European Pharmacopoeia Chapter 5.1.6
Alternative methods for control of microbiological quality

Sébastien Jouette, Ph. D.

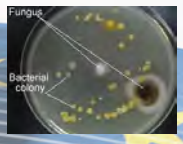
Scientific programme officer, European Pharmacopoeia Department

European Directorate for the Quality of Medicines and HealthCare

5.1.6 Alternative methods for control of microbiological quality

- **AIM**: to facilitate the implementation and use of alternative microbiological methods (AMM) where this can lead to cost-effective microbiological control and improved assurance for the quality of pharmaceutical products.

- **CONTENT**:
  - basic principles for detection, enumeration, isolation and identification of the methods which have successfully been used in QC of pharmaceuticals;
  - guidance on validation of alternative methods against compendial methods.



5.1.6 and Ph. Eur. General Notices

5.1.6. ALTERNATIVE METHODS FOR CONTROL OF MICROBIOLOGICAL QUALITY

The following chapter is published for information.

1. GENERAL INTRODUCTION

The objective of this chapter is to facilitate the implementation and use of alternative microbiological methods where this can lead to efficient microbiological control and improved assurance for the quality of pharmaceutical products.

- ✓ **Monographs**: mandatory requirements, unless otherwise indicated
- ✓ **General chapters**:
  - become mandatory when referred to in a monograph,
  - unless such reference is made in a way that indicates that it is not the intention to make the text referred to mandatory but rather to cite it for information.

5.1.6 and Ph. Eur. General Notices

5.1.6. ALTERNATIVE METHODS FOR CONTROL OF MICROBIOLOGICAL QUALITY

The following chapter is published for information.

1. GENERAL INTRODUCTION

The objective of this chapter is to facilitate the implementation and use of alternative microbiological methods where this can lead to efficient microbiological control and improved assurance for the quality of pharmaceutical products.

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

Revision of Chapter 5.1.6

First version published in 2006

Enquiry on modern microbiological methods by Ph. Eur. (2010)

Input sought from manufacturers and NPAs

➢ **KEY ELEMENTS**:

- To give a general view on the use of alternative microbiological methods in Europe
- **To find out if the contents of the chapter were appropriate and see if the chapter needed revision**
- Had the chapter facilitated the use of alternative methods?

➢ **OUTCOME**:

- Chapter 5.1.6 helpful
- Lots of proposals for improvements
- Validation examples useful
- Further **validation examples** should be given

Elaboration of the revised chapter
(meetings, drafting, data)

Pharmeuropa 27.1, 2015:
> 200 comments

Improvement

Published in **supplement 9.2**
(January 2017)

Scope of revision

❖ Relevance of alternative methods to Process Analytical Technology

❖ Update and improved description of the methods

❖ Improvement of the section dealing with Process validation :

-Clearer structure for primary validation and validation of intended use

-Description of tasks to be undertaken for the validation process between supplier and user

What's new: Chapter Structure

Former version

GENERAL INTRODUCTION

1. GENERAL PRINCIPLES OF ALTERNATIVE METHODS
2. GENERAL VALIDATION REQUIREMENTS
3. SPECIFIC VALIDATION REQUIREMENTS

Annex: Example validation of an alternative method: detailed protocol followed by a laboratory for the implementation of bioluminescence

New version

1. GENERAL INTRODUCTION
2. GENERAL PRINCIPLES OF ALTERNATIVE METHODS
3. VALIDATION OF ALTERNATIVE MICROBIOLOGICAL METHODS

EDQM-Website: EXAMPLES OF VALIDATION FOR ALTERNATIVE METHODS *

* After approval by Ph. Eur commission

To eliminate potential misinterpretation of the purpose of examples

To keep it updated with new validated alternatives methods

What's new

1. GENERAL INTRODUCTION

❖ Adding AMMs for application of Process Analytical Technology (PAT) – in-process control and environmental monitoring

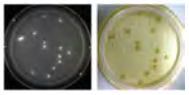
❖ 3 major types of determination specific to microbiological tests:
- qualitative tests for the presence or absence of micro-organisms;
- quantitative tests for enumeration of micro-organisms;
- identification tests.

The choice of test is key – not all technologies are suitable for all determinations
– the output signal of the test may be different and requires validation and analysis to determine equivalence
– validation requirements are determined by the test used

2. GENERAL PRINCIPLES OF ALTERNATIVE METHODS

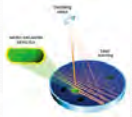
- Growth-based methods, where a detectable signal is usually achieved by a period of culture

  e.g.: -Measurement of consumption or production of gas
  -Bioluminescence (ATP)



- Direct measurement, where individual cells are differentiated and/or imaged

  e.g.: -Solid phase cytometry, flow cytometry
  -Autofluorescence



- Cell component analysis, where the expression of specific cell components offers an indirect measure of microbial presence and identification of micro-organisms

Is the method suitable?

- Is the method destructive ? Identification may not be possible
- Can non-viable or viable non-culturable micro-organisms be detected ?
- Can pure colonies be found ?
- Will the micro-organisms be present in the reference database used ?

What's new

2. GENERAL PRINCIPLES OF ALTERNATIVE METHODS

- Some **methods** have been **removed** (*e.g.* micro-calorimetry, phage-based method)

- **Revision** or **addition** of methods
  - autofluorescence, LAL as an alternative to gram staining
  - extensive revision of genotypic methods
  - modification of paragraphs "Critical Aspects" and "Potential Uses"

- General information on identification methods:
  - **Databases** (included in the primary validation, customisation)
  - Traditional Biochemical & Phenotypic versus Genotypic techniques

What's new:
Validation of Alternative Microbiological Methods

Restructuring the chapter:

3-1. *INTRODUCTION*

3-2. *VALIDATION PROCESS*

Risk-benefit analysis
- Very important to consider the limitations of both the conventional and alternative method
- To decide on which alternative method to be used
- To understand the impact of the new method

3-3. *TYPES OF MICROBIOLOGICAL TESTS*

Primary validation
- Primary validation sets out criteria that should be selected by the supplier using a panel of test micro-organisms

Validation for the intended use
- Comprehensive validation for the intended use is set out and includes URS, DQ, IQ, OQ, PQ

Validation Process:
tasks and responsability

Table 5.1.6.-1 – Tasks to be undertaken during the validation process

Activity	normally carried out by	
	Supplier	User
Primary validation	+	-[1]
URS (instrument, application)	-	+
Description of the technique	+	-[2]
Risk benefit analysis	-[3]	+
Design Qualification	-	+
Installation Qualification	-[4]	+
Operational Qualification	-[4]	+
Performance Qualification:		
- Verification of primary performance qualification	-	+
- Verification for the intended use (e.g. sterility testing, TAMC/TYMC, ...)	-	+
Method Suitability Test	-	+

(1) The user performs primary validation if they employ the alternative method for an use other than that defined by the supplier.
(2) The user shall critically review information provided by the supplier.
(3) As part of commercialisation, the supplier may list advantages of the alternative method over conventional techniques.
(4) IQ / OQ for complex equipment, IQ/OQ is often outsourced to supplier.

Validation Process

Primary validation

3-2-3. **Primary validation**

The supplier, using a panel of test micro-organisms appropriate for the intended use, must characterise the principle of detection. Depending on the type of alternative method, relevant validation criteria shall be selected from those listed below:

- prerequisite treatment of sample or micro-organisms;
- type of response;
- specificity;
- detection limit;
- quantitation limit;
- range;
- linearity;
- accuracy and precision;
- robustness of the method in a model system.

1) Supplier 2) User

Validation Process

Validation for the intended use

3-2-4. **Validation for the intended use**

Validation for the intended use should encompass the entire process, from the decision to change any aspects of a microbiological testing programme to on-going routine use. It should consist of the following phases:

- user requirement specification (URS);
- design qualification (DQ);
- installation qualification (IQ);
- operational qualification (OQ);
- performance qualification (PQ).

Describes the functions that the method must be capable of performing:

- Which kind of analysis are performed?
- Which sensitivity is needed?
- Which kind of microorganisms should be detected?
- Number and kind of analysis
- Interface with LIMS, CSV, etc. needed?

- Same results according to predefined criteria
- Use of a model-system
- Use of pharmacopoeial test strains, in-house isolates, stressed microorganisms

Validation parameters depending on the types of microbiological tests

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

Table 5.1.6.-2 – *Validation criteria for qualitative, quantitative and identification tests*

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

Validation for the intended use

Suitability Testing

- Show the suitability of the AMM in the presence of the product

Suitability testing
- It must show that the test sample does not interfere with the technology's detection capacity or microbial recovery (*i.e* detection or enumeration)
- Acceptance criteria for the method are defined as a function of the application and of the validation data

3-3-1-4. *Suitability testing*

The alternative method must be applied according to the specified procedure and with the samples to be analysed under the responsibility of the user. It must be shown that the test sample does not interfere with the system's detection capacity or microbial recovery. Specific points to be addressed are:

- the ability of the test to detect micro-organisms in the presence of the sample matrix;
- verifying if the sample matrix interferes with the alternative system (e.g. background signal or inhibiting chemical reactions).

Acceptance criteria for the method in routine use will need to be defined as a function of the application and the validation data.

3-3-2-8. *Suitability testing*

The alternative method must be applied according to the specified procedure and with the samples to be analysed under the responsibility of the user. It must be shown that the test sample does not interfere with the system's enumeration capacity or microbial recovery. Specific points to be addressed are:

- the ability of the test to detect micro-organisms in the presence of the sample matrix;
- verifying if the sample matrix interferes with the alternative system (e.g. background signal or inhibiting chemical reactions).

Acceptance criteria for the method are defined as a function of the application and of the validation data.

Validation for the intended use

Equivalence Testing

- Conducted directly on the validation parameters
- Parallel testing with the alternative method and the compendial method.
- The result must be equivalent

Equivalence testing

- Sufficient numbers of replicates for relevant strains of test micro-organisms are required
- Parallel testing is justified by a risk assessment and requires parallel testing of samples for a predefined period of time or a predefined number of samples
- Results of the alternative method are equivalent to those of the pharmacopoeial method
- Further analysis may be required

Validation for the intended use

Equivalence Testing (qualitative method)

Example of equivalence testing for a rapid sterility test based on ATP bioluminescence:

- Contaminated test samples are prepared by inoculation with less than 5 CFU of three different test micro-organisms
- Parallel testing with the alternative method and the compendial method
- 3 test runs with 10 replicates each are performed. Results are statistically evaluated.

Validation for the intended use

Equivalence Testing (quantitative method)

If the result of the alternative method can be expressed as the number of CFUs per weight or per volume, statistical analysis of the results shall demonstrate that the results of the alternative method enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

If the result of the alternative method cannot be expressed as the number of CFUs, equivalence testing is performed using suitable parameters, followed by statistical analysis to demonstrate that the results of the alternative method enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

→ use of statistics to demonstrate equivalency between an alternative and a growth-based compendial method

Examples of AMM where equivalence testing was successfuly performed even if its result cannot be expressed as the number of CFUs

A solid phase cytometry:
The result is presented as the number of viable cells per volume tested and not in CFU per volume tested

NAT Method (e.g for mycoplasma detection):
The result may be presented as gene copies/cell

What's new: Examples of validation

- The detailed laboratory protocol for the implementation of a bioluminescence method has been removed
- 3 **new examples** will be outlined on EDQM website* soon
  - 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.

* After approval by Ph. Eur commission

Acknowledgements

- Group of Experts n°1 of the Ph. Eur., Chaired by V'lain Fenton-May

Thank you for your attention!

Welcome

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Current USP Perspectives on Rapid Microbiological Methods

Radhakrishna S.Tirumalai, Ph.D.
Principal Scientific Liaison-General Chapters
US Pharmacopeial Convention
Rockville,MD. U.S.A.

Topics

- Recent Revisions to Validation of Alternative Microbiological Methods <1223>
- Rapid Sterility Tests

Validation of Alternative Microbiological Methods <12223>

Alternate Methods

USP General Notices 6.30:

"Alternate methods may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction or in other special circumstances. Such alternate methods shall be validated ……and must be shown to give equivalent or better results…."

USP<1223>

- This chapter provides guidance on the selection, evaluation, and use of microbiological methods as alternatives to compendial methods.
- To properly implement alternative methods, one must consider a number of important issues before selecting the analytical technology and qualifying that method with the actual product.
- These issues include, but are not limited to, identification of suitable alternative methodology, development of user specifications for equipment selection, demonstration of the applicability of the method as a replacement for a standard compendial method, and qualification of the method in the laboratory.

Factors to Consider

These factors include the following

- Identification of suitable alternative assay methodologies
- Development of user specifications for equipment selection
- Demonstration of the applicability of the method as a replacement for a standard compendial method
- Installation and operational qualification of the equipment
- Laboratory performance qualification of the method
- Re-alignment for decision making to release product

Microbiology is a Logarithmic Science

Microbiology is a logarithmic science. While we can distinguish between 100 and 1000 cfu (a difference of 1 $\log_{10}$), it may be not possible to discern smaller differences (less than 0.3–0.5 $\log_{10}$). The inherent variability of these methods is substantially greater than analytical chemistry methods. This inherent analytical variability must always be considered in the selection, development, and validation of alternative methods. The expectation of a degree of agreement between alternate microbiological methods and traditional growth-based methods beyond what is technically feasible could complicate the implementation of newer analytical technologies regardless of their specific mode of analysis

Limitations of the CFU

- The appearance of a visible colony requires significant growth of the initial cells plated - at the time of counting the colonies it is not possible to determine if the colony arose from one cell or 1,000 cells.
- Therefore, the results are given as CFU/mL (colony-forming units per milliliter) for liquids, and CFU/g (colony-forming units per gram) for solids to reflect this uncertainty (rather than cells/mL or cells/g).

Limitations of the CFU

- The colony-forming unit (CFU) is an estimate of viable bacterial or fungal numbers in a water, air, soil, food or drug sample.
- Unlike direct microscopic counts where all cells, dead and living, are counted, CFU estimates viable cells that are capable of dividing in the solid plate count medium under the incubation conditions employed.

USP <1223>

- “The units of measurement (signal) of a microbiological quality assessment performed using alternative microbiological test methods will generally not be a CFU, but rather a different approach to obtaining a cell count estimate.”
- Direct detection of higher cell counts as compared to CFU is not necessarily indicative of a greater microbiological safety risk”

Signals other than CFU

- Signals other than colony-forming units that may be used for microbial enumeration include:
- Vital stained cells detected by solid-phase and fluid fluorescent microscopy
- ATP levels measured by bioluminescence
- Laser-induced cell fluorescence technologies

New USP<1223> refers to Non- inferiority

- "To demonstrate the acceptability of the alternate procedure relative to the current microbiological procedure, the laboratory must demonstrate that the new procedure is as good as or better than the current procedure in terms of the ability to detect presence of microorganisms."
- "In general, a recommended approach for comparing the alternate procedure to the compendial procedure is to use a **non-inferiority test** (one-sided, as in non-inferiority tests conducted in clinical trials for the evaluation of new drug products rather than two-sided equivalence (as in bioequivalence)."

Non-inferiority

- Concepts of equivalence/non-inferiority are adapted from clinical studies of new therapies where it is increasingly more difficult to develop therapies with higher efficacy than the standard of care.
- However, these new therapies may offer advantages of fewer side effects, low overall treatment costs, better treatment outcomes, easier application, or few drug interactions.

Non-inferiority Clinical Trials

A non-inferiority trial is a comparison with an active control (existing treatment) to determine whether the difference in response between a new drug and the active control is small enough (less than some pre-specified margin) to demonstrate that the new treatment is not less effective (or is slightly less effective) than the control in achieving the primary clinical outcome.

Determining Equivalence

- First step is define the limits of equivalence (± δ) for the alternative method.
- Calculate the 95% confidence intervals for the difference between the control, i.e. compendial and alternative test method.
- If the confidence interval is entirely within ± δ then equivalence is established.

Equivalence Options

- Four options are available to establish the equivalence of an alternative method are:
  - Acceptable procedures, i.e. merely meeting a minimum performance requirement without demonstrating equivalence to the compendial method
  - Performance equivalence to the compendial method
  - Results equivalent to the compendial method
  - Decision equivalence to the compendial method

Equivalence Options

Option	Demonstration	Comparison to Offficial Compendial Method	Based on Numerical Results or Conclusion	Number of Characteristics
Acceptable procedures	Acceptable	No	Results	Multiple
Performance equivalent	Equivalent	Yes	Results	Multiple
Results equivalent	Equivalent	Yes	Results	Single
Decision equivalent	Equivalent	Yes	Conclusions	Single

Method Suitability

After an alternative method has been shown to be equivalent to the compendial test with one product, it is not necessary to repeat the equivalency parameters for every new product; it is merely necessary to verify the method suitability for each additional product.

Rapid Sterility Tests

Issues on hand…

- The currently required 14-day incubation period for the compendial sterility tests imposes a significant burden on the manufacturer, who must quarantine product until successful completion of the test.
- The long incubation time (14 days) is unsuitable for numerous small lot size products including cytotherapy, radiopharmaceuticals, pharmacy compounded sterile products, and some clinical trial materials.

Expert Panel

- An expert panel composed of representative stakeholders from the sterile compounding, PET, cell therapy, pharmaceutical and contract testing industries were recruited to establish user requirement specifications and recommend to the USP Microbiology Expert Committee suitable technologies for the rapid sterility testing of short-life injectable products**.**
- Expert panel also included technology experts.

Expert Panel Approach

- A two-phased approach was decided upon with the user requirement specifications for the different stakeholders established first.
- Based on these user requirements, the most appropriate technologies for a compendial rapid sterility test(s) would be recommended to the USP General Chapters-Microbiology Expert Committee.

Critical User Requirement Specifications

The most critical user requirements for candidate rapid sterility tests were

- Specificity (i.e. the ability to detect a wide range of species)
- Limit of detection (i.e. the ability to detect a low number of microorganisms)
- Time to result
- Improved patient safety
- Sample preparation
- Sample quantity (i.e. minimum number of articles tested and quantity per container tested).

Ability to detect a wide range of microorganisms

- Although all the analytical platforms should have the ability to detect a wide range of bacteria, yeast and mold, it is equally important to demonstrate that a rapid sterility test technology chosen is capable of detecting microorganisms implicated in sterility test failures, infection outbreaks and product recalls associated with either compounded sterile preparations, radiopharmaceuticals, cell therapies or manufactured pharmaceuticals.
- This is especially true if the technology, after risk analysis, is shown to improve patient safety with the administration of the products unique to that stakeholder group.

Limit of Detection

- This user requirement is perhaps the most challenging
- Within the limitations of preparing inocula with one or more colony-forming units, growth-based sterility tests can be shown to have at least a theoretical Limit of Detection (LOD) of 1 CFU or 1 to 3 CFU based on a Poisson distribution.
- Setting a LOD of a single viable cell of all technologies is a huge barrier of entry for a rapid sterility test especially when the signal is not the colony-forming unit that is amplified by cultural enrichment.

Time to result

- The incubation time for growth-based USP <71> Sterility Tests is at least 14 days, which makes it unsuitable for positron emission tomography and cell therapy as these short-life products would be administered prior to the completion of the test, marginal for sterile compounding, but generally suitable for pharmaceutical manufacturing.
- PET radiopharmaceuticals are usually administered within several hours of preparation due to the short half-life, so candidates for a rapid sterility test need to be real time.
- For compounded sterile preparations and cell therapies, rapid sterility tests need to be completed at best within 24 hours or within 48 hours at the most, whereas manufactured pharmaceuticals can be tested within 5-7 days to shorten the release cycle time.

Time to result

Signals employed by different technologies may be amplified by enrichment culture with a 24-48 hour incubation or by concentration e.g. filtration, selective adsorption and elution or centrifugation, to reduce the time to result and lower the limit of detection.

Patient Safety

- It is widely accepted that a rapid sterility test for compounded sterile preparations, radiopharmaceuticals and cell therapies will improve patient safety, especially if contaminated materials can be detected prior to administration to patients.
- Furthermore, sterility test methods that continuously monitor for the presence of viable microorganisms and report when a failure is detected would enable the laboratory to report to the clinician who could intervene with patient will provide an additional advantage.

Sample Quantity

- The minimum number of articles tested and quantity per container tested per media is defined in Tables 2 and 3 of USP <71> Sterility Tests.
- Whereas this sampling plan is suitable for manufactured pharmaceuticals, it is unsuitable for products generated by sterile compounding pharmacies, PET facilities, and cell therapy manufacturing facilities due to the small batch size of their products and the volume of the products to the individual patients. A further consideration is the sample size limitation of the advanced technology.

Sample Quantity

- Alternative sampling plans have been proposed in compendial chapters. The recommended approaches to sterility testing of compounded sterile preparation (CSPs) and cell therapy products as found in USP <797> and Ph. Eur. 2.6.27.
- In the proposed revision to USP <797>, if a Category 2 CSP is assigned a beyond-use dating that requires a sterility test, the testing must be performed as specified in USP <71> *Sterility Tests*. An exception to *Table 3: Minimum Number of Articles to be Tested in Relation to the Number of Articles in the Batch* is that the sterility test may be performed with 10% of the batch, rounded up to the next whole number, when the batch size is less than 40 units.

A Risk Based Approach

- There are obvious trade-offs between limit of detection, sample size and time to results. Detecting a contaminated unit prior to administration will improve patient safety.
- It is therefore pragmatic to develop risk-based rapid sterility tests that provide a certain level of LOD suitable for different stakeholders.
- For example, for a short lived product with a shelf life of less than a day, the time to result is a critical user requirement specification from a patient safety perspective.
- So a rapid sterility test with a time to test of less than a day would promote patient safety even if it had a limit of detection greater than one viable microbial cell.

Candidate Technologies

- Solid Phase LASER Scanning Cytometry
- Flow Cytometry
- CO_2 sensoring
- pH sensor
- ATP bioluminescence
- RT-PCR
- Isothermal Microcalorimetry

Candidate Technologies

It is acknowledged that no single method will work for all types of products/ product matrices and one or more of these analytical platforms may be found to have insurmountable technical limitations, which may prevent them from becoming compendial test methods.

Next Steps

- Immediate steps include the development of a Stimuli Article to explain the perspectives. This has been proposed in PF 43(5); September-October 2017 issue.
- General Information Chapter.
- This would be followed by proof of concept testing of candidate technologies and a test methods chapter based on technologies that can be advanced as compendial methods.

Questions

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Thank You

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Japanese Pharmacopoeia (JP) perspective
The overview of the new chapter of General Information entitled “Rapid Microbial Methods (G4 Microorganisms)”

October 10, 2017
at EDQM Premises, Strasbourg, France

Yutaka Kikuchi
National Institute of Health Sciences, Japan
Masao Nasu
Osaka Ohtani University, Japan

国立医薬品食品衛生研究所
National Institute of Health Sciences

Today’s presentation is an overview of the Japanese Pharmacopeia’s (JP) new informational chapter on **rapid microbial methods (RMMs).**

The chapter was published in the 17th edition of the JP (JP17) with a Japanese version in March and an English version in April of 2016.

The English version is **free to download** from the website URL of following:

Ministry of Health, Labour and Welfare (MHLW)
http://www.mhlw.go.jp/file/06-Seisakujouhou-11120000-Iyakushokuhinkyoku/JP17_REV.pdf

Pharmaceuticals and Medical Devices Agency (PMDA)
http://www.pmda.go.jp/files/000217649.zip

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Four RMMs are listed in the JP17 (as *General information*)

1. **Mycoplasma Testing for Cell Substrates used for the Production of Biotechnological/Biological Products This document describes the currently (revised a part of JP15)**
2. **Rapid Counting of Microbes using Fluorescent Staining (since supplement 2 to JP15)**
3. **Rapid Identification of Microorganisms Based on Molecular Biological Method (since JP16)**
4. **Rapid Microbial Methods (new chapter)**

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Rapid Microbial Methods / *General information*

Introduction

1. **Detection targets and principles**
2. **Validation**
3. **Applications and particular considerations**

4

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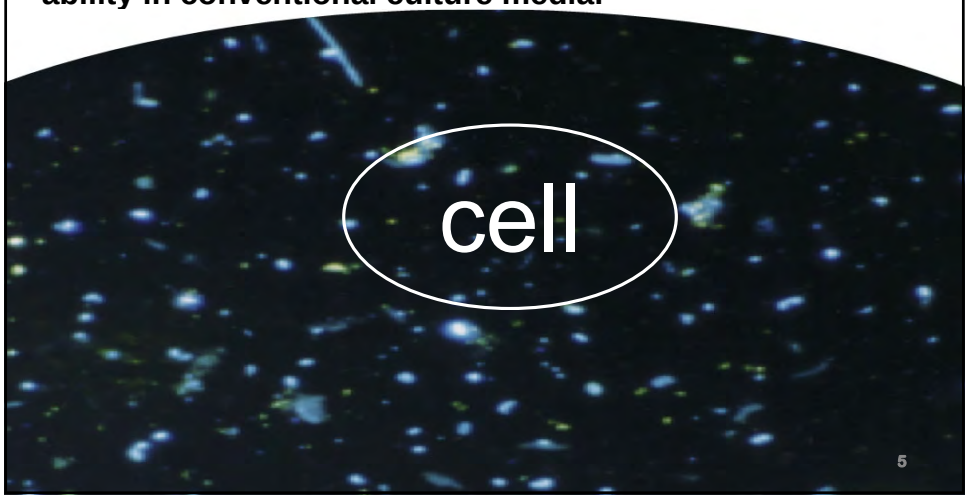
Introduction

Since the 1980s it has become clear that the majority of bacteria in the natural environment have low growth ability in conventional culture media.



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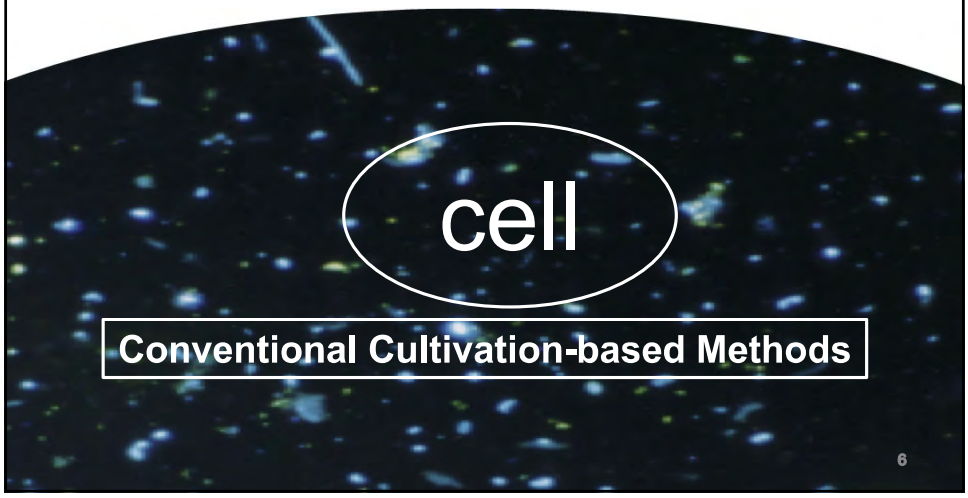
Introduction

The detection, enumeration and identification of these bacteria are difficult by means of culture methods alone.



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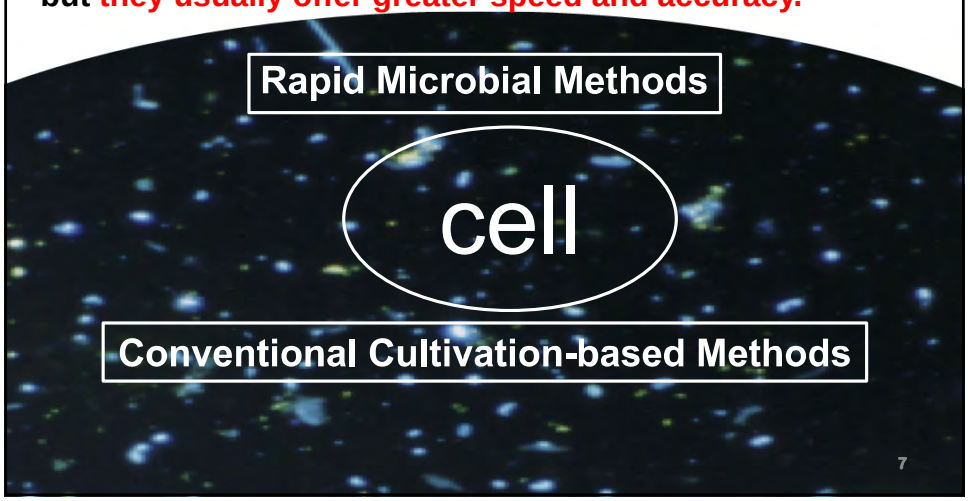
Introduction

Compared to the conventional methods, these new methods are not necessarily superior in every respect, but they usually offer greater speed and accuracy.



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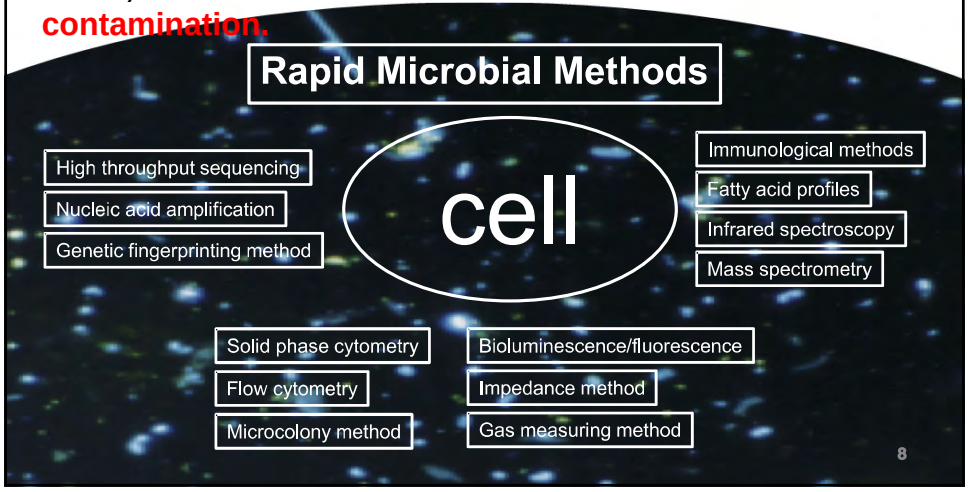
Introduction

Therefore, these new methods are very useful to improve the standards of microbial control in critical areas, and to decrease the risk of hazardous microbial contamination.



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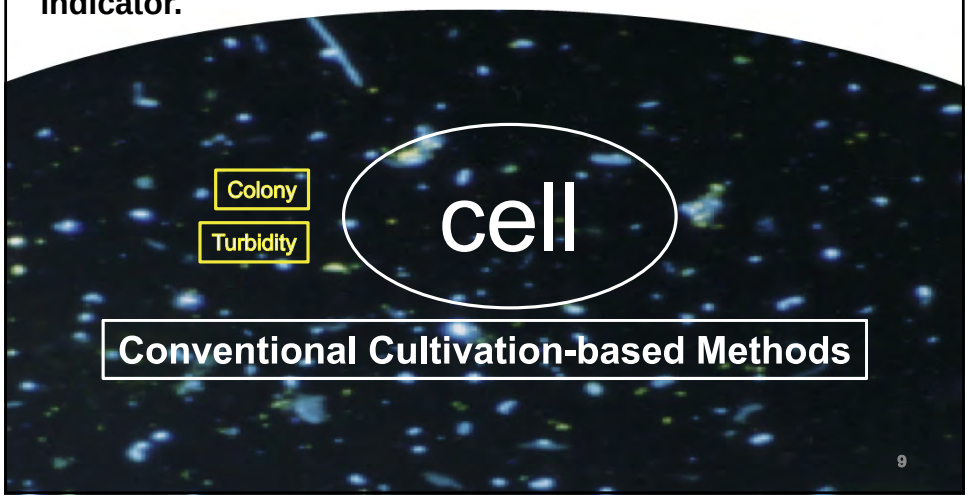
Introduction

The conventional cultivation-based methods use colony formation or turbidity change due to cell growth as an indicator.



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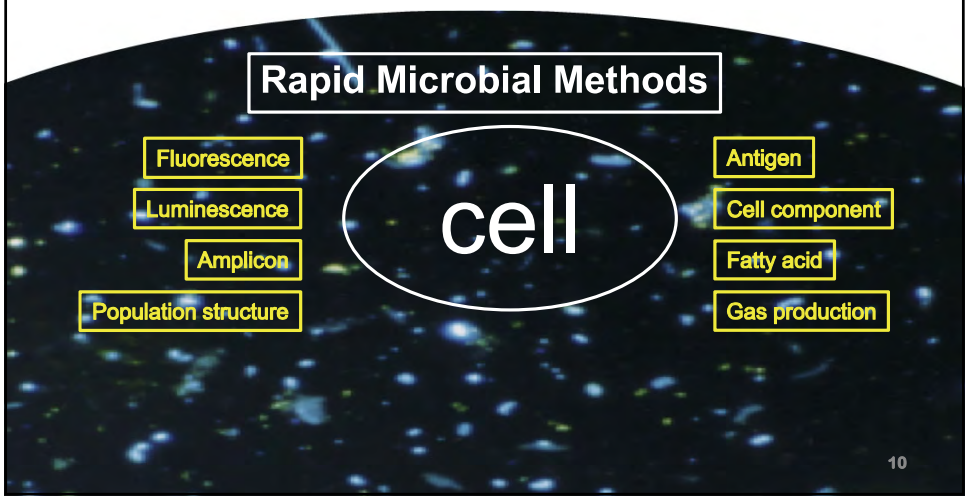
Introduction

The new methods vary greatly as regards the detection target and the detection principle.



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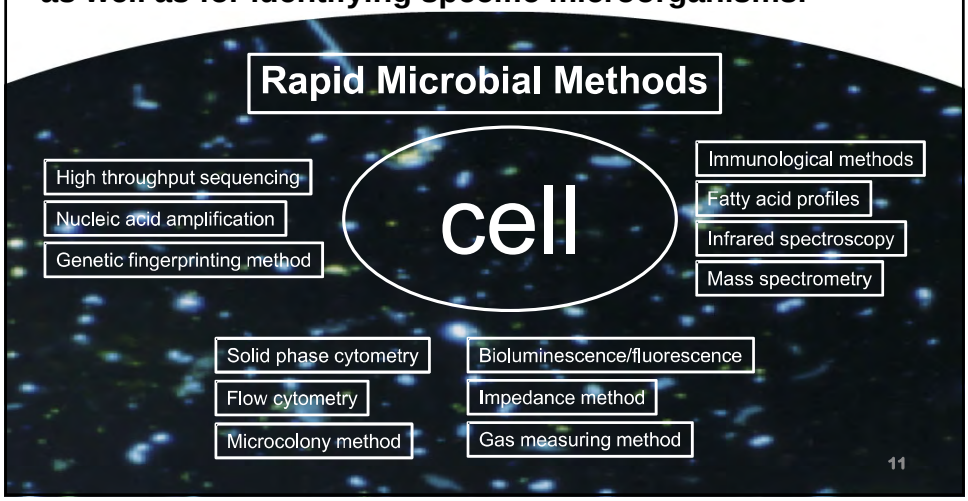
Introduction

The new methods may be more suitable for obtaining a comprehensive understanding of the microbial community, as well as for identifying specific microorganisms.



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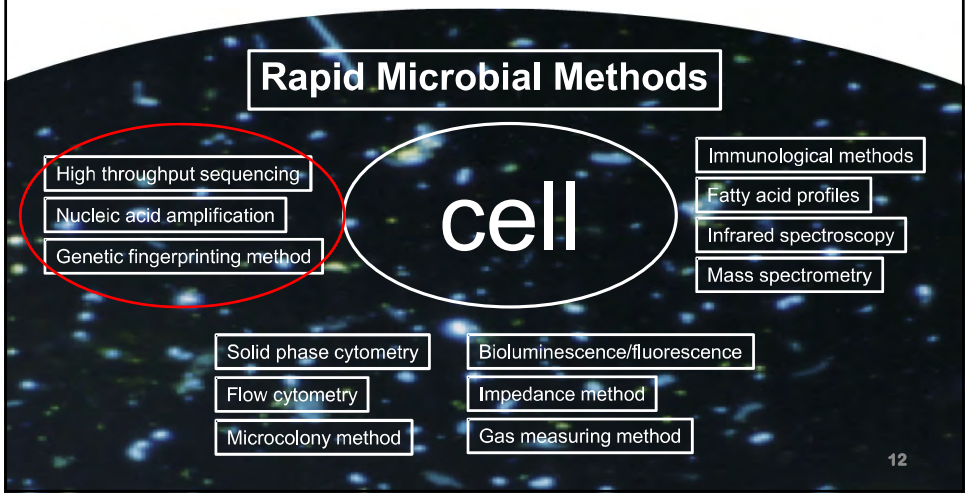
Introduction

Among these methods, phylogenic analysis based on gene sequences has become popular.



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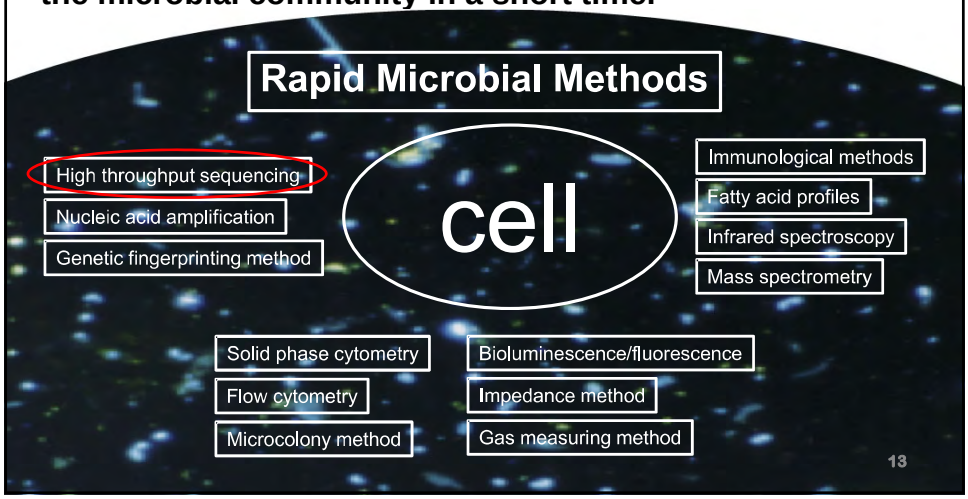
Introduction

The dramatic development of sequencing techniques in recent years now allows us to analyze the composition of the microbial community in a short time.



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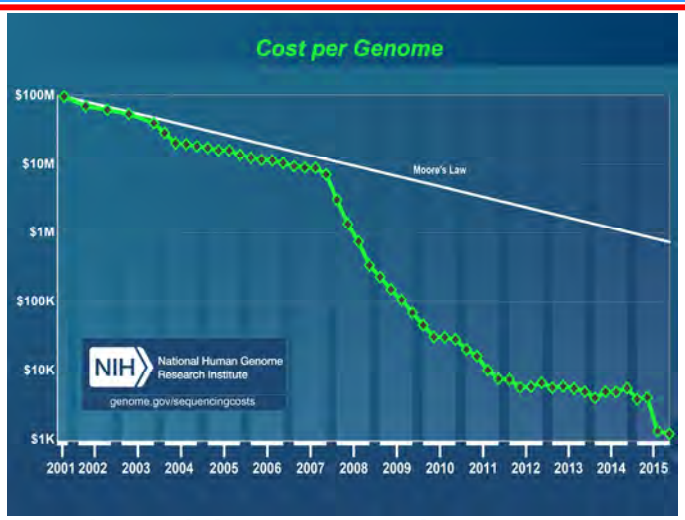

The cost of determining one megabase of DNA sequence of a specified quality.

https://www.genome.gov/27541954/dna-sequencing-costs-data/

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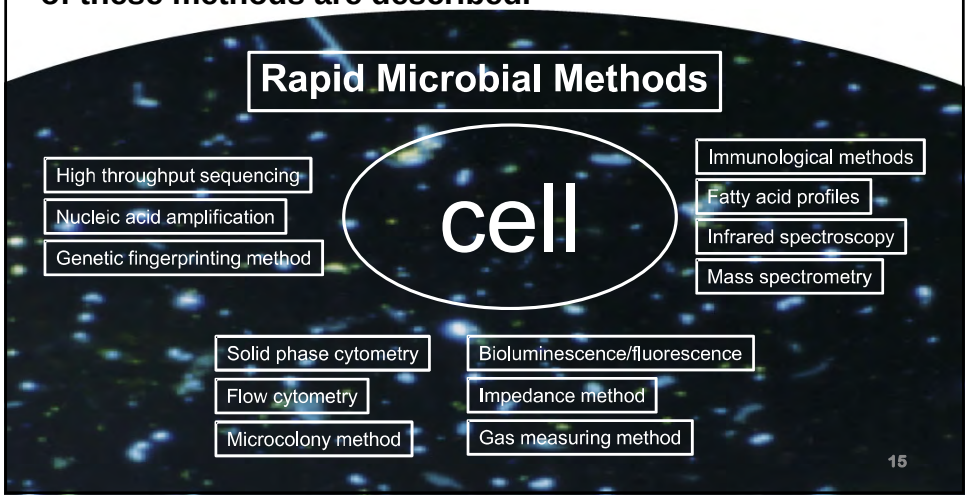
Introduction

The principles of the new methods and their range of applicability are introduced, and key points in the usage of these methods are described.



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Rapid Microbial Methods / *General information*

Introduction

1. Detection targets and principles

2. Validation

3. Applications and particular considerations

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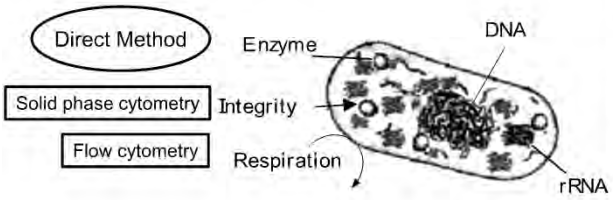
1. Detection targets and principles

1) Direct Method

2) Indirect Method



Indirect Method

- Immunological methods
- Nucleic acid amplification
- Bioluminescence/fluorescence
- Microcolony method
- Impedance method
- Gas measuring method
- Fatty acid profiles
- Infrared spectroscopy
- Mass spectrometry
- Genetic fingerprinting method
- High throughput sequencing

1. Detection targets and principles

Name	Target	Principles of measurement	Examples of measurement device
1) Direct Method			
Solid phase cytometry	Microorganism	Directly detect the signals from the bacteria trapped onto a filter. The signals on their physiological activities can be obtained by choosing suitable dyes. Autofluorescence may also be used. To selectively detect specific bacteria, gene probe, antibody or fluorescent-labeled phage may be utilized. Various optical devices including a fluorescent microscope and laser microscope are used as detection/measurement apparatus.	Fluorescence microscope, Laser scanning cytometer, etc.
Flow cytometry	Microorganism	Directly detect the signals given by the bacteria passing through fluid or air. The signals on their physiological activities can be obtained by choosing suitable dyes. Autofluorescence may also be used. To selectively detect specific bacteria, gene probe, antibody or fluorescent-labeled phage may be utilized. Various optical devices are used as detection/measurement apparatus.	Flow cytometer, etc.

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1. Detection targets and principles

Name	Target	Principles of measurement	Examples of measurement device
2) Indirect Method			
Immunological methods	Antigen	React the antigen of bacteria with the specific antibody, and detect the color or fluorescence visually or by a microplate reader. Immunochromatography is a simple and easy method for the purpose.	Immunochromatography, Micro plate reader, etc.
Nucleic acid amplification	Nucleic acid	Amplify a nucleic acid of microorganism by using the primers specific to the target microorganism, and analyze the amplified nucleic acid fragments. Quantitative determination is possible by performing of quantitative PCR.	Electrophoresis apparatus, Quantitative PCR
Bioluminescence/ fluorescence	ATP, etc.	Measure ATP which is released from microorganisms on the basis of luminous or fluorescence phenomena occurred by enzyme reaction.	luminescence detector, fluorescence detector, etc.
Micro colony method	Growth (Micro colony)	Detect and count the micro colony that appears in early stage of colonization. The same culture conditions (medium composition, temperature, etc.) as the plate culture method can be used.	Fluorescence microscopy etc.
Impedance method	Growth (Electrical characteristic)	Utilize the change in electrical properties of medium due to the metabolites produced by the growth of microorganisms.	Electrodes

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1. Detection targets and principles

Name	Target	Principles of measurement	Examples of measurement device
2) Indirect Method			
Gas measuring method	Growth (Gas production, etc.)	Utilize the change in amount of gases caused by CO_2 production, O_2 consumption, etc. with the growth of microorganisms.	Gas measuring instrument Color change of medium
Fatty acid profiles	Fatty acid	Utilize the fatty acid profile of cell components that differs depending on the taxonomic groups of microorganism.	Gas chromatography
Infrared spectroscopy	Cell component	Utilize the pattern of infrared spectrum obtained by infrared light irradiation to whole microorganism.	Fourier transformation infrared spectroscope
Mass spectrometry	Cell component	Measure the cell component by means of a mass spectrometer, and identify it by database.	Mass spectrometry
Genetic fingerprinting method	DNA	Utilize the electrophoresis pattern of DNA fragments obtained by cleaving the DNA extracted from sample with a restriction enzyme. It can be identified by database. Analysis of community structure is possible by T-RFLP.	Electrophoresis apparatus
High throughput sequencing	Nucleic acid	Determine the sequence of nucleic acids extracted from bacteria exist in sample, and analyze the community structure phylogenetically.	Sequencer, etc.

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1. Detection targets and principles

Detection of a bacterial cell by gene sequence-based method

target	indicator
Conventional cultivation-based method Determine the number of bacterial cells as colony formation.	colony
Nucleic acid amplification Extract chromosomal DNA harboring 16S rRNA gene*, and analyze the amplified nucleic acid fragments.	amplicon
High throughput sequencing Determine the sequence of nucleic acids extracted from bacteria exist in sample, and analyze the community structure phylogenetically.	population structure

* 1 to 15 copies per cell

Rapid Microbial Methods / *General information*

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1. Detection targets and principles

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2. Validation

To qualify introduced equipment, a standard component or strain, which represents the target of each method, should be utilized.

In direct measurement, type strains should be used.

In indirect measurement, standard components, etc., of the target bacteria are used.

2. Validation

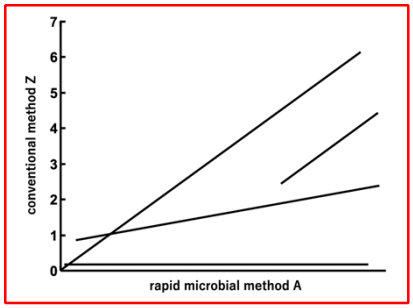
To validate a protocol / procedure, it is required to demonstrate that the detection target is a suitable index / indicator for bacterial number or quantity.

It is also important to state whether any special precautions are necessary in applying the protocol / procedure.

2. Validation

When **using a type strain**, the result of **validation should be equivalent to or better** than that of the conventional method.

However, because the **detection principles** of new methods are **usually different** from that of conventional methods, **the correlation between them is not always required.**



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2. Validation

For detection of environmental bacteria, it is important that the **physiological state of the type strain should be maintained as close as possible to that of environmental bacteria,** in order to obtain reliable results.

Quality Control of Water for Pharmaceutical Use/*General information* (JP17)

4.4.2. Media Growth Promotion Test

In the media growth promotion test with the R2A Agar Medium, use the strains listed below or other strains considered equivalent to these strains.

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2. Validation

Quality Control of Water for Pharmaceutical Use/*General information* (JP17)

Prior to the test, inoculate these strains into sterile purified water and starve them at 20-25°C for 3 days.

***Methylobacterium extorquens*: NBRC 15911**
***Pseudomonas fluorescens*: NBRC 15842, ATCC 17386, etc.**

Dilute the fluid containing the strain starved with sterile purified water to prepare a fluid containing about $5x10^1$-$2x10^2$ CFU/mL of viable counts. When pipetting 1 mL of the diluted fluid onto the R2A Agar Medium and incubating at 20-25°C for 4-7 days, sufficient proliferation of the inoculated strain must be observed.

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Rapid Microbial Methods / *General information*

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1. Detection targets and principles

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3. Applications and particular considerations

New methods are expected to find application in a variety of fields.

However, since their detection targets and detection protocols / procedures are different from the conventional methods, the resulting data may not show a good correlation with existing data.

3. Applications and particular considerations

Although, it is important in principle that a new method should have an equal or greater capability than the conventional method, a new method may be used after verifying their validity, even in the absence of equivalence to conventional methods.

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3. Applications and particular considerations

Because the new methods are rapid, **product testing, environmental monitoring, bioburden evaluation, raw materials control, etc. can be performed in real-time,** and this is highly advantageous for process control, allowing alert levels, action levels and so on to be set up based on trend analysis of the obtained data.

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3. Applications and particular considerations

Because the new methods are rapid, product testing, environmental monitoring, bioburden evaluation, raw materials control, etc. can be performed **in real-time,** and this is highly advantageous for process control, allowing alert levels, action levels and so on **to be set up based on trend analysis of the obtained data.**

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3. Applications and particular considerations

These new rapid methods may be applied to;

- Quality control of pharmaceutical manufacturing water
- Microbial evaluation of processing areas
- Sterility test
- Microbial limit test
- Antimicrobial and preservatives effectiveness test
- Raw material acceptance test

etc.

Conclusions and future directions:

- RMMs are using a different signal instead of CFU.
- The detection principles of RMMs are different from that of conventional methods, the correlation between them is not always required.
- RMMs should be performed in short time with accuracy.
- RMMs may be applied to quality control of the production acceptance test (PAT) and so on.
- Phylogenic analysis based on the high throughput sequencing shows real microbial world in the pharmaceutical manufacturing facilities.