THE EUROPEAN DIRECTORATE FOR THE QUALITY OF MEDICINES & HEALTHCARE (EDQM)

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2024 EDQM virtual training programme:

Independent modules on European Pharmacopoeia texts related to Biologicals and on Microbiology chapters

(Live Webinars)
Date: 30 January 2024 – 01 February 2024

Module 4
Microbiology

1 February 2024

Module 4 Agenda

Microbiology chapters

Emmanuelle Charton and Thuy Bourgeois, EDQM, Council of Europe

Rapid microbiological methods and Mycoplasmas

Solène Le Maux and Thuy Bourgeois, EDQM, Council of Europe

Pyrogenicity (BET, rFC, MAT, pyrogenicity strategy)

Gwenaël Cirefice, EDQM, Council of Europe

Microbiology chapters

Ph. Eur. Microbiology Chapters

2.6. Biological tests

2.6.1*	Sterility
2.6.2	Mycobacteria
2.6.7	Mycoplasmas
2.6.8	Pyrogens
2.6.12*	Microbiological examination of non-sterile products: microbial enumeration tests
2.6.13*	Microbiological examination of non-sterile products: test for specified micro-organisms
2.6.14*	Bacterial endotoxins
2.6.16	Tests for extraneous agents in viral vaccines for human use
2.6.27	Microbiological examination of cell-based preparations
2.6.30	Monocyte-activation test
2.6.31	Microbiological examination of herbal products and extracts
2.6.32	Test for bacterial endotoxins using recombinant factor C
2.6.36	Microbiological examination of live biotherapeutic products: tests for enumeration of microbial contaminants
2.6.37	Principles for the detection of extraneous viruses in immunological veterinary medicinal products using culture methods
2.6.38	Microbiological examination of live biotherapeutic products: tests for specified micro-organisms
2.6.39	Microbiological examination of human tissues
2.6.40	Monocyte-activation test for vaccines containing inherently pyrogenic components

2.7. Biological assay

2.7.2	Microbiological assay of antibiotics

5.1. General texts on microbiology

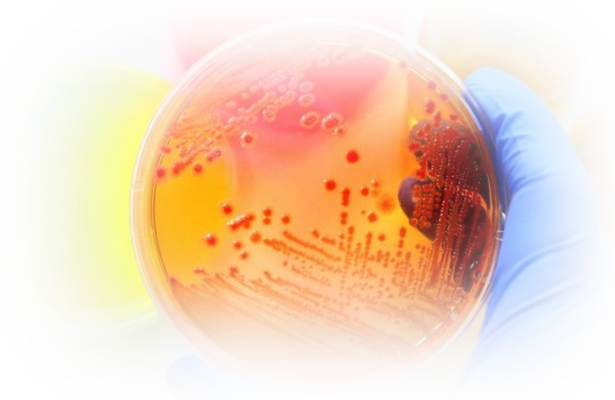
5.1.1	Methods of preparation of sterile products
5.1.2	Biological indicators and related microbial preparations used in the manufacture of sterile products
5.1.3	Efficacy of antimicrobial preservation
5.1.4*	Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use
5.1.5	Application of the F0 concept to steam sterilisation of aqueous preparations
5.1.6	Alternative methods for control of microbiological quality
5.1.7	Viral safety
5.1.8	Microbiological quality of herbal medicinal products for oral use and extracts used in their preparation
5.1.9	Guidelines for using the test for sterility
5.1.10	Guidelines for using the test for bacterial endotoxins
5.1.11	Determination of bactericidal, fungicidal or yeasticidal activity of antiseptic medicinal products

Non exhaustive list

*internationally harmonised chapters

Outline

- **Sterility**
- **Microbiological quality of non-sterile products**
- **Efficacy of antimicrobial preservation**



2.6.1 Sterility

2.6.1 International Harmonisation (see Q4B Annex 8)

- *"NOTE (1) This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8. Pharmacopoeial harmonisation."*

ICH Q4B

Evaluation and recommendation of pharmacopoeial tests for use in the ICH regions

Implementation status

Q4B Annex 8(R1) Sterility Test General Chapter

The ICH Harmonised Annex was finalised under *Step 4* in June 2009. This annex is the result of the Q4B process for Sterility Test General Chapter. This annex was revised (R1) on 27 September 2010 to include the Interchangeability Statement from Health Canada, Canada.

Date of *Step 4*: 27 September 2010

Status: *Step 5*

Implementation status:

ANVISA, Brazil - Implemented; Date: 1 November 2010; Reference: RDC 49/2010 RDC 298/2019

COFEPRIS, Mexico - Implemented; Date: 25 February 2022; Reference: Pharmacopeia of the United Mexican States 13.0.

EC, Europe - Implemented; Date: 1 September 2010; Reference: CHMP/ICH/645592/2008

EDA, Egypt - In the process of implementation;

FDA, United States - Implemented; Date: 1 June 2017; Reference: Vol. 74, No. 244, p. 68068-9

HSA, Singapore - Not applicable;

Health Canada, Canada - Implemented; Date: 26 October 2010; Reference: File #: 10-121878-840

MFDS, Republic of Korea - Implemented; Date: 27 December 2012; Reference: Korean Pharmacopoeia : Sterility Test [MFDS, Republic of Korea Notification]

MHLW/PMDA, Japan - Implemented; Date: 1 September 2010; Reference: PFSB/ELD Notification No. 0917-1

MHRA, UK - Implemented; Date: 1 September 2010;

NMPA, China - Not yet implemented; Reference: Chinese Pharmacopoeia (2020 edition) volume IV, general notes and related general chapters, rules of developing and revision process for national pharmaceutical standards

SFDA, Saudi Arabia - Not yet implemented;

Swissmedic, Switzerland - Implemented; Date: 27 September 2010; Reference: ICH Guidelines apply in Switzerland automatically upon reaching Step 4: Swissmedic Journal 05/2006, p. 504

TFDA, Chinese Taipei - Implemented; Date: 16 October 2011; Reference: 1. Chinese Phamacopeia 2. Public Announcement for " List for ICH Guidelines Adopted."

TITCK, Türkiye - Not yet implemented;

2.6.1 The steps of the sterility test

- Sample preparation
- Inoculation of sample to the two different liquid media
  - Membrane filtration (0.45 µm)
  - Or: direct inoculation

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.

- Incubation (14 days)
- Observation and interpretation of results

"*If no evidence of growth is found, the product to be examined complies with the test for sterility*"

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  - Membrane filtration (0.45 µm)
  - Or: direct inoculation

If the product has antimicrobial properties, wash the membrane not less than 3 times by filtering through it each time the volume ... st. Do ... if ... that such a cycle does not fully eliminate the antimicrobial activity.

Specific instructions to be followed if the preparation has antimicrobial properties

- Incubation (14 days)
- Observation and interpretation of results

"*If no evidence of growth is found, the product to be examined complies with the test for sterility*"

Observation and results/Invalidity of the test

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.

2.6.1 Culture media

- **Two fluid media: Fluid Thioglycollate medium** and **Soya-bean casein digest medium**
- **Sterility**
- **Growth promotion**

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

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Pseudomonas aeruginosa	
Anaerobic bacterium	
Clostridium sporogenes	
Fungi	
Candida albicans	
Aspergillus brasiliensis	

Inoculum: a small number (not more than 100 CFU)

Incubation
Not more than 3 days for bacteria
Not more than 5 days for fungi

The media are suitable if a clearly visible growth of the micro-organisms occurs.

SLIDO

Can I skip the growth promotion test if I use a commercial medium which indicates, in its CoA, that it complies?

✓Yes

✓No

Correct answer in green!

2.6.1 Culture media

- **Two fluid media: Fluid Thioglycollate medium** and **Soya-bean casein digest medium**
- **Sterility**
- **Growth promotion** 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.

Table 2.6.1.-1. – *Strains of the test micro-organisms suitable for use in the growth promotion test and the method suitability test*

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Pseudomonas aeruginosa
Anaerobic bacterium
Clostridium sporogenes
Fungi
Candida albicans
Aspergillus brasiliensis

Incubation
Not more than 3 days for bacteria
Not more than 5 days for fungi

Inoculum: a small number (not more than 100 CFU)

The media are suitable if a clearly visible growth of the micro-organisms occurs.

2.6.1 Method suitability

Method suitability: the aim is to verify that the product will not interfere with the test: the product is tested in the presence of the test micro-organisms in the same conditions as for the test for the product to be examined. The micro-organisms should grow.

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.

SLIDO

- **My product – claimed to be sterile- has antimicrobial properties, that I have not been able to eliminate by the 5 times 100 ml washing per filter: what should I do?**

  - ✓I should perform the sterility test, even if the antimicrobial activity has not been fully eliminated
  - ✓My product has antimicrobial properties and therefore will not be subject to contamination. Sterility is not a requirement
  - ✓My product has antimicrobial properties and therefore will not be subject to contamination. I decide not to perform the test in routine based on my control strategy (waiving of tests Per the General Notices)
  - ✓I should continue to try to eliminate the antimicrobial activity, for example by further washings of the membrane

Correct answers in green!

Response

- Sterility (2.6.1) remains a requirement (the preparation is *sterile*)

2.6.1. "The test is applied to substances, preparations or articles which, according to the Pharmacopoeia, are required to be sterile."

- Waiving of tests is always possible (see General Notices)
- Further washings are not allowed per 2.6.1

2.6.1 Neutralisation

"If the product has antimicrobial properties, wash the membrane not less than three 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 method suitability it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity."

2.6.1 Method suitability

Method suitability 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.

2.6.1 Minimum number of items to be tested

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.

2.6.1 Minimum quantity to be used for each medium

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)

Example

Quantity to be tested for a batch of a parenteral preparation consisting of 500 vials filled with 10 ml

2.6.1 Minimum number of items to be tested

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Number of items in the batch*	Minimum number of items to be tested for each medium, unless otherwise justified and authorised**
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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.

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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
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– 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)

Example

Quantity to be tested for a batch of a parenteral preparation consisting of 500 vials filled with 10 ml

- According to Table 2.6.1.3: 10 vials
- According to 2.6.1.2: 5 ml per vial

→ Minimum 50 ml per medium

SLIDO

- **In order to obtain these 50 ml….**

  ✓I pull all vials together, I obtain 10 ml x 10 vials = 100 ml. From these 100 ml, I extract 50 ml for the test

  ✓I extract 5 ml from each vial and I pull these quantities together, which gives 50 ml, that I use for the test

  ✓From each of the 10 vials, I use 5 ml to inoculate medium 1 and 5 ml to inoculate medium 2

Correct answers in green!

SLIDO

- **Can I use less than the quantities given in Tables 2.6.1-2 and 2.6.1-3?**

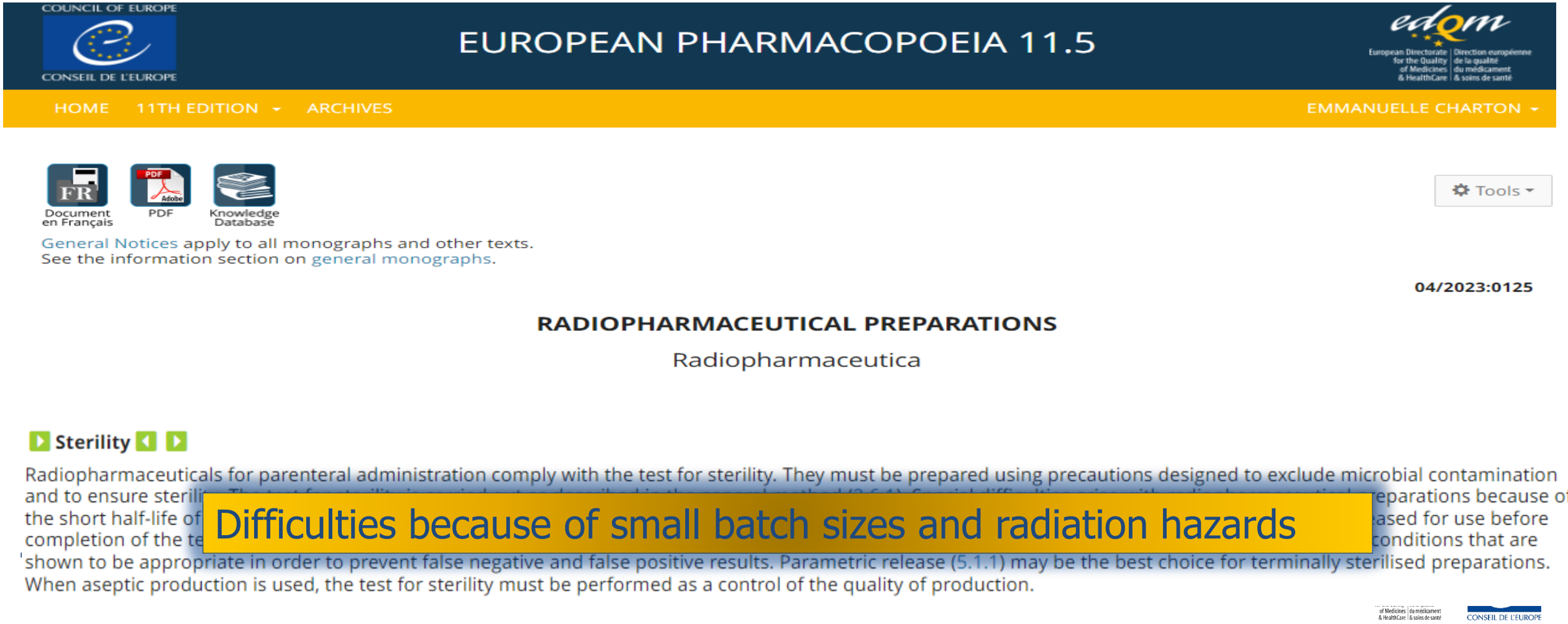
✓No

✓Yes

Correct answers in green!

Response: These are *Minimum quantities*, but

- The General Notices allow you to use alternative methods
- Specific cases are allowed: Cell-based preparations (cf chapter 2.6.27), Radiopharmaceutical preparations



Testing for the sterility of empty containers

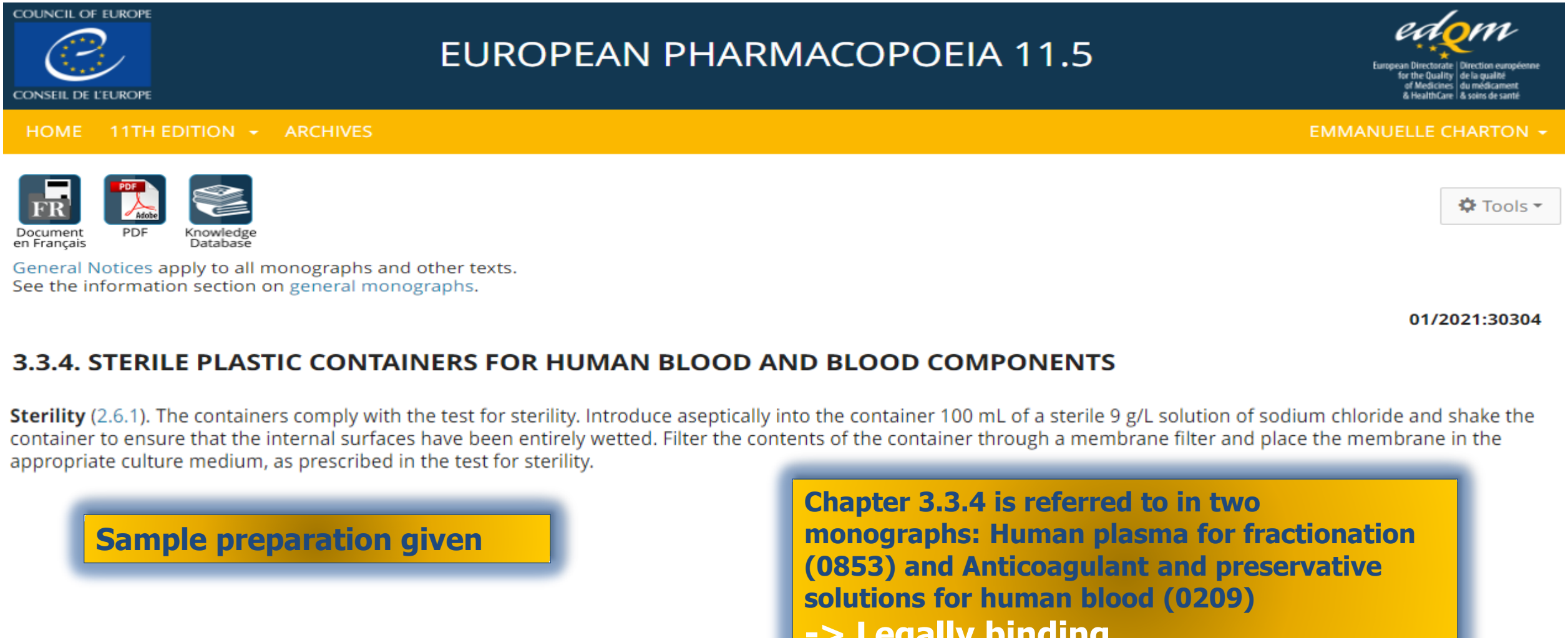
- **Sterile plastic syringes**

Sample preparation given

Look in section 3!

Testing for the sterility of empty containers

- **Sterile plastic containers for blood**



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EUROPEAN PHARMACOPOEIA 11.5

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Tools

General Notices apply to all monographs and other texts.
See the information section on general monographs.

01/2021:30304

3.3.4. STERILE PLASTIC CONTAINERS FOR HUMAN BLOOD AND BLOOD COMPONENTS

Sterility (2.6.1). The containers comply with the test for sterility. Introduce aseptically into the container 100 mL of a sterile 9 g/L solution of sodium chloride and shake the container to ensure that the internal surfaces have been entirely wetted. Filter the contents of the container through a membrane filter and place the membrane in the appropriate culture medium, as prescribed in the test for sterility.

Sample preparation given

Chapter 3.3.4 is referred to in two monographs: Human plasma for fractionation (0853) and Anticoagulant and preservative solutions for human blood (0209)
-> Legally binding

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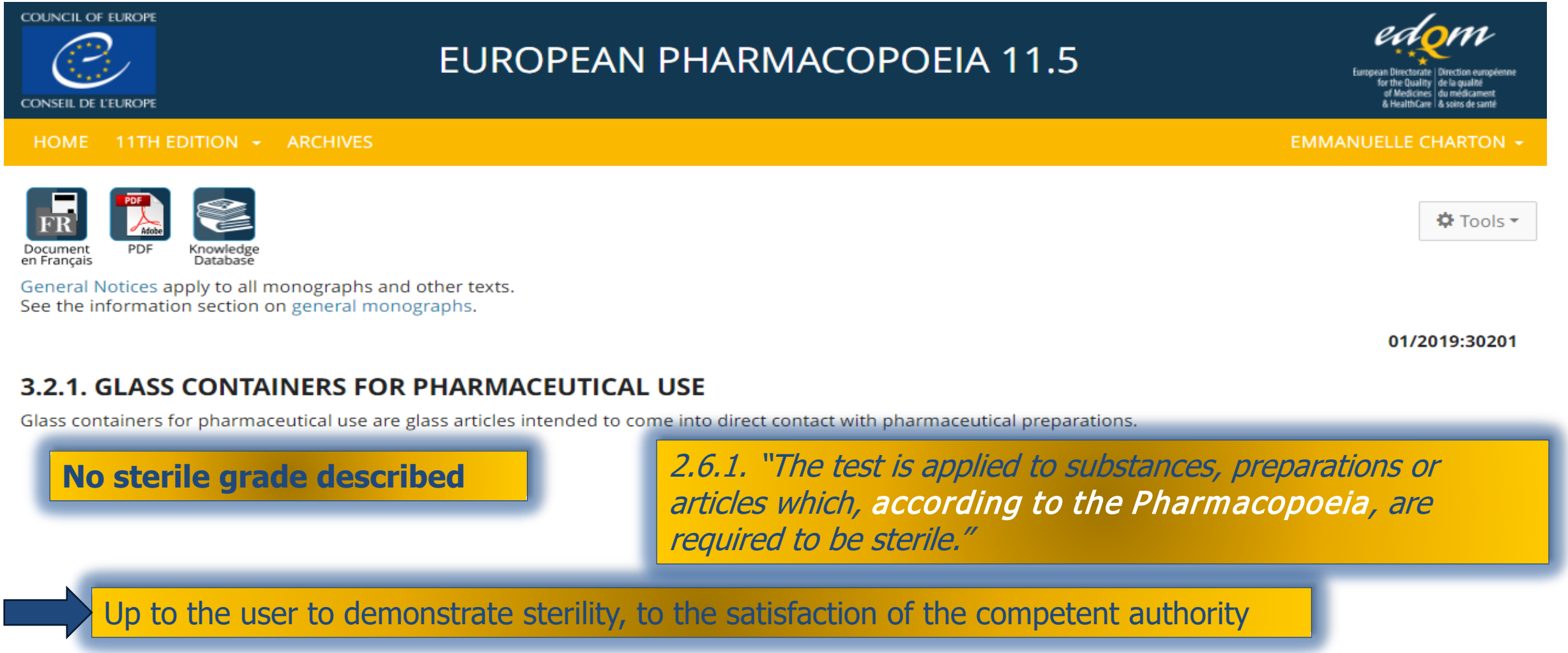
Testing for the sterility of empty containers

- **Glass containers**



No sterile grade described

*2.6.1. "The test is applied to substances, preparations or articles which, **according to the Pharmacopoeia**, are required to be sterile."*

Up to the user to demonstrate sterility, to the satisfaction of the competent authority

Slido

- **My product is a parenteral preparation, consisting of a lyophilisate accompanied with a diluent. What should comply with 2.6.1?**

✓The lyophilisate?
✓The diluent?
✓The reconstituted product?

Correct answers in green!

Response

*2.6.1. "The test is applied to substances, preparations or articles which, **according to the Pharmacopoeia**, are required to be sterile."*

"The reconstituted vaccine complies with the test for sterility (2.6.1)"

"The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph Vaccines for veterinary use (0062)."

The reconstituted product is a Parenteral preparation, which has to comply with the monograph 0520, which requires compliance with 2.6.1

The lyophilisate? The diluent?

Powders for injections or infusions

DEFINITION

Powders for injections or infusions are sterile parenteral preparations intended for injection or infusion after reconstitution with the prescribed volume of a prescribed sterile liquid. They may be prepared by lyophilisation.

They are supplied in their final containers and, when reconstituted, rapidly form either practically particle-free solutions or uniform dispersions. After dissolution or dispersion, they comply with the requirements for injections or for infusions.

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

Sterility requirements in individual monographs on vaccines

Vaccine for human use

Bacterial and fungal contamination

It complies with the test for sterility (2.6.1).

Vaccine for veterinary use

Bacteria and fungi

The vaccine complies with the test for sterility prescribed in the monograph Vaccines for veterinary use (0062).

3-8. **Bacteria and fungi**. Vaccines comply with the test for sterility (*2.6.1*). Where the volume of liquid in a container is greater than 100 mL, the membrane filtration method is used wherever possible. Where the membrane filtration method cannot be used, the direct inoculation method may be used. Where the volume of liquid in each container is at least 20 mL, the minimum volume to be used for each culture medium is 10 per cent of the contents or 5 mL, whichever is less. The appropriate number of items to be tested (*2.6.1*) is 1 per cent of the batch with a minimum of 4 and a maximum of 10.

For live bacterial and for live fungal vaccines, the absence of micro-organisms other than the vaccine strain is demonstrated by suitable methods such as microscopic examination and inoculation of suitable media.

For frozen or freeze-dried avian live viral vaccines produced in embryonated eggs, for non-parenteral use only, the requirement for sterility is usually replaced by requirements for absence of pathogenic micro-organisms and for a maximum of 1 non-pathogenic micro-organism per dose.

For other vaccines presented in a non-liquid form for non-parenteral use only, in agreement with the competent authority and provided that the product remains stable throughout its shelf life, the requirement for sterility may be replaced by requirements for absence of relevant pathogenic micro-organisms and an appropriately low number of micro-organisms per dose, based on batch data and process validation.

Sterility requirements in individual monographs on vaccines

Vaccine for human use

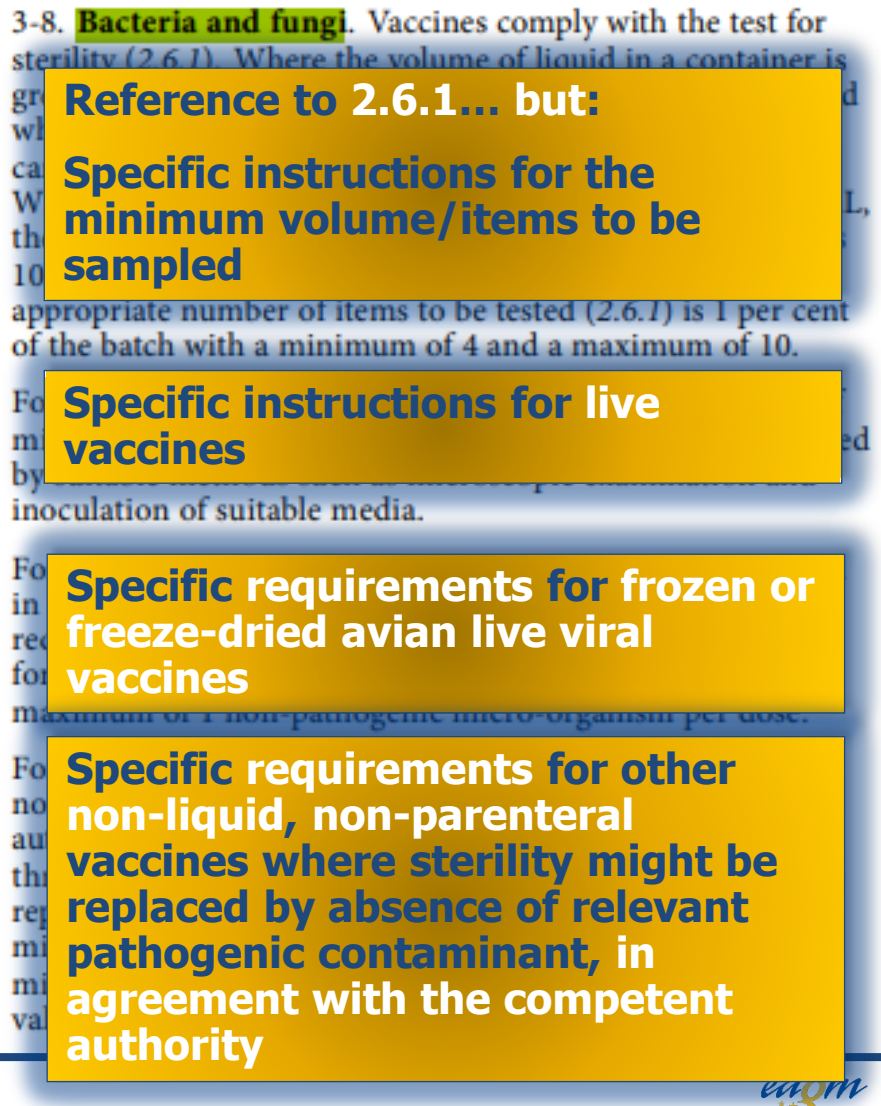
Bacterial and fungal contamination

It complies with the test for sterility (2.6.1).

Vaccine for veterinary use

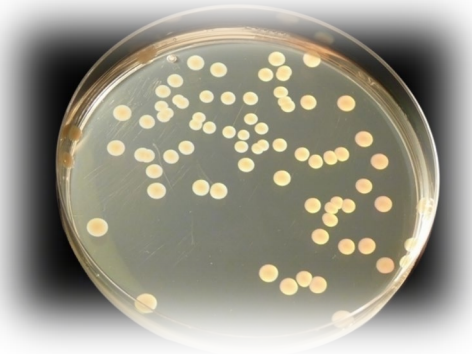
Bacteria and fungi

The vaccine complies with the test for sterility prescribed in the monograph Vaccines for veterinary use (0062).



2.6.12, 2.6.13 and 5.1.4 Microbiolgical quality of non sterile preparations

"NOTE (1) This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8. Pharmacopoeial harmonisation."



FAQs on Microbiology chapters

2.6.12 Microbiological examination of non-sterile products: microbial enumeration tests

2.6.13 Microbiological examination of non-sterile products: test for specified micro-organisms

5.1.1 Methods of preparation of sterile products

5.1.2 Biological indicators and related microbial preparations used in the manufacture of sterile products

5.1.3 Efficacy of antimicrobial preservation

5.1.4 Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use

FAQs on Microbiology chapters

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Knowledge Database

Tools

General Notices apply to all monographs and other texts.
See the information section on general monographs.

01/2021:20612

2.6.12. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS: MICROBIAL ENUMERATION TESTS(1) (SEE NOTE)

1. INTRODUCTION

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi that may grow under aerobic conditions.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

The methods are not applicable to products containing viable micro-organisms as active ingredients.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopoeia method has been demonstrated.

2. GENERAL PROCEDURES

Carry out the determination under conditions designed to avoid extrinsic microbial contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any micro-organisms that are to be revealed in the test.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralised. If inactivators are used for this purpose, their efficacy and their absence of toxicity for micro-organisms must be demonstrated.

If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated.

3. ENUMERATION METHODS

Use the membrane filtration method or the plate-count methods, as prescribed. The most-probable-number (MPN) method is generally the least accurate method for microbial counts, however, for certain product groups with a very low bioburden, it may be the most appropriate method.

The choice of method is based on factors such as the nature of the product and the required limit of micro-organisms. The chosen method must allow testing of a sufficient sample size to judge compliance with the specification. The suitability of the method chosen must be established.

4. GROWTH PROMOTION TEST, SUITABILITY OF THE COUNTING METHOD AND NEGATIVE CONTROLS

FAQs on Microbiological chapters

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Detailed view of Microbiological examination of non-sterile products: microbial enumeration tests (2.6.12.).

Status	In use
Monograph Number	20612
English Name	Microbiological examination of non-sterile products: microbial enumeration tests (2.6.12.)
French Name	Contrôle microbiologique des produits non stériles : essais de dénombrement microbien (2.6.12.)
Latin Name	
Pinyin Name	
Chinese Name	
Pharmeuropa	30.1
Published in English Supplement	10.3
Published in French Supplement	10.3
On-going	Minor revision
State of work	4 - DEF
Pharmeuropa	
Description	Results and interpretation: clarification of the reading procedure to be performed when verifying the suitability of the membrane filtration method.
Chromatogram	Not available
Additional information	Available
	View history
Interchangeable (ICH_Q4B)	NO
Pharmacopoeial harmonisation	YES (10.3)
Reference standards	
Practical Information	Test(s) | Brand Name/Information
CEP	

FAQs on Microbiological chapters

Frequently asked questions on chapter *2.6.12*

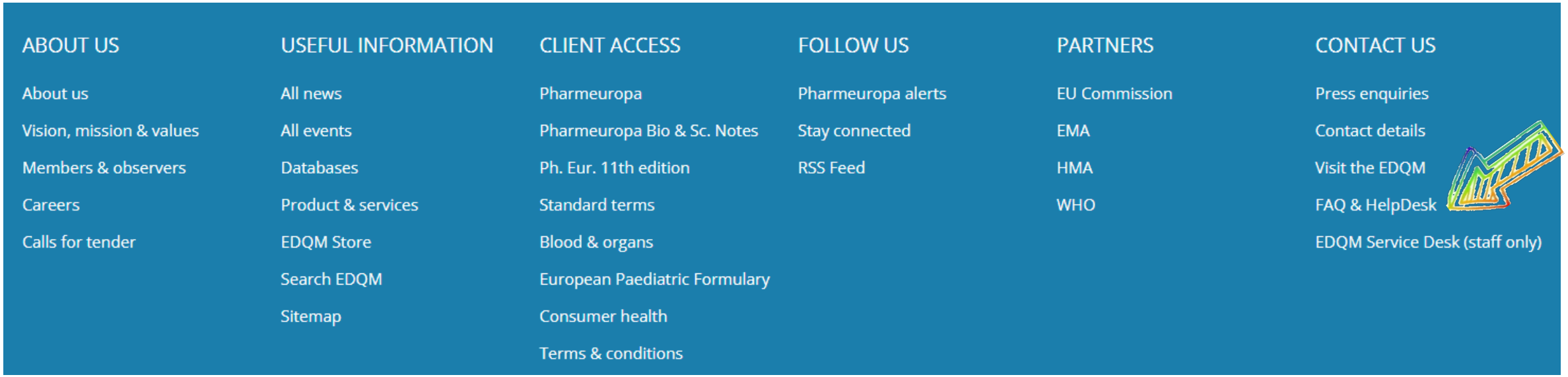
Microbiological examination of non-sterile products: microbial enumeration tests

SECTION CONCERNED	QUESTIONS	ANSWERS
4-2. Preparation of test strains	Can microbial strains other than those that are cited in the Ph. Eur. be used?	The micro-organisms that are prescribed in this chapter must be used. Strains from other culture collections may be used if they have been shown to be equivalent to those prescribed in the Ph. Eur.
	Is there a method that allows for the verification of the presence of 100 CFU in the inoculum?	In general, a membrane filtration or plate-count method is used to enumerate the correct inoculum.
	Can ready-prepared certified strains be used?	Yes, provided that you can demonstrate they are suitable for the intended use.
	What is meant by micro-organisms being not more than 5 passages removed from the original master seed-lot?	The text in the chapter is based on standard microbiological laboratory methods. A "passage" is whenever a micro-organism is (sub)cultured in a liquid or on a solid medium. To explain by example, a lyophilised culture is received from a national repository (= passage 0), resuscitated and grown in broth. This growth step is the first passage. The broth culture is then prepared for cryopreservation. The prepared culture is split into many different vials which are frozen individually. The next passage for the micro-organism occurs when the frozen vial is thawed and streaked on nutrient plates or tube slants. If the micro-organisms are passaged twice more you are still only 4 passages removed from the national repository strain.

FAQs on Microbiological chapters

https://www.edqm.eu/en/

FAQ



FAQs on Microbiological chapters

FAQ & HelpDesk - EDQM all activities

How to consult the EDQM FAQs?

To consult the FAQs, click on the following link: **EDQM FAQs**

The Frequently Asked Questions (FAQs) cover all the EDQM's activities. More than 200 FAQs have been identified and organised into 11 different topics.

Top 5 Most Frequently Asked Questions

- The Content of a European Pharmacopoeia monograph or a General Chapter
- EDQM electronic Publications – Technical Support
- Ordering an EDQM Reference Standard
- Certification of Pharmaceutical Substances (CEP)
- General information on Reference Standards

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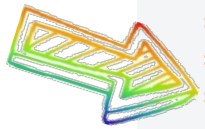
To access the HelpDesk portal click on the following link: **EDQM HelpDesk**

For more information on using the HelpDesk, please consult the **Manual**.

Despite the current healthcare crisis and the preventive measures taken at national level, we will continue to do our utmost to provide you with the best possible service. However, please note that there may nonetheless be delays in the processing of your requests.
We apologise for any inconvenience this may cause and thank you in advance for your understanding.

FAQs on Microbiological chapters

- **EDQM FAQs in English**
  - EDQM MISSION AND ROLE
  - QUALITY & SAFETY
  - HEALTHCARE
  - EUROPEAN PHARMACOPOEIA & INTERNATIONAL HARMONISATION
    - General Chapters and Monographs
      - ELABORATION AND REVISION
      - COMPLIANCE WITH A MONOGRAPH
      - MEASURING QUANTITIES
      - REAGENTS AND SUPPLIERS
      - CHARACTERS AND IDENTIFICATION
      - IMPURITIES AND CHROMATOGRAPHY
      - GENERAL CHAPTER 2.2.46 (11.0)
      - WATER - LOSS ON DRYING - SOLVENTS
      - PHARMACEUTICAL TECHNICAL PROCEDURES
      - MICROBIOLOGY
      - ELEMENTAL IMPURITIES
      - MEDICINAL PRODUCT MONOGRAPHS
      - MISCELLANEOUS
    - Pharmacopoeial Harmonisation (Pharmacopoeial Discussion Group (PDG))
  - BIOLOGICAL STANDARDISATION PROGRAMME
  - CONTROL OF MEDICINES (OFFICIAL MEDICINES CONTROL LABORATORIES, OMCL
  - CERTIFICATION OF SUBSTANCES FOR PHARMACEUTICAL USE
  - PRODUCTS AND SERVICES (Publications, Reference standards, CombiStats)
  - EDQM ELECTRONIC PUBLICATIONS - TECHNICAL SUPPORT
  - COMMUNICATIONS & EVENTS
  - EDQM HelpDesk User Manual
- FAQ de l'EDQM en français



MICROBIOLOGY

FAQs on Microbiological chapters

/... / MICROBIOLOGY

General chapters 2.6.12, 2.6.13, 5.1.1, 5.1.2, 5.1.3 and 5.1.4

You are here:
EDQM FAQs / EDQM FAQs in English / EUROPEAN PHARMACOPOEIA & INTERNATIONAL HARMONISATION / General Chapters and Monographs / MICROBIOLOGY / General chapters 2.6.12, 2.6.13, 5.1.1, 5.1.2, 5.1.3 and 5.1.4

Answer:

In response to the high number of questions raised on these general chapters, a detailed formulary is now available in the Knowledge Database.

Once you are in the database, in the drop-down menu, select 'search a number' and type the chapter number (e.g. '20612' or '50103') Click the link in the 'Number' column to open the detailed view, then click 'Available' next to 'Additional information'.

You can also directly access the detailed formularies using the following links:

- 2.6.12
- 2.6.13
- 5.1.1 and 5.1.2
- 5.1.3
- 5.1.4

Microbiological quality of non-sterile pharmaceutical preparations

Case study: Deferiprone oral solution (soluble in water)

Monograph 01/2021:2987

- It complies with the monograph *Liquid preparations for oral use (0672)*
- This dosage form monograph contains:
  - ✓A statement that liquid preparations for oral use may contain suitable antimicrobial preservatives
  - ✓A reference to general chapter *5.1.4 Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*

5.1.4 *Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*

- Table 5.1.4.-1. gives acceptance criteria for TAMC, TYMC and specified micro-organisms for all Ph. Eur. routes of administrations

Table 5.1.4.-1. – *Acceptance criteria for microbiological quality of non-sterile dosage forms*

Route of administration	TAMC (CFU/g or CFU/mL)	TYMC (CFU/g or CFU/mL)	Specified micro-organisms
Non-aqueous preparations for oral use	10^3	10^2	Absence of *Escherichia coli* (1 g or 1 mL)
Aqueous preparations for oral use	10^2	10^1	Absence of *Escherichia coli* (1 g or 1 mL)

2.6.12

2.6.13

2.6.12 Microbiological examination of non-sterile products: enumeration

Describes the tests allowing for quantitative enumeration of mesophilic bacteria and fungi that may grow under aerobic conditions

Applies to a substance or preparation

Not applicable to products containing viable micro-organisms as active ingredients

Testing of products

- Amount used
- Examination of the product (Membrane filtration, Plate-count methods, Most-probable-number method)
- Test conditions
  - Plates of casein soya bean digest agar: 30-35 °C for 3-5 days
  - Plates of Sabouraud-dextrose agar: 20-25 °C for 5-7 days

Interpretation of results

- Total Aerobic Microbial Count (TAMC): number of Conlony Forming Units (CFU) found using casein soya bean digest agar
- Total combined yeasts/moulds count (TYMC): number of CFU found on Sabouraud-dextrose agar

2.6.12 Microbiological examination of non-sterile products: enumeration

Describes the tests allowing for quantitative enumeration of mesophilic bacteria and fungi that may grow under aerobic conditions

Applies to a substance or preparation

Not applicable to products containing viable micro-organisms as active ingredients

Growth promotion test

- Each batch tested
- Test conditions
- Acceptance criteria

e.g. Sabouraud-dextrose agar: ≤ 5 days

Method suitability

Suitability of the method to be confirmed **in the presence of test sample**

- Sample preparation. Neutralisation / removal of antimicrobial activity
- Test: 5 micro-organisms; microbial suspension added to the sample and to a control; incubation conditions
- Acceptance criteria

e.g. Sabouraud-dextrose agar: ≤ 5 days

Testing of products

- Amount used
- Examination of the product (Membrane filtration, Plate-count methods, Most-probable-number method)
- Test conditions

2.6.13 Microbiological examination of non-sterile products: specified micro-organisms

Describes the tests allowing for determination of the absence or limited occurrence of specified micro-organisms under the conditions described

Applies to a substance or preparation

Testing of products

Escherichia coli

- Sample preparation as in *2.6.12* and pre-incubation on casein soya bean digest broth
- Selection in MacConkey broth (42-44°C for 24-48 h) and sub-culture on MacConkey agar (30-35 °C for 18-72 h)

Interpretation of results

Growth of colonies indicates the possible presence of E. coli. This is confirmed by identification tests. The product complies with the test if no colonies are present or if the identification tests are negative.

2.6.13 Microbiological examination of non-sterile products: specified micro-organisms

Describes the tests allowing for determination of the absence or limited occurrence of specified micro-organisms under the conditions described

Applies to a substance or preparation

Growth promotion and inhibitory properties of media

- Each batch tested
- Test conditions and acceptance criteria

Method suitability

Suitability of the method to be confirmed **in the presence of test sample**

- Sample preparation and test conditions as in Testing of products using the shortest incubation period prescribed, ≤100 CFU of *E. coli*

Testing of products

Escherichia coli

- Sample preparation as in 2.6.12 and pre-incubation on casein soya bean digest broth
- Selection in MacConkey broth (42-44°C for 24-48 h) and sub-culture on MacConkey agar (30-35 °C for 18-72 h)

Acceptance criteria

E. coli must be detected with the indication reactions as described in section 4.

2.6.13 Microbiological examination of non-sterile products: specified micro-organisms

Testing of products: MacConkey broth (42-44°C for 24-48 h); MacConkey agar (30-35 °C for 18-72 h)

Table 2.6.13.-1 – *Growth promoting, inhibitory and indicative properties of media*

	Medium	Property	Test strains
Test for bile-tolerant gram-negative bacteria	Enterobacteria enrichment broth-Mossel	Growth promoting	*E. coli* *P. aeruginosa*
		Inhibitory	*S. aureus*
	Violet red bile glucose agar	Growth promoting + indicative	*E. coli* *P. aeruginosa*
Test for *Escherichia coli*	MacConkey broth	Growth promoting	*E. coli*
		Inhibitory	*S. aureus*
	MacConkey agar	Growth promoting + indicative	*E. coli*

Growth promoting property of MacConkey agar

- ≤100 CFU of *E. coli*
- 30-35 °C for not more than 18 h
- Clearly visible growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Indicative property of MacConkey agar

- ≤100 CFU of E. coli
- 30-35 °C for 18-72 h
- Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

2.6.13 Microbiological examination of non-sterile products: specified micro-organisms

Testing of products: MacConkey broth (42-44°C for 24-48 h); MacConkey agar (30-35 °C for 18-72 h)

Table 2.6.13.-1 – *Growth promoting, inhibitory and indicative properties of media*

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		Inhibitory	*S. aureus*
	Violet red bile glucose agar	Growth promoting + indicative	*E. coli* *P. aeruginosa*
Test for *Escherichia coli*	MacConkey broth	Growth promoting	*E. coli*
		Inhibitory	*S. aureus*
	MacConkey a…	Growth promoting + indicative	*E. coli*

Inhibitory property of MacConkey broth

- ≤100 CFU of *S. aureus*
- 42-44°C for not less than 48 h
- No growth of the test micro-organism occurs.

Growth promoting property of MacConkey broth

- ≤100 CFU of *E. coli*
- 42-44 °C for not more than 24 h
- Clearly visible growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Growth promoting property of MacConkey agar

- ≤100 CFU of *E. coli*
- 30-35 °C for not more than 18 h
- Clearly visible growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Indicative property of MacConkey agar

- ≤100 CFU of E. coli
- 30-35 °C for 18-72 h
- Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

2.6.12 / 2.6.13 SLIDO

Question 1: My Deferiprone oral solution has an antimicrobial activity on *Escherichia coli*. Which of the following options can I proceed to overcome the inhibitory effect?

- ❑ 1. Increase the volume of the diluent or culture medium
- ❑ 2. Try to find a suitable neutraliser
- ❑ 3. Use membrane filtration method
- ❑ 4. Combine the previous options
- ❑ 5. I don’t know

Correct answers in green!

2.6.12 / 2.6.13 Neutralisation / removal of antimicrobial activity

2.6.13

General procedure: If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralised as described in general chapter 2.6.12.

Suitability of the test method: Any antimicrobial activity of the product necessitates a modification of the test procedure (see 4-5-3 of general chapter 2.6.12).

2.6.12

4-5-3. **Neutralisation/removal of antimicrobial activity.** The number of micro-organisms recovered from the prepared sample diluted as described in 4-5-2 and incubated following the procedure described in 4-5-4, is compared to the number of micro-organisms recovered from the control preparation.

If growth is inhibited (reduction by a factor greater than 2), then modify the procedure for the particular enumeration test to ensure the validity of the results. Modification of the procedure may include, for example, (1) an increase in the volume of the diluent or culture medium, (2) incorporation of specific or general neutralising agents into the diluent, (3) membrane filtration, or (4) a combination of the above measures.

Neutralising agents. Neutralising agents may be used to neutralise the activity of antimicrobial agents (Table 2.6.12.-2). They may be added to the chosen diluent or the medium preferably before sterilisation. If used, their efficacy and their absence of toxicity for micro-organisms must be demonstrated by carrying out a blank with neutraliser and without product.

2.6.12 / 2.6.13 SLIDO

Question 2: The inhibitory effect on *E. coli* is removed when I dilute 1 mL of my product to a 1 in 100 dilution, then use 10 mL of this dilution (i.e. 0.1 mL) to inoculate a suitable volume of casein soya bean digest broth. Is my result of "absence of *E. coli* in 0.1 mL" compliant with the Ph. Eur.?

- ✓ Yes
- ✓ No
- ✓ I don't know

Correct answer in green!

2.6.12 / 2.6.13 Quiz

4-2. *ESCHERICHIA COLI*

4-2-1. **Sample preparation and pre-incubation**. Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

◊When testing orodispersible films, filter the volume of sample corresponding to 1 film of the preparation described under 4-5-1 in general chapter *2.6.12* through a sterile filter membrane and place in 100 mL of casein soya bean digest broth. Incubate at 30-35 °C for 18-24 h.◊

Question 2: The inhibitory effect on *E. coli* is removed when I dilute 1 mL of my product to a 1 in 100 dilution, then then use 10 mL of this dilution (i.e. 0.1 mL) to inoculate a suitable volume of casein soya bean digest broth. Is my result of "absence of *E. coli* in 0.1 mL" compliant with the Ph. Eur.?

No

2.6.12/5.1.4 Acceptance criteria

When an acceptance criterion for microbiological quality is prescribed it is interpreted as follows:

- 10^1 CFU: maximum acceptable count = 20;
- 10^2 CFU: maximum acceptable count = 200;
- 10^3 CFU: maximum acceptable count = 2000, and so forth.

Specification of Deferiprone oral solution:

- TAMC: 10^2 CFU/mL
- TYMC: 10^1 CFU/mL

The product could be released if up to 200 CFU/mL for TAMC and 20 CFU/mL for TYMC are counted.

5.1.4 SLIDO

Question 3: When testing of my Deferiprone oral solution for *E. coli*, the presence of another micro-organism was observed. Can I conclude that my product complies with the European Pharmacopoeia?

Answer:

- ✓ Yes
- ✓ No
- ✓ It depends
- ✓ I don't know

Correct answer in green!

5.1.4 Other micro-organisms

In addition to the micro-organisms listed in Table 5.1.4.-1, the significance of other micro-organisms recovered is evaluated in terms of:

- use of the product: hazard varies according to the route of administration (eye, nose, respiratory tract);
- nature of the product: its ability to support growth, the presence of adequate antimicrobial preservation;
- method of application;
- intended recipient: risk may differ for neonates, infants, the debilitated;
- use of immunosuppressive agents, corticosteroids;
- presence of disease, wounds, organ damage.

Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialised training in microbiology and the interpretation of microbiological data. For raw materials, the assessment takes account of processing to which the product is subjected, the current technology of testing and the availability of materials of the desired quality.

Risk-based assessment of the relevant factors

5.1.4 *Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*

Table 5.1.4.-2. – *Acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use*

	TAMC (CFU/g or CFU/mL)	TYMC (CFU/g or CFU/mL)
Substances for pharmaceutical use	10^3	10^2

5.1.4 SLIDO

Question 4: I am a manufacturer of a parenteral preparation, which uses sucrose as an excipient. The monograph on sucrose (0204) does not include a test for microbial contamination. According to table 5.1.4-2, do I still have to comply with the limits for TAMC and TYMC?

Answer

✓Yes

✓No

Correct answers in green!

Response (1/2)

General Monograph 2034 Substances for pharmaceutical use

- **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.

The general monograph does not state that such control is **not** necessary when **not** indicated in the monograph!

Response (2/2)

Pharmaceutical preparations 2619

PRODUCTION

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.

Parenteral preparations 0520

PRODUCTION

During the development of parenteral 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*.

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 general chapter 5.1.1. *Methods of preparation of sterile products*.

- Stricto senso, a microbial quality requirement is not needed to confirm compliance with the sucrose monograph, **but**….
- Specific additional microbial contamination requirements might be asked for, to reach an appropriate grade according to the intended use – as indicated in relevant general monographs

Efficacy of antimicrobial preservation (5.1.3)

5.1.3 Scope

- Aimed at verifying the efficacy of preservatives in pharmaceutical preparations
- Referred to in the Production section of
  - ✓General monograph 2619 Pharmaceutical preparations
  - ✓Other relevant general monographs (e.g. Vaccines, Allergen products, Immunosera)
  - ✓Dosage form monographs (e.g. Parenteral preparations 0520)
- *The test is not intended to be used for routine control purposes.*

Pharmaceutical preparations 2619

PRODUCTION

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*.

- 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*.

Parenteral preparations (0520)

PRODUCTION

During the development of parenteral 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*.

During the development of parenteral 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.

5.1.3 The steps of the test

The efficacy of the antimicrobial activity may be demonstrated by the test described below.

- "Challenge" of the sample by inoculation of micro-organisms

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.

- Incubation
- Sampling at different time intervals (the preservative should reduce the level/prevent the proliferation of the micro-organisms)
- Acceptance criteria at each time of testing: fall of the count or "no increase" of the count

5.1.3 Acceptance criteria

In chapter 5.1.3, the criteria for evaluation of antimicrobial activity are given in terms of the $\log_{10}$ reduction of viable micro-organisms

Table 5.1.3-1 Acceptance criteria for parenteral preparations

		Log 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.

Vaccines for human use (general monograph 0153)

During development studies, the effectiveness of the antimicrobial preservative throughout the shelf life 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.

- If neither the A criteria nor the B criteria (in chapter 5.1.3) 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.

Vaccines for human use (general monograph 0153)

Same additional requirements in the general monograph **Vaccines for veterinary use** (0062)

- If neither the A criteria nor the B criteria (in chapter 5.1.3) 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.

FAQ

Frequently asked questions on chapter *5.1.3*
Efficacy of antimicrobial preservation

SECTION CONCERNED	QUESTIONS	ANSWERS
Test for efficacy of antimicrobial preservation	It is recommended to use test micro-organism *Zygosaccharomyces rouxii* if the sugar concentration of the product tested is high. At what sugar concentration is use of this test micro-organism mandatory?	The use of *Zygosaccharomyces rouxii* is given as an example in the chapter. No prescribed concentration is given for oral preparations containing a high concentration of sugar. Therefore a decision on the use of *Zygosaccharomyces rouxii*, taking into account the risk of having osmophilic micro-organisms in your product, has to be made when testing the efficacy of an antimicrobial preservative.
Acceptance criteria Tables 5.1.3.-1/2/3	Can you define the terms "no recovery" and "no increase"?	There is no specification in the Ph. Eur. of what is considered as "an increase": this means that you have to make your own interpretation. This interpretation will depend on the variability of your counting method. *"**No increase**: no increase in number of viable micro-organisms compared to the previous reading"* may be specified as not more than 0.5 $\log_{10}$ higher than the value to which it is compared; however, this is not a specific Ph. Eur. requirement. **No recovery**, may be specified at LOQ level, e.g. <10 CFU/mL or g, as this interpretation of "no recovery" would be consistent with the prescribed method.

Latest update: October 2023

FAQ

Frequently asked questions on chapter *5.1.3*

Can you define the terms “no recovery” and “no increase”

There is no specification in the Ph. Eur. of what is considered as “an increase”: this means that you have to make your own interpretation. This interpretation will depend on the variability of your counting method.

“No increase”: no increase in number of viable micro-organisms compared to the previous reading may be specified as not more than 0.5 $\log_{10}$ higher than the value to which it is compared; however, this is not a specific Ph. Eur. requirement.

Slido

Question: *In order to fulfil the A criteria, 3 log reductions for bacteria at 24 hours should be achieved. Can a reduction of 2.8 log_{10} be rounded up to 3 log_{10} and therefore be considered acceptable? Can I still release my product which such result?*

✓Yes

✓No

Correct answer in green!

Response (Part of EDQM FAQs)

- The test is not aimed at releasing a lot, it is carried out during development of the product
- "Strictly speaking, logarithmic values should not be rounded.
- We recommend you to approach this problem on a case-by-case basis, a specific borderline result might be considered acceptable when taking into account the preservative efficacy test as a whole and the precision of the method. As part of a laboratory investigation, you may repeat testing and avoid reacting on a single potentially faulty figure."

Module 4 Agenda

Microbiology chapters

Emmanuelle Charton and Thuy Bourgeois, EDQM, Council of Europe

Rapid microbiological methods and Mycoplasmas

Solène Le Maux and Thuy Bourgeois, EDQM, Council of Europe

Pyrogenicity (BET, rFC, MAT, pyrogenicity strategy)

Gwenaël Cirefice, EDQM, Council of Europe

Rapid microbiological methods and Mycoplasmas

Ph. Eur. Microbiology Chapters

2.6. Biological tests

2.6.1*	Sterility
2.6.2	Mycobacteria
2.6.7	Mycoplasmas
2.6.8	Pyrogens
2.6.12*	Microbiological examination of non-sterile products: microbial enumeration tests
2.6.13*	Microbiological examination of non-sterile products: test for specified micro-organisms
2.6.14*	Bacterial endotoxins
2.6.16	Tests for extraneous agents in viral vaccines for human use
2.6.27	Microbiological examination of cell-based preparations
2.6.30	Monocyte-activation test
2.6.31	Microbiological examination of herbal products and extracts
2.6.32	Test for bacterial endotoxins using recombinant factor C
2.6.36	Microbiological examination of live biotherapeutic products: tests for enumeration of microbial contaminants
2.6.37	Principles for the detection of extraneous viruses in immunological veterinary medicinal products using culture methods
2.6.38	Microbiological examination of live biotherapeutic products: tests for specified micro-organisms
2.6.39	Microbiological examination of human tissues
2.6.40	Monocyte-activation test for vaccines containing inherently pyrogenic components

2.7. Biological assay

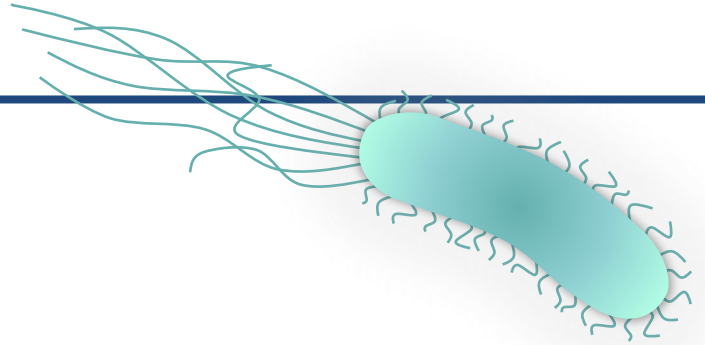
2.7.2	Microbiological assay of antibiotics

5.1. General texts on microbiology

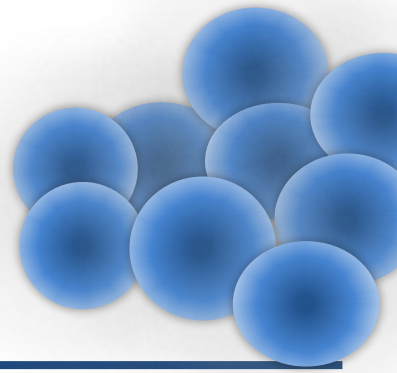
5.1.1	Methods of preparation of sterile products
5.1.2	Biological indicators and related microbial preparations used in the manufacture of sterile products
5.1.3	Efficacy of antimicrobial preservation
5.1.4*	Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use
5.1.5	Application of the F0 concept to steam sterilisation of aqueous preparations
5.1.6	Alternative methods for control of microbiological quality
5.1.7	Viral safety
5.1.8	Microbiological quality of herbal medicinal products for oral use and extracts used in their preparation
5.1.9	Guidelines for using the test for sterility
5.1.10	Guidelines for using the test for bacterial endotoxins
5.1.11	Determination of bactericidal, fungicidal or yeasticidal activity of antiseptic medicinal products

Non exhaustive list

*internationally harmonised chapters



Rapid microbiological methods in the Ph. Eur.



5.1.6. Alternative methods for control of microbiological quality

Objective: Facilitate the implementation and use of alternative microbiological methods where this can lead to cost-effective microbiological control and improved assurance for the quality of pharmaceutical products

Definition of alternative analytical procedure in General Notices:

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

- ✓ Users' responsibility to demonstrate comparability to the satisfaction of the *competent authority*
- ✓ Compliance required, but alternative procedures may be used: same pass/fail decision
- ✓ The pharmacopoeial procedure remains the reference procedure

5.1.6. Alternative methods for control of microbiological quality

Objective: Facilitate the implementation and use of alternative microbiological methods where this can lead to cost-effective microbiological control and improved assurance for the quality of pharmaceutical products

Introduction

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

General principles of alternative microbiological methods

- 3 categories
- Basic principles of methods, critical aspects and potential uses of methods which have successfully been used in the QC of pharmaceuticals

- **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

- No recommendation of one method over another
- Not an exclusive or exhaustive list
- Other methods may be applicable

5.1.6. Alternative methods for control of microbiological quality

Information

Objective: Facilitate the implementation and use of alternative microbiological methods where this can lead to cost-effective microbiological control and improved assurance for the quality of pharmaceutical products

Introduction

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

General principles of alternative microbiological methods

- 3 categories
- Basic principles of methods, critical aspects and potential uses of methods which have successfully been used in the QC of pharmaceuticals

Guidance on how to implement alternative microbiological methods

Selection of the method

Equipment qualification

Two levels of validation

Primary validation
Not product specific

Validation for the intended use
Use in routine, for the product

Equivalence testing

Selection of the method

Essential to understand and define what the procedure is intended to achieve in order to select the method

- Presence/absence, number, viability and/or identity of micro-organisms
- Measure obtained
  - in traditional method: total number and viability indicated by the number of colonies
  - other parameters used as viability measure: e.g. the level of ATP, the accumulation or metabolism of substrates in living cells.

Results from different viability-indicating methods not always identical

- micro-organisms may not be able to reproduce on a given medium, but may still accumulate and metabolise a substrate
- micro-organisms may be unable, at a given state of damage, to accumulate a substrate, but may still be able to recover and reproduce

Selection of the method

Essential to understand and define what the procedure is intended to achieve in order to select the method

- Description of the technique
  - Principle of detection clearly described SUPPLIERS
  - Method must be fully detailed SUPPLIERS
  - Review of these information, **by the user**, to select the method
- Risk-benefit analysis
  - Information obtained by, and the limitations of, the pharmacopoeial method and the alternative method must be considered and compared in a risk-benefit analysis SUPPLIERS
  - 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

SUPPLIERS As part of commercialisation, the supplier may list advantages of the alternative method over conventional techniques.

Equipment qualification

Where specific equipment is critical for the application of a method, the equipment, including computer hardware and software, must be fully qualified

- user requirement specification (URS)
- design qualification (DQ)
- installation qualification (IQ) SUPPLIERS
- operational qualification (OQ) SUPPLIERS
- performance qualification (PQ)
  - ➢verification of primary validation data given by the supplier
  - ➢verification for the intended use (e.g. sterility testing, TAMC/TYMC, …)

Typically done with a panel of micro-organisms (e.g. pharmacopoeial test strains, in-house isolates or stressed/slow-growing micro-organisms)

SUPPLIERS IQ/OQ can be often outsourced to supplier.

SLIDO

- **Who can perform the primary validation?**

✓Who can perform the primary validation?
✓Equipment supplier
✓User
✓I don’t know

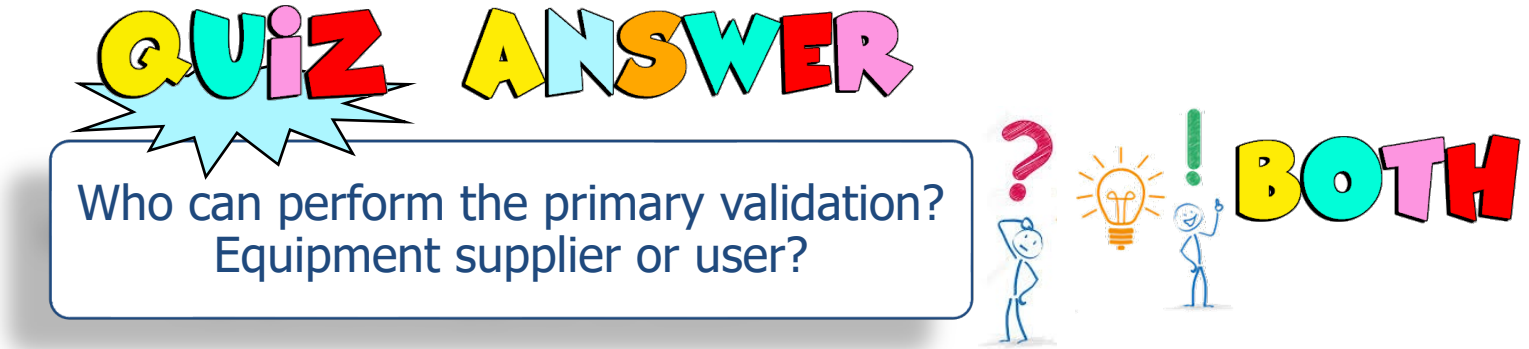
Correct answers in green!

Primary validation

Principle of detection characterisation – not product specific

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



SUPPLIERS

The user performs primary validation if they employ the equipment for a use other than that defined by the supplier.

Validation for the intended use

Experimentally established by the user that the performance characteristics of the method meet the requirements of the intended application – use in routine, product specific

Criteria	Qualitative test	Quantitative test	Identification test
Specificity	+	+	+
Accuracy	+[1]	+	+
Precision	-	+	-
Detection limit	+	-[2]	-
Quantitation limit	-	+	-
Linearity	-	+	-
Range	-	+	-
Robustness	+	+	+
Suitability testing	+	+	-

(1) Performing an accuracy test of the alternate method with respect to the compendial method can be used instead of the validation of the limit of detection test.
(2) May be needed in some cases

Equivalence testing

To demonstrate that the alternative method is equivalent to the official method

To the satisfaction of the competent authorities

Criterion	Qualitative test	Quantitative test	Identification test
Equivalence testing	+	+	-

Can be conducted:

- directly on the validation parameters (sufficient numbers of replicates for relevant strains of test micro-organisms are required)
- parallel testing of samples for a predefined period of time or a predefined number of samples

→ Same pass/fail decision

Examples of validation protocols

- 3 examples of alternative methods developed, validated and used by various laboratories
  - rapid sterility test based on membrane filtration
  - quantitative test for the enumeration of micro-organisms using solid phase cytometry
  - a molecular-based microbial identification method
- Support to the users on what may be performed during the validation of an alternative microbiological methods as described in chapter *5.1.6*
- Not intended to be a compilation of all available equipment used for alternative microbiological methods on the market

Access to example booklet:
https://pharmeuropa.edqm.eu/media/homepage/documents/2020/01/09/brochure-pheur-2018.pdf

Reference to 5.1.6 and ARMM in the Ph. Eur.

Ph. Eur. texts referencing 5.1.6

2.6.27	Microbiological examination of cell-based preparations
2.6.36	Microbiological examination of live biotherapeutic products: tests for enumeration of microbial contaminants
2.6.39	Microbiological examination of human tissues
5.25	Process analytical technology
2537	3-O-Desacyl-4′-monophosphoryl lipid A
5.27	**Comparability of alternative analytical procedures**
2.7.24	*Flow cytometry*
5.1.9	*Guidelines for using the test for sterility*

NEW!

PROPOSAL

Revision on-going

General update of the chapter to reflect the techniques currently in use and update of the validation guidance, incl.

- Update the methods description
- Clarify supplier and user responsibilities
- Clarify guidance and complement the information

REVISION

Ph. Eur. texts referencing ARMM

2.6.12*	Microbiological examination of non-sterile products: microbial enumeration tests
2.6.13*	Microbiological examination of non-sterile products: test for specified micro-organisms
2.6.31	Microbiological examination of herbal products and extracts
2.6.36	Microbiological examination of live biotherapeutic products: tests for enumeration of microbial contaminants
2.6.38	Microbiological examination of live biotherapeutic products: tests for specified micro-organisms

"**Alternative microbiological procedures,** including **automated methods**, may be used, provided that their equivalence to the Pharmacopoeia method has been demonstrated."

Supplement 11.5 (published in January 2024)
Pharmeuropa 35.4 (public deadline: 31 December 2023)
Pharmeuropa 36.1 (public deadline: 31 March 2024)

Non exhaustive list

*internationally harmonised chapters
ARMM: alternative rapid microbiological method

5.1.9 Guidelines for using the test for sterility

Non-mandatory guidelines for using the test for sterility 2.6.1

- Precautions against microbial contamination
- Guidance to manufacturers:
  - ➢ importance of homogeneity of the batch, conditions of manufacture and appropriate sampling plan
  - ➢ case of aseptic production
- Observation and interpretation of results
- Only referenced in 2.6.1 for information

Revision on-going

General update of the chapter to reflect the use of alternative sterility methods

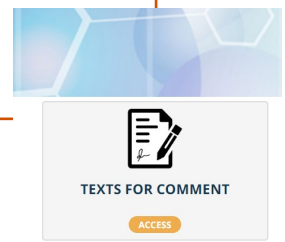
2.6.1 Sterility

- Official sterility test
- Harmonised chapter, see chapter *5.8*
- Visual detection of micro-organisms
  - ➢ Membrane filtration
  - ➢ Direct inoculation
  - ➢ Incubation for at least 14 days

Pharmeuropa 36.1

Commenting period from January 2024:

- to 31 March 2024 for the public
- to 31 May 2024 for the NPA



5.1.9 Guidelines for using the test for sterility

Proposal

CURRENT TEXT

A manufacturer is neither obliged to carry out such tests nor precluded from using modifications of, or **alternatives** to, the stated method, provided he is satisfied that, if tested by the official method, the material in question would comply with the requirements of the European Pharmacopoeia.

REVISED DRAFT

Indication of the possibility to use the official method, *2.6.1 Sterility*, or an alternative method in accordance with the principles provided in Chapter *5.1.6 Alternative methods for control of microbiological quality*.

REVISION

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. […] In this rapidly developing field, **other methods** are likely to appear and the guidance offered herein **may be equally applicable** in these cases.

Ph. Eur. 5.1.6

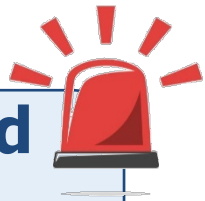
2.6.27 Microbiological examination of cell-based preparations

Outlines approaches to microbiological examination of cell-based preparations

Ph. Eur. texts referencing 2.6.27

- *2323 Human haematopoietic stem cells*
- *5.14 Gene transfer medicinal products for human use*
- *2.6.39 Microbiological examination of human tissues*
- Proposed to be referred in new texts

- Chapter 2.6.1 Sterility required but might not be performed for technical reasons or due to the characteristics of the specific cell-based preparation
- Method selection must be
  - based on the **characteristics** of the final preparation and the manufacturing process
  - supported by a **risk analysis**



Specificity of cell-based preparations

- Limited shelf life - often cannot be cryopreserved
- Microbial contaminants may be found either inside or on the surface of cells or other components of the cell-based preparation → importance of representative sample to detect contamination
- Small batch size - limited sample volume
- Need for short analytical lead time - "Wait time" critical for patient

ARMM: alternative rapid microbiological method

2.6.27 Microbiological examination of cell-based preparations

Outline approaches to microbiological examination of cell-based preparations

The following approaches to microbiological examination may be applied:

- methods based on the sterility test prescribed in general chapter 2.6.1
- automated growth-based method described in 2.6.27
- a combination of preculturing and detection by alternative methods (5.1.6)
- direct detection by alternative methods (5.1.6)

'Negative-to-date' readout

- *Understood as an intermediate reading of a test method that has not yet been completed*
- *When a preparation with a very short shelf life and when justified, 'negative-to-date' results may be used as the readout*

2.6.27 Microbiological examination of cell-based preparations

Outline approaches to microbiological examination of cell-based preparations

The following approaches to microbiological examination may be applied:

- methods based on the sterility test prescribed in general chapter 2.6.1
- automated growth-based method described in 2.6.27
- a combination of preculturing and detection by alternative methods (5.1.6)
- direct detection by alternative methods (5.1.6)

'Negative-to-date' readout

Automated growth-based method

Growth promotion test

- Confirming the **suitability** of the culture **media** used for microbiological examination
- Each batch tested

Method suitability

- **Suitability** of the method to be confirmed **in the presence of test sample**
- For a **validated** automated growth-based method, only a confirmation of the suitability of the method for the given cell-based preparation must be performed

Testing of the preparation to be examined

- Inoculation volume
- Incubation: at least 7 days, up to 14 days
- Inoculation temperature

Observation and interpretation of results

- Media examined visually or with automated systems
- At least daily and at the end of the observation period for evidence of microbial growth

2.6.27 Microbiological examination of cell-based preparations

Outline approaches to microbiological examination of cell-based preparations

The following approaches to microbiological examination may be applied:
- methods based on the sterility test prescribed in general chapter 2.6.1
- automated [illegible] method described in 2.6.27
- a co[illegible]n of preculturing [illegible] detection by alternative methods (5.1.6)
- [illegible] detection by alternative met[illegible]ds (5.1.6)

'Negative-to-date' readout

Automated growth-based method

Growth promotion test
- Confirming the **suitability** of the culture **media** used for microbiological examination
Each batch tested

Method suitability
- **Suitability** of the method to be c[illegible]firmed **in the presence of test sample**
- For a **validated** automated grow[illegible]-based method, only a confirmation [illegible]f the suitability of the method for the [illegible] cell-based preparation must be perf[illegible]

Testing of the preparation to be examined
- Inoculation volume
- Incubation: at least 7 days, up to 14 days
- Inoculation [illegible]nperature

Observation and interpretation of results
- Media examined visually or with automated systems
- At least daily and at the end of the observation period for evidence of microbial growth

SLIDO

- **Can I use the same culture conditions for the growth promotion test and for the method suitability?**

  ✓ Yes
  ✓ No
  ✓ I don’t know

Correct answer in green!

2.6.27 Microbiological examination of cell-based preparations

Growth promotion test

Aim: to confirm the suitability of the culture media used for microbiological examination

- Each media batch tested
- To ensure media sensitivity
- Insufficient sensitivity could increase the risk of not detecting a contaminant
- If growth medium doesn't meet the release criteria → not sensitive enough

Culture conditions

- Test at least 2 suitable culture media
- **List of microorganisms given**
  - **Indicator culture to confirm conditions**
- Inoculation: not more than 100 CFU of each of the strains listed
  - Sensitivity
- **Incubation for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi**
- Other conditions as defined for testing (incl. temperature)

Outcome

- Test media are satisfactory if there is clear evidence of growth in all inoculated media containers

To ensure sensitivity of the media
Harmonised with chapter 2.6.1. Sterility

Method suitability

Aim: to confirm the suitability of the method in presence of test sample

- For a validated automated growth-based method, only a confirmation of the suitability of the method for the given cell-based preparation must be performed with respect to specificity (absence of false positive results), sensitivity, reproducibility and robustness

Culture conditions

- Presence of test sample
- Test at least 3 replicates
  - Reproducibility
- **List of microorganisms given:**
  - **list of the most common contaminants - to be adapted** depending on origin of cells and any micro-organisms previously found or associated with the particular type of cells
- Inoculation: not more than 100 CFU of each of the strains listed
  - Sensitivity
- **Incubation for at least 7 days, up to 14 days**
- Other conditions as defined for testing (incl. temperature)

Outcome

- Method is suitable for the intended test sample if between 1 and 100 CFU are detected for each strain

Supplement 11.5 (published in January 2024, implementation date 1 July 2024)

Rapid microbiological methods in the Ph. Eur.

The Ph. Eur. facilitates the use of rapid methods

Revisions (5.1.6 and 5.1.9): Additional support from the Ph. Eur. to facilitate the implementation of ARMM

2.6.7 MYCOPLASMAS

Examples of validation protocols booklet
edited in 2018

5.1.6 Alternative methods for control of microbiological quality
first publication in Supplement 5.5 (published in January 2006)

2.6.27 Microbiological examination of cell-based preparations
first publication in Supplement 5.6 (published in July 2006)

Reference to these alternative methods in a number of texts

Mycoplasmas

Mycoplasmas (2.6.7)

CURRENT TEXT

Indication on the method(s) to be applied to production stages

Methods description:

- ✓ Culture method
- ✓ Indicator cell culture method
- ✓ Nucleic acid amplification techniques (NAT)

Guidelines for Mycoplasma NAT validation

53 texts prescribing for the mycoplasma test in the Ph. Eur.

PROPOSAL

ON-GOING REVISION

Extensive revision in order to reflect the current knowledge and practices in the field of mycoplasma testing

Pharmeuropa 34.2 (Apr 2022)

Pharmeuropa 36.1 (Jan 2024)

Mycoplasmas (2.6.7)

PROPOSAL

CURRENT TEXT

Indication on the method(s) to be applied to production stages

Methods description:

- ✓ Culture method
- ✓ Indicator cell culture method
- ✓ Nucleic acid amplification techniques (NAT)

Guidelines for Mycoplasma NAT validation

ON-GOING REVISION

Major revison

Introduction section: overarching information applicable to the rest of the chapter (e.g. control strategy based on a risk assessment, sample should contain cells and supernatant.)

Methods description:

- ✓ Culture method
- ✓ Indicator cell culture method
- ✓ Nucleic acid amplification techniques (NAT)

Major revison

Guidelines for Mycoplasma NAT validation (e.g. strains selection / characterisation, acceptance criterion for the GC/CFU ratio)

On-going revision of Ph. Eur. texts related to Mycoplasma project

Chapter 2.6.7

Human Vaccines and Sera:

1107 . Hepatitis A vaccine (inactivated, adsorbed)
0158 . Influenza vaccine (split virion, inactivated)
0159. Influenza vaccine (whole virion, inactivated)
0869. Influenza vaccine (surface antigen, inactivated)
2053. Influenza vaccine (surface antigen, inactivated, virosome)
2149. Influenza vaccine (surface antigen, inactivated, prepared in cell cultures)
0214. Poliomyelitis vaccine (inactivated)
0164. Smallpox vaccine (live)
1375. Tick-borne encephalitis vaccine (inactivated)
0537. Yellow fever vaccine (live)

Veterinary Vaccines and Sera:

0062. Vaccines for veterinary use

Pharmeuropa 36.1

Commenting period from January 2024:

- to 31 March 2024 for the public
- to 31 May 2024 for the National Pharmacopoeia Authority

Mycoplasmas (2.6.7)

Question: Chapter 2.6.7 Mycoplasmas requires 10 mL of sample into 100 mL of each liquid medium for the culture method. However, only a small volume is available. Which options can be proceeded?

Responses:

- ✓ Using 1 mL of sample but keeping the same dilution (1 mL in 10 mL of each liquid medium)
- ✓ Using an alternative NAT method
- ✓ Using an alternative method per the General Notices
- ✓ I do not know

Correct answers in green!

Mycoplasmas (2.6.7) QUIZ ANSWER

CULTURE METHOD

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.

Question: Chapter 2.6.7 Mycoplasmas requires 10 mL of sample into 100 mL of each liquid medium for the culture method. However, only a small volume is available. Which options can be proceeded?

Using a smaller volume could affect the limit of detection.

The claimed sensitivity of the culture method using 10 mL of sample is 10 CFU/mL. If 1 mL of sample is used, the sensitivity would be 100 CFU/mL, which is considered not appropriate.

In justified cases, a smaller sample volume may be used if authorised by the competent authority.

NAT as an alternative method may be used where only a small sample volume is available.

Mycoplasmas (2.6.7)

Question: In the culture method, the solid media are incubated under microaerophilic condition (nitrogen containing 5-10 per cent of carbon dioxide and sufficient humidity to prevent desiccation of the agar surface). How is the oxygen concentration allowed in the Ph. Eur.?

Responses:

- ✓ A low but undefined oxygen content
- ✓ < 0.5%
- ✓ < 5%
- ✓ I do not know

Correct answers in green!

Mycoplasmas (2.6.7) QUIZ ANSWER

CULTURE METHOD

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.

Question: In the culture method, it is described that the solid media are incubated under microaerophilic condition (nitrogen containing 5-10 per cent of carbon dioxide and sufficient humidity to prevent desiccation of the agar surface). How is the oxygen concentration allowed in the Ph. Eur.?

Microaerophilic condition means any oxygen concentration lower than the atmospheric oxygen level. In the mycoplasma test, it indicates a mixture of nitrogen and carbon dioxide, which implies a low but undefined oxygen content.

Mycoplasmas (2.6.7)

The Ph. Eur. facilitates the use of rapid methods

2.6.7 MYCOPLASMAS

Module 4 Agenda

Microbiology chapters

Emmanuelle Charton and Thuy Bourgeois, EDQM, Council of Europe

Rapid microbiological methods and Mycoplasmas

Solène Le Maux and Thuy Bourgeois, EDQM, Council of Europe

Pyrogenicity (BET, rFC, MAT, pyrogenicity strategy)

Gwenaël Cirefice, EDQM, Council of Europe

Endotoxin and Pyrogen testing

Ph. Eur. Microbiology Chapters

2.6. Biological tests

2.6.1*	Sterility
2.6.2	Mycobacteria
2.6.7	Mycoplasmas
2.6.8	Pyrogens
2.6.12*	Microbiological examination of non-sterile products: microbial enumeration tests
2.6.13*	Microbiological examination of non-sterile products: test for specified micro-organisms
2.6.14*	Bacterial endotoxins
2.6.16	Tests for extraneous agents in viral vaccines for human use
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2.6.30	Monocyte-activation test
2.6.31	Microbiological examination of herbal products and extracts
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2.6.37	Principles for the detection of extraneous viruses in immunological veterinary medicinal products using culture methods
2.6.38	Microbiological examination of live biotherapeutic products: tests for specified micro-organisms
2.6.39	Microbiological examination of human tissues
2.6.40	Monocyte-activation test for vaccines containing inherently pyrogenic components

2.7. Biological assay

2.7.2	Microbiological assay of antibiotics

5.1. General texts on microbiology

5.1.1	Methods of preparation of sterile products
5.1.2	Biological indicators and related microbial preparations used in the manufacture of sterile products
5.1.3	Efficacy of antimicrobial preservation
5.1.4*	Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use
5.1.5	Application of the F0 concept to steam sterilisation of aqueous preparations
5.1.6	Alternative methods for control of microbiological quality
5.1.7	Viral safety
5.1.8	Microbiological quality of herbal medicinal products for oral use and extracts used in their preparation
5.1.9	Guidelines for using the test for sterility
5.1.10	Guidelines for using the test for bacterial endotoxins
5.1.11	Determination of bactericidal, fungicidal or yeasticidal activity of antiseptic medicinal products

Non exhaustive list

*internationally harmonised chapters

Assays for pyrogens / endotoxins in the Ph. Eur.

1971

Pyrogens (2.6.8)
(*"Rabbit Pyrogen Test"*)

Pyrogen detection

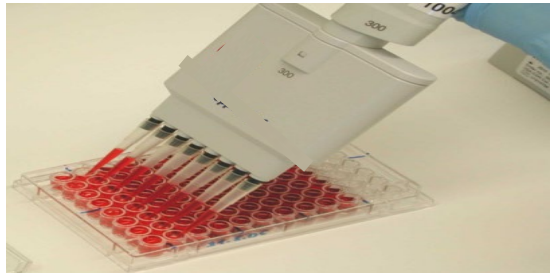
1987

LAL is a lyophilised amoebocyte lysate obtained from the horseshoe crab (*L. polyphemus* or *T. tridentatus*)

BET (2.6.14) &
Guidelines for using the BET (5.1.10)

Endotoxin detection

2010



Monocyte-activation test (2.6.30)

- *MAT for vaccines containing inherently pyrogenic components (2.6.40) [NEW]*

Pyrogen detection

2020

BET using recombinant Factor C (2.6.32)

Endotoxin detection

2.6.14 Bacterial endotoxins

(General Chapter harmonised with JP and USP, see Q4B Annex 14)

Test for bacterial endotoxins (BET)

- To detect or quantify endotoxins from gram-negative bacteria
- Uses amoebocyte lysate from the horseshoe crab ("LAL" reagent)
- Principle: cascade reaction of LAL in the presence of endotoxin.
- 3 techniques:
  - Gel-clot (gel formation)
  - Turbidimetric (development of turbidity after cleavage of a substrate)
  - Chromogenic (development of colour after cleavage of a substrate)

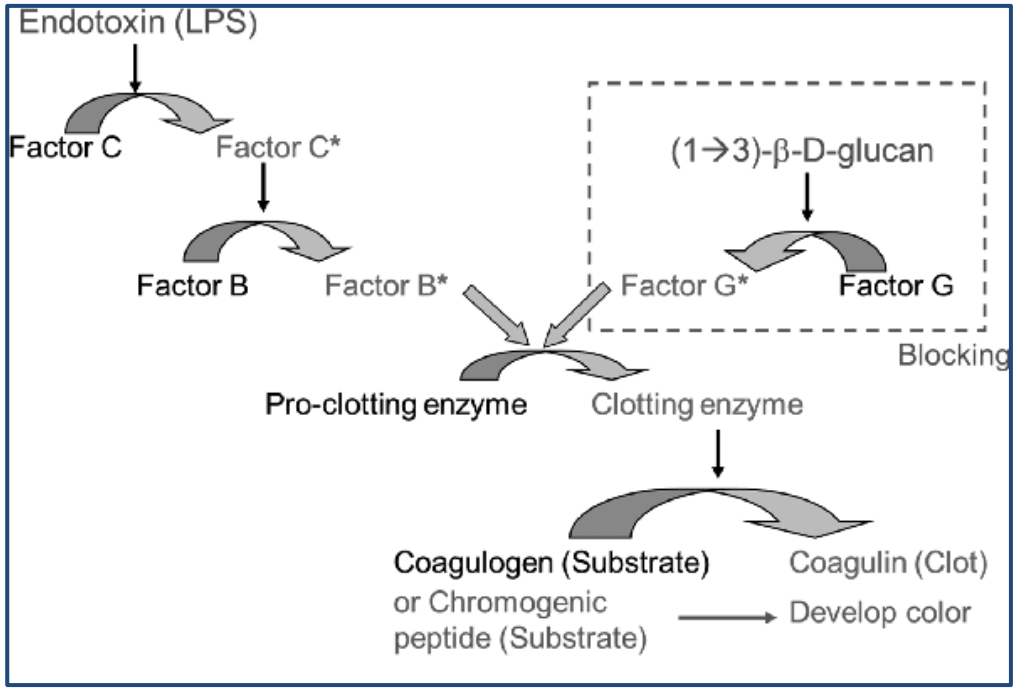


Figure: LAL cascade of endotoxin detection. *Source: JH Park, J Environ Health Sci, 2014; 40(4): 265-278*

Test for bacterial endotoxins (BET)

→ 6 methods are described in chapter 2.6.14:

- Method A. Gel-clot method: limit test
- Method B. Gel-clot method: semi-quantitative test

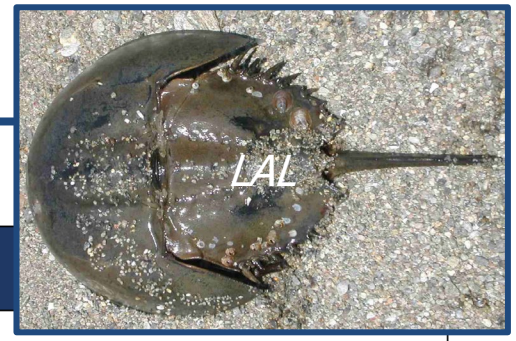
Gel-clot technique

- Method C. Turbidimetric kinetic method
- Method D. Chromogenic kinetic method
- Method E. Chromogenic end-point method
- Method F. Turbidimetric end-point method

Photometric quantitative techniques

"*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.*"

2.6.14 BET… and 5.1.10 Guidelines for using the BET



Chapter 2.6.14 is to be read in conjunction with chapter 5.1.10 Guidelines for using the BET

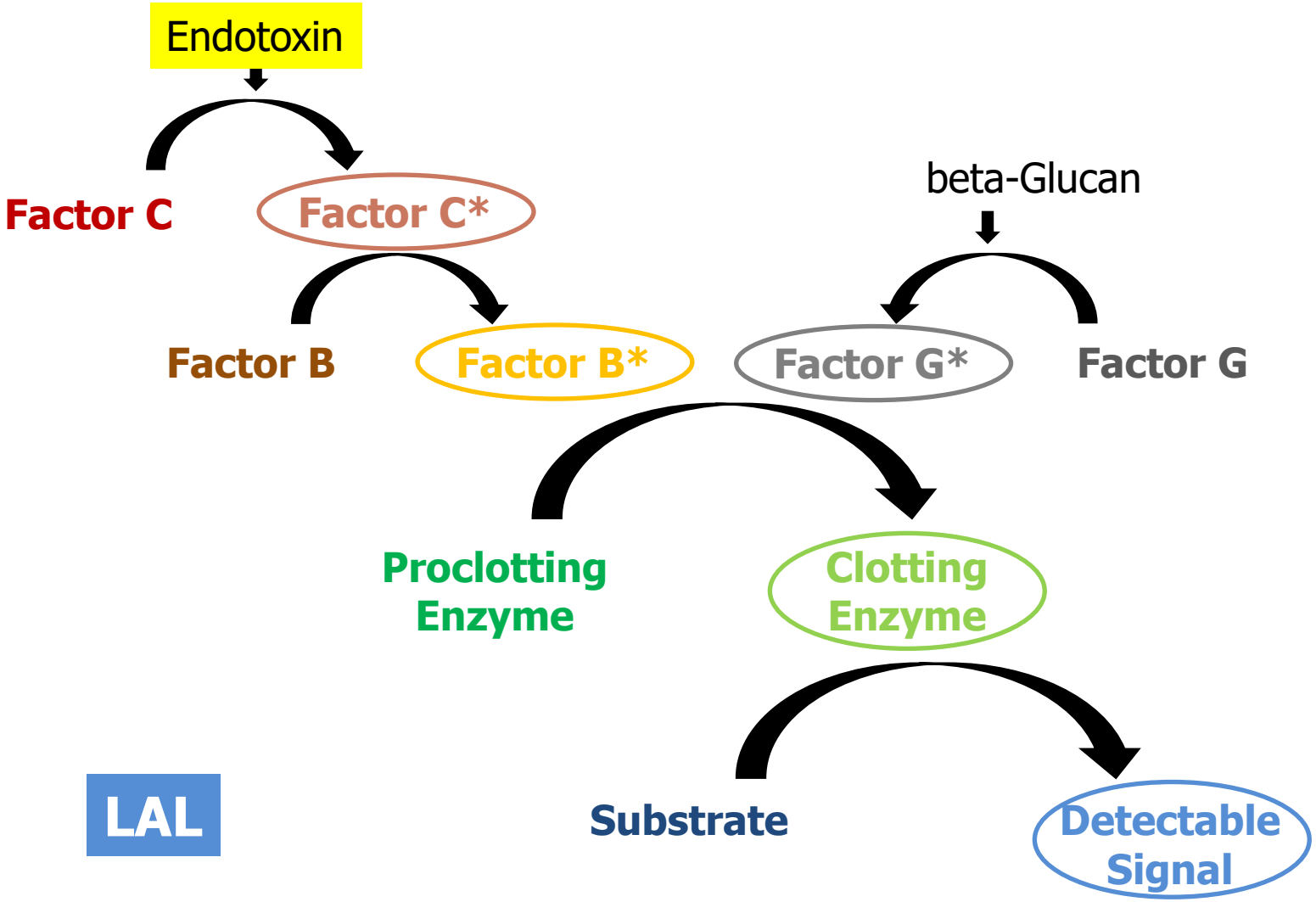
2.6.14. BACTERIAL ENDOTOXINS	
1. APPARATUS	
2. REAGENTS	
3. PREPARATION OF THE STANDARD ENDOTOXIN STOCK SOLUTION	
4. PREPARATION OF THE STANDARD ENDOTOXIN SOLUTIONS	
5. PREPARATION OF THE TEST SOLUTIONS	
6. DETERMINATION OF THE MAXIMUM VALID DILUTION	
7. GEL-CLOT TECHNIQUE • *PREPARATORY TESTING* - Confirmation of the labelled lysate sensitivity - Test for interfering factors • *LIMIT TEST (METHOD A)* - Procedure - Interpretation • *QUANTITATIVE TEST (METHOD B)* - Procedure - Calculation and interpretation	8. PHOTOMETRIC QUANTITATIVE TECHNIQUES • *TURBIDIMETRIC TECHNIQUE (METHODS C AND F)* • *CHROMOGENIC TECHNIQUE (METHODS D AND E)* • *PREPARATORY TESTING* - Assurance of criteria for the standard curve - Test for interfering factors • *TEST* - Procedure - Calculation - Interpretation

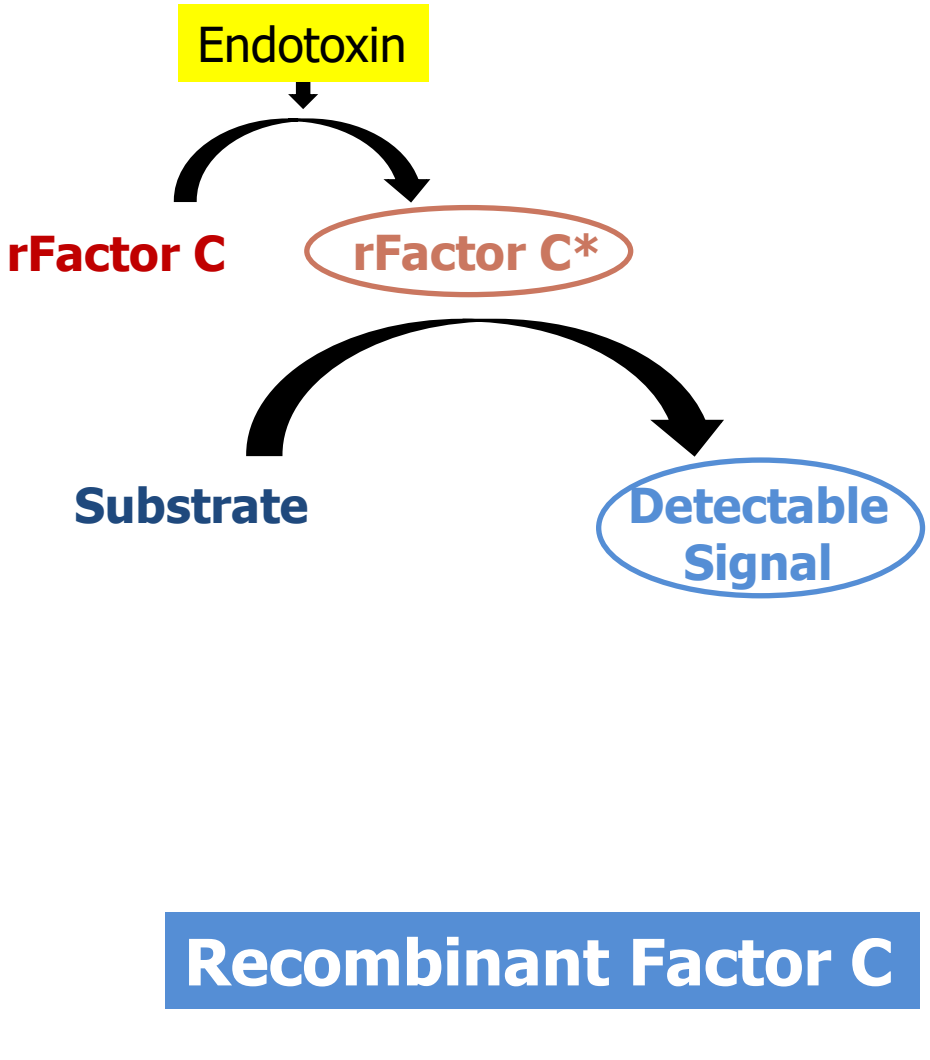
5.1.10 GUIDELNES FOR USING THE BET
1. INTRODUCTION
2. METHOD AND ACCEPTANCE CRITERIA *2-1. METHODS AND PRECAUTIONS TO BE TAKEN* *2-2. ENDOTOXIN LIMIT CONCENTRATION* *2-3. CALCULATION OF THE ENDOTOXIN LIMIT* *2-4. CONSIDERATIONS WHEN ESTABLISHING AN ENDOTOXIN LIMIT FOR A SPECIFIC SUBSTANCE OR PRODUCT* *2-5. MAXIMUM VALID DILUTION*
3. RISK ASSESSMENT
4. REFERENCE MATERIAL
5. WATER FOR BET
6. pH OF THE MIXTURE
7. VALIDATION OF THE LYSATE
8. PRELIMINARY TEST FOR INTERFERING FACTORS
9. REMOVAL OF INTERFERING FACTORS
10. THE PURPOSE OF THE CONTROLS
11. READING AND INTERPRETATION OF RESULTS
12. IMPLEMENTATION OF METHODS DESCRIBED IN THE PH. EUR.
13. REPLACEMENT OF A METHOD PRESCRIBED IN A MONOGRAPH *13-1. BY ANOTHER METHOD DESCRIBED IN THE PH. EUR.* *13-2. BY AN ALTERNATIVE METHOD NOT DESCRIBED IN THE PH. EUR.*

Chapter 5.1.10:
- Explains the reason for requirements in 2.6.14
- Deals with reading and interpretation of results

2.6.32 Recombinant Factor C

LAL vs rFC





2.6.32 BET using recombinant Factor C

01/2021:20632
corrected 11.0

2.6.32. TEST FOR BACTERIAL ENDOTOXINS USING RECOMBINANT FACTOR C

The test for bacterial endotoxins using recombinant factor C (rFC) is carried out to quantify endotoxins from gram-negative bacteria. It is performed using rFC based on the gene sequence of the horseshoe crab (*Limulus polyphemus, Tachypleus tridentatus, Tachypleus gigas* or *Carcinoscorpius rotundicauda*), using a fluorimetric method.

The test is carried out in a manner that avoids bacterial endotoxin contamination.

1. EQUIPMENT

Depyrogenate all glassware and other heat-stable equipment in a dry-heat oven using a validated process. A commonly used minimum time and temperature is 30 min at 250 °C. Where plastic equipment (such as microplates and pipette tips for automatic pipettes) is employed, it must be shown to be free of detectable endotoxin and not to interfere with the test.

2. REAGENTS

Reagents

Recombinant factor C is based on the gene sequence of the horseshoe crab (*Limulus polyphemus, Tachypleus tridentatus, Tachypleus gigas* or *Carcinoscorpius rotundicauda*). All reagents, including the fluorogenic substrate and assay buffer, must be free of detectable endotoxin.

- General chapter 2.6.32 published in 2020 (Supplement 10.3), implemented on 1 January 2021
- Recognised as an official method by the 39 member states of the Ph. Eur. and the EU
- Describes a BET that uses a rFC based on the gene sequence of the horseshoe crab, and a fluorimetric end-point detection method
- Chapter 2.6.32 was a significant development in a context where the world relies on horseshoe crabs as a single source of reagent

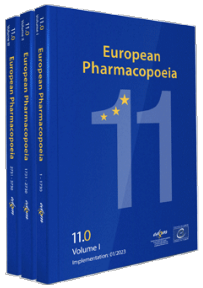
2.6.32 BET using recombinant Factor C

Table of Content

5.1.10 Guidelines for using the BET (revised)

- Revised in 2020 to clarify requirements for the introduction of rFC by users of the Ph. Eur.
- Implication for users of chapter 2.6.32: facilitated implementation
  - With chapter 2.6.32, rFC assays are described in the Ph. Eur. As a Ph. Eur. method, they don't have to re-validated, other than in consideration of their use for a specific substance or product. → i.e. product-specific validation only
  - *Replacement of BET method prescribed in monograph by an rFC assay is regarded as the use of an alternative method, as per the General Notices.*

Use of chapter *2.6.32* as a replacement for *2.6.14*



Alternative method: demonstration of equivalence as per General notices

Replacement by an official method of the Ph. Eur.?

Yes

No

To replace *2.6.14* prescribed in a monograph by another method

Chapter *2.6.32*

Other method

Minimum required:

Full validation: accuracy, precision, specificity, detection limit, quantitation limit, linearity, range, robustness.

« Chapter 5.1.10 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. »

Ph. Eur. webinar on rFC (April 2021)

- rFC was the subject of a dedicated Ph. Eur. webinar in April 2021
  - Detailed the (long) history of chapter 2.6.32 and rFC in Ph. Eur.
  - Explained in further details how to implement chapter 2.6.32
    - What conditions need to be met?
    - What needs to be verified?
    - Is validation required?
    - What needs to be done when using rFc instead of LAL? What is an alternative method in this context?

- The webinar is available on demand at: https://www.edqm.eu/en/-/webinar-on-using-recombinant-factor-c-for-bacterial-endotoxin-testing-in-the-european-pharmacopoeia-how-far-have-we-come-how-far-have-we-to-go-

NEW! Revision of Water monographs (rFC)

04/2024:0169

WATER FOR INJECTIONS

Aqua ad iniectabile

H_2O

DEFINITION

Water for the prep
administration wh
injections in bulk)
or preparations fo
for injections).

Wate

PRODUCTION

Water for injectio
complies with the
consumption laid
purified water. It i

- by distillation i
with the water
metal and whic
the entrainmen
- by a purificatio
Reverse osmosi
coupled with ot
electro-deionisa

04/2024:0008

WATER, PURIFIED

Aqua purificata

H_2O M_r 18.02

DEFINITION

Water for the preparation of medicines other than those that are required to be both sterile and apyrogenic, unless otherwise justified and authorised.

Purified water in bulk

PRODUCTION

Purified water in bulk is prepared by distillation, by ion exchange, by reverse osmosis or by any other suitable method from water that complies with the regulations on water intended for human consumption laid down by the competent authority.

Purified water in bulk is stored and distributed in conditions designed to prevent growth of micro-organisms and to avoid any other contamination.

Microbiological monitoring. During production and subsequent storage, appropriate measures are taken to ensure that the microbial count is adequately controlled and monitored. Appropriate alert and action levels are set so as to detect adverse trends. Under normal conditions, an appropriate action level is a microbial count of 100 CFU/mL, determined by filtration through a membrane with a nominal pore size not greater than 0.45 µm, using R2A agar and incubating at 30-35 °C for not less than 5 days. The size of the

- Revision of 2 fundamental Ph. Eur. monographs: *Water for injections* (0169) and *Purified water* (0008) to allow the use of rFC to test for endotoxins

→ Implication for users: users can select the test described in 2.6.32 (i.e. rFC) directly when testing pharmaceutical waters, i.e. without a side-by-side comparison against the tests described in chapter 2.6.14 (i.e. LAL)

WATER, PURIFIED

Purified water in bulk

Bacterial endotoxins (*2.6.14* or *2.6.32*): less than 0.25 IU/mL, if intended for use in the manufacture of dialysis solutions without a further appropriate procedure for removal of bacterial endotoxins.

WATER FOR INJECTIONS

Water for injections in bulk

Bacterial endotoxins (*2.6.14* or *2.6.32*): less than 0.25 IU/mL.

Sterilised water for injections

Bacterial endotoxins (*2.6.14* or *2.6.32*): less than 0.25 IU/mL.

- Revised water monographs published in the Ph. Eur. in October 2023 (Supplement 11.4), implementation date: 1 April 2024

rFC: scenario 1

The individual monograph for an API refers to chapter 2.6.14. Can I use rFC instead?

- Yes
- No
- I don’t know

Correct answer in green!

rFC: scenario 1

The individual monograph for an API refers to chapter 2.6.14. Can I use rFC instead?

Yes. Through the reference to chapter 2.6.14, the monograph prescribes the use of LAL. However, alternative methods may be used as per the General Notices. Requirements for the introduction of rFC (case of replacement of LAL prescribed in a monograph) are given in chapter 5.1.10 *Guidelines for using the BET*:

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.

rFC: scenario 2

I would like to use rFC to test water for injections in bulk. Is it a requirement to show its equivalence to LAL?

- Yes
- No
- I don’t know

Correct answer in green!

rFC: scenario 2

I would like to use rFC to test water for injections in bulk. Is it a requirement to show its equivalence to LAL?

No. The revised monograph on *Water for injections* (0169) prescribes the use of *LAL or rFC*. rFC may thus be used directly, i.e. without a side-by-side comparison against LAL as alternative method.

rFC: scenario 3

I would like to use rFC to test a new API, which is not covered by an individual monograph. What are the requirement(s)?

- ○ Full method validation
- ● Product-specific validation
- ● Demonstration of equivalence to LAL
- ○ I don’t know

Correct answers in green!

rFC: scenario 3

I would like to use rFC to test a new API, which is not covered by an individual monograph. What are the requirements?

- The General monograph *Substances for pharmaceutical use* (2034) prescribes the use of LAL (through a reference to chapter 2.6.14). Users would thus have to follow the same requirements as in scenario 1.

2.6.30 Monocyte-Activation Test (MAT)

Monocyte-Activation Test

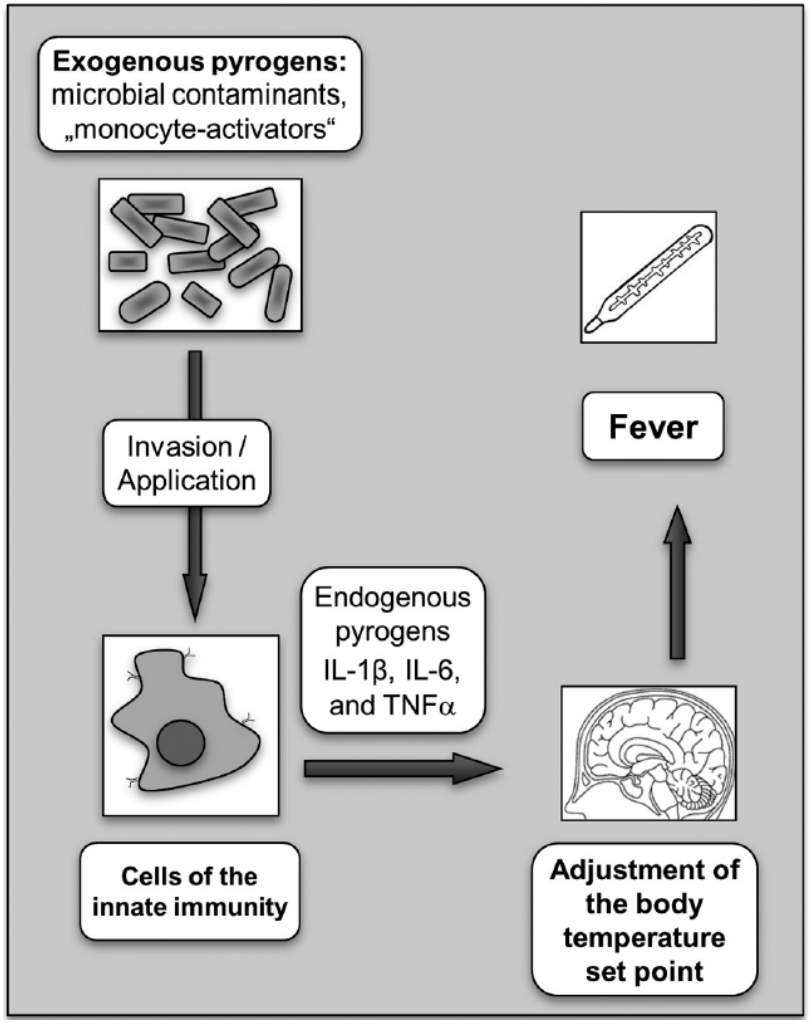


Figure: Human fever reaction.
Source: Hasiwa et al. ALTEX 30, 2/13 2013

- Principle: Upon activation by pyrogens, human monocytes release mediators such as pro-inflammatory cytokines (e.g. IL-6, IL-1β, TNF-α), which are detected in an immunoassay (ELISA)
- Can detect **endotoxin and non-endotoxin pyrogens**
- Based on the human fever response (better prediction of pyrogenic activity in humans)
- Non-animal test

Monocyte-Activation Test

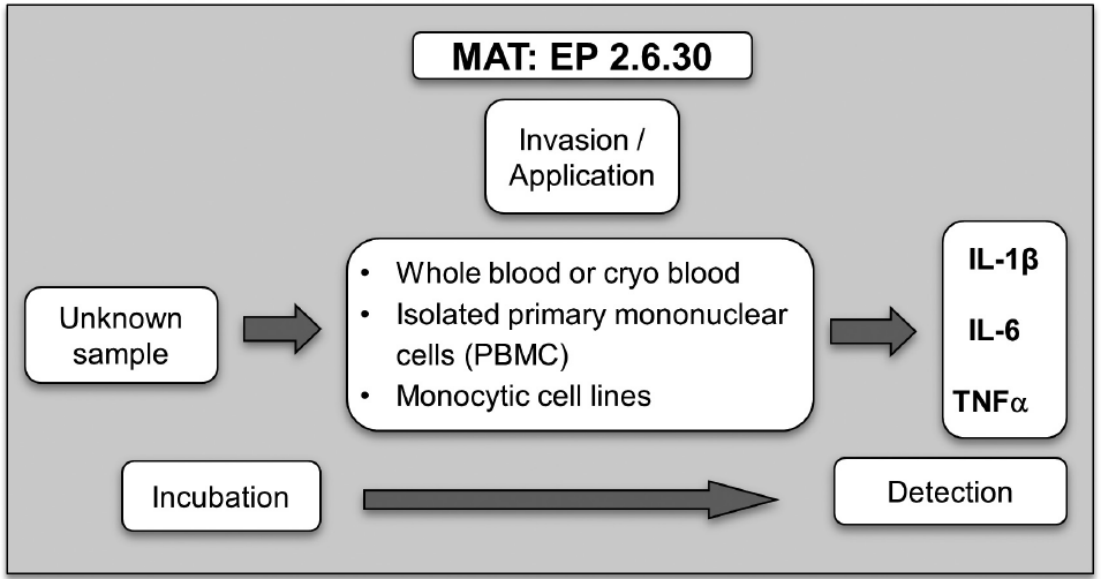


Figure: Principle of MAT.
Source: Hasiwa et al. ALTEX 30, 2/13 2013

- Different variants of MAT depending on:
  - Source of human monocyte: whole blood (fresh or cryopreserved), PBMCs (fresh or cryo-preserved), human monocytic cell line
  - ELISA read-out: IL-6, IL-1β, TNF-α…

- 2 methods described in chapter 2.6.30:

NEW

  - Method 1 (Semi-quantitative test): comparison of the preparation being examined with a standard endotoxin dose-response curve
  - Method 2 (Reference lot comparison test): comparison of the preparation being examined with a validated reference lot of that preparation

Chapter 2.6.30 - Overview

NEW • 07/2024:20630

2.6.30. MONOCYTE-ACTIVATION TEST

1. INTRODUCTION

The monocyte-activation test (MAT) is used to detect substances that activate human monocytes or monocytic cells to release endogenous mediators such as pro-inflammatory cytokines, for example tumour necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6). These cytokines play a role in fever pathogenesis. Therefore, the MAT detects the presence of pyrogens in the test sample.

Pharmaceutical products that contain non-endotoxin pyrogenic or pro-inflammatory contaminants (hereinafter referred to collectively as 'non-endotoxin contaminants') often show steep dose-response curves in comparison with endotoxin dose- curves. Preparations that contain or may contain ts have to be tested at a range of di um dilution.

The followin d in the present general chap

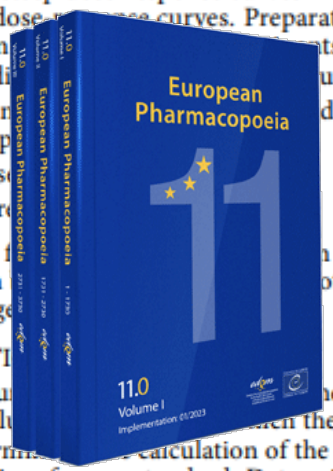
Method 1: s

Method 2: r

In addition, n practical aspects of the tests can otes' section at the end of this ge

2. DEFINITI

The maximu e maximum allowable dil en the contaminant limit can be determ calculation of the MVD is based on the endotoxin reference standard. Determine the MVD using



2.6.30 MONOCYTE-ACTIVATION TEST
1. INTRODUCTION
2. DEFINITIONS
3. GENERAL PROCEDURE
4. EQUIPMENT
5. CELL SOURCES AND QUALIFICATION *5-1. WHOLE BLOOD* *5-2. PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)* *5-3. QUALIFICATION OF BLOOD DONORS* *5-4. QUALIFICATION OF FRESH CELLS* *5-5. QUALIFICATION OF CRYOPRESERVED CELLS* *5-6. MONOCYTIC CONTINUOUS CELL LINES*
6. PREPARATORY TESTING *6-1. ASSURANCE OF CRITERIA FOR THE ENDOTOXIN STANDARD CURVE* *6-2. TEST FOR INTERFERING FACTORS (for method 1)* *6-3. DETERMINATION OF THE OPTIMAL DILUTIONS OF THE TEST AND REFERENCE LOTS (for method 2)* *6-4. INTERFERENCE IN THE DETECTION SYSTEM* *6-5. METHOD VALIDATION FOR THE NON-ENDOTOXIN MONOCYTE-ACTIVATING CONTAMINANTS*
7. METHODS *7-1. METHOD 1: SEMI-QUANTITATIVE TEST* *7-2. METHOD 2: REFERENCE LOT COMPARISON TEST*
Guidance notes

Guidance notes
1. INTRODUCTION
2. METHODS *2-1. INFORMATION REGARDING THE CHOICE OF METHODS* *2-2. EXPRESSION OF CONCENTRATIONS AND PRODUCT DILUTIONS* *2-3. CALCULATION OF CONTAMINANT LIMIT CONCENTRATION* *2-4. INTERFERENCE TESTING* *2-5. CROSS-VALIDATION*

Evolution of Ph. Eur. chapter 2.6.30 MAT

Recommendations of ECVAM Workshop 43 (2001)

First version published in 2010, (*Supplement 6.7*)

EDQM survey (2013)
on implementation of MAT & applicability of 2.6.30

➢ **Outcome**:

- MAT uses: for product release, to rule out the presence of NEPs, for in-process testing, for trouble-shooting
- Chapter 2.6.30 is useful however some technical guidance for successful performance of the test are required

Revised chapter published in 2017 (*Supplement 9.2*)

Improvements

- Situation in the field has evolved since 2010 (e.g. accrued user experience, kits based on different approaches are available…)
- **New revision of chapter 2.6.30 to reflect accrued experience, take into account user feedback**

NEW!

Revised chapter published in January 2024 (*Supplement 11.5*). Implementation date: 1 July 2024

Improvements

Determination of the MVD

- *Maximum Valid Dilution (MVD):* the maximum allowable dilution of a sample at which the contaminant limit can be determined

NEW •

$$MVD = \frac{CLC \times C}{Test\ sensitivity}$$

- CLC = contaminant limit concentration
- C = concentration of test solution

→ *MVD is calculated for each product*

- *CLC* (endotoxin equivalents): acceptance criterion for a pass/fail decision

CLC = K / M → *Guidance on how to calculate the limit is given in the guidance notes*

  - K= threshold pyrogenic dose of endotoxin per kilogram of body mass → *Values for K are given in the guidance notes*
  - M = maximum recommended bolus dose of product per kilogram of body mass

- *Test sensitivity* : the lowest endotoxin reference standard concentration on the standard curve whose response exceeds the cut-off value ($\bar{x}$ + 3s)
  - $\bar{x}$ = mean of the responses obtained for the 4 blank replicates
  - s = standard deviation of the responses obtained for the 4 blank replicates

Cell sources and qualification

- Qualification of blood donors
  - *Qualification criteria for blood donors (health, medication…)*

- Qualification of cell sources
  - Whole blood, PBMCs: obtained from single donors or from pooled whole blood, qualified according to the requirements described in sections […] 5-4 (*Qualification of fresh cells*) or 5-5 (*Qualification of cryopreserved cells*) and where applicable, section 6-5 (*Method validation for non-endotoxin monocyte-activating contaminants*)
  - Pools: *minimum number of donors*
  - Fresh cells, cryopreserved cells: *timeframe for use of blood after collection, criteria for dose-response curve, qualification for use for the detection of non-endotoxin contaminants, averaging effect (for pooled cells)*

Cell sources and qualification (cont'd)

- Qualification of cell sources
  - Monocytic cell lines:

NEW

- Cell lines meeting the requirements of chapter 2.6.30 are appropriate for the detection of endotoxins and NEPs, after successful qualification (→ as per section 6-5)
- Maintained under aseptic conditions, and regularly tested the absence of microbial and viral contamination & checked for identity and stability
- Functional stability: *criteria for functional stability, testing the receptor expression*
- *Criteria for dose-response curve, qualification for use for the detection of non-endotoxin contaminants*

Preparatory testing

- Assurance of criteria for the endotoxin standard curve
  - The basal content of the chosen read-out (blank) is optimised to be as low as possible
  - Appropriate regression model (e.g. a linear regression model or 4-/5-PL model) depending on the number of concentrations prepared and the dose-response relationship observed
  - NEW • Standard curve: at least 4 endotoxin concentrations (linear model), at least 5 (4-PL) or at least 6 (5-PL). At least 4 replicates of each concentration
  - Acceptance criteria for the standard curve: 1) good fit between the data points and the chosen regression model (evaluated by statistical test ($p > 0.05$) or visually); 2) coefficient of determination is not less than 0.975
- Test for interfering factors *(for method 1)*
  - Aim: ensure that the preparation being examined does not interfere with the test
  - Concentration of endotoxin spike: usually equal to or near the estimated middle of the endotoxin standard curve
  - Test solution is considered free of interfering factors if the mean recovery of the spike is within 50-200%
- Determination of the optimal dilutions of the test and reference lots *(for method 2)*
  - Dilutions of the test and reference lots depends on the type of analysis to make the comparison between the two (to be justified and validated for each product). *An example is given*

Preparatory testing (cont'd)

- Interference in the detection system
  - Aim: ensure that the preparation being examined does not interfere in the detection system
  - Preparation is tested for interference in the detection system (e.g. ELISA) for the chosen read-out (e.g. IL-6)
  - Agreement between a dilution series of the standard for the chosen read-out, in the presence and absence of the preparation being examined, is to be within, for example ± 20 per cent of the optical density

- Method validation for non-endotoxin monocyte-activating contaminants
  - Aim: show that the test system detects non-endotoxin pyrogens
  - Using at least 2 non-endotoxin ligands for PRRs, at least 1 of which is to be spiked into the preparation examined. If available, historic batches found to be contaminated with non-endotoxin contaminants that caused positive responses in the RPT or adverse events in man are also included.

NEW

  - Spike recovery must be within 50-200% (in case of synergism, sufficient to be >50%)
  - The test system should ensure that at least TLR4 and 2 other TLR ligands that reflect the most likely contaminant(s) of the preparation tested are detected

Method 1: Semi-quantitative test NEW

- Comparison of the preparation being examined with a standard endotoxin dose-response curve
- To pass the test, the contaminant concentration of the prep. is to be < CLC

- Sol. A: dilution at which the test for interfering factors was carried out (highest concentration for which endotoxin recovery is consistently within 50-200%)
- NEW Sol. B & C: dilutions chosen after review of data from product-specific validation, not exceeding the MVD (e.g. 1:2 x MVD & MVD)
- Sol. AS, BS, CS: sol. A, B & C spiked with standard endotoxin at a concentration equal to or near the middle dose from the endotoxin standard curve
- Sol. R_0: negative control
- Sol. R_1-R_x: sol. of standard endotoxin at the concentrations used in the test for interfering factors

Solution	Solution/dilution factor	Added endotoxin	Number of replicates
A	Test solution/f	None	4
B	Test solution/f_1	None	4
C	Test solution/f_2	None	4
AS	Test solution/f	Equal to or near the middle of the endotoxin standard curve	4
BS	Test solution/f_1	Equal to or near the middle of the endotoxin standard curve	4
CS	Test solution/f_2	Equal to or near the middle of the endotoxin standard curve	4
R_0	Pyrogen-free saline or test diluent	None (negative control)	4
R_1-R_x	Standard endotoxin diluted in pyrogen-free saline or test diluent	≥ 4 concentrations of standard endotoxin	4 of each concentration

Method 1: Semi-quantitative test (cont'd)

- Data included in the analysis relate to cells for which the criteria for the endotoxin standard curve are satisfied
- Calculate the concentration of endotoxin equivalents in each of the replicates of solutions A, B and C and solutions AS, BS and CS using the endotoxin standard curve
- Validity criteria: endotoxin recovery for spiked samples (AS, BS & CS) is within 50-200%. The test is not valid unless at least one of the dilutions displays a spike recovery within 50-200%
- The preparation complies with the test if the mean concentrations of endotoxin equivalents in the replicates of sol. A, B and C, after correction for dilution and concentration, are all < CLC. Conversely, the preparation does not comply if the mean concentration of any of the solutions exceeds the CLC, regardless of the spike recovery

NEW

Method 2: Reference lot comparison test

- Comparison of the preparation being examined with a validated reference lot
  - The type of analysis to compare the two is to be justified and validated for each product
  - NEW • Reference lot: lot of the preparation that has been found to be safe and efficacious through clinical studies, or is representative thereof
  - Method intended to be performed where a prep. shows marked interference but cannot be diluted within the MVD to overcome the interference or because it contains or is believed to contain non-endotoxin contaminants

Solution	Solution/dilution factor	Number of replicates
A	Solution of reference lot/f_1	4
B	Solution of reference lot/f_2	4
C	Solution of reference lot/f_3	4
D	Solution of preparation to be examined/f_1	4
E	Solution of preparation to be examined/f_2	4
F	Solution of preparation to be examined/f_3	4
G	Positive control (standard endotoxin)	4
R_0	Diluent (negative control)	4

- Sol. A, B and C: reference lot diluted by dilution factors determined during preparatory testing
- Sol. D, E and F: prep. being examined diluted by the same dilution factors
- Sol. G: positive test control for the viability of the cells (standard endotoxin concentration that gives a clear positive response)
- Sol. R_0: negative control (diluent used to dilute the prep.)

Method 2: Reference lot comparison test (cont'd)

- Data included in the analysis relate to cells for which sol. G and at least one of sol. A, B and C give a response that is greater than the basal release of the read-out (sol. R_0)

- Calculate the mean responses of the replicates of sol. A-F using the standard curve for the read-out. Divide the sum of the mean responses to solutions D, E and F by the sum of the mean responses to solutions A, B and C. The preparation complies if the resulting value complies with a defined acceptance criterion not exceeding a justified value

Phasing out the Rabbit Pyrogen Test from the Ph. Eur.

New Ph. Eur. Pyrogenicity strategy

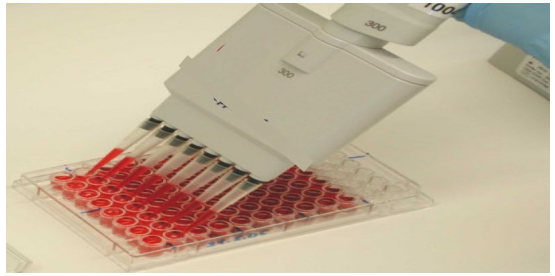
1971

Pyrogens (2.6.8)

1987

BET (2.6.14)

2010



MAT (2.6.30)

2020

BET using rFC (2.6.32)

The RPT continues to be widely performed

Experts of the Ph. Eur.

The European Partnership for Alternative Approaches to Animal Testing

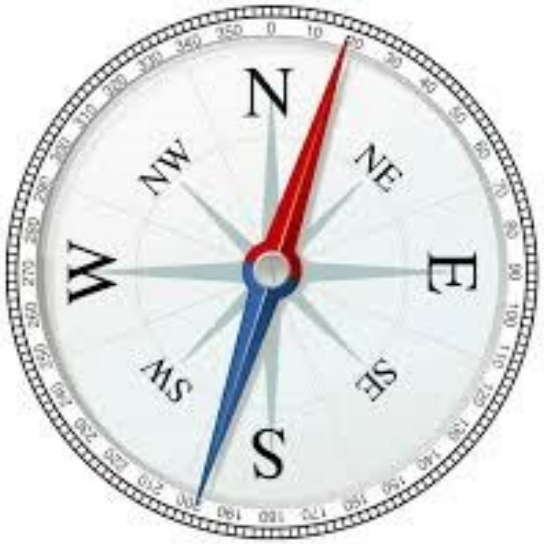
Proposal

- **New chapter *5.1.13* Pyrogenicity**
- **Deletion of the rabbit pyrogen test from 60 Ph. Eur. texts by 2025 and suppression of chapter *2.6.8* from the Ph. Eur. by 2026**

Public consultation in Pharmeuropa 35.1

New Ph. Eur. Pyrogenicity strategy

→ Published on Pharmeuropa webpage: https://go.edqm.eu/NewPyrogenicityStrategy



Strategy for removing or replacing the rabbit pyrogen test:
New pyrogenicity strategy of the European Pharmacopoeia Commission
September 2022

Replacement of chapter *2.6.8: proposed strategy*

Consolidated strategy approved by the European Pharmacopoeia Commission in June 2022

2.6.8 in 60 texts of the PhEur

European Pharmacopoeia 12 ?

2.6.8 in 60 texts of the PhEur

X

5.1.13
Pyrogenicity

New! Chapter
***5.1.13.* Pyrogenicity**

USER

Potential pyrogens other than endotoxins can be ruled out

Risk analysis, potential presence of non-endotoxin pyrogens Stage of manufacturing process

Exclusion of potential pyrogens other than endotoxins not possible

Chapters
***2.6.14/ 2.6.32.* BET**

or both

Chapter
***2.6.30.* MAT**

Decision on a testing strategy
Decision on the limits

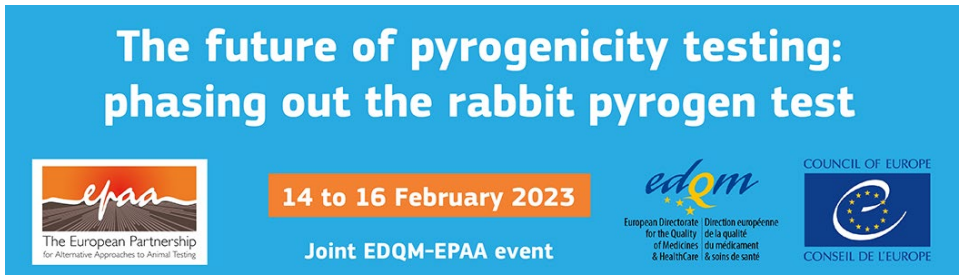
Explanatory notes in the revised Ph. Eur. texts (selected extracts)

- *"It should be noted that the exercise will ultimately lead to the suppression of general chapter 2.6.8 from the Ph. Eur. Manufacturers still using the rabbit pyrogen test are strongly encouraged to take the necessary steps to proceed with its replacement by a suitable in vitro alternative (e.g. the monocyte-activation test), in line with the new requirements of this general monograph."*

- *"Importantly, the revision of this text does not call into question strategies involving the test for bacterial endotoxins that are already used by manufacturers to control the pyrogenicity of their products and have been authorised by the competent authority, nor is it intended to prompt a retrospective assessment of pyrogenicity."*

EPAA/EDQM International Public Conference

To mark the publication of revised Ph. Eur. texts omitting the RPT in Pharmeuropa 35.1 *(Jan 2023)*



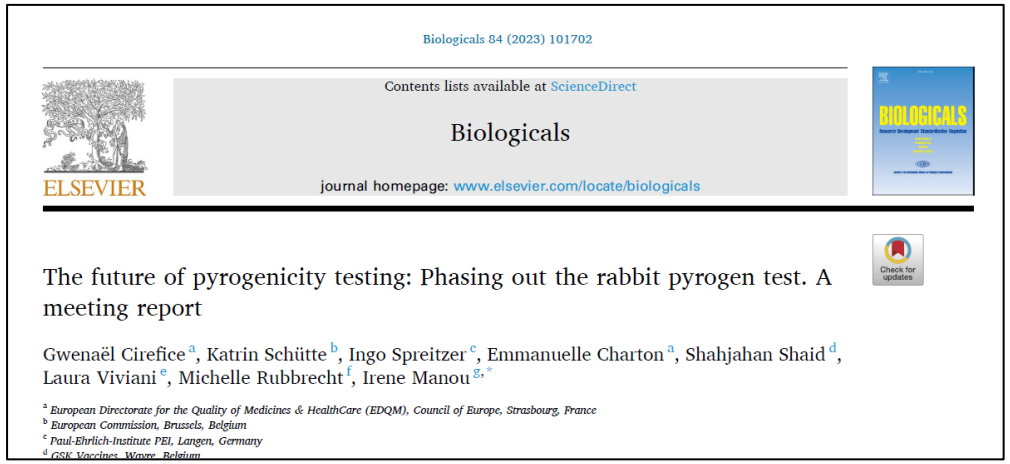
- Hosted by the European Commission in Brussels
- 250 participants from Industry, Academia, Regulatory Authorities (worldwide), WHO, Pharmacopoeias (worldwide), National Control Laboratories, MAT kit manufacturers and developers, service providers

Take home messages:

- In Europe, stakeholders are showing great enthusiasm towards the Ph. Eur. strategy aimed at phasing out the RPT
- Outside Europe, the strategy is generally seen positively, however, alternative methods such as MAT are not described in detail nor even mentioned in most Pharmacopoeias, where the RPT is still required in monographs. The journey towards complete removal might therefore take longer
- International convergence toward the same goal is important
- Implementing the MAT has been facilitated greatly in the last years by the standardisation of reagents and the increase in available kits
- **The time has come to switch from *in vivo* RPT to *in vitro***

Useful resources!

- Recording of live event: https://single-market-economy.ec.europa.eu/events/epaa-edqm-event-future-pyrogenicity-testing-2023-02-14_en
- Article in biologicals: https://www.sciencedirect.com/science/article/pii/S1045105623000404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The future of pyrogenicity testing: Phasing out the rabbit pyrogen test. A meeting report

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Timelines

WHAT	WHO	WHEN: Publication in PhPa	WHEN: Envisaged implementation date
Elaboration of new chapter on *Pyrogenicity* (*5.1.13*) (and revision of chapter *5.1.10*)	BET WP	●	●
REVISION			
Chapter 2.6.30	BET WP	●	●
Gen. monograph 2034	BET WP	●	●
Gen. monograph 0520	G12 with BET WP support	●	●
All other Ph. Eur. texts	GoE/WP with BET WP support	●	●
DELETE	Pyrogens (*2.6.8*)		●

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