20. PARTICULATE CONTAMINATION: VISIBLE PARTICLES

Particulate contamination consists of mobile undissolved substances, other than gas bubbles, unintentionally present in liquid preparations.

The test is intended to provide a simple procedure for the visual assessment of the quality of liquid preparations, if applicable after reconstitution, as regards visible particles.

EQUIPMENT

The equipment (see Figure 2.9.20.-1) consists of a viewing station comprising:

- a matt black panel (A) of appropriate size held in a vertical position;
- a non-glare white panel (B) of appropriate size held in a vertical position next to the black panel;
- a non-glare white panel (C) of appropriate size held in a horizontal position next to (A) and (B);
- a lampholder (D) fitted with a suitable, shaded, white-light source and with a suitable light diffuser (e.g. a viewing illuminator containing two 13 W fluorescent tubes, each 525 mm in length, or an appropriate light-emitting diode (LED) light source). The intensity of illumination at the viewing point is maintained between 2000 lux and 3750 lux, although higher values may be required for coloured glass or plastic containers and for coloured or turbid preparations.

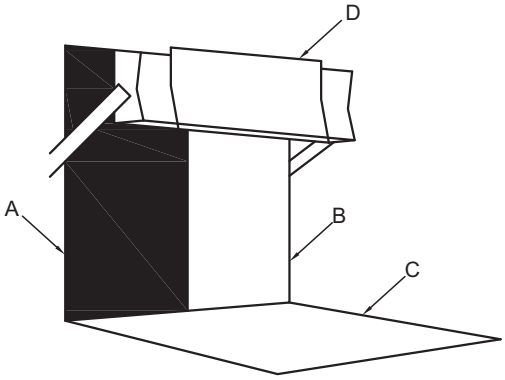

Figure 2.9.20.-1. – *Equipment for visible particles*

METHOD

Adequate visual inspection of the container and contents is necessary, which may require the removal of any adherent labels from the container. Gently swirl or invert the container, ensuring that air bubbles are not introduced, and observe without magnification for about 5 s in front of the white panel (B), although longer observation times may be required for coloured glass or plastic containers and for coloured or turbid preparations. Where inspection in the primary container is not possible, the contents may be transferred for inspection into a sample container that is free from visible particles, taking precautions to prevent contamination during transfer. Repeat the inspection in front of the black panel (A). Record the presence of any visible particles.

Individual vaccine monographs

Examples of individual monographs for various types of viral vaccines.
The following examples demonstrate the applicable requirements for specific products. They can be taken as guidance and should be considered for vaccines in a similar class without a specific monograph.

TEXT N°	TITLE	PRODUCT TYPE(S)
2441	Human papillomavirus vaccine (rDNA)	Recombinant protein-based vaccines. Production in an insect cell / baculovirus expression vector system.
1056	Hepatitis B vaccine (rDNA)	Recombinant protein-based vaccines. Production in CHO cells.
0214	Poliomyelitis vaccine (inactivated)	Inactivated viral vaccines.
0537	Yellow fever vaccine (live)	Live attenuated viral vaccines. *Certain considerations may be relevant to recombinant viral vectored vaccines using yellow fever virus or other viruses as backbone.*
0213	Measles vaccine (live)	Live attenuated viral vaccines. *Certain considerations may be relevant to recombinant viral vectored vaccines using measles virus or other viruses as backbone.*
2772	Influenza vaccine (live, nasal)	Live attenuated viral vaccines for nasal administration. *Certain considerations may be relevant to recombinant viral vectored vaccines using influenza virus or other viruses as backbone.*

01/2019:2441

HUMAN PAPILLOMAVIRUS VACCINE (rDNA)

Vaccinum papillomaviri humani (ADNr)

DEFINITION

Human papillomavirus vaccine (rDNA) is a preparation of purified virus-like particles (VLPs) composed of the major capsid protein (L1) of one or more human papillomavirus (HPV) genotypes; the antigens may be adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. The vaccine may also contain the adjuvant 3-*O*-desacyl-4′-monophosphoryl lipid A. The antigens are obtained by recombinant DNA technology.

PRODUCTION

GENERAL PROVISIONS

The vaccine shall have been shown to induce specific neutralising antibodies in man. The production method shall have been shown to yield consistently vaccines comparable in quality with the vaccine of proven clinical efficacy and safety in man.

The vaccine is produced by the expression of the viral genes coding for the capsid proteins in yeast or in an insect cell/baculovirus expression vector system, purification of the resulting VLPs and the rendering of these particles into an immunogenic preparation. The suitability and safety of the expression systems are approved by the competent authority. Production of the vaccine is based on a seed lot/cell bank system. Unless otherwise justified and authorised, the virus and cells used for vaccine production shall not have undergone more passages from the master seed lot/cell bank than was used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

Reference preparation. A batch of vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. The reference vaccine is preferably stabilised and the stabilisation method shall have been shown to have no significant effect on the assay validity.

CHARACTERISATION

Characterisation of the VLPs is performed on lots produced during vaccine development, including the process validation batches. Characterisation includes protein composition, for example using techniques such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting or mass spectrometry, peptide mapping and/or terminal amino acid sequence analysis. Morphological characteristics of the VLPs and degree of aggregation are determined to confirm the presence of the conformational epitopes that are essential for efficacy. VLP characterisation may be done by atomic force microscopy and transmission electron microscopy, dynamic light scattering, epitope mapping and reactivity with neutralising monoclonal antibodies. In addition, the protein, lipid, nucleic acid and carbohydrate content are measured where applicable. The level of residual host-cell protein derived from insect cells meets acceptable safety criteria as set by the competent authority.

CELL BANKS AND SEED LOTS

Production in recombinant yeast cells. Only cell banks that have been satisfactorily characterised for identity, microbial purity, growth characteristics and stability shall be used for production. Gene homogeneity is studied for the master and working cell banks. A full description of the biological characteristics of the host cell and expression vectors is given. The physiological measures used to promote and control the expression of the cloned gene in the host cell are described in detail. This includes genetic markers of the host cell, the construction, genetics and structure of the expression vector, and the origin and identification of the gene that is being cloned. The nucleotide sequence of the gene insert and of adjacent segments of the vector and restriction-enzyme mapping of the vector containing the gene insert are provided. Data that demonstrates the stability of the expression system during storage of the recombinant working cell bank up to or beyond the passage level used for production is provided.

Production in an insect cell/baculovirus expression vector system

– *Insect cell substrate*. Only cell banks that have been satisfactorily characterised for identity, purity, growth characteristics, stability, extraneous agents and tumorigenicity shall be used for production. Such characterisation is performed at suitable stages of production in accordance with general chapters *5.2.3. Cell substrates for the production of vaccines for human use* and *2.6.16. Tests for extraneous agents in viral vaccines for human use*. Special attention is given to insect-borne viruses, in particular insect-borne potential human pathogens (e.g. arboviruses). Adventitious infectious agents of insect cells may be without cytopathic effect. Tests therefore include nucleic acid amplification techniques, and other tests such as electron microscopy and co-cultivation.
– *Recombinant baculovirus*. The use of the recombinant baculovirus vector is based on a seed-lot system with a defined number of passages between the original virus and the master and the working seed-lots, as approved by the competent authorities. The recombinant baculovirus expression vector contains the coding sequence of the HPV L1 antigen. Segments of the expression construct are analysed using nucleic acid amplification techniques in conjunction with other tests performed on the purified recombinant protein for assuring the quality and consistency of the expressed HPV L1 antigens. The recombinant baculovirus used in the production of HPV vaccines is identified by historical records, which include information on the origin and identity of the gene being cloned as well as the construction, genetics and structure of the baculovirus expression vector(s). The genetic stability of the expression construct is demonstrated from the baculovirus master seed up to at least the highest level used in production and preferably beyond this level.

Recombinant baculovirus seed lots are prepared in large quantities and stored at temperatures favourable for stability.

Only a seed lot that complies with the following requirements may be used for virus propagation.

Identification. The master and working seed lots are identified by the HPV type of the inserted gene of origin, by an appropriate method such as nucleic acid amplification techniques (NAT) (*2.6.21*).

Virus concentration. The virus concentration of the master and working seed lots is determined to monitor consistency of production.

Extraneous agents (*2.6.16*). The working seed lot complies with the requirements for seed lots and control cells. Special attention is given to *Spiroplasma* spp. and insect-borne viruses, in particular insect-borne potential human pathogens (e.g. arboviruses).

PROPAGATION AND HARVEST

All processing of the cell banks and baculovirus seed lots and subsequent cell cultures is done under aseptic conditions in an area where no other cells are being handled.

Where justified and authorised for production in an insect cell/baculovirus expression vector system, a stored virus intermediate culture that complies with the 5 following tests may be used for virus propagation.

Identification. Each stored virus intermediate culture is identified by HPV type, by an immunological assay using specific antibodies or by a molecular identity test such as NAT (*2.6.21*).

Bacterial and fungal contamination. Each stored virus intermediate culture complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

Virus concentration. The virus concentration of each stored baculovirus intermediate culture is determined by a suitable method such as plaque assay or NAT (*2.6.21*) in order to monitor consistency of production.

Extraneous agents (*2.6.16*). Each stored virus intermediate culture complies with the tests for extraneous agents.

Control cells. The control cells of the production cell culture from which each stored virus intermediate is derived comply with a test for identity and with the requirements for extraneous agents (*2.6.16*).

Production in recombinant yeast cells. Identity, microbial purity, plasmid retention and consistency of yield are determined at suitable production stages.

Production in an insect cell/baculovirus expression vector system. Insect cell cultures are inoculated with recombinant baculovirus at a defined multiplicity of infection as approved by the competent authority. Several single harvests may be pooled before testing. No antibiotics are added at the time of harvesting or at any later stage of manufacturing.

SINGLE HARVESTS

Only a single harvest or a pool of single harvests that complies with the following requirements may be used in the preparation of the purified monovalent antigen.

Identification. Each single harvest is identified as the appropriate HPV type by immunological assay or by a molecular biology-based assay, for example hybridisation or polymerase chain reaction (PCR).

Bacterial and fungal contamination. In case of production in an insect cell/baculovirus expression vector system the single harvest complies with the test for sterility (*2.6.1*). In case of production in yeast cells the single harvest is tested for culture purity by inoculation of suitable medium to ensure no growth other than the host organism.

Extraneous agents (*2.6.16*). In case of production in an insect cell/baculovirus expression vector system the single harvest complies with the tests for extraneous agents. Special attention is given to insect-borne viruses as mentioned under Cell banks and seed lots.

Control cells. In case of production in an insect cell/baculovirus expression vector system the control cells comply with a test for identification and with the requirements for extraneous agents (*2.6.16*). Special attention is given to insect-borne viruses as mentioned under Cell banks and seed lots.

PURIFIED MONOVALENT ANTIGEN

Harvests are purified using validated methods. When an insect cell/baculovirus expression vector system substrate is used, the production process is validated for its capacity to eliminate (by removal and/or inactivation) adventitious viruses and recombinant baculoviruses.

Only a purified monovalent antigen that complies with the following requirements may be used in the preparation of the final bulk. In agreement with the competent authority one or more of the tests mentioned below may be omitted if performed on the adsorbed monovalent antigen.

Total protein. The total protein is determined by a validated method. The content is within the limits approved for the particular product.

Antigen content and identification. The quantity and specificity of each antigen type is determined by a suitable immunochemical method (*2.7.1*) such as radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot (preferably using a monoclonal antibody directed against a protective epitope) or single radial diffusion. The antigen/protein ratio may be determined and is within the limits approved for the particular product.

Antigenic purity. The purity of each purified monovalent antigen is determined by a suitable method, such as SDS-PAGE with quantification by densitometric analysis, the limit of detection being 1 per cent of impurities or better with respect to total protein. A reference preparation is used to validate each test. The protein purity is calculated as the ratio of the L1 protein-related bands relative to the total protein bands, expressed as a percentage. For the genotypes included in the vaccine, the value calculated for purity is within the limits approved for the particular product.

Percent intact L1 monomer. The antigenic purity assay serves also to assess the integrity of the L1 monomer. The percent intact L1 monomer is the ratio of the intact L1 monomer to the total protein, expressed as a percentage.

VLP size and structure. The size and structure of the VLPs is established and monitored by a suitable method such as dynamic light scattering. The size is within the limits approved for the particular product.

Composition. The protein, lipid, nucleic acid and carbohydrate contents are determined, where applicable.

Host-cell and vector-derived DNA: maximum 10 ng of DNA in a quantity of purified antigen equivalent to a single human dose of vaccine, determined in each monovalent purified antigen by sensitive methods.

Residual host-cell proteins. Tests for residual host-cell proteins are carried out. The content is within the limits approved for the particular product.

Chemicals used for disruption and purification. Tests for the chemicals used for purification or other stages of production are carried out. The content is within the limits approved for the particular products.

Sterility (*2.6.1*). Each purified monovalent antigen complies with the test, carried out using 10 mL for each medium.

ADSORBED MONOVALENT ANTIGEN

The purified monovalent antigens may be adsorbed onto a mineral vehicle such as an aluminium salt.

Only an adsorbed monovalent antigen that complies with the following requirements may be used in the preparation of the final bulk.

Sterility (*2.6.1*). Each adsorbed monovalent antigen complies with the test, carried out using 10 mL for each medium.

Bacterial endotoxins (*2.6.14*). Each adsorbed monovalent antigen is tested for bacterial endotoxins. The content is within the limits approved for the particular product.

Antigen content and identification. Each antigen type is identified by a suitable immunochemical method (*2.7.1*) such as radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot (preferably using a monoclonal antibody directed against a protective epitope) or single radial diffusion. The antigen/protein ratio is determined.

Mineral vehicle concentration. Where applicable, each adsorbed monovalent antigen is tested for the content of mineral vehicle. The content is within the limits approved for the particular product.

ADSORBED 3-O-DESACYL-4′-MONOPHOSPHORYL LIPID A BULK

If 3-*O*-desacyl-4′-monophosphoryl lipid A is included in the vaccine it complies with the monograph *3-O-desacyl-4′-monophosphoryl lipid A (2537)*. Where 3-*O*-desacyl-4′-monophosphoryl lipid A is adsorbed prior to inclusion in the vaccine, the adsorbed 3-*O*-desacyl-4′-monophosphoryl lipid A bulk complies with the following requirements.

Degree of adsorption of 3-*O*-desacyl-4′-monophosphoryl lipid A. The content of non-adsorbed 3-*O*-desacyl-4′-monophosphoryl lipid A in the adsorbed 3-*O*-desacyl-4′-monophosphoryl lipid A bulk is determined by a suitable method, for example gas chromatographic quantification of the *3-O-desacyl-4′-monophosphoryl lipid A (2537)* fatty acids in the supernatant, evaporated to dryness, after centrifugation.

pH (*2.2.3*). The pH is within the limits approved for the particular preparation.

Sterility (*2.6.1*). It complies with the test, carried out using 10 mL for each medium.

FINAL BULK VACCINE

The final bulk vaccine is prepared directly from each purified monovalent antigen HPV type or adsorbed purified monovalent antigen HPV type. An antimicrobial preservative, a mineral vehicle such as an aluminium salt and the adjuvant 3-*O*-desacyl-4′-monophosphoryl lipid A may be included in the formulation of the final bulk.

Only a final bulk that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

Sterility (*2.6.1*). The final bulk vaccine complies with the test, carried out using 10 mL for each medium.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for antimicrobial preservative content (where applicable) has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot. If an *in vivo* assay is carried out, then provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Adjuvants. If the vaccine contains an adjuvant, the amount is determined and shown to be within acceptable limits with respect to the expected amount. A suitable method for 3-*O*-desacyl-4′-monophosphoryl lipid A is, for example, gas chromatography.

Degree of adsorption. The degree of adsorption of each antigen and, where applicable, 3-*O*-desacyl-4′-monophosphoryl lipid A is assessed.

IDENTIFICATION

The vaccine is shown to contain the different types of HPV antigen by a suitable immunochemical method (*2.7.1*). The *in vitro* assay may serve to identify the vaccine. In addition, where applicable, the test for 3-*O*-desacyl-4′-monophosphoryl lipid A content also serves to identify the 3-*O*-desacyl-4′-monophosphoryl lipid A-containing vaccine.

TESTS

Aluminium (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

3-*O*-Desacyl-4′-monophosphoryl lipid A: minimum 80 per cent and maximum 120 per cent of the intended amount.

Where applicable, determine the content of 3-*O*-desacyl-4′-monophosphoryl lipid A by a suitable method, for example gas chromatography (*2.2.28*).

Antimicrobial preservative. Where applicable, determine the content of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

Sterility (*2.6.1*). The vaccine complies with the test.

Bacterial endotoxins (*2.6.14*): maximum 5 IU per single human dose. If the adjuvant prevents the determination of endotoxin, a suitable in-process test is carried out.

ASSAY

The assay is performed by an *in vivo* test or an *in vitro* test having acceptance criteria established by correlation studies against an *in vivo* test.

***In vivo* test**. A suitable *in vivo* assay method consists of the injection of not fewer than 3 dilutions of the vaccine to be examined and of a reference vaccine preparation, using for each dilution a group of a suitable number of female mice of a suitable strain. The vaccine is diluted in a solution of *sodium chloride R* containing the aluminium adjuvant used for the vaccine production. The mice are 6-8 weeks old at the time of injection, and each mouse is given a 0.5 mL injection. A preimmunisation serum sample is taken prior to inoculation, and a final serum sample is taken at a defined time between days 21 and 28. Assay the individual sera for specific neutralising antibodies against each HPV type by a suitable immunochemical method (*2.7.1*).

The test is not valid unless:

- for both the vaccine to be examined and the reference vaccine, the ED_{50} lies between the smallest and the largest doses given to the animals;
- the statistical analysis shows no significant deviation from linearity or parallelism;
- the confidence limits ($P = 0.95$) are within the limits approved for the particular product.

***In vitro* test**. Carry out an immunochemical determination (*2.7.1*) of each antigen genotype content. Enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA) using monoclonal antibodies specific for protection-inducing epitopes of the L1 protein have been shown to be suitable. Suitable numbers of dilutions of the vaccine to be examined and a manufacturer's reference preparation are used and a suitable model is used to analyse the data. For each type, the antigen content is within the limits approved for the particular product.

LABELLING

The label states:

- the amount of L1 proteins and the genotype of HPV contained in the vaccine;
- the cell substrate used for production of the vaccine;
- the name and amount of the adjuvant and/or adsorbent used;
- that the vaccine must not be frozen.

01/2019:1056

HEPATITIS B VACCINE (rDNA)

Vaccinum hepatitidis B (ADNr)

DEFINITION

Hepatitis B vaccine (rDNA) is a preparation of hepatitis B surface antigen (HBsAg), a component protein of hepatitis B virus; the antigen may be adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. The vaccine may also contain the adjuvant 3-*O*-desacyl-4′-monophosphoryl lipid A. The antigen is obtained by recombinant DNA technology.

PRODUCTION

GENERAL PROVISIONS

The vaccine shall have been shown to induce specific, protective antibodies in man. The production method shall have been shown to yield consistently vaccines that comply with the requirements for immunogenicity and safety.

Hepatitis B vaccine (rDNA) is produced by the expression of the viral gene coding for HBsAg in yeast (*Saccharomyces cerevisiae*) or mammalian cells (Chinese hamster ovary (CHO) cells or other suitable cell lines), purification of the resulting HBsAg and the rendering of this antigen into an immunogenic preparation. The suitability and safety of the cells are approved by the competent authority.

The vaccine may contain the product of the S gene (major protein), a combination of the S gene and pre-S2 gene products (middle protein) or a combination of the S gene, the pre-S2 gene and pre-S1 gene products (large protein).

Reference preparation: part of a representative batch shown to be at least as immunogenic in animals as a batch that, in clinical studies in young, healthy adults, produced not less than 95 per cent seroconversion, corresponding to a level of HBsAg neutralising antibody recognised to be protective, after a full-course primary immunisation. An antibody level not less than 10 mIU/mL is recognised as being protective.

CHARACTERISATION OF THE SUBSTANCE

Development studies are carried out to characterise the antigen. The complete protein, lipid and carbohydrate structure of the antigen is established. The morphological characteristics of the antigen particles are established by electron microscopy. The mean buoyant density of the antigen particles is determined by a physico-chemical method, such as gradient centrifugation. The antigenic epitopes are characterised. The protein fraction of the antigen is characterised in terms of the primary structure (for example, by determination of the amino-acid composition, by partial amino-acid sequence analysis and by peptide mapping).

CULTURE AND HARVEST

Identity, microbial purity, plasmid retention and consistency of yield are determined at suitable production stages. If mammalian cells are used, tests for extraneous agents and mycoplasmas are performed in accordance with general chapter *2.6.16. Tests for extraneous agents in viral vaccines for human use*, but using 200 mL of harvest in the test in cell culture for other extraneous agents.

PURIFIED ANTIGEN

Only a purified antigen that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Total protein. The total protein is determined by a validated method. The content is within the limits approved for the specific product.

Antigen content and identification. The quantity and specificity of HBsAg is determined in comparison with the International Standard for HBsAg subtype *ad* or an in-house reference, by a suitable immunochemical method (*2.7.1*) such as radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot (preferably using a monoclonal antibody directed against a protective epitope) or single radial diffusion. The antigen/protein ratio is within the limits approved for the specific product.

The molecular weight of the major band revealed following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed under reducing conditions corresponds to the value expected from the known nucleic acid and polypeptide sequences and possible glycosylation.

Antigenic purity. The purity of the antigen is determined by comparison with a reference preparation using liquid chromatography or other suitable methods such as SDS-PAGE with staining by acid blue 92 and silver. A suitable method is sensitive enough to detect a potential contaminant at a concentration of 1 per cent of total protein. Not less than 95 per cent of the total protein consists of hepatitis B surface antigen.

Composition. The content of proteins, lipids, nucleic acids and carbohydrates is determined.

Host-cell- and vector-derived DNA. If mammalian cells are used for production, not more than 10 pg of DNA in the quantity of purified antigen equivalent to a single human dose of vaccine.

Caesium. If a caesium salt is used during production, a test for residual caesium is carried out on the purified antigen. The content is within the limits approved for the specific product.

Sterility (*2.6.1*). The purified antigen complies with the test, carried out using 10 mL for each medium.

Additional tests on the purified antigen may be required depending on the production method used: for example, a test for residual animal serum where mammalian cells are used for production or tests for residual chemicals used during extraction and purification.

ADSORBED 3-O-DESACYL-4′-MONOPHOSPHORYL LIPID A BULK

If 3-*O*-desacyl-4′-monophosphoryl lipid A is included in the vaccine it complies with the monograph *3-O-desacyl-4′-monophosphoryl lipid A (2537)*. Where 3-*O*-desacyl-4′-monophosphoryl lipid A liquid bulk is adsorbed prior to inclusion in the vaccine, the adsorbed 3-*O*-desacyl-4′-monophosphoryl lipid A bulk complies with the following requirements.

Degree of adsorption of 3-*O*-desacyl-4′-monophosphoryl lipid A. The content of non-adsorbed 3-*O*-desacyl-4′-monophosphoryl lipid A in the adsorbed 3-*O*-desacyl-4′-monophosphoryl lipid A bulk is determined by a suitable method, for example gas chromatographic quantification of the *3-O-desacyl-4′-monophosphoryl lipid A (2537)* fatty acids in the supernatant, evaporated to dryness, after centrifugation.

pH (*2.2.3*). The pH is within the limits approved for the particular preparation.

Sterility (*2.6.1*). It complies with the test, carried out using 10 mL for each medium.

FINAL BULK VACCINE

An antimicrobial preservative, a mineral carrier, such as aluminium hydroxide or hydrated aluminium phosphate, and the adjuvant 3-*O*-desacyl-4′-monophosphoryl lipid A may be included in the formulation of the final bulk.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (*2.6.1*). The final bulk vaccine complies with the test, carried out using 10 mL for each medium.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde (where applicable) and antimicrobial preservative content (where applicable) have been carried out on the final bulk vaccine with satisfactory results, they may be omitted on the final lot. If the assay is carried out *in vivo*, then provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Degree of adsorption. The degree of adsorption of the antigen and, where applicable, 3-*O*-desacyl-4′-monophosphoryl lipid A is assessed.

IDENTIFICATION

The assay or, where applicable, the electrophoretic profile, serves also to identify the vaccine. In addition, where applicable, the test for 3-*O*-desacyl-4′-monophosphoryl lipid A content also serves to identify the 3-*O*-desacyl-4′-monophosphoryl lipid A-containing vaccine.

TESTS

Aluminium (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

3-*O*-Desacyl-4′-monophosphoryl lipid A: minimum 80 per cent and maximum 120 per cent of the intended amount.

Where applicable, determine the content of 3-*O*-desacyl-4′-monophosphoryl lipid A by a suitable method, for example gas chromatography (*2.2.28*).

Free formaldehyde (*2.4.18*): maximum 0.2 g/L.

Antimicrobial preservative. Where applicable, determine the content of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

Sterility (*2.6.1*). The vaccine complies with the test.

Pyrogens (*2.6.8*). The vaccine complies with the test for pyrogens. Inject the equivalent of one human dose into each rabbit or, if the vaccine contains 3-*O*-desacyl-4′-monophosphoryl lipid A, inject per kilogram of the rabbit's mass an amount of the vaccine containing 2.5 µg of 3-*O*-desacyl-4′-monophosphoryl lipid A.

ASSAY

The vaccine complies with the assay of hepatitis B vaccine (rDNA) (*2.7.15*).

LABELLING

The label states:

- the amount of HBsAg per container;
- the type of cells used for production of the vaccine;
- the name and amount of the adjuvant and/or adsorbent used;
- that the vaccine must be shaken before use;
- that the vaccine must not be frozen.

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POLIOMYELITIS VACCINE (INACTIVATED)

Vaccinum poliomyelitidis inactivatum

DEFINITION

Poliomyelitis vaccine (inactivated) is a liquid preparation of suitable strains of human poliovirus types 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method. It is a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production method shall have been shown to yield consistently vaccines of acceptable safety and immunogenicity in man.

Production of the vaccine is based on a virus seed-lot system. Cell lines are used according to a cell-bank system. If primary, secondary or tertiary monkey kidney cells are used, production complies with the requirements indicated below.

Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more passages from the master seed lot than was used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

SUBSTRATE FOR VIRUS PROPAGATION

The virus is propagated in a human diploid cell line (*5.2.3*), in a continuous cell line (*5.2.3*) or in primary, secondary or tertiary monkey kidney cells.

Primary, secondary or tertiary monkey kidney cells. The following special requirements for the substrate for virus propagation apply to primary, secondary or tertiary monkey kidney cells.

Monkeys used in the preparation of kidney cell cultures for production and control of the vaccine. The animals used are of a species approved by the competent authority, in good health and, unless otherwise justified and authorised, have not been previously employed for experimental purposes. Kidney cells used for vaccine production and control are derived from monitored, closed colonies of monkeys bred in captivity, not from animals caught in the wild; a previously approved seed lot prepared using virus passaged in cells from wild monkeys may, subject to approval by the competent authority, be used for vaccine production if historical data on safety justify this.

Monitored, closed colonies of monkeys. The monkeys are kept in groups in cages. Freedom from extraneous agents is achieved by the use of animals maintained in closed colonies that are subject to continuous and systematic veterinary and laboratory monitoring for the presence of infectious agents. The supplier of animals is certified by the competent authority. Each monkey is tested serologically at regular intervals during a quarantine period of not less than 6 weeks imposed before entering the colony, and then during its stay in the colony.

The monkeys used are shown to be tuberculin-negative and free from antibodies to simian virus 40 (SV40) and simian immunodeficiency virus. The blood sample used in testing for SV40 antibodies must be taken as close as possible to the time of removal of the kidneys. If *Macaca* sp. monkeys are used for production, the monkeys are also shown to be free from antibodies to herpesvirus B (cercopithecine herpesvirus 1) infection. Human herpesvirus 1 has been used as an indicator for freedom from herpesvirus B antibodies on account of the danger of handling herpesvirus B (cercopithecine herpesvirus 1).

Monkeys from which kidneys are to be removed are thoroughly examined, particularly for evidence of tuberculosis and herpesvirus B (cercopithecine herpesvirus 1) infection. If a monkey shows any pathological lesion relevant to the use of its kidneys in the preparation of a seed lot or vaccine, it is not to be used nor are any of the remaining monkeys of the group concerned unless it is evident that their use will not impair the safety of the product.

All the operations described in this section are conducted outside the area where the vaccine is produced.

Monkey cell cultures for vaccine production. Kidneys that show no pathological signs are used for preparing cell cultures. Each group of cell cultures derived from a single monkey forms a separate production cell culture giving rise to a separate single harvest.

The primary monkey kidney cell suspension complies with the test for mycobacteria (*2.6.2*); disrupt the cells before carrying out the test.

If secondary or tertiary cells are used, it shall be demonstrated by suitable validation tests that cell cultures beyond the passage level used for production are free from tumorigenicity.

SEED LOTS

Each of the 3 strains of poliovirus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

Only a working seed lot that complies with the following requirements may be used for virus propagation.

Identification. Each working seed lot is identified as human poliovirus types 1, 2 or 3 by virus neutralisation in cell cultures using specific antibodies.

Virus concentration. The virus concentration of each working seed lot is determined to define the quantity of virus to be used for inoculation of production cell cultures.

Extraneous agents. The working seed lot complies with the requirements for seed lots for virus vaccines (*2.6.16*). In addition, if primary, secondary or tertiary monkey kidney cells have been used for isolation of the strain, measures are taken to ensure that the strain is not contaminated with simian viruses such as simian immunodeficiency virus, simian virus 40, filoviruses and herpesvirus B (cercopithecine herpesvirus 1). A working seed lot produced in primary, secondary or tertiary monkey kidney cells complies with the requirements given below under Virus propagation and harvest for single harvests produced in such cells.

VIRUS PROPAGATION AND HARVEST

All processing of the cell bank and cell cultures is done under aseptic conditions in an area where no other cells or viruses are being handled. Approved animal serum (but not human serum) may be used in the cell culture media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. Not less than 500 mL of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells); where continuous cell lines in a fermenter are used for production, 200×10^6 cells are set aside to prepare control cells; where primary, secondary or tertiary monkey kidney cells are used for production, a cell sample equivalent to at least 500 mL of the cell suspension, at the concentration employed for vaccine production, is taken to prepare control cells.

Only a single harvest that complies with the following requirements may be used in the preparation of the vaccine. The tests for identification and bacterial and fungal contamination may be carried out instead on the purified, pooled monovalent harvest. After demonstration of consistency of production at the stage of the single harvest, the test for virus concentration may be carried out instead on the purified, pooled monovalent harvest.

Control cells. The control cells of the production cell culture comply with a test for identification (if a cell-bank system is used for production) and with the requirements for extraneous agents (*2.6.16*; where primary, secondary or tertiary monkey kidney cells are used, the tests in cell cultures are carried out as shown below under Test in rabbit kidney cell cultures and Test in cercopithecus kidney cell cultures).

Test in rabbit kidney cell cultures. Test a sample of at least 10 mL of the pooled supernatant fluid from the control cultures for the absence of herpesvirus B (cercopithecine herpesvirus 1) and other viruses by inoculation onto rabbit kidney cell cultures. The dilution of supernatant in the nutrient medium is not greater than 1/4 and the area of the cell layer is at least 3 cm^2 per millilitre of inoculum. Set aside one or more containers of each batch of cells with the same medium as non-inoculated control cells. Incubate the cultures at 37 °C and observe for at least 2 weeks. The test is not valid if more than 20 per cent of the control cell cultures are discarded for non-specific, accidental reasons.

Test in cercopithecus kidney cell cultures. Test a sample of at least 10 mL of the pooled supernatant fluid from the control cultures for the absence of SV40 virus and other extraneous agents by inoculation onto cell cultures prepared from the kidneys of cercopithecus monkeys, or other cells shown to be at least as sensitive for SV40, by the method described under Test in rabbit kidney cell cultures. The test is not valid if more than 20 per cent of the control cell cultures are discarded for non-specific, accidental reasons.

Identification. The single harvest is identified as containing human poliovirus types 1, 2 or 3 by virus neutralisation in cell cultures using specific antibodies.

Virus concentration. The virus concentration of each single harvest is determined by titration of infectious virus in cell cultures.

Bacterial and fungal contamination. The single harvest complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

Mycoplasmas (*2.6.7*). The single harvest complies with the test for mycoplasmas, carried out using 10 mL.

Test in rabbit kidney cell cultures. Where primary, secondary or tertiary monkey kidney cells are used for production, test a sample of at least 10 mL of the single harvest for the absence of herpesvirus B (cercopithecine herpesvirus 1) and other viruses by inoculation onto rabbit kidney cell cultures as described above for the control cells.

Test in cercopithecus kidney cell cultures. Where primary, secondary or tertiary monkey kidney cells are used for production, test a sample of at least 10 mL of the single harvest for the absence of SV40 virus and other extraneous agents. Neutralise the sample by a high-titre antiserum against the specific type of poliovirus. Test the sample in primary cercopithecus kidney cell cultures or cells that have been demonstrated to be at least as susceptible for SV40. Incubate the cultures at 37 °C and observe for 14 days. At the end of this period, make at least one subculture of fluid in the same cell culture system and observe both primary cultures and subcultures for an additional 14 days.

PURIFICATION AND PURIFIED MONOVALENT HARVEST

Several single harvests of the same type may be pooled and may be concentrated. The monovalent harvest or pooled monovalent harvest is purified by validated methods. If continuous cell lines are used for production, the purification process shall have been shown to reduce consistently the content of substrate-cell DNA to not more than 100 pg per single human dose.

Only a purified monovalent harvest that complies with the following requirements may be used for the preparation of the inactivated monovalent harvest.

Identification. The virus is identified by virus neutralisation in cell cultures using specific antibodies or by determination of D-antigen.

Virus concentration. The virus concentration is determined by titration of infectious virus.

Specific activity. The ratio of the virus concentration or the D-antigen content, determined by a suitable immunochemical method (*2.7.1*), to the total protein content (specific activity) of the purified monovalent harvest is within the limits approved for the particular product.

INACTIVATION AND INACTIVATED MONOVALENT HARVEST

Several purified monovalent harvests of the same type may be mixed before inactivation. To avoid failures in inactivation caused by the presence of virus aggregates, filtration is carried out before and during inactivation; inactivation is started within a suitable period, preferably not more than 24 h and in any case not more than 72 h, of the prior filtration. The virus suspension is inactivated by a validated method that has been shown to inactivate poliovirus without destruction of immunogenicity; during validation studies, an inactivation curve with at least 4 points (for example, time 0 h, 24 h, 48 h and 96 h) is established showing the decrease in concentration of live virus with time. If formaldehyde is used for inactivation, the presence of an excess of formaldehyde at the end of the inactivation period is verified. The inactivation kinetics tests mentioned below are carried out on each batch to ensure consistency of the inactivation process.

Only an inactivated monovalent harvest that complies with the following requirements may be used in the preparation of a trivalent pool of inactivated monovalent harvests or a final bulk vaccine.

Test for effective inactivation. After neutralisation of the formaldehyde with sodium bisulfite (where applicable), verify the absence of residual live poliovirus by inoculation on suitable cell cultures of 2 samples of each inactivated monovalent harvest, corresponding to at least 1500 human doses. Cells used for the test must be of optimal sensitivity regarding residual infectious poliovirus, for example kidney cells from certain monkey species (*Macaca*, *Cercopithecus* or *Papio*), or Hep-2 cells. If other cells are used, they must have been shown to possess at least the same sensitivity as those specified above. Take one sample not later than 3/4 of the way through the inactivation period and the other at the end. Inoculate the samples in cell cultures such that the dilution of vaccine in the nutrient medium is not greater than 1/4 and the area of the cell layer is at least 3 cm^2 per millilitre of inoculum. Set aside one or more containers with the same medium as non-inoculated control cells. Observe the cell cultures for at least 3 weeks. Make not fewer than 2 passages from each container, one at the end of the observation period and the other 1 week before; for the passages, use cell culture supernatant and inoculate as for the initial sample. Observe the subcultures for at least 2 weeks. No sign of poliovirus multiplication is present in the cell cultures. At the end of the observation period, test the susceptibility of the cell culture used by inoculation of live poliovirus of the same type as that present in the inactivated monovalent harvest.

Inactivation kinetics. Kinetics of inactivation are established and approved by the competent authority. Adequate data on inactivation kinetics are obtained and consistency of the inactivation process is monitored.

Sterility (*2.6.1*). The inactivated monovalent harvest complies with the test for sterility, carried out using 10 mL for each medium.

D-antigen content. The content of D-antigen determined by a suitable immunochemical method (*2.7.1*) is within the limits approved for the particular preparation.

FINAL BULK VACCINE

The final bulk vaccine is prepared directly from the inactivated monovalent harvests of human poliovirus types 1, 2 and 3 or from a trivalent pool of inactivated monovalent harvests. A suitable stabiliser and a suitable antimicrobial preservative may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Sterility (*2.6.1*). The final bulk vaccine complies with the test for sterility, carried out using 10 mL for each medium.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde and antimicrobial preservative and the *in vivo* assay have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

The *in vivo* assay may be omitted once it has been demonstrated for a given product and for each poliovirus type that the acceptance criteria for the D-antigen determination are such that it yields the same result as the *in vivo* assay in terms of acceptance or rejection of a batch. This demonstration must include testing of subpotent batches, produced experimentally if necessary, for example by heat treatment or other means of diminishing the immunogenic activity. Where there is a significant change in the manufacturing process of the antigens or their formulation, any impact on the *in vivo* and *in vitro* assays must be evaluated, and the need for revalidation considered.

Provided that the protein content has been determined on the purified monovalent harvests or on the inactivated monovalent harvests and that it has been shown that the content in the final lot will not exceed 10 µg per single human dose, the test for protein content may be omitted on the final lot.

Provided that the test for bovine serum albumin has been performed with satisfactory results on the trivalent pool of inactivated monovalent harvests or on the final bulk vaccine, it may be omitted on the final lot.

IDENTIFICATION

The vaccine is shown to contain human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

TESTS

Free formaldehyde (*2.4.18*): maximum 0.2 g/L.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

Protein content (*2.5.33, Method 2*): maximum 10 µg per single human dose.

Bovine serum albumin: maximum 50 ng per single human dose, determined by a suitable immunochemical method (*2.7.1*).

Sterility (*2.6.1*). It complies with the test.

Bacterial endotoxins (*2.6.14*): less than 5 IU per single human dose.

ASSAY

D-antigen content. As a measure of consistency of production, determine the D-antigen content for human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) using a reference preparation calibrated in European Pharmacopoeia Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in European Pharmacopoeia Units and intended for use in the assay of D-antigen. The European Pharmacopoeia Unit and the International Unit are equivalent.

***In vivo* test**. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (*2.7.20*).

LABELLING

The label states:

- the types of poliovirus contained in the vaccine;
- the nominal amount of virus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose;
- the cell substrate used to prepare the vaccine.

07/2020:0537

YELLOW FEVER VACCINE (LIVE)

Vaccinum febris flavae vivum

DEFINITION

Yellow fever vaccine (live) is a freeze-dried preparation of yellow fever virus derived from the 17D strain and grown in fertilised hen eggs. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid.

PRODUCTION

The production of vaccine is based on a virus seed-lot system. The production method shall have been shown to yield consistently yellow fever vaccine (live) of acceptable immunogenicity and safety for man.

Reference preparation. In the test for neurotropism, a suitable batch of vaccine known to have satisfactory properties in man is used as the reference preparation.

A reference preparation calibrated in International Units per ampoule is used to verify the titre of the virus inoculum in the tests for viraemia (viscerotropism) and immunogenicity, and to titrate the vaccine batch in the potency assay.

The International Unit is the activity contained in a stated quantity of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

SUBSTRATE FOR VIRUS PROPAGATION

Virus for the preparation of master and working seed lots and of all vaccine batches is grown in the tissues of chick embryos from a flock free from specified pathogens (SPF) (*5.2.2*).

SEED LOTS

The 17D strain shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are prepared in large quantities and stored at a temperature below – 60 °C. Master and working seed lots shall not contain any human protein, added serum or antibiotics.

Unless otherwise justified and authorised, the virus in the final vaccine shall be between passage levels 204 and 239 from the original isolate of strain 17D. A working seed lot shall be only 1 passage from a master seed lot. A working seed lot shall be used without intervening passage as the inoculum for infecting the tissues used in the production of a vaccine lot, so that no vaccine virus is more than 1 passage from a seed lot that has passed all the safety tests.

Only a virus seed lot that complies with the following requirements may be used for virus propagation.

Identification. The master and working seed lots are identified as containing yellow fever virus by serum neutralisation in cell culture using specific antibodies, or by molecular methods (e.g. nucleic acid amplification techniques (NAT), sequencing).

Extraneous agents (*2.6.16*). Each master seed lot complies with the following tests:

- bacterial and fungal sterility (as described in chapter *2.6.16* under Virus seed lot and virus harvests);
- mycoplasmas (as described in chapter *2.6.16* under Virus seed lot and virus harvests);
- mycobacteria (as described in chapter *2.6.16* under Virus seed lot and virus harvests).

Avian leucosis viruses. For each master seed lot, the absence of avian leucosis viruses is verified using a suitable method.

Extraneous agents (*2.6.16*). Each working seed lot complies with the following tests:

- bacterial and fungal sterility (as described in chapter *2.6.16* under Virus seed lot and virus harvests);
- mycoplasmas (as described in chapter *2.6.16* under Virus seed lot and virus harvests);
- mycobacteria (as described in chapter *2.6.16* under Virus seed lot and virus harvests);
- test in cell culture for other extraneous agents: a neutralised sample of 5 mL of working seed lot, representing at least 500 000 (5.7 $\log_{10}$) IU, is tested for the presence of extraneous agents by inoculation into continuous simian kidney and human cell cultures as well as into primary chick-embryo-fibroblast cells; the cells are incubated at 36 ± 1 °C and observed for a period of 14 days; the working seed lot passes the test if there is no evidence of the presence of any extraneous agents; the test is not valid unless at least 80 per cent of the cell cultures remain viable;
- avian viruses: a neutralised sample of 1 mL of working seed lot, representing at least 100 000 (5.0 $\log_{10}$) IU, is tested for the presence of avian viruses by inoculation by the allantoic route into a group of at least 20 fertilised, 9- to 11-day-old, SPF eggs (*5.2.2*), and by inoculation into the yolk sac of a group of at least 20 fertilised, 5- to 7-day-old, SPF eggs (*5.2.2*); incubate for 7 days; the working seed lot complies if the allantoic and yolk sac fluids show no signs of haemagglutinating agents and if the embryos and chorio-allantoic membranes examined to detect any macroscopic pathology are typical; the test is not valid unless at least 80 per cent of the inoculated eggs survive during the 7-day observation period.

Avian leucosis viruses. For each working seed lot, the absence of avian leucosis viruses is verified using a suitable method.

Tests in monkeys. Each master and working seed lot complies with the following tests in monkeys for viraemia (viscerotropism), immunogenicity and neurotropism.

The monkeys shall be *Macaca* sp. susceptible to yellow fever virus and shall have been shown to be non-immune to yellow fever at the time of injecting the seed virus. They shall be healthy and shall not have received previously intracerebral or intraspinal inoculation. Furthermore, they shall not have been inoculated by other routes with neurotropic viruses or with antigens related to yellow fever virus. Not fewer than 10 monkeys are used for each test.

Use a test dose of 0.25 mL containing the equivalent of not less than 5000 (3.7 $\log_{10}$) IU and not more than 50 000 (4.7 $\log_{10}$) IU, determined by an *in vitro* titration for infectious virus in cell culture. Inject the test dose into 1 frontal lobe of each monkey under anaesthesia and observe the monkeys for not less than 30 days.

Viraemia (Viscerotropism). Viscerotropism is indicated by the amount of virus present in serum. Take blood from each of the test monkeys on the 2nd, 4th and 6th days after inoculation and prepare serum from each sample. Prepare 1:10, 1:100 and 1:1000 dilutions from each serum and inoculate each dilution into a group of at least 4 cell culture vessels used for the determination of the virus concentration. The seed lot complies with the test if none of the sera contains more than the equivalent of 500 (2.7 $\log_{10}$) IU in 0.03 mL and at most 1 serum contains more than the equivalent of 100 (2.0 $\log_{10}$) IU in 0.03 mL.

Immunogenicity. Take blood from each monkey 30 days after the injection of the test dose and prepare serum from each sample. The seed lot complies with the test if at least 90 per cent of the test monkeys are shown to be immune, as determined by examining their sera in the test for neutralisation of yellow fever virus described below.

It has been shown that a low dilution of serum (for example, 1:10) may contain non-specific inhibitors that influence this test; such serum shall be treated to remove inhibitors. Mix dilutions of at least 1:10, 1:40 and 1:160 of serum from each monkey with an equal volume of

17D vaccine virus at a dilution that will yield an optimum number of plaques with the titration method used. Incubate the serum-virus mixtures in a water-bath at 37 °C for 1 h and then cool in iced water; add 0.2 mL of each serum-virus mixture to each of 4 cell culture plates and proceed as for the determination of virus concentration. Inoculate similarly 10 plates with the same amount of virus, plus an equal volume of a 1:10 dilution of monkey serum known to contain no neutralising antibodies to yellow fever virus. At the end of the observation period, compare the mean number of plaques in the plates receiving virus plus non-immune serum with the mean number of plaques in the plates receiving virus plus dilutions of each monkey serum. Not more than 10 per cent of the test monkeys have serum that fails to reduce the number of plaques by 50 per cent at the 1:10 dilution.

Neurotropism. Neurotropism is assessed from clinical evidence of encephalitis, from incidence of clinical manifestations and by evaluation of histological lesions, in comparison with 10 monkeys injected with the reference preparation. The seed lot is not acceptable if either the onset and duration of the febrile reaction or the clinical signs of encephalitis and pathological findings are such as to indicate a change in the properties of the virus.

Clinical evaluation

The monkeys are examined daily for 30 days by personnel familiar with clinical signs of encephalitis in primates (if necessary, the monkeys are removed from their cage and examined for signs of motor weakness or spasticity). The seed lot is not acceptable if in the monkeys injected with it the incidence of severe signs of encephalitis, such as paralysis or inability to stand when stimulated, or mortality is greater than for the reference vaccine. These and other signs of encephalitis, such as paresis, incoordination, lethargy, tremors or spasticity are assigned numerical values for the severity of symptoms by a grading method. Each day each monkey in the test is given a score based on the following scale:

- grade 1: rough coat, not eating;
- grade 2: high-pitched voice, inactive, slow moving;
- grade 3: shaky movements, tremors, incoordination, limb weakness;
- grade 4: inability to stand, limb paralysis or death (a dead monkey receives a daily score of 4 from the day of death until day 30).

A clinical score for a particular monkey is the average of its daily scores; the clinical score for the group is the arithmetic mean of the individual monkey scores. The seed lot is not acceptable if the mean of the clinical severity scores for the group of monkeys inoculated with it is significantly greater ($P = 0.95$) than the mean for the group of monkeys injected with the reference preparation. In addition, special consideration is given to any animal showing unusually severe signs when deciding on the acceptability of the seed lot.

Histological evaluation

5 levels of the brain are examined including:

- block I: the corpus striatum at the level of the optic chiasma;
- block II: the thalamus at the level of the mamillary bodies;
- block III: the mesencephalon at the level of the superior colliculi;
- block IV: the pons and cerebellum at the level of the superior olives;
- block V: the medulla oblongata and cerebellum at the level of the mid-inferior olivary nuclei.

Cervical and lumbar enlargements of the spinal cord are each divided equally into 6 blocks; 15 µm sections are cut from the tissue blocks embedded in paraffin wax and stained with gallocyanin. Numerical scores are given to each hemisection of the cord and to structures in each hemisection of the brain as listed below. Lesions are scored as follows:

- grade 1 - minimal: 1 to 3 small focal inflammatory infiltrates; degeneration or loss of a few neurons;
- grade 2 - moderate: 4 or more focal inflammatory infiltrates; degeneration or loss of neurons affecting not more than one third of cells;
- grade 3 - severe: moderate focal or diffuse inflammatory infiltration; degeneration or loss of 33-90 per cent of the neurons;
- grade 4 - overwhelming: variable but often severe inflammatory reaction; degeneration or loss of more than 90 per cent of neurons.

It has been found that inoculation of yellow fever vaccine into the monkey brain causes histological lesions in different anatomical formations of the central nervous system with varying frequency and severity (I. S. Levenbook *et al*., *Journal of Biological Standardization*, 1987, 15, 305-313). Based on these 2 indicators, the anatomical structures can be divided into target, spared and discriminator areas. Target areas are those that show more severe specific lesions in a majority of monkeys irrespective of the degree of neurovirulence of the seed lot. Spared areas are those that show only minimal specific lesions and in a minority of monkeys. Discriminator areas are those where there is a significant increase in the frequency of more severe specific lesions with seed lots having a higher degree of neurovirulence. Discriminator and target areas for *Macaca cynomolgus* and *Macaca rhesus* monkeys are shown in the table below.

Type of monkey	Discriminator areas	Target areas
Macaca cynomolgus	Globus pallidus	Substantia nigra
	Putamen	
	Anterior/median thalamic nucleus	
	Lateral thalamic nucleus	
Macaca rhesus	Caudate nucleus	Substantia nigra
	Globus pallidus	Cervical enlargement
	Putamen	Lumbar enlargement
	Anterior/median thalamic nucleus	
	Lateral thalamic nucleus	
	Cervical enlargement	
	Lumbar enlargement	

Scores for discriminator and target areas are used for the final evaluation of the seed lot. The individual monkey score is calculated from the sum of individual target area scores in each hemisection divided by the number of areas examined. A separate score is calculated similarly for the discriminator areas.

Mean scores for the test group are calculated in 2 ways: (1) by dividing the sum of the individual monkey discriminator scores by the number of monkeys; and (2) by dividing the sum of the individual monkey target and discriminator scores by the number of monkeys. These 2 mean scores are taken into account when deciding on the acceptability of the seed lot. The seed lot is not acceptable if either of the mean lesion scores is significantly greater ($P = 0.95$) than for the reference preparation.

PROPAGATION AND HARVEST

All processing of the fertilised eggs is done under aseptic conditions in an area where no other infectious agents or cells are handled at the same time. At least 2 per cent but not fewer than 20 and not more than 80 eggs are maintained as uninfected control eggs. After inoculation and incubation at a controlled temperature, only living and typical chick embryos are harvested. At the time of harvest, the control eggs are treated in the same way as the inoculated eggs to obtain a

control embryonic pulp. The age of the embryo at the time of virus harvest is reckoned from the initial introduction of the egg into the incubator and shall be not more than 12 days. After homogenisation and clarification by centrifugation, the extract of embryonic pulp is tested as described below and kept at − 70 °C or colder until further processing. Virus harvests may be pooled. No human protein is added to the virus suspension at any stage during production. If stabilisers are added, they shall have been shown to have no antigenic or sensitising properties for man.

Only a single harvest or, where applicable, a pool of single harvests that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification. The single harvest or pool of single harvests contains virus that is identified as yellow fever virus by serum neutralisation in cell culture using specific antibodies, or by molecular methods (e.g. NAT, sequencing).

Bacterial and fungal contamination. The single harvest complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

Mycoplasmas (*2.6.7*). The single harvest or pool of single harvests complies with the test for mycoplasmas, carried out using 10 mL.

Mycobacteria (*2.6.2*). A 5 mL sample of the single harvest or pool of single harvests is tested for the presence of *Mycobacterium* spp. by culture methods known to be sensitive for the detection of these organisms.

Embryonic pulp of control eggs. The extract of the control eggs shows no evidence of the presence of any extraneous agents in the tests described below.

Test in cell culture for other extraneous agents. Inoculate a 5 mL sample of embryonic pulp of the control eggs into continuous simian kidney and human cell cultures as well as into primary chick-embryo-fibroblast cells. The cells are incubated at 36 ± 1 °C and observed for a period of 14 days. The embryonic pulp of the control eggs passes the test if there is no evidence of the presence of any extraneous agents. The test is not valid unless at least 80 per cent of the cell cultures remain viable.

Avian viruses. Using 0.1 mL per egg, inoculate the embryonic pulp of control eggs: by the allantoic route into a group of 10 fertilised, 9- to 11-day-old, SPF eggs (*5.2.2*); and into the yolk sac of a group of 10 fertilised, 5- to 7-day-old, SPF eggs (*5.2.2*). Incubate for 7 days. The embryonic pulp lot of the control eggs complies if the allantoic and yolk sac fluids show no signs of haemagglutinating agents and if the embryos and chorio-allantoic membranes examined to detect any macroscopic pathology are typical. The test is not valid unless at least 80 per cent of the inoculated eggs survive during the 7 day observation period.

Virus concentration. In order to calculate the dilution for formulation of the final bulk, each single harvest is titrated as described under Assay.

FINAL BULK VACCINE

Single harvests or pools of single harvests that comply with the tests prescribed above are pooled and clarified again. A test for protein nitrogen content is carried out. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. The final bulk vaccine complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

Protein nitrogen content: maximum 0.25 mg per human dose before the addition of any stabiliser.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers and freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to prevent contamination and the introduction of moisture.

Only a final lot that is satisfactory with respect to thermal stability and each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for ovalbumin has been performed with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Thermal stability. Maintain at least 3 containers of the final lot of freeze-dried vaccine in the dry state at 37 ± 1 °C for 14 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for vaccine stored at the temperature recommended for storage. The virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific yellow fever virus antibodies, there is a significant reduction in its ability to infect susceptible cell cultures. Alternatively, the vaccine reconstituted as stated on the label contains virus that is identified as yellow fever virus by molecular methods (e.g. NAT, sequencing).

TESTS

Ovalbumin: maximum 5 µg of ovalbumin per human dose, determined by a suitable immunochemical method (*2.7.1*).

Water (*2.5.12*): maximum 3.0 per cent.

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (*2.6.1*).

Bacterial endotoxins (*2.6.14*): less than 5 IU per single human dose.

ASSAY

Titrate for infective virus in cell cultures using at least 3 separate containers of vaccine. Titrate 1 container of an appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. Calculate the individual virus concentration for each container of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations using the usual statistical methods (for example, *5.3*). The combined virus concentration for the 3 containers of vaccine is compared to the results of the reference preparation titrated in parallel, to obtain results in International Units. The combined virus concentration of the vaccine is not less than 3.0 $\log_{10}$ IU per human dose and not more than the upper limit approved for the particular product by the competent authority.

The assay is not valid if:

- the confidence interval (P = 0.95) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ IU;
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ IU from the established value.

The assay is repeated if the confidence interval (P = 0.95) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ IU; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval (P = 0.95) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ IU.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

LABELLING

The label states:

- the strain of virus used in preparation of the vaccine;
- that the vaccine has been prepared in chick embryos;
- the minimum virus concentration;
- that contact between the vaccine and disinfectants is to be avoided.

01/2019:0213

MEASLES VACCINE (LIVE)

Vaccinum morbillorum vivum

DEFINITION

Measles vaccine (live) is a freeze-dried preparation of a suitable attenuated strain of measles virus. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production of vaccine is based on a virus seed-lot system and, if the virus is propagated in human diploid cells, a cell-bank system. The production method shall have been shown to yield consistently live measles vaccines of adequate immunogenicity and safety in man. Unless otherwise justified and authorised, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy; even with authorised exceptions, the number of passages beyond the level used for clinical studies shall not exceed 5.

The potential neurovirulence of the vaccine strain is considered during preclinical development, based on available epidemiological data on neurovirulence and neurotropism, primarily for the wild-type virus. In light of this, a risk analysis is carried out. Where necessary and if available, a test is carried out on the vaccine strain using an animal model that differentiates wild-type and attenuated virus; tests on strains of intermediate attenuation may also be needed.

SUBSTRATE FOR VIRUS PROPAGATION

The virus is propagated in human diploid cells (*5.2.3*) or in cultures of chick-embryo cells derived from a chicken flock free from specified pathogens (*5.2.2*).

SEED LOT

The strain of measles virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are prepared in large quantities and stored at temperatures below – 20 °C if freeze-dried, or below – 60 °C if not freeze-dried.

Only a seed lot that complies with the following requirements may be used for virus propagation.

Identification. The master and working seed lots are identified as measles virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The virus concentration of the master and working seed lots is determined to monitor consistency of production.

Extraneous agents (*2.6.16*). The working seed lot complies with the requirements for seed lots.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled during production. Suitable animal (but not human) serum may be used in the growth medium, but the final medium for maintaining cells during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. Not less than 500 mL of the production cell cultures is set aside as uninfected cell cultures (control cells). The viral suspensions are harvested at a time appropriate to the strain of virus being used.

Only a single harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification. The single harvest contains virus that is identified as measles virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The virus concentration in the single harvest is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

Extraneous agents (*2.6.16*). The single harvest complies with the tests for extraneous agents.

Control cells. If human diploid cells are used for production, the control cells comply with a test for identification. They comply with the tests for extraneous agents (*2.6.16*).

FINAL BULK VACCINE

Virus harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Bacterial and fungal contamination. The final bulk vaccine complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

FINAL LOT

A minimum virus concentration for release of the product is established such as to ensure, in light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the requirements for minimum virus concentration for release, with the following requirement for thermal stability and with each of the requirements given below under Identification and Tests may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Thermal stability. Maintain at least 3 vials of the final lot of freeze-dried vaccine in the dry state at 37 ± 1 °C for 7 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for vaccine stored at the temperature recommended for storage. The virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific measles antibodies, it is no longer able to infect susceptible cell cultures.

TESTS

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (*2.6.1*).

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (*2.7.1*).

Water (*2.5.12*). Not more than 3.0 per cent, determined by the semi-micro determination of water.

ASSAY

Titrate the vaccine for infective virus, using at least 3 separate vials of vaccine and inoculating a suitable number of wells for each dilution step. Titrate 1 vial of an appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. The relation with the

appropriate European Pharmacopoeia Biological Reference Preparation is established and monitored at regular intervals if a manufacturer's reference preparation is used. Calculate the individual virus concentration for each vial of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*). The combined estimate of the virus concentration for the 3 vials of vaccine is not less than that stated on the label; the minimum virus concentration stated on the label is not less than 3.0 $\log_{10}$ $CCID_{50}$ per single human dose.

The assay is not valid if:

- the confidence interval ($P = 0.95$) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ $CCID_{50}$;
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ $CCID_{50}$ from the established value.

The assay is repeated if the confidence interval ($P = 0.95$) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ $CCID_{50}$; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P = 0.95$) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ $CCID_{50}$.

Measles vaccine (live) BRP is suitable for use as a reference preparation.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

LABELLING

The label states:

- the strain of virus used for the preparation of the vaccine;
- the type and origin of the cells used for the preparation of the vaccine;
- the minimum virus concentration;
- that contact between the vaccine and disinfectants is to be avoided.

07/2020:2772

INFLUENZA VACCINE (LIVE, NASAL)

Vaccinum influenzae vivum pernasale

DEFINITION

Influenza vaccine (live, nasal) is an aqueous suspension of a live attenuated strain or strains of influenza virus, type A or B, or a mixture of strains of the 2 types grown individually in fertilised hens' eggs. The vaccine is presented in a form suitable for nasal administration. The vaccine is a colourless slightly opalescent liquid and may contain white particles.

PRODUCTION

GENERAL PROVISIONS

Production of the vaccine is based on a virus seed-lot system. The production method shall have been shown to consistently yield influenza vaccine (live) that complies with the requirements for immunogenicity, safety and stability.

CHOICE OF VACCINE STRAIN

The World Health Organization reviews the world epidemiological situation annually and if necessary recommends new strains corresponding to this epidemiological evidence.

Such strains are used in accordance with the regulations in force in the signatory States of the Convention on the Elaboration of a European Pharmacopoeia.

The attenuated donor virus strain and the attenuated vaccine virus strain may be generated by the manufacturer itself by classical reassortment methods or reverse genetics (e.g. plasmid rescue). The wild type virus strains used for the production of the attenuated vaccine virus seed lots must have been approved by the competent authority.

The complete history of production of the attenuated vaccine virus strain including description of the derivation of the seeds from the attenuated donor virus strain(s) and the WHO recommended wild virus strain(s) shall be approved by the competent authority.

During development studies and whenever a new HA subtype of influenza A virus (i.e. non-H1, non-H3 subtype) or a new influenza B virus type differing from the currently circulating genetic lineages is included in the vaccine, the neurovirulence of the master virus seed lots is assessed using suitable animal models (e.g. in mice) with the attenuated donor virus strain as a comparator. The new strain shall not be more neurovirulent than the comparator.

Genotypic and phenotypic characterisations of attenuated donor virus strain(s) are undertaken using techniques for identification of attenuation markers and nucleotide sequences.

SUBSTRATE FOR VIRUS PROPAGATION

Influenza virus seed and all vaccine batches are propagated in fertilised eggs from chicken flocks free from specified pathogens (SPF) (*5.2.2*).

VIRUS SEED LOTS

The production of vaccine is based on a seed-lot system. The attenuated donor virus strains and the wild-type virus strains used for the production of the attenuated master seed lots are identified by historical records that include information on their origins and the tests used in their characterisations. Only an attenuated master donor virus strain that has been demonstrated by a suitable method (e.g. multiplex PCR assay) to be free from human respiratory pathogens which are able to replicate in eggs could be used for the production of attenuated master virus seed lots. This assay is omitted if reverse genetics method (e.g. plasmid rescue) is used.

The production of the attenuated master virus seed lot must be approved by the competent authority. The attenuated master virus seed lot must have the same characteristics as the attenuated donor virus strain. The number of passages required to produce the attenuated master virus seed lot from the attenuated donor virus strain is limited and approved by the competent authority. Unless otherwise justified and authorised, the inoculum for infecting the eggs used in the production of a vaccine lot shall be a virus harvest without intermediate passage, so that no vaccine virus is more than 1 passage from an attenuated master virus seed lot that has passed all safety tests.

Each virus seed lot used for propagation must have been filtrated through a bacteria retentive filter.

The attenuated master virus seed lot must express the haemagglutinin and neuraminidase from the wild type virus strain and other proteins from the attenuated donor virus strain.

The attenuated master virus seed lot characterisation shall include the following tests:

- genotype analyses using validated nucleic acid amplification techniques (*2.6.21*);
- virus sequencing of the seed lot and comparison of the coding sequences as follows; the sequences of the haemagglutinin and neuraminidase genes with those of the recommended strains and the sequences of the 6 remaining genes with those of the attenuated donor strain;
- genetic stability by sequencing, cold adapted and temperature sensitive phenotypes determination and attenuation test upon several passages in the substrate.

Only an attenuated master virus seed lot that complies with the following requirements may be used in the preparation of the harvest.

Identification. For each attenuated master virus seed lot, the haemagglutinin and neuraminidase antigens are identified using suitable methods.

Cold adapted and temperature sensitive phenotype. For each attenuated master virus seed lot a test is carried out in cell cultures to demonstrate the cold adapted and temperature sensitive phenotypes of the seed lot. The attenuated master virus seed lot complies with the test:

- For the cold adaptation, if the loss of virus titre between the incubation at + 25 °C and + 33 °C is not more than 2.0 $\log_{10}$ of infectious units as expressed in fluorescent focus units (FFU).
- For the temperature sensitivity, if the loss of virus titre between the incubation at + 33 °C and +37 °C (for strains B) or 39 °C (for strains A) is not less than 2.0 $\log_{10}$ of infectious units as expressed in FFU.

Attenuation. For each attenuated master virus seed lot, an *in vivo* attenuation test is carried out on ferrets. The conditions of the test such as inoculation dose and observation period are established in validation studies. The attenuation test is performed by intranasal inoculation of ferrets, free from antibodies against influenza virus, with the attenuated master virus seed lot. The animals are monitored for a defined number of days post-inoculation for signs of influenza-like illness, including nasal discharge, frequent sneezing, severe lethargy, or fever.

At the conclusion of the monitoring period, animals are euthanized. Nasal turbinate and lung tissues are collected and analysed for the presence of infectious virus using a suitable infectivity assay.

For a master virus seed lot to be identified as attenuated, the virus must be detected in samples of nasal turbinate tissues and samples from lung tissues from individual animals, and must demonstrate that the virus growth is restricted or shows no virus replication. In addition, there are no signs of influenza-like illness in the inoculated animals.

Virus concentration. The virus concentration of each attenuated master virus seed lot is determined by titration in cell cultures using a suitable validated *in vitro* cell-based assay (e.g. fluorescent focus assay) to monitor the consistency of production.

Extraneous agents (*2.6.16*). Each attenuated master virus seed lot complies with the requirements for virus seed lots.

Avian leucosis viruses. For each attenuated master virus seed lot, the absence of avian leucosis viruses is verified using a suitable method.

PROPAGATION AND HARVEST

All processing of the fertilised eggs is done under aseptic conditions in an area where no other infectious agents or cells are handled at the same time. After inoculation and incubation at a controlled temperature, only eggs containing living and typical chick embryos are harvested. The percentage of rejected eggs is recorded. After homogenisation and clarification by centrifugation, the clarified allantoic fluid is tested as described below and kept at − 70 °C or colder until further processing. No human protein is added to the virus suspension at any stage during production. If stabilisers are added, they shall have been shown to have no antigenic or sensitising properties for man.

Only a single harvest or a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the monovalent bulk.

Extraneous agents (*2.6.16*). Each single harvest or monovalent pooled harvest complies with the tests for extraneous agents with the exception of the tests for mycobacteria and sterility which are not required at this stage of production.

Avian leucosis viruses. For each single harvest or monovalent pooled harvest, the absence of avian leucosis viruses is verified using a suitable method.

Microbiological contamination. The bioburden test using a membrane filtration is carried out on each single harvest or on each monovalent pooled harvest to determine the total viable aerobic count and to verify the absence of yeast and mould using selective media. The total viable aerobic count is within the limit approved by the competent authority. Verification of absence of *Vibrio*, *Shigella* and *Salmonella* is carried out using supplementary specific validated techniques approved by the competent authority.

MONOVALENT BULK

Monovalent bulks are prepared by pooling a number of satisfactory single harvests or monovalent pooled harvests of the same virus type. The monovalent bulk is concentrated and purified by high-speed centrifugation or another suitable method then filtered through a bacteria retentive filter.

Only a monovalent bulk that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification. Each monovalent bulk is identified as influenza virus of the given type using suitable haemagglutinin type specific assay.

Virus concentration. The virus concentration of each monovalent bulk is determined by titration using a suitable validated *in vitro* assay (e.g. fluorescent focus assay).

Cold adapted and temperature sensitive phenotype. Each monovalent bulk complies with the test as described under Virus seed lots.

Attenuation test. The attenuation test is performed by intranasal inoculation of ferrets, free from antibodies against influenza virus, with each monovalent bulk test sample as described under Virus seed lots.

If sufficient consistency data are available, and approved by the competent authority, only the first 3 monovalent bulks following the introduction of a new attenuated master virus seed lot are tested on ferrets.

Wherever possible, in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, manufacturers are encouraged to develop validated *in vitro* alternative methods to the animal test for monovalent bulks using appropriate tools such as molecular methods or other suitable methods for determination of viral attenuation markers.

Genotyping. The genotype of each monovalent bulk is verified using suitable validated nucleic acid amplification techniques (*2.6.21*).

Bacterial and fungal contamination. Each monovalent bulk complies with the test for sterility (*2.6.1*), carried out using 10 mL of each medium.

Total protein content: maximum 0.25 mg per human dose before the addition of any stabiliser.

FINAL BULK VACCINE

A final bulk vaccine is formulated aseptically from appropriate quantities of the monovalent bulks of each virus strain. The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. Where a final bulk vaccine is formulated as a release intermediate, it complies with the following requirements and is within the limits approved for the particular product. A suitable stabiliser may be added. Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. The final bulk vaccine complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

FINAL LOT

An approved minimum virus concentration for release of the product is established for each virus strain to ensure, in light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity. Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Thermal stability. Maintain not fewer than 3 containers of the final lot at an elevated temperature for a defined period of time, using conditions found suitable for the particular product as approved by the competent authority. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for vaccine maintained at the temperature recommended for storage. For each virus strain, the virus concentration of the containers that have been heated does not decrease by more than an approved amount during the period of exposure.

IDENTIFICATION

The assay serves to confirm the antigenic specificity of the vaccine.

TESTS

Ovalbumin. Not more than the quantity stated on the label and in any case not more than 1 µg per human dose, determined by a suitable immunochemical method (*2.7.1*) using a suitable reference preparation of ovalbumin.

Total protein. Not more than the quantity stated on the label and in any case not more than 2.2 mg per human dose.

Bacterial and fungal contamination. It complies with the test for sterility (*2.6.1*).

Bacterial endotoxins (*2.6.14*): less than 6 IU per single human dose.

ASSAY

Titrate the vaccine for infective virus in cell cultures using at least 3 separate containers of vaccine and inoculating a suitable number of wells for each dilution step. Titrate 1 container of an appropriate virus reference preparation in triplicate to

validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established for each virus strain on a historical basis by each laboratory. If the vaccine contains more than one influenza virus strain, titrate each virus strain separately, using an appropriate type-specific antiserum.

Calculate the individual virus concentration for each container of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*). For each virus strain, the combined virus concentration for the 3 containers of vaccine is within the range stated on the label.

The assay is not valid if:

- for each virus strain, the confidence interval ($P = 0.95$) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ infectious units as expressed in FFU;
- for each virus strain, the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ infectious units as expressed in FFU from the established value.

The assay is repeated if the confidence interval ($P = 0.95$) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ infectious units as expressed in FFU; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P = 0.95$) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ infectious units as expressed in FFU.

LABELLING

The label states:

- that the vaccine has been prepared in eggs;
- the strain or strains of influenza virus used in preparation of the vaccine;
- the minimum and maximum virus strain concentration per human dose;
- the maximum amount of ovalbumin;
- the season during which the vaccine is intended to protect.

Monographs on adjuvants

TEXT N°	TITLE	PRODUCT TYPE(S)
1664	Aluminium hydroxide, hydrated, for adsorption	Vaccines containing aluminium hydroxide as adjuvant.
2805	Squalene	Vaccines containing a squalene-based adjuvant.

01/2017:1664
corrected 10.0

ALUMINIUM HYDROXIDE, HYDRATED, FOR ADSORPTION

Aluminii hydroxidum hydricum ad adsorptionem

$[AlO(OH)],xH_2O$

DEFINITION

Content: 90.0 per cent to 110.0 per cent of the content of aluminium stated on the label.

NOTE: *shake the gel vigorously for at least 30 s immediately before examining.*

CHARACTERS

Appearance: white or almost white, translucent, viscous, colloidal gel. A supernatant may be formed upon standing.

Solubility: a clear or almost clear solution is obtained with alkali hydroxide solutions and mineral acids.

IDENTIFICATION

Solution S (see Tests) gives the reaction of aluminium.

To 10 mL of solution S add about 0.5 mL of *dilute hydrochloric acid R* and about 0.5 mL of *thioacetamide reagent R*. No precipitate is formed. Add dropwise 5 mL of *dilute sodium hydroxide solution R*. Allow to stand for 1 h. A gelatinous white precipitate is formed which dissolves upon addition of 5 mL of *dilute sodium hydroxide solution R*. Gradually add 5 mL of *ammonium chloride solution R* and allow to stand for 30 min. The gelatinous white precipitate is re-formed.

TESTS

Solution S. Add 1 g to 4 mL of *hydrochloric acid R*. Heat at 60 °C for 1 h, cool, dilute to 50 mL with *distilled water R* and filter if necessary.

pH (*2.2.3*): 5.5 to 8.5.

Adsorption power. Dilute the substance to be examined with *distilled water R* to obtain an aluminium concentration of 5 mg/mL. Prepare *bovine albumin R* solutions with the following concentrations of bovine albumin: 0.5 mg/mL, 1 mg/mL, 2 mg/mL, 3 mg/mL, 5 mg/mL and 10 mg/mL. If necessary, adjust the gel and the *bovine albumin R* solutions to pH 6.0 with *dilute hydrochloric acid R* or *dilute sodium hydroxide solution R*.

For adsorption, mix 1 part of the diluted gel with 4 parts of each of the solutions of *bovine albumin R* and allow to stand at room temperature for 1 h. During this time shake the mixture vigorously at least 5 times. Centrifuge or filter through a non-protein-retaining filter. Immediately determine the protein content (*2.5.33, Method 2*) of either the supernatant or the filtrate.

It complies with the test if no bovine albumin is detectable in the supernatant or filtrate of the 2 mg/mL *bovine albumin R* solution (maximum level of adsorption) and in the supernatant or filtrate of *bovine albumin R* solutions of lower concentrations. Solutions containing 3 mg/mL, 5 mg/mL and 10 mg/mL *bovine albumin R* may show bovine albumin in the supernatant or filtrate, proportional to the amount of bovine albumin in the solutions.

Sedimentation. If necessary, adjust the substance to be examined to pH 6.0 using *dilute hydrochloric acid R* or *dilute sodium hydroxide solution R*. Dilute with *distilled water R* to obtain an aluminium concentration of approximately 5 mg/mL. If the aluminium content of the substance to be examined is lower than 5 mg/mL, adjust to pH 6.0 and dilute with a 9 g/L solution of *sodium chloride R* to obtain an aluminium concentration of about 1 mg/mL. After shaking for at least 30 s, place 25 mL of the preparation in a 25 mL graduated cylinder and allow to stand for 24 h.

It complies with the test if the volume of the clear supernatant is less than 5 mL for the gel with an aluminium content of about 5 mg/mL.

It complies with the test if the volume of the clear supernatant is less than 20 mL for the gel with an aluminium content of about 1 mg/mL.

Chlorides (*2.4.4*): maximum 0.33 per cent.

Dissolve 0.5 g in 10 mL of *dilute nitric acid R* and dilute to 500 mL with *water R*.

Nitrates: maximum 100 ppm.

Place 5 g in a test-tube immersed in ice-water, add 0.4 mL of a 100 g/L solution of *potassium chloride R*, 0.1 mL of *diphenylamine solution R* and, dropwise with shaking, 5 mL of *sulfuric acid R*. Transfer the tube to a water-bath at 50 °C. After 15 min, any blue colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 5 mL of *nitrate standard solution (100 ppm NO_3) R*.

Sulfates (*2.4.13*): maximum 0.5 per cent.

Dilute 2 mL of solution S to 20 mL with *water R*.

Ammonium (*2.4.1, Method B*): maximum 50 ppm, determined on 1.0 g.

Prepare the standard using 0.5 mL of *ammonium standard solution (100 ppm NH_4) R*.

Arsenic (*2.4.2, Method A*): maximum 1 ppm, determined on 1 g.

Iron (*2.4.9*): maximum 15 ppm, determined on 0.67 g.

Bacterial endotoxins (*2.6.14*): less than 5 IU of endotoxin per milligram of aluminium, if intended for use in the manufacture of an adsorbed product without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 2.50 g in 10 mL of *hydrochloric acid R*, heating for 30 min at 100 °C on a water-bath. Cool and dilute to 20 mL with *water R*. To 10 mL of the solution, add *concentrated ammonia R* until a precipitate is obtained. Add the smallest quantity of *hydrochloric acid R* needed to dissolve the precipitate and dilute to 20 mL with *water R*. Carry out the complexometric titration of aluminium (*2.5.11*). Carry out a blank titration.

STORAGE

At a temperature not exceeding 30 °C. Do not allow to freeze. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

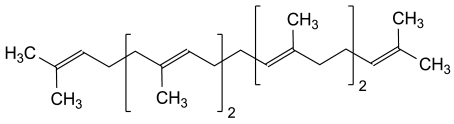
LABELLING

The label states the declared content of aluminium.

01/2020:2805

SQUALENE

Squalenum



$C_{30}H_{50}$ M_r 410.7

[111-02-4]

DEFINITION

(6*E*,10*E*,14*E*,18*E*)-2,6,10,15,19,23-Hexamethyltetracosa-2,6,10,14,18,22-hexaene. It may be of vegetable or animal origin.

Content: 97.0 per cent to 103.0 per cent (anhydrous substance).

This monograph applies to squalene used as an adjuvant in vaccines.

CHARACTERS

Appearance: clear, colourless or light yellow, oily liquid.

Solubility: practically insoluble in water, freely soluble in acetone and in cyclohexane, soluble in ethanol (96 per cent).

Relative density: about 0.86.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

Comparison: *squalene CRS*.

TESTS

Appearance. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution Y_6 (*2.2.2, Method II*).

Refractive index (*2.2.6*): 1.491 to 1.499.

Acid value (*2.5.1*): maximum 1.0.

Iodine value (*2.5.4, Method B*): 350 to 450, determined on 0.06 g.

Peroxide value (*2.5.5, Method A*): maximum 5.0.

Saponification value (*2.5.6*): maximum 5.0.

Water (*2.5.32*): maximum 0.2 per cent, determined on 2.00 g.

ASSAY

Gas chromatography (*2.2.28*). *Prepare the solutions immediately before use.*

Test solution. Dissolve 0.100 g of the substance to be examined in *heptane R* and dilute to 100.0 mL with the same solvent.

Reference solution (a). Dissolve 0.100 g of *squalene CRS* in *heptane R* and dilute to 100.0 mL with the same solvent.

Reference solution (b). Dissolve 0.100 g of the substance to be examined and 0.100 g of *methyl lignocerate R* in *heptane R* and dilute to 100.0 mL with the same solvent.

Column:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 1.7 mL/min.

Split ratio: 1:12.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 12	290
Injection port		275
Detector		300

Detection: flame ionisation.

Injection: 1 µL.

Relative retention with reference to squalene (retention time = about 8.1 min): methyl lignocerate = about 0.9.

System suitability: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to methyl lignocerate and squalene.

Calculate the percentage content of $C_{30}H_{50}$ taking into account the assigned content of *squalene CRS*.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states the origin of squalene (vegetable or animal).