



EXAMPLES OF VALIDATION PROTOCOLS OF THE ALTERNATIVE MICROBIOLOGICAL METHODS ACCORDING TO CHAPTER 5.1.6 "Alternative methods for control of micro- biological quality"



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EXAMPLES OF VALIDATION PROTOCOLS OF THE ALTERNATIVE MICROBIOLOGICAL METHODS ACCORDING TO CHAPTER 5.1.6“Alternative methods for control of microbiological quality”

The following are examples of alternative methods developed, validated and used by various laboratories. The examples are based on the methods and equipment used by the laboratories involved and are published for information only and not for general application. They are not provided as a recommendation from the Pharmacopoeia and cannot be cited as references.

Although the proprietary names of the instruments are not given, the characteristics of some are so specific that they may be identifiable. It is not the intention here to advocate the use of a specific technology, or to give preference to one manufacturer over another. The sole intention of this section is to give users an insight into the level of information required for regulatory acceptance.

1. EXAMPLE OF A VALIDATION PROTOCOL FOR A RAPID STERILITY TEST

1.1 PRINCIPLE OF THE ALTERNATIVE METHOD

This alternative sterility test method is based on membrane filtration. Following a pooling step, the sample is filtered through filtration funnels. The membranes are subsequently transferred onto cassettes containing suitable solid nutrient media and incubated. Incubation conditions are chosen in order to reproduce the incubation conditions of the pharmacopoeial sterility test. Incubation time is defined taking into account the time required to ensure detection of a worst-case stressed micro-organism, while including an adequate safety margin. After incubation, the membranes are sprayed with the appropriate reagents and examined using a system that detects (micro)colonies by adenosine triphosphate (ATP) bioluminescence.

1.2 VALIDATION OF THE ALTERNATIVE METHOD

1.2.1 Specificity

The specificity of a qualitative microbiological method is defined as its ability to detect only the micro-organisms sought (i.e., it does not generate false positive results). In the case of a sterility test, this implies that the method is capable of detecting all kinds of micro-organisms without producing a positive test result in the absence of micro-organisms.

To demonstrate this ability, both methods, alternative and pharmacopoeial, are performed in parallel with an inoculum containing less than 100 CFU of a representative panel of micro-organisms, including pharmacopoeial reference strains (see chapter 2.6.1, table 2.6.1.-1) and in-house isolates.

The in-house isolates are selected to represent the flora obtained during environmental monitoring, and include slow-growing micro-organisms.

If necessary, specific stress conditions, such as heat treatment (for non-spore-forming micro-organisms) or nutrient depletion (for spore-forming micro-organisms), may be applied before the suspensions are used¹.

Acceptance criterion: all the micro-organisms in each run show growth.

In order to demonstrate that no false positive results are obtained in the absence of micro-organisms, the background bioluminescence is evaluated by examining at least 3 sterile membranes through which the product to be examined has been filtered. A risk analysis must have previously shown that ATP from other sources can be excluded as a cause of false positive results.

Acceptance criterion: no false positive results occur due to background bioluminescence.

1.2.2 Detection limit

The detection limit is evaluated using the pharmacopoeial reference strains recommended in chapter 2.6.1, and stressed in-house isolates representative of a wide variety of potential contaminants (including endospores and very slow-growing micro-organisms). Three 10-fold dilutions are prepared from a starting quantity of 50 CFU for each micro-organism (i.e., a range from approximately 50 CFU to 0.05 CFU). A significant number of replicates (e.g., minimum 10 replicates of each dilution) are tested using the alternative method and the pharmacopoeial method, respectively. The 95 per cent confidence limits of the most probable number (MPN) per gram is deduced from a MPN table for each method. The 95 per cent confidence limits for the ratio between the respective proportions of micro-organisms detected by the methods is also estimated.

Acceptance criterion: The detection limit value of the alternative method must not be greater than that of the pharmacopoeial method.

The detection limit is evaluated based on the results of at least two independent test runs per micro-organism.

1.2.3 Robustness

Two parameters have been found to have a major impact on test results and may also present significant variability: the incubation time and the time required to transfer the filter membrane from the spraying station to the detection system. As regards materials and environments, robustness is tested using different filter funnel lots, reagent lots, media lots, rinsing fluid lots, anaerobic incubation systems, analysts and laboratories. Robustness experiments are conducted with both a fast-growing and a slow-growing micro-organism in inocula containing 10-100 CFU. Three test runs with 3 replicates are performed for each micro-organism and robustness parameter.

Acceptance criterion: no statistically significant difference is observed between the conditions of interest (e.g., reagent lot A and reagent lot B) for a predefined micro-organism recovery after 14 days of incubation.

¹Gray, J.C., Staerk, A., Berchtold, M., Hecker, W., Neuhaus, G., Wirth, A. Growth-promoting Properties of Different Solid Nutrient Media Evaluated with Stressed and Unstressed Micro-organisms: Prestudy for the Validation of a Rapid Sterility Test. PDA Journal of Pharmaceutical Science and Technology 2010, 64: 249-263

1.2.4 Suitability testing

The product-specific suitability study focuses on identification of an adequate rinse protocol and demonstration that the product to be examined does not interfere with the alternative method by inducing high background bioluminescence or inhibiting the bioluminescence reaction. To demonstrate absence of a residual inhibitory effect after rinsing the membrane, 3 test runs are carried out with the pharmacopoeial reference strains recommended in chapter 2.6.1 and with in-house isolates. A control (identically inoculated membrane through which no product has been filtered) is prepared for comparison. Possible inhibition of the bioluminescence reaction is excluded by inoculating 3 membranes through which the product to be examined has been filtered with 10-100 CFU of a stressed, slow-growing micro-organism.

Acceptance criterion: an equivalent number of bioluminescent (micro)colonies is recovered compared to the control.

1.2.5 Equivalence testing

Contaminated test samples are prepared by inoculation with fewer than 5 CFU of 3 different test micro-organisms. Three test runs are then performed with 10 replicates of each of the three contaminated samples, with the alternative and the pharmacopoeial method in parallel. Routine test conditions are simulated as closely as possible, for example by also applying a standard rinse protocol. The results obtained are subjected to statistical evaluation.

Acceptance criterion: statistical analysis demonstrates 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 pharmacopoeial method was used.

2. EXAMPLE OF A VALIDATION PROTOCOL FOR AN ALTERNATIVE QUANTITATIVE METHOD (ENUMERATION OF MICRO-ORGANISMS)

2.1 PRINCIPLE OF THE ALTERNATIVE METHOD

The alternative method uses solid phase cytometry which combines 3 well-known technologies: membrane filtration, fluorescent cell staining and laser scanning. After standard membrane filtration, all viable micro-organisms retained on the membrane are fluorescently stained through enzymatic cleavage of a non-fluorescent substrate to liberate free fluorochrome into the cytoplasm. Only metabolically active (viable) cells and spores with intact membranes will appear fluorescent. . After viability staining, a fully overlapping laser scan is performed and any viable cells present on the membrane are individually detected and automatically counted. Given that the solid phase cytometry test for the enumeration of micro-organisms counts viable cells individually, the result is expressed as the number of viable cells per unit volume and not in CFU per unit volume.

2.2 VALIDATION OF THE ALTERNATIVE METHOD

2.2.1 Accuracy, precision, quantitation limit, linearity and range

The alternative method is validated using a representative panel of different micro-organisms, including pharmacopoeial reference strains (see chapter 2.6.12, table 2.6.12.-1) and in-house isolates.

The accuracy, precision, quantitation limit, linearity and range of both the alternative method and the pharmacopoeial quantitative method (membrane filtration method) for the enumeration of micro-organisms are determined using an independent dilution series – 300 CFU/mL, 100 CFU/mL, 50 CFU/mL, 10 CFU/mL, 5 CFU/mL and 1 CFU/mL – individually prepared for each micro-organism by three different analysts. Five replicates (*Note: a 6th replicate of the 100 CFU/mL dilution is prepared for the determination of precision*) of each concentration of the independent dilution series are tested in parallel by 3 analysts using the alternative and the pharmacopoeial methods. The experiments are performed using different equipment and different lots of reagents.

Acceptance criteria:

- (i) *Accuracy:* the alternative method is shown to recover at least as many micro-organisms as the pharmacopoeial method, using appropriate statistical analysis;
- (ii) *Precision:* the coefficient of variation is calculated and the precision of the alternative method is comparable to that of the pharmacopoeial method.
- (iii) *Linearity:* the regression line is calculated and the method is linear if the estimated slope is significant and the deviation from linearity is non-significant (see general chapter 5.3).
- (iv) *Range:* the range of the alternative method is equal or greater to the range of the pharmacopoeial method.
- (v) *Quantitation limit:* the lowest concentration in the linear range is considered to be the quantitation limit of the method. The value obtained for this limit with the alternative method must not be greater than that of the pharmacopoeial method.

2.2.2 *Specificity*

To determine the specificity of both methods, a suspension of *S. aureus* with a titre of approximately 100 CFU/mL is prepared and divided into 2 aliquots for each assay. One aliquot of the suspension is treated to inactivate/kill all viable micro-organisms using conditions that avoid lysis of the non-viable cells (for example, by treatment with 100 per cent ethanol). In both methods (solid phase cytometry and membrane filtration, for the alternative and pharmacopoeial methods, respectively), 3 replicates of 1 mL each of the treated suspension, representing approximately 100 non-viable cells are filtered individually using membrane filtration equipment. After filtration, 3 replicates of 1 mL each of the untreated suspension, representing approximately 100 viable cells or CFUs, are filtered using the same membranes. Consequently, about 100 non-viable cells and about 100 viable cells or CFUs are retained by each of the membranes.

Acceptance criterion: regardless of the different principles on which the alternative and the pharmacopoeial methods for enumeration of micro-organisms are based, the final test result for both these methods is about 100 viable cells or CFUs per membrane.

2.2.3 *Robustness*

The repeatability, intermediate precision and reproducibility of the alternative and the pharmacopoeial methods are determined by deliberately varying a number of key parameters, for example, the validation experiments can be performed on different days, by different analysts, using different systems or different reagent batches and in different laboratories.

Acceptance criterion: these deliberate variations do not have a significant effect on the test results.

2.2.4 Suitability testing

Suitability testing on the alternative and pharmacopoeial methods for enumeration of micro-organisms is performed in accordance with section 4-5 of general chapter 2.6.12. It must therefore be proven that the product does not inhibit the growth or detection of viable micro-organisms under the conditions of the test. In order to do so, test suspensions of not more than 100 CFU for each test micro-organism are added separately into the rinse during the membrane filtration step of both methods (at the same rinsing step and in the same manner).

Acceptance criterion: a recovery of 50-200 per cent is obtained in accordance with general chapter 2.6.12.

2.2.5 Equivalence testing

The equivalence of the enumeration tests is demonstrated directly by performing the assays side-by-side for a predetermined period of time (or number of samples) and then carrying out statistical analysis to compare the results.

Acceptance criterion: statistical analysis demonstrates 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 pharmacopoeial method was used.

3. EXAMPLE OF A VALIDATION PROTOCOL FOR A MOLECULAR-BASED MICROBIAL IDENTIFICATION METHOD

3.1 PRINCIPLE OF THE ALTERNATIVE METHOD

The gene that encodes rRNA (rDNA) is the most conserved DNA in all cells and is extensively used to identify micro-organisms and determine their taxonomy and phylogeny. The 16S rDNA sequence has hypervariable regions that have diverged during evolution, flanked by strongly conserved regions. Primers for the identification of micro-organisms are designed to bind to these conserved regions and to amplify the variable regions between them. The amplicons of the PCR reaction are purified and subsequently forward- and reverse-sequenced. Based on these forward and reverse sequences, a consensus sequence is assembled and compared to sequence databases.

3.2 VALIDATION OF THE ALTERNATIVE METHOD

3.2.1 Accuracy

The accuracy of the method is investigated using several well-characterised bacterial-type, yeast and mould strains, which are found in the database.

Different subcultures of well-characterised micro-organisms are harvested (at least 3 individual colonies are collected), prepared and subjected to identification. All strains are sequenced 3 times on 3 successive days by 1 analyst.

Acceptance criterion: Micro-organisms are identified correctly to species level and above the pre-defined reporting level for sequence homology (for example, see Table 1).

3.2.2 Specificity

The specificity of an identification method is its ability to discriminate micro-organisms actually present from interfering factors that generate false identification results, e.g.,

mixtures of micro-organisms that give positive identification results for micro-organisms that are not present in the sample. Mixtures of appropriate micro-organisms can be used to demonstrate the ability of the test to reveal either an invalid result or a valid result for a micro-organism actually present in the mixture. The assay must be able to differentiate between valid and invalid results which are due to mixed cultures or DNA.

Acceptance criterion: the results of the specificity test using mixed cultures (quantified and titred based on CFU or using an analytical method to standardise the DNA titre) lead to valid identifications. These results must clearly demonstrate that the analysis of mixed cultures does not result in false identifications (validation example, see Table 2).

3.2.3 **Robustness**

Method robustness can be determined at the same time as method accuracy and precision. During multiple identifications of the same micro-organism, several critical parameters can be deliberately modified.

An intra-laboratory study can be performed using modifications of the critical parameters for microbial identification. These include carrying out the identification on different working days; with different analysts within one laboratory; using different of test-kit and reagent lots; using cultures of different ages (e.g. 3- and 7-day-old cultures of the reference strains).

Different subcultures of well-characterised micro-organisms are harvested (at least 3 individual colonies are collected), prepared and subjected to identification. All strains are sequenced 3 times on 3 successive days by the same analyst (accuracy and intra-laboratory robustness) and on 3 successive days by 2 analysts (intra-laboratory robustness). Sample preparation and instrument set-up are carried out according to the internal procedures of the laboratory.

It is also possible to modify this study to include an inter-laboratory study, in which case the microbial identification is performed on the same strains by different laboratories using:

- different types of equipment, if available;
- slightly different procedures for the purification of the target DNA;
- different test-kits and test-kit lots (for the purification of the PCR-amplicons as well as for purification of the sequencing products);
- different versions of the data-processing software, if applicable.

Acceptance criterion: Micro-organisms are identified correctly to species level, with a pre-defined percentage match between the database and the test strains (validation example, see Table 1).

If feasible, identical acceptance criteria for accuracy and robustness are applied by each collaborating laboratory.

Table 1 – Summary of the validation studies for accuracy and robustness for a molecular-based microbial identification method

Species	Repeatability						Reproducibility				
	Sequence homology/ [% match]			Max. difference in sequence homology/ [%]	Average of sequence homology/ [% match]	Standard deviation	Worst sequence homology/ [% match]	Best sequence homology/ [% match]	Max. difference in sequence homology/ [%]	Average of sequence homology/ [%]	Standard deviation
	Run 1	Run 2	Run 3								
<i>Pseudomonas aeruginosa</i> (ATCC 10145 T/DSM 50071T)	100.0	99.8	99.7	0.3	99.8	0.15	99.68	100.00	0.32	99.94	0.12
<i>Stenotrophomonas maltophilia</i> (ATCC 13637 T/ DSM 50170 T)	100.0	99.8	100.0	0.2	99.9	0.12	99.79	100.00	0.21	99.98	0.07
<i>Escherichia coli</i> (ATCC 11775 T/ DSM 30083T)	99.6	99.9	99.8	0.3	99.8	0.15	99.58	99.90	0.32	99.80	0.11
<i>Salmonella choleraesuis</i> (ATCC 13312 T/DSM 14846 T)	99.8	100.0	100.0	0.2	99.9	0.12	99.79	100.00	0.21	99.98	0.07
<i>Staphylococcus aureus</i> (ATCC 12600 T/DSM 20231T)	99.8	100.0	100.0	0.2	99.9	0.12	99.79	100.00	0.21	99.98	0.07
<i>Staphylococcus epidermidis</i> (ATCC 14990 T/ DSM 20044T)	100.0	100.0	100.0	0	100.0	0	99.70	100.00	0.30	99.91	0.13
<i>Streptococcus agalactiae</i> (ATCC 13813 T/DSM 2134 T)	100.0	100.0	99.9	0.1	100.0	0.06	99.38	100.00	0.62	99.85	0.20
<i>Enterococcus faecalis</i> (ATCC 19433 T/ DSM 20478 T)	100.0	99.9	99.9	0.1	99.9	0.06	99.80	100.00	0.20	99.88	0.06
<i>Bacillus cereus</i> (ATCC 14579 T/ DSM 31T)	100.0	100.0	100.0	0	100.00	0	99.60	100.00	0.40	99.93	0.14
<i>Bacillus subtilis subsp. Subtilis</i> (ATCC 6051 T/ DSM 10T)	99.8	99.8	99.8	0.2	99.8	0	99.60	99.80	0.20	99.77	0.07
<i>Corynebacterium bovis</i> (ATCC 7715 T/DSM 20582T)	100.0	100.0	100.0	0	100.0	0	100.00	100.00	0	100.00	0
<i>Carnobacterium gallinarum</i> (ATCC 49517 T/ DSM 4847 T)	99.9	99.9	99.9	0.1	99.0	0	99.70	99.90	0.20	99.81	0.11
<i>Clostridium perfringens</i> (ATCC 13124 T/DSM 756 T)	99.7	99.7	99.7	0.3	99.7	0	99.67	99.78	0.11	99.71	0.05
<i>Candida albicans</i> (ATCC 18804 T/ CBS 562T) or (ATCC 10231/CBS6431)	100.0	100.0	100.0	0	100	0	100.00	100.00	0	100.00	0
<i>Aspergillus brasiliensis</i> (ATCC 16404/CBS 733.88)	100.0	100.0	100.0	0	100	0	100.00	100.00	0	100.00	0

Table 2 – Summary of specificity validation studies (mixed cultures quantified and standardised using a DNA quantification method)

Experiment	Micro-organism(s)	Ratio	ID Result
Pure Culture 1	<i>S. aureus</i>		<i>S. aureus</i>
Pure Culture 2	<i>M. luteus</i>		<i>M. luteus</i>
Pure Culture 3	<i>R. pickettii</i>		<i>R. pickettii</i>
Pure Culture 4	<i>P. aeruginosa</i>		<i>P. aeruginosa</i>
Mixed Culture 1	<i>S. aureus</i> + <i>M. luteus</i>	1:1	<i>S. aureus</i>
Mixed Culture 2	<i>S. aureus</i> + <i>M. luteus</i>	1:9	<i>S. aureus</i>
Mixed Culture 3	<i>S. aureus</i> + <i>M. luteus</i>	9:1	<i>S. aureus</i>
Mixed Culture 4	<i>R. pickettii</i> + <i>P. aeruginosa</i>	1:1	<i>R. pickettii</i>
Mixed Culture 5	<i>R. pickettii</i> + <i>P. aeruginosa</i>	1:9	<i>P. aeruginosa</i>
Mixed Culture 6	<i>R. pickettii</i> + <i>P. aeruginosa</i>	9:1	<i>R. pickettii</i>
Mixed Culture 7	<i>S. aureus</i> + <i>R. pickettii</i>	1:1	<i>R. pickettii</i>
Mixed Culture 8	<i>S. aureus</i> + <i>R. pickettii</i>	1:9	<i>R. pickettii</i>
Mixed Culture 9	<i>S. aureus</i> + <i>R. pickettii</i>	9:1	<i>B. gladioli</i> *
Mixed Culture 10	<i>S. aureus</i> + <i>P. aeruginosa</i>	1:1	<i>P. aeruginosa</i>
Mixed Culture 11	<i>S. aureus</i> + <i>P. aeruginosa</i>	1:9	<i>P. aeruginosa</i>
Mixed Culture 12	<i>S. aureus</i> + <i>P. aeruginosa</i>	9:1	<i>P. aeruginosa</i>
Mixed Culture 13	<i>M. luteus</i> + <i>R. pickettii</i>	1:1	<i>R. pickettii</i>
Mixed Culture 14	<i>M. luteus</i> + <i>R. pickettii</i>	1:9	<i>R. pickettii</i>
Mixed Culture 15	<i>M. luteus</i> + <i>R. pickettii</i>	9:1	<i>R. pickettii</i>
Mixed Culture 16	<i>M. luteus</i> + <i>P. aeruginosa</i>	1:1	<i>P. aeruginosa</i>
Mixed Culture 17	<i>M. luteus</i> + <i>P. aeruginosa</i>	1:9	<i>P. aeruginosa</i>
Mixed Culture 18	<i>M. luteus</i> + <i>P. aeruginosa</i>	9:1	<i>P. aeruginosa</i>
Mixed Culture 19	<i>S. aureus</i> + <i>M. luteus</i> + <i>R. pickettii</i> + <i>P. aeruginosa</i>	1:1:1:1	<i>R. pickettii</i>

*ID does not fulfil test acceptance criteria - consensus length is only 188 bp → invalid result

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