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Biotechnology — Analytical methods — Risk-based approach for method selection and validation for rapid microbial detection in bioprocesses

Biotechnologie — Méthodes d'analyse — Approche basée sur les risques pour la sélection et la validation de méthodes pour la détection microbienne rapide dans les bioprocédés

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Foreword

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This document was prepared by Technical Committee ISO/TC 276, *Biotechnology*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Patient safety is essential in providing cell-based therapies. However, novel cell-based therapies present many challenges with respect to the timely assessment of microbial contamination. Since many cell-based therapies have short shelf lives, they are administered to patients within hours after formulation. In addition to final product testing, testing on cell banks and product intermediates is common. Microbiological testing includes bacteria, fungi, mycoplasma and viral adventitious agents. Culture-based testing methods (e.g. pharmacopeia methods) have been widely adopted by industry. However, culture-based testing methods can take days to weeks to obtain a result. More rapid methods for microbiological testing are needed to ensure patient safety prior to product administration. The development and use of rapid, validated methods that are sensitive and accurate, and that allow for the detection of a broad range of microorganisms are therefore desired and supported by this document.

Biotechnology — Analytical methods — Risk-based approach for method selection and validation for rapid microbial detection in bioprocesses

1 Scope

This document provides guidance, a framework and a risk-based approach for the selection and validation of methods for rapid microbial detection in cellular therapeutic product manufacturing.

This document provides a flexible risk-based framework for the detection of microbial contamination in cellular therapeutic products and cellular intermediates.

This document provides general requirements and risks associated with cellular therapeutic product manufacturing, with flexibility to address differences in specific manufacturing processes of each unique cellular therapeutic product.

This document primarily addresses sterility testing in cellular therapeutic product manufacturing. This document is applicable to other cell-derived therapeutic product manufacturing.

This document focuses on rapid microbial test methods (RMTMs) used for both in-process and final product testing.

Viral testing in cellular therapeutic product manufacturing is not included in this document.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
acceptance criteria
numerical limits, ranges, or other attributes or variables meeting predefined performance for the assays described

Note 1 to entry: Acceptance criteria are specified by the *user requirement specifications* (3.30).

3.2
accuracy
measurement accuracy
closeness of agreement between a measured quantity value and an assigned quantity value of a measurand

Note 1 to entry: The concept “measurement accuracy” is not a quantity and is not given a numerical quantity value. A measurement is said to be more accurate when it offers a smaller measurement error.

Note 2 to entry: The term “measurement accuracy” should not be used for measurement trueness and the term measurement precision should not be used for “measurement accuracy”, which, however, is related to both these concepts.

Note 3 to entry: “Measurement accuracy” is sometimes understood as closeness of agreement between measured quantity values that are being attributed to the measurand.

[SOURCE: ISO 16140-1:2016, 2.2]

3.3
analytical sensitivity
quotient of the change in measurement indication and the corresponding change in value of a quantity being measured

Note 1 to entry: Analytical sensitivity should not be used to mean *detection limit* (3.8) or quantitation limit and should not be confused with *diagnostic sensitivity* (3.9).

[SOURCE: ISO 18113-1:2022, 3.2.4, modified — Admitted term “sensitivity of a measurement procedure” deleted. Notes 1 to 3 to entry deleted. Note 4 to entry renumbered as Note 1 to entry.]

3.4
analytical specificity
capability of a measuring system, using a specified measurement procedure, to provide measurement results for one or more measurands which do not depend on each other nor on any other quantity in the system undergoing measurement

Note 1 to entry: Lack of analytical specificity is called analytical interference.

Note 2 to entry: Analytical specificity should not be confused with *diagnostic specificity* (3.10).

Note 3 to entry: ISO/IEC Guide 99:2007 uses the term “selectivity” for this concept instead of “specificity”.

[SOURCE: ISO 18113-1:2022, 3.2.5, modified — Admitted term “selectivity of a measurement procedure” deleted. Notes to entry replaced.]

3.5
aseptic
conditions and procedures used to exclude the introduction of microbial contamination

[SOURCE: ISO 18362:2016, 3.3, modified — “aseptic” replaced “aseptic technique” as the term.]

3.6
cellular therapeutic product
product containing cells as the active substance

EXAMPLE Cell and gene therapy products, tissue engineered products, drug products.

Note 1 to entry: Products produced from cells for gene therapies are included in the definition of cellular therapeutic product, as cells are not necessarily the active substance for all gene therapies.

Note 2 to entry: Recombinant proteins are not included in this definition of cellular therapeutic product.

[SOURCE: ISO 20399:2022, 3.9, modified — “used for cell therapy or gene therapy” deleted from the definition.]

3.7
design qualification
DQ
process for *verification* (3.32) that the proposed specification for the facility, equipment or system of the assay meets the expectation for the *user requirement specifications (URS)* (3.30)

[SOURCE: ISO 11139:2018, 3.220.1, modified — Abbreviated term “DQ” and “of the assay” added. “user requirement specifications (URS)” replaced “intended use”.]

3.8
detection limit
limit of detection
measured quantity value, obtained by a given measurement procedure, for which the probability of falsely claiming the absence of a component in a material is β, given a probability α of falsely claiming its presence

Note 1 to entry: IUPAC recommends default values for α and β equal to 0,05.

Note 2 to entry: The abbreviation LOD is sometimes used.

Note 3 to entry: The term "sensitivity" is discouraged for "detection limit".

[SOURCE: ISO/IEC Guide 99:2007, 4.18]

3.9
diagnostic sensitivity
ability of an *in vitro* diagnostic examination procedure to identify the presence of a target marker associated with a particular disease or condition

Note 1 to entry: Also defined as percent positivity in samples where the target marker is known to be present.

Note 2 to entry: Diagnostic sensitivity is expressed as a percentage (number fraction multiplied by 100), calculated as 100 × the number of true positive values (TP) divided by the sum of the number of true positive values (TP) plus the number of false negative values (FN), or 100 × TP/(TP + FN). This calculation is based on a study design where only one sample is taken from each subject.

Note 3 to entry: For microbial detection, diagnostic sensitivity represents the fraction of target organisms that were detected correctly.

[SOURCE: ISO 18113-1:2022, 3.2.17, modified — "identify the presence of a target marker" replaced "have positive results". Second sentence of Note 1 to entry deleted. Note 3 to entry replaced.]

3.10
diagnostic specificity
ability of an *in vitro* diagnostic examination procedure to recognize the absence of a target marker associated with a particular disease or condition

Note 1 to entry: Also defined as percent negativity in samples where the target marker is known to be absent.

Note 2 to entry: Diagnostic specificity is expressed as a percentage (number fraction multiplied by 100), calculated as 100 × the number of true negative values (TN) divided by the sum of the number of true negative values (TN) plus the number of false positive values (FP), or 100 × TN/(TN+FP). This calculation is based on a study design where only one sample is taken from each subject.

[SOURCE: ISO 18113-1:2022, 3.2.18, modified — "recognize the absence of a target marker associated with a" replaced "have negative results associated with an absence of". Second sentence of Note 1 to entry deleted. Note 3 to entry deleted.]

3.11
false negative
result indicated by the test method to be *negative* (3.15) which has subsequently been shown to contain the target microorganisms

[SOURCE: ISO 13843:2017, 3.14, modified — "microorganisms" replaced "organism".]

3.12
false positive
result indicated by the test method to be *positive* (3.19) which was subsequently shown not to contain the target microorganisms

[SOURCE: ISO 13843:2017, 3.15, modified — "microorganisms" replaced "organism".]

3.13
fit for purpose
in line with prearranged requirements for an intended use

[SOURCE: ISO 20387:2018, 3.24, modified — Admitted term "fitness for the intended purpose" and Note 1 to entry deleted.]

3.14
installation qualification
IQ
process of establishing by objective evidence that all key aspects of the process equipment and ancillary system for the assay instrument installation comply with the approved *user requirement specifications (URS)* (3.30)

[SOURCE: ISO 11139:2018, 3.220.2, modified — "for the assay instrument" added and "user requirement specifications (URS)" replaced "specification".]

3.15
negative
test result indicating the absence of the analyte in a given test portion as defined by the procedure of the method

[SOURCE: ISO 16140-1:2016, 2.43, modified — "negative" replaced "negative test result" as the term. "the absence of the analyte" replaced "the analyte was not detected" and "qualitative" deleted before "method".]

3.16
nucleic acid amplification techniques
NAT
biochemistry and molecular biology methods that involve the *in vitro* synthesis of many copies of DNA or RNA from one original template

Note 1 to entry: NAT is characterized by existence of reverse transcription, amplification method and type of determination (qualitative or quantitative)

Note 2 to entry: Examples of amplification methods are PCR and iso thermal amplification (NEAR, TMA, LAMP, HAD, CRISPER, SDA)

3.17
operational qualification
OQ
process of obtaining and documenting evidence that installed equipment operates within predetermined limits when used in accordance with its operational procedures

[SOURCE: ISO 11139:2018, 3.220.3]

3.18
performance qualification
PQ
process of establishing by objective evidence that the assay process, under anticipated conditions, consistently produces a result which meets all predetermined *user requirement specifications (URS)* (3.30)

[SOURCE: ISO 11139:2018, 3.220.4, modified — "assay" added before "process", "result" replaced "product" and "user requirement specifications (URS)" replaced "requirements".]

3.19
positive
test result indicating the presence of the analyte in a given test portion as defined by the procedure of the method

Note 1 to entry: When the reference method or alternative method provides a preliminary positive test result requiring further testing to confirm this result, this test result can be considered as a presumptive positive test result. If the further testing specified by the method's procedure confirms that the test result can indeed be considered as being positive, the test result can be considered as a confirmed positive test result.

[SOURCE: ISO 16140-1:2016, 2.50, modified — "positive" replaced "positive test result" as the term.]

3.20
precision
closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions

Note 1 to entry: Measurement precision is usually expressed numerically by measures of imprecision, such as standard deviation, variance, or coefficient of variation under the specified conditions of measurement.

Note 2 to entry: The "specified conditions" can be, for example, repeatability conditions of measurement, intermediate precision conditions of measurement, or reproducibility conditions of measurement (see ISO 5725-1).

[SOURCE: ISO/IEC Guide 99:2007, 2.15, modified — "precision" replaced "measurement precision" as the term. Notes 3 and 4 to entry deleted.]

3.21
qualification
activities undertaken to demonstrate that utilities, equipment and methods are suitable for their intended use and perform properly

Note 1 to entry: Qualification of either equipment or processes, or both, generally includes *installation qualification* (3.14), *operational qualification* (3.17) and *performance qualification* (3.18).

[SOURCE: ISO 11139:2018, 3.220, modified — "or modes" deleted after "methods".]

3.22
rapid microbial test method
RMTM
analytical method that allows the user to get microbiology test results faster compared with traditional visual observation methods using direct inoculation and culture-plating

Note 1 to entry: Generally, this means in a significantly reduced time as compared with the traditional method (e.g. hours or days).

3.23
reference material
material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in measurement or in examination of nominal properties

[SOURCE: ISO/IEC Guide 99:2007, 5.13, modified — Abbreviated term "RM", notes to entry and examples deleted.]

3.24
risk assessment
overall process of risk identification, risk analysis and risk evaluation

[SOURCE: ISO Guide 73:2009, 3.4.1]

3.25
risk control
process in which decisions are made and measures implemented by which risks are reduced to, or maintained within, specified levels

[SOURCE: ISO 14971:2019, 3.21]

3.26
risk-based approach
methodology that allows the prioritization of activities based on a previous analysis of data and according to the biosafety level

3.27
robustness
measure of a test method's capacity to remain unaffected by small, but deliberate, variations in method parameters and to provide an indication of its reliability during normal usage

[SOURCE: ICH Q2(R1)[11]]

3.28
shelf life
period of time after production during which a product that is kept under specified conditions retains its specified properties

[SOURCE: ISO 1382:2020, 3.485, modified — The term "storage life" deleted. "material or" deleted before "product" and "that is" added.]

3.29
sterility
state of being free from *viable microorganisms* (3.33)

Note 1 to entry: In practice, no such absolute statement regarding the absence of microorganisms can be proven.

[SOURCE: ISO 11139:2018, 3.274]

3.30
user requirement specifications
URS
requirements specific to a user or requirements that are not covered in general requirements

3.31
validation
confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled

[SOURCE: ISO 9000:2015, 3.8.13, modified — Notes to entry deleted.]

3.32
verification
confirmation, through the provision of objective evidence, that specified requirements have been fulfilled

[SOURCE: ISO 9000:2015, 3.8.12, modified — Notes to entry deleted.]

3.33
viable microorganism
microorganism within a sample that has at least one attribute of being alive (e.g. metabolically active, capable of reproduction, possession of an intact cell membrane, with the capacity to resume these functions) defined based on the intended measurement purpose

4 General considerations

Prior to patient administration, cellular therapeutic products should be tested for microbial contamination. Many of these products rely on the activity of viable cells for a therapeutic effect. Viable cells cannot be terminally sterilized and rely on a combination of aseptic techniques and closed-system manufacturing to ensure sterility of the final product. These products typically have a relatively short shelf life and are manufactured as single lots or small lots, presenting challenges for utilizing compendial or culture-based methods for detecting microbial contamination.[35][36][37][38]

When selecting a rapid method, the following should be taken into account:

a) the shelf life of the sample;

b) the volume of sample available for testing;

c) the number of samples to be tested;

d) the manufacturing step from which the sample will be collected;

e) the time to result;

f) the microorganisms to be detected;

g) how to distinguish viable from non-viable microorganisms;

h) the ability to speciate microbes that are identified in the sample.

In addition, it is important to take the availability of resources to conduct the tests into account such as trained personnel and required instrumentation.

NOTE 1 Sampling can introduce microbial contamination into the manufacturing process.

NOTE 2 The amount of material available for testing can be limited, especially for autologous cellular therapeutic products. In some cases, parallel cellular therapeutic products can be manufactured to assess microbial contamination.

NOTE 3 Test methods can require specific training and experience to be conducted and analysed.

It is recommended to consider adding cell supernatants (e.g. culture solution, washing solution, frozen stock solution) instead of cell containing solutions for sterility testing, to solve the problem of small samples of cell products that cannot be sampled for testing.

5 Risk management for microbiological contamination

5.1 Risk management in manufacturing process

A risk-based approach for determining methods to detect microbial contamination in cellular therapeutic product manufacturing should be used. It should take into account the source and method used for the collection of the cellular starting material.[1][2][3][4]

Potential sources of microbial contamination of cellular therapeutic products include, but are not limited to, cellular starting material, raw materials and consumables, and the manufacturing environment.[5]

Apheresis products are the most common source of cellular starting materials. Sources of microbial contamination can be associated with the incomplete disinfection of the skin, sterility failures in kits and bags used to collect and store the apheresis products, and technician/operator error. Donor bacteraemia can also be a source of contamination for the apheresis product.

Microbial contamination and infectious viruses in reagents, ancillary (raw) materials and recommendations for ancillary materials are described in ISO 20399. Consumables should be pre-sterilized and single use to reduce the risk of microbial contamination.[6]

Cell processing/manufacturing should be performed in a closed system or an appropriate clean room (e.g. ISO 6 to ISO 7) to prevent microbial contamination.

NOTE Open or benchtop processes increase the risk of contamination from the air and surfaces that possibly have not been adequately cleaned or disinfected.

Some general factors to be taken into account in a risk assessment for RMTMs are outlined in Annex A. Detailed points to take into account in some critical decisions for the use of RMTMs in cellular therapeutic product manufacturing that require a risk assessment can be found in Annexes B, C, D and E.[6][7]

Environmental controls can minimize the risk of microbial contamination. Examples of environmental controls are:

— sanitization procedures;

— high efficiency particulate air (HEPA) filtration and air flow;

— gowning procedures;

— aseptic technique;

— clean-room procedures and classifications (in accordance with ISO 14644-1).

Points to take into account when developing risk control can include, but are not limited to:

a) input materials:
   1) collection process and donor selection (see Annex B);
   2) autologous or allogenic, fresh or frozen;
   3) conditions;
b) ancillary materials in accordance with ISO 20399 and consumables (pre-sterilized, single use, etc.);
c) environmental factors;
d) equipment;
e) process steps:
   1) closed or open process steps;
   2) cell banking;
   3) culture or expansion;
   4) purification;
   5) final product;
f) containment strategy (see Annex C);
g) monitoring (see Annex C);
h) storage, packaging and administration (see Annex D);
i) in-process and final-release testing (see Annex E).

5.2 Risk management in microbial testing

The use of a risk assessment approach to rapid microbial testing in cellular therapeutic product manufacturing can limit the risk of validation of a rapid microbial testing system.[8][9][10][11] A risk-

based approach can be used to establish the most appropriate rapid microbial testing mechanism for intended use. This often focuses on determining the user requirement specifications (URS) as a foundation.

NOTE The following documents discuss risk assessment and give general guidance on how to implement it in manufacturing processes:

— ISO 31000;

— ISO 13022;

— ISO Guide 73.

6 Selection of a fit-for-purpose assay

6.1 General

Well- defined user requirements and a clear understanding of an assay's intended use(s) guide the design of assays with biological relevance and sufficient performance (e.g. analytical sensitivity, analytical specificity, precision, accuracy, robustness) to enable subsequent decision-making (fit for the intended purpose or fit for purpose).

To identify or develop an appropriate assay for detecting microbial contamination in cellular therapeutic products, the goal of testing should be established and documented. For example, detection versus quantification. If a test is needed to detect and identify "every" bacterial or fungal contaminant, then a sequencing approach should be used. If a test is needed to determine taxonomic or quantity resolution of only certain reference microorganisms or a limited list of compendial microorganisms,[35][36][37][38] then multiplexed PCR or a similar targeted approach is most suitable.

Appropriate assay design shall include specifications for the test method and strategies to ensure measurement quality and reproducibility of results. This can include incorporating replicate measurements, using sample randomization to reduce biases, and the inclusion of appropriate measurement controls.

Appropriate assay design shall also include approaches to ensure an appropriate analytical sensitivity and an established and documented detection limit. The uncertainty of the measurement should also be established and documented.

The assay shall have a high analytical specificity for the measurement target without significant interference from other components in the cell preparation.

The intended use of the assay should guide the fit-for-purpose requirements of the measurement. The uncertainty of measurement should be taken into account.

To determine the appropriate assays, users shall assess the issues of the number and types of microorganisms required for testing. The extent necessary for identification shall be determined. There shall be an assessment made as to whether a determination between viable and non-viable microbial cells is needed.

The assay should be sufficiently robust so that the results are not significantly affected by small changes in the measurement process (e.g. temperature fluctuations, minor sample handling fluctuations) as defined by the user for the intended purpose.

The assay should be sufficiently robust for the measurement target so that the results are not significantly affected by small changes in other components of the cell preparation (e.g. serum concentration, presence of preservation agents). When using non-compendial methods, the alternative method should yield results equivalent or better than compendial methods.[35][36][37][38]

6.2 Assay selection

Points to take into account when choosing a method include, but are not limited to, whether a method:[5][9]

a) is based on sound underlying scientific principles;

b) can detect all the microorganisms which need to be detected;

c) can identify all such microorganisms to the required taxonomic level;

d) can reproducibly quantitate such identified microorganisms, if necessary;

e) can detect analytes in the concentration range of interest;

f) has sufficient analytical specificity and analytical sensitivity for the intended use;

g) can meet specific method performance criteria;

h) has adequate quality assurance (QA) and quality control (QC);

i) can be performed with readily available equipment;

j) uses appropriate resources;

k) has sufficient robustness;

l) addresses the level of expertise required (e.g. technique-sensitive areas, need of specialized training);

m) contains necessary aspects of QA (e.g. calibrated equipment, media quality, incubation conditions, amplicon quality, sequence data quality);

n) addresses biosafety concerns (e.g. necessity of specific biosafety practices to handle the test pathogens);

o) meets specific requirements for the intended use (e.g. appropriate test parameters for materials that contain bacteria from starting materials that cannot be sterilized through the manufacturing process);

p) accounts for clinical conditions and natural cell variations within the patient population;

q) is a general microbial testing method or a specific microbial testing method;

r) is fit to detect bacteria, fungi and mycoplasma as appropriate;

s) can distinguish viable from non-viable microorganisms.

6.3 Kit or system selection

A vendor can refer to a contract laboratory that performs the test or does the validation or the supplier of the kit or system.[12][13][14][15] In release testing, a kit or system vendor should have proper quality assessment and should follow guidelines for manufacturers of testing kits, or ISO 13485 when applicable. The kit or system should be accompanied by applicable validation documents and services.

If a manufacturer is using an external kit or system vendor of RMTMs, the following points for the kit or system vendor selection should be taken into account:

a) required QA certification of kit or system vendor;

b) QA standards employed by the kit or system vendor (e.g. ISO 9001);

c) audit history and plan (i.e. auditing by regulators, pharmaceutical companies or accreditation bodies);

d) economic viability of kit or system vendor;

e) references for credibility of the test method (i.e. current user list, scientific publications or accreditation);

f) technical support services available from the kit or system vendor;

g) documentation supplied by the kit or system vendor (i.e. evaluation and validation documentation).

6.4 Considerations for various test types

The microbial tests are risk-based, so the user can select the preferred technology for their intended use and balance competing URS including the time to result, specificity, detection limit, sample size and product attributes.

Recommended approach for microbial contamination detection options includes:

a) after completion of a batch, record the step that includes aseptic manipulations (e.g. manual replenishment of the cell culture medium), and a sample should be taken to screen for microbial contamination (i.e. in-process testing);

b) a sample should be taken in advance of the final step with the timing reflecting the screening method employed, e.g. 48 h to 72 h prior to release for a growth-based contamination check or shorter for a more rapid microbial test (i.e. surrogate finished product testing);

c) the final product can be subjected to a compendial or growth-based microbial test and released before the completion of the test with the clinician responsible for the treatment of the patient being notified if the test becomes positive (i.e. traditional finished product testing);

d) the final product should be tested for microbial contamination using a rapid microbial test and released at the successful completion of the test (i.e. real-time finished product testing).

Table 1 describes these different release testing strategies with the detection options.

Further information and guidance on specification of various RMTM types can be found in Annex H.

Table 1 — In-process and release testing strategies[6]

Technology	Basis of the test	Detection option	Viable versus non-viable distinction
Gram stain	Differential staining of bacterial cells	Final product testing	no
USP <71> sterility test	Growth in soybean-casein digest and fluid thioglycolate medium	Final product testing	yes
Respiration	CO_2 production in proprietary aerobic	In-process monitoring, timed pre-release testing, final product testing	yes
Nucleic acid method	Nucleic acid amplification	Rapid release testing of final product	no
Flow cytometry	Vital staining	Rapid release testing of final product	yes
Solid phase cytometry	Vital staining	Rapid release testing of final product	yes

Table 1 *(continued)*

Technology	Basis of the test	Detection option	Viable versus non-viable distinction
Adenosine triphosphate (ATP) bioluminescence	ATP production in soybean casein digest and fluid thioglycolate medium	In-process monitoring, timed pre-release testing, final product testing	yes

6.5 User requirement specifications

6.5.1 General

URS concerning RMTM should be identified. URS depend on the factors outlined in Clause 4. Annexes B, C, D, E, F and G provide information on factors for consideration.

6.5.2 Speed

Prior to patient administration, a cellular therapeutic product shall be suitably tested for microbial contamination. The selected method should provide results within the appropriate time frame generally associated within the short shelf life of the cellular therapeutic product. This allows for a sufficient testing review without delaying the cellular therapeutic product release.[16]

Microbiological tests with results obtained within 3 days to 4 days or a shorter time frame are preferable to tests that take 7 days to 14 days for results.

NOTE 1 For non-frozen cell products, sterility testing often yields results within 1 day.

NOTE 2 For frozen cell products, acceptable validated methods can require 7 days to 14 days.

NOTE 3 For typical frozen resuscitated cell products, the preferred testing period is within 1 h.

NOTE 4 For a cell culture process prior to product canning, the preferred testing time is within 1 day.

6.5.3 Sample volume

A sample is often defined on a per test basis and sampling frequency requirements are based on the likelihood of contamination. Sample volumes available for microbial testing vary depending on the manufacturing lot size/volume and the volume of each product unit. The test method selected should take into account the volume of the sample available for testing. Cellular therapeutic products are often single lots or small lots; hence, only small volumes are available for microbial testing.

6.5.4 In-process versus final release testing

Assays performed on in-process samples and final product samples can differ based on the length of the employed manufacturing processes. Due to the long cycle times of traditional visual observation methods using direct inoculation, membrane filtration and culture-plating, microbial testing can be difficult to carry out as an in-process control. URS for in-process testing and final release tests can differ. A risk-based approach for defining the requirements of in-process testing and final product testing should be used. In-process testing shall be conducted as part of any change control procedure (e.g. media exchanges or after the addition of growth factors).

6.5.5 Specificity

A clear distinction between the analytical specificity characterizing the capability related to the presence or absence of microorganisms and the analytical specificity related to species identification in measurement shall be determined.[17][18][19]

Diagnostic specificity is mathematically expressed as shown by Formula (1):

$$R_{TN} = [N_{TNC}/(N_{TNC}+N_{TPI})] \quad (1)$$

where

R_{TN} is the true negative rate;

N_{TNC} is the number of samples tested negative correctly;

N_{TPI} is the number of samples tested positive incorrectly.

Diagnostic specificity for microbiology methods is traditionally demonstrated through the use of pure positive and negative control cultures. Appropriate target and non-target control cultures should be carefully selected.

The definition of appropriate target and non-target control cultures or other standards for use in both validation and routine QC shall be done in the development of any new microbial method.[12] In a robust method, a single target microorganism should be discernible in complex matrices containing potentially millions of non-target organisms. The selection of the microorganism for the analytical specificity validation should comply with the concerning microbiology population.

6.5.6 Sensitivity

Diagnostic sensitivity is mathematically expressed as shown by Formula (2):

$$R_{TP} = N_{TPC} / (N_{TPC} + N_{TNI}) \quad (2)$$

where

R_{TP} is the true positive rate;

N_{TPC} is the number of samples tested positive correctly;

N_{TNI} is the number of samples tested negative incorrectly.

Each manufacturer should perform a risk assessment to determine the type of environmental monitoring programme and determine acceptability of product testing strategy. Further information on environmental monitoring can be found in Annex I.

7 Validation

7.1 General concepts

For quantitative measurements, validation can include, but is not limited to:[12][17][18][19][20]

a) analytical sensitivity;

b) analytical specificity;

c) limit of quantification;

d) linearity;

e) accuracy;

f) precision;

g) robustness;

h) ruggedness;

i) reproducibility.

For qualitative measurements validation can include but is not limited to:

— diagnostic specificity;

— detection limit;

— ruggedness;

— robustness.

When appropriate, RMTMs should be compared with traditional methods. A plan for validation of an RMTM should take into account which step in the manufacturing process the sample is being taken from, the components of the sample that can interfere with the accuracy of the test, and the volume of the sample that is available for testing. Cell culture medium containing antibiotics or anti-fungal reagents can interfere with the results; therefore, studies on bacteriostasis and fungistasis shall be conducted.

Assays should be demonstrated to meet predefined method performance criteria required for the application of the cellular therapeutic product.[21]

A representative sample should be used for assay validation and verification.

When cellular samples with unknown characteristics are to be measured, the qualification of the test method should be carried out first.

A multi-variate approach to evaluate effects of various factors on the method performance can be used.[21]

EXAMPLE The experimental design can incorporate the identification of operating parameters, and potential variations in the test sample.

Sound and appropriate statistical methods for the design and analysis of laboratory experiments should be incorporated into the design of validation protocols.

NOTE 1 Guidelines for proper validation can be found in the ISO 5725 series, ICH Q2(R1)[11], ISO/IEC 17025 and ISO/TS 23565.

NOTE 2 Laboratory documentation, including complete data derived from all assays necessary to ensure compliance with established specifications and standards, is maintained.

NOTE 3 When defining the quantitative assay control space, cell measurements can have the following properties, which can require further consideration:

— there is a case if no reference material;

— it is difficult to secure the amount of measurement sample;

— the measurement target is unstable;

— the state of the object to be measured cannot be determined unambiguously.

7.2 Selection of microorganisms for validation

The RMTMs shall be tested using the microorganism relevant to the cellular therapeutic product and manufacturing method.

Examples of microorganisms and samples for validation belong to several of the following categories:

a) gram-negative bacteria;

b) gram-positive bacteria;

c) aerobic bacteria;

d) anaerobic bacteria;

e) yeast;

f) fungi;

g) isolates detected in starting materials;

h) isolates detected by in-process testing or during preliminary cellular therapeutic product testing;

i) isolates detected by environmental monitoring of the manufacturing facility;

j) isolates from production areas that represent low-nutrient and high-stress environments;

k) microorganisms from commercial sources that have continually been exposed to high-nutrient growth media;

l) slow-growing bacteria.

The validation should include the study of growth under aerobic and anaerobic conditions, especially for cellular therapeutic products manufactured in closed systems.

7.3 Quality by design of method validation

The composition of the test sample should be representative of the cellular therapeutic product samples that RMTMs are intended to be tested. Some test parameters to take into account are the cell concentration, media and additives, as well as preservation and antimicrobial agents with a bacteriostatic or fungistatic effect. Several matrix compositions should be tested to validate the method for multiple sample types of the same cellular therapeutic product.

The application of RMTMs shall be validated for in-process use and the final cellular therapeutic product in a cell culture production. The cell culture medium can contain antibiotics in different stages of the in-process, which can contain a preservation agent as well as a higher cell concentration. Thus, the different steps of cell production shall be separately validated, because of their different compositions.

To validate the performance of a commercially available testing system, studies using characteristic microorganisms under prescribed assay conditions should be performed, and the results should be compared with those provided by the supplier to show sufficient performance.

In the validation study design, the potential for the materials being tested to generate false positive or false negative results should be evaluated using appropriate controls. This will depend on the product matrix, additives and preservatives, and unique characteristics of the sample. The use of additional positive and negative controls containing cellular therapeutic product components, but not cells, is recommended.

7.4 Revalidation method

Revalidation should be completed when there any changes in cellular therapeutic product manufacturing, including the formulation, or any changes in the RMTMs that can potentially inhibit or enhance the detection of viable microorganisms.[22] Initial validation can be designed to minimize the effect of some changes by designing assay conditions that encompass known or proposed changes (e.g. worst case, matrix approach). Revalidation of the RMTM should be performed whenever there are changes in the process that can potentially inhibit (e.g. the addition of antimicrobials) or enhance the detection of viable microorganisms.

Verification of critical parameters of the test method post-process, or cellular therapeutic product changes using the microorganisms most difficult to detect, can serve as an indication of the need to revalidate the method.

7.5 System validation

System validation should encompass the entire process, from the decision to change any aspect of the testing programme to ongoing use. It should consist of the following phases:[23]

a) URS;

b) design qualification (DQ);

c) installation qualification (IQ);

d) operational qualification (OQ);

e) performance qualification (PQ).

7.6 Use of reference material in validation

When an appropriate certified reference material is available, it should be used in the validation of the test method.

When an appropriate certified reference material is not available, in-house reference materials can be used. In-house reference materials should be homogeneous and stable with an appropriate shelf life. Their established properties should be regularly verified.

Reference materials can be used as comparison control for measurement, when appropriate.

The use of reference materials in validation shall be documented. Reference materials are appropriate when the intended use of a test is to detect and identify any bacterial and fungal contaminants in a sample.

NOTE Cases can exist where no suitable reference material is available for validation. In these cases, alternative approaches can be applied. For example, for cell counting, practices described in ISO 20391-2 can be applied.

7.7 Acceptance criteria of targeted validation parameters

Acceptance criteria shall be consistent with:

a) the intended use;

b) the validation of the instrument;

c) the computerized supporting platform;

d) the validation of the new technology as compared to compendial methods;[35]

e) the general test method;

f) the test method specification.

When the test method specification is not compatible with the intended use of the measurement, then the test method should be reconsidered. Changes based on reconsiderations can include a change of instrument(s), reagent(s) or protocol(s), or multiple of these.[24]

Acceptance criteria can be predefined from external requirements (e.g. tests for endotoxin level).

Acceptance criteria can be:

— either quantitative or qualitative;

— defined by a performance index or URS, or both;

— defined from scientific papers;

— defined by local regulations.

Acceptance criteria shall be determined before validation, established and documented. Documentation should include justification and rationale, and should contain sufficient information to permit evaluation of the results.

If the RMTM involves bacterial growth and the growth is inhibited, modifications (e.g. increased dilution, additional membrane filter washes, addition of inactivating agents) to the test method should be implemented to optimize recovery.

Media used to perform sterility testing should be rendered sterile. If the RMTM involves bacterial growth, the media should be demonstrated as growth promoting.

Validation should be more than a study conducted on a new method or cellular therapeutic product. Instead, validation should encompass the entire process that commences with the decision to change some aspect of the microbiological testing programme and continues through ongoing routine use. It follows, therefore, that validation starts from the outset, and the validation plan shall be designed to include each stage of the process required to implement a new test method. Adoption of this approach can streamline and expedite the introduction of the new method by ensuring that each step in the process is taken into account and documented before moving onto a subsequent stage.[25]

7.8 Precision

Depending on the stipulated conditions, precision can be divided into method repeatability, intermediate precision and reproducibility. Within the frame of a single laboratory validation, both the method repeatability and intermediate precision should be determined.

7.9 Detection limit

The detection limit measurements establish a baseline detection value under optimal conditions. It is the lowest number of microorganisms in a sample that can be detected, but not necessarily quantified, under the stated experimental conditions.

NOTE 1 Colony counting methods can be capable of detecting single target microorganisms in large samples. In this method, the result is reported as “not detected” when no microorganisms are detected. False negative testing is possible when the pathogen is difficult to cultivate. A significant example is the “accidental” cultivation of *Helicobacter pylori*. In cases where RMTMs are independent of culture, colony counting is not applicable.

The detection limit for cell-based therapies should be established through industry needs, appropriateness and patient safety. Local regulations can apply. This should be determined through a fit-for-purpose approach.[25]

NOTE 2 If the user does not know where to expect the detection limit, a good strategy is to estimate it by first performing a pilot study with just a few replicates at each concentration and covering a wider concentration range, and then following up with a larger number of replicates over a narrow concentration range.

7.10 Accuracy

Frequently, accuracy can be improved, taking into account the mean of a number of results produced by the method. There are four general approaches to obtain a suitable estimate of the true quantity value:

a) the use of certified reference materials;

b) the comparison with results obtained from higher order reference measurement procedures (further information is provided in ISO 17511);

c) recovery experiments using spiked samples;

d) the comparison with results obtained from interlaboratory comparison tests.

Accuracy is critical for repeatability and reproducibility. The use of appropriate calibrators can increase accuracy. Guidance for accuracy can be found in ISO 5725-2.

7.11 Robustness

The robustness of a microbiological method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters. During the robustness test, the effect of small deviations in relevant method parameters on the method performance and the measurement results should be investigated. Relevant method parameters that are likely to influence the method outcome should be monitored and include, but are not limited to, critical reagent concentrations, instrument operation parameters and incubation temperatures.

7.12 Ruggedness

Ruggedness is the reproducibility of a method achieved when the same sample is tested under a variety of normal test conditions (e.g. different analyst, reagents, batches).

Ruggedness should be assessed for RMTMs in the manufacturing process.

8 Use and application of rapid microbial tests

8.1 Number and type of samples

Factors to determine the number of samples should include, but are not limited to:

a) the type of samples to be tested;

b) the number of samples per work shift;

c) the ability of the test method to be extended to other types of samples in the process;

d) the value of samples;

e) the volume of samples needed;

f) the amount of natural variation within patient populations.

The samples shall represent the entire batch and processing conditions. Samples should be taken:

— at the beginning, middle and end of the aseptic processing operation;

— in conjunction with processing interventions or excursions;

— after any point in the manufacturing process that has an increased risk of contamination.

8.2 Testing environment

Certain aspects of sterility testing are of particular importance, including the control of the testing environment, understanding the test limitations, and investigating the sources of contamination in manufacturing systems following a positive test result.

The testing laboratory environment should maintain facilities and controls in a manner that is comparable to those sterility conditions used for aseptic filling operations. Poor or deficient sterility conditions in test facilities or controls can result in false positive results.[26]

Significantly better production facilities and controls than those for sterility testing can result in mistakenly attributing a positive sterility test result to a faulty laboratory even when the product tested could have, in fact, been non-sterile. Therefore, a manufacturing deficiency can go undetected.

Isolators (e.g. glove boxes, laminar flow hoods for sterility testing) should be used in order to minimize the chance of a false positive test result.

8.3 Sensitivity

Points to be taken into account for the diagnostic sensitivity to help determine which test method to use can include, but are not limited to:

a) the required level of diagnostic sensitivity for the needed application;

b) the needed analytical sensitivity level that depends on the current specification(s) for the test method being replaced (e.g. the detection limit for traditional plate count and turbidity methods can be < 10 CFU per ml);

c) the comparison of the detection limit to the compendial method;

d) the results evaluated for the intended application;

e) detection of variation(s) between test batches.

8.4 Analytical specificity (microorganism detection)

Points to be taken into account for the analytical specificity to help determine which test method to use should include the evaluation of which microorganisms the novel test method is required to detect or identify based on the needed application (more information is given in Annex A). This determination should be based on the historical data generated from the compendial test method[35] and complemented by information compiled through the research and manufacturing process.

8.5 Comparable test data

Points to be taken into account for comparable test data to help determine which test method to use should include, but are not limited to:

a) the needed requirement of the ability to demonstrate comparable test data;

b) validation studies and acceptance criteria designed to reflect the needs of novel, more sensitive technology.

If the RMTM is based on technology that can readily demonstrate comparable data, then the validation studies and acceptance criteria detailed in Clause 7 can be used.

Points to be taken into account for the needed degree of operator qualification to help determine which test method to use should include, but are not limited to:

— the complexity of operation of the RMTM;

— the skill level required to run an assay and interpret results;

— the educational background and experience of laboratory personnel involved;

— the various areas that personnel will be required to have a background in (i.e. analytical chemistry, nucleic acid biochemistry, computer skills).

9 Investigation of positive sterility results

A plan for investigating positive sterility results should be in place (e.g. confirmation of the test result by either repeating the assay or conducting a secondary assay, or both). If a positive test for microbial contamination is confirmed, the manufacturer should investigate the source of the contamination. See requirements for the local regulatory agency with oversight for cellular therapeutic products.[11][12][13][14]

The plan in cases of unsatisfactory results should include identifying whether the cause is manufacturing or testing. Some examples of plans for the identification can include:

— the repetition of original test;

— the performance of secondary tests for confirmation of results.

Some of the factors that should be involved in the determinations of the secondary tests are:

— analytical sensitivity;

— diagnostic sensitivity;

— diagnostic specificity.

Repetition of the original test or execution of a secondary test should be performed when microbial growth is found in the repeat test, which indicates the sample examined does not meet the test requirements for sterility.

In order to declare a not-sterile test invalid, it should clearly be demonstrated that the failure of the test was unrelated to the product being examined.

Possible reasons to declare a test invalid:

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 microorganisms isolated from the test, the growth of this species (or these species) can be ascribed unequivocally to faults with respect to the material or the technique, or both, used in conducting the sterility test procedure.

The plan in cases where the sample does not meet the requirement is that the risk assessment should be repeated. The plan can also include:

— the performance of investigations for risk levels associated with the specific steps during the manufacturing process;

— the performance of investigations to evaluate the cause or microorganism of contamination.

10 Training

Personnel performing sterility testing shall be qualified and trained for the task. A written programme should be in place to maintain updated training of personnel and confirm acceptable sterility testing practices.

Where warranted, a risk-based assessment of the relevant factors shall be performed by personnel with specialized training in appropriate disciplines based on the test method (e.g. microbiology, molecular biology, immunology) and in the interpretation of microbiological data. For raw materials, the assessment shall take account of the processing to which the product is subjected, the current technology of testing, and the availability of materials of the desired quality.

A qualification programme shall be in place to train personnel to conduct testing according to an established protocol.

11 Documentation

Detailed protocols and experimental designs for validation studies shall be documented.

Examples of items to be included in the documentation:

a) the make and model of the instrument and kit used, and relevant settings;

b) the measurement results with appropriate units and uncertainty;

c) the equipment calibration record;

d) the sample preparation procedures and conditions;

e) the data analysis procedure;

f) the time when the sample is taken and the time to result.

Standard operating procedures (SOPs) for the conducting of tests shall be established and documented to ensure that the appropriate test parameters are followed. Data generated from validation studies as well as data analyses shall be documented and made available to appropriate personnel.

12 Test report

Testing results shall be documented and should be included in the batch records for the cellular therapeutic product. Test reports should contain sufficient detail to allow for independent assessment of testing results.[28] If possible, data should be findable, accessible, interoperable and reusable (FAIR). ISO 20691 and ISO/TR 3985 provide resources on data management.

The test report shall include, but is not limited to, the following:

a) the sample;

b) the International Standard used (including its year of publication);

c) the method used (if the standard includes several);

d) the result(s), including a reference to the clause which explains how the results were calculated;

e) any deviations from the procedure;

f) any unusual features observed;

g) the date of the test.

Examples of further reporting elements include, but are not limited to, the following:

— the data analysis procedure;

— the make and model of the instrument and kit used, and relevant settings;

— the source of the sample (step in manufacturing process).

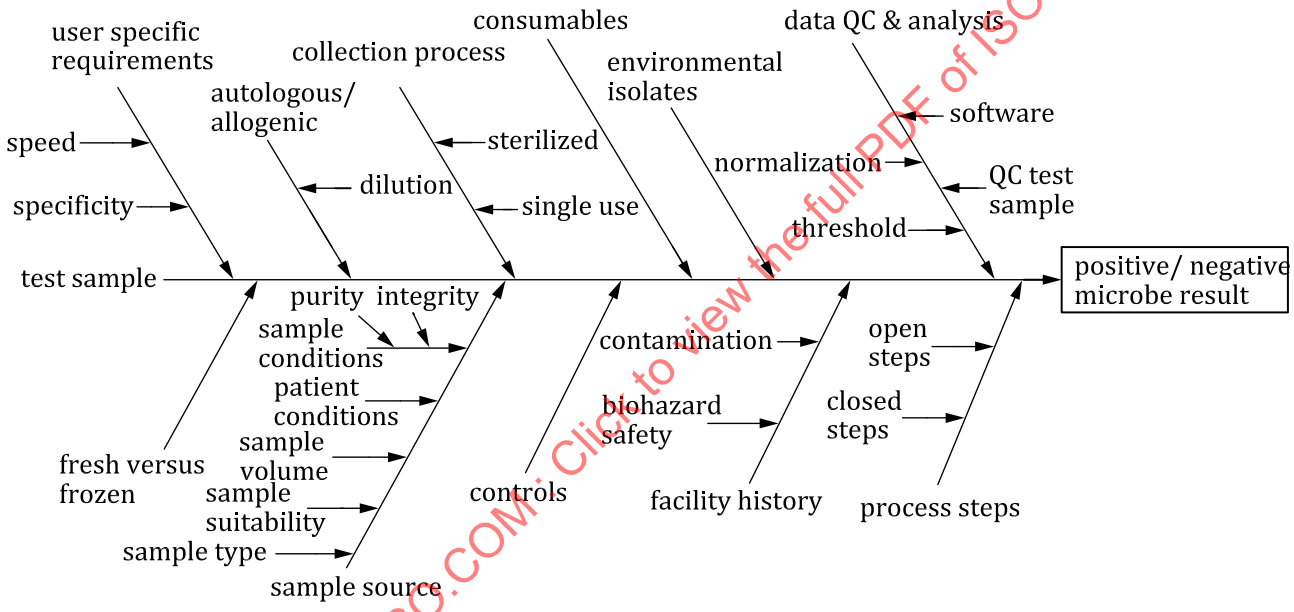
Annex A
(informative)

Exemplary framework for identifying microbial contamination

Determining the appropriate use of rapid microbial tests in biological samples requires many different factors to be taken into account. A thorough method selection based on risk assessment is used to determine the requirements for development and methods of use for rapid microbial testing in biological products.

An overview of influences on the risk assessment is provided in Clause 5.

This annex will help rapid microbial test designers and users to consider factors that can be relevant when developing and using an appropriate test method risk assessment plan, see Figure A.1.



NOTE The central arrow represents the process from test sample to result. Branches feeding into experimental progression characterize the sources for consideration for the risk assessment when determining the development and use of rapid microbial tests in biological products.

Figure A.1 — Cause and effect diagram — Whole process uncertainty for contributions to risk assessment for use and development of rapid microbial test methods

Annex B
(informative)

Risk analysis with cellular therapeutic products related to input materials — Donor selection

Table B.1 — Risk analysis with cellular therapeutic products related to input materials — Donor selection[6]

Input materials	Risk identification	Risk rating	Risk mitigation/critical process controls
Donor qualification	Autologous donation	Low	Donor interview; medical examination
	Allogenic donation	Moderate to high	Donor interview; medical examination
Source of the cells	Peripheral blood	Low[a]	Skin sanitization; diversion of the first 10 ml during collection
	Adipose tissue	Moderate to high	Surgical suite
	Bone marrow	High	Surgical suite
	Cord blood	Moderate	Maternal risk screening; vaginal birth; use of cord-retrieval technicians
Collection process	Systems with a high probability of microbial ingress	High	Eliminate “open” systems
	Systems with a low probability of microbial ingress	Low	Promote “closed” systems

[a] Can be high when the patient has a pre-existing microbial infection.

Annex C
(informative)

Risk analysis with cellular therapeutic products related to input materials — Cell transformation and expansion

Table C.1 — Risk analysis with cellular therapeutic products related to input materials — Cell transformation and expansion[6]

Input materials	Risk identification	Risk rating	Risk mitigation/critical process controls
Single-use consumables	Open (e.g. transfer bags, roller bags, cassettes)	High	Sterile, disposable consumables
	Closed	Low	
Cell banks	Contamination	Moderate	Sterility, mycoplasma and infectious agent screening
Transduction	Viral and bacterial vectors	Low to moderate	Infection agent screening; sterile filling/sterility testing
Cell expansion	Multiple aseptic manipulations	Low to moderate	Biological safety cabinets, restricted access barrier systems, and isolator systems; bioburden monitoring
Culture media	Foetal bovine serum and other components	Low to moderate	Source certification; adventitious agent screening; terminal sterilization (pasteurization, chemical treatment or irradiation)
Micro-carrier	Immunomagnetic beads	Low	Terminal sterilization
Utilities	Pharmaceutical-grade water	Moderate	Good manufacturing practice compendial compliance; microbial monitoring; endotoxin monitoring
	Compressed gasses	Moderate	Sterile filtration at point of use
Facility	Containment	Low to moderate	BSC, isolators, RABS, etc.
	Segregation of process steps		Labelling consideration for dedicated work areas for high-risk containment steps, appropriate disinfection between steps, process flows and HVAC cascades

Table C.1 *(continued)*

Input materials	Risk identification	Risk rating	Risk mitigation/critical process controls
	Personnel and materials flow		Clearly defined and physically separate or proceduralized segregated entry and exit; transfer disinfection procedures for materials; adequate protection of sterile materials for transfer
Cell harvesting	Digestive enzymes (e.g. trypsin, collagenase, DNase/RNase, restriction endonucleases, growth factors, cytokines, monoclonal antibodies)	Low to moderate	Terminal sterilization; use of non-animal derived materials
Cell storage solution	Buffers; saline; surfactants; cryoprotectants	Low	Sterile filtration

Annex D
(informative)

Risk analysis with cellular therapeutic products related to input materials — Packaging storage and administration

Table D.1 — Risk analysis with cellular therapeutic products related to input materials — Packaging storage and administration[6]

Input materials	Risk identification	Risk rating	Risk mitigation/critical process controls
Packaging	Intravenous (IV) bags	Low	Irradiation sterilization; aseptic transfer, container-closure integrity
Storage	Ambient temperature; refrigeration; temperature; frozen/cryopreserved	Low	Frozen storage should include liquid nitrogen vapour not liquid nitrogen
Shipping	Refrigerated or frozen	Low	Shipping frozen in liquid nitrogen or controlled, refrigerated transport
Donor/recipient	Allogenic only	Moderate-high	Vaccination: pre- and post-antibiotic treatment

Annex E
(informative)

Risk-based classification for monitoring practices for cellular therapeutic product manufacturing

Table E.1 — Risk-based classifications for monitoring practices for cellular therapeutic product manufacturing[6]

Manufacturing environment	Air cleanliness standards	Environmental monitoring frequency	Environmental monitoring action levels (CFU)	Aseptic process simulation requirements	Microbial testing
BSC in a classified area	ISO 5/ISO 7	Each shift/each operator	< 1/< 5	Initial 3 batches/ semi-annual	In-process and final product testing
Barrier system in a classified area	ISO 5/ISO 7	Each shift	< 1/< 5	Initial 3 batches/ semi-annual	In-process and final product testing
Open isolator system	ISO 5/ISO 7	Each shift	< 1/< 5	Initial 3 batches/ semi-annual	In-process and final product testing
Closed isolator system	ISO 5/ISO 8	Periodic	< 1/< 50	Initial 3 batches/ semi-annual	Final product testing
Gloveless, robotic, isolator system	ISO 5/ISO 8	None	None	None	None

Annex F
(informative)

Validation of rapid microbial test methods

F.1 General

When a commercial kit is used, certain elements of the validation can be carried out by the manufacturer and information provided to the user. However, different results can be obtained by the user depending on the equipment used and the target (sample) tested. Therefore, the user can confirm the manufacturer's validation results by using their own facilities. This can be especially relevant when the measurement sample is different from the sample validated by manufacturer. The detection limit and reproducibility of the kit can be confirmed with the targets of interest. When the user's sample preparation or measurement methods are different from the method specified by the instrument manufacturer, the employed method for validation of the instrument should be used. In addition, when information on the kit reagents is not available from the manufacturer, a countermeasure is required to obtain information from the manufacturer about how to modify the kit production if required. If the composition of the kit reagents is modified, the user confirms that the detection limit and the detection accuracy of the modified kit for target microorganisms is comparable to the previous one, as needed.

Method validation shows sufficient analytical and diagnostic sensitivity for the microbial species listed in Annex G. Here the species are selected from the viewpoint of the user's facility environment, the source of product and the location of administration to the patient.

The tests include both a positive control (run control) of 100 CFU or less and a negative control. The microbial strains used for the positive control tests are those within a low number of passages obtained from an official or suitably accredited agency, and handled appropriately. The unit of inoculation is determined before use. When cell suspension is used as a test sample, a preliminary test is performed for the effect of the cells on detecting microorganisms. The cell suspension used for the preliminary test confirms bacteria/fungi-free. The cell suspension itself is used as a negative control, and the cell suspension spiked with test microorganisms is used as a positive control. On the other hand, when a supernatant of a cell sample is used as test samples, method validation implies that the method employed is able to fully detect the microbial contamination in the cell-contained sample.

The most important parameters for method validation of the analytical procedure are the analytical and diagnostic specificity and the detection limit. In addition, the robustness of the analytical procedure should be evaluated. The range of validation is established as the complete procedure from sample preparation to detection.

Where commercial kits and instruments are used for a part or all of the analytical procedure, documented full validation data already covered by the manufacturer can replace validation data by the user, and a full validation by the user is unnecessary. Nevertheless, the performance of the kit and instrument with respect to its intended use and user's test system is demonstrated by the user (e.g. specificity, detection limit).

Reference strains evaluated for concentration in CFUs/ml are used at various stages during validation of analytical or diagnostic specificity or the detection limit. During routine application of the test, microbial reference strains or the test sample calibrated for concentration using reference strains are used as positive controls. In the test, microorganisms for validation of the procedure are included in sample preparation. As for evaluation parameters, three parameters are evaluated: specificity, detection limit and robustness.

F.2 Detection limit

The detection limit of an individual analytical procedure is the lowest amount of target in a sample that can be detected, but not necessarily quantitated as an exact value. For the establishment of the detection limit, a positive cut-off point is determined for the analytical procedure. The positive cut-off point is the target (bacterial cells, nucleic acid, ATP, etc.) per volume of sample that can be detected in 95 % of test runs. To determine the positive cut-off point, a dilution series of characterized and calibrated (CFUs) microbial reference strains or international standards are tested on different days to examine variation between test runs.

For validation of the detection limit, the microbial species listed in Annex G can be used. These species represent an optimal selection in terms of the frequency of occurrence as contaminants of mammalian culture cells used for production of biotechnological/biological products, phylogenetic relationships, and animal-derived components used during culture and production processes. The list is only for method validation and not for use as positive run controls in routine tests.

For establishment of the detection limit, an appropriate dilution series (10-fold or $10^{0,5}$-fold dilution) is prepared from the undiluted microorganism evaluated for concentration (CFU/ml, etc.), and performed for each dilution. Based on the dilution factor that shows the detection limit, a positive cut-off point is determined as the minimum number of CFUs in the test sample. For each microbial strain selected from reference strains described in Annex G, at least three independent 10-fold dilution series is tested, with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution, to enable a statistical analysis of the results.

EXAMPLE A laboratory can test three dilution series on different days with eight replicates for each dilution, four dilution series on different days with six replicates for each dilution, or six dilution series on different days with four replicates for each dilution.

In order to keep the number of dilutions at a manageable level, a preliminary test is performed to obtain a preliminary value for the positive cut-off point (i.e. the highest dilution giving a positive signal). The range of dilutions can then be chosen around the determined preliminary cut-off point. The concentration of microorganism (CFUs/ml, etc.) that can be detected in 95 % of test runs can then be calculated using an appropriate statistical evaluation. These results can also serve to evaluate the variability of the analytical procedure.

If an alternative method is proposed to replace the conventional culture method, the system is shown to detect 10 CFU, spiked in target sample for each microbial reference species.

F.3 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and provides an indication of its reliability during normal usage. The evaluation of robustness is taken into account during the development phase. This evaluation demonstrates the reliability of the analytical procedure with respect to deliberate variations in method parameters.

As for nucleic acid testing, for example, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. $MgCl_2$, primers, deoxyribonucleotides) are tested. Modifications of extraction kits or extraction procedures as well as different thermal cycler types can also be evaluated.

Annex G
(informative)

Microorganisms for validation of rapid microbial test methods

It can be helpful to have a list of microorganisms to use for validation of rapid microbial tests intended for cell-based therapies. Table G.1 provides a non-exclusive list of microorganisms for the validation of rapid microbial tests in cell-based therapies.

For more information on the microbial species and strains given in Table G.1, see References [30] and [31] for bacteria, and References [32], [33] and [34] for yeast and mould.

Table G.1 — Microorganisms for validation

No.[a,b,c,d]	Species name	Strains	Type	More potential sources
1	*Aspergillus brasiliensis*	ATCC 16404, IMI 149007, IP 1431.83, NBRC 9455	Yeast and mould	Environmental/air
2	*Bacillus spizizenii (Bacillus subtilis* subsp. *spizizenii)*	ATCC 6633, CIP 52.62, NBRC 3134, NCIMB 8054	Aerobic and facultative bacteria	Environmental/air
3	*Candida albicans*	ATCC 10231, IP 48.72, NBRC 1594, NCPF 3179	Yeast and mould	Process specific/ bronchus
4	*Clostridium sporogenes*	ATCC 11437, CIP 100651, NBRC 14293, NCIMB 12343, ATCC 19404, CIP 79.3, NCTC 532	Anaerobic bacteria	Environmental/air
5	*Pseudomonas aeruginosa*	ATCC 9027, CIP 82.118, NBRC 13275, NCIMB 8626	Aerobic and facultative bacteria	Environmental/water

[a] No. 1 to 6: Microbial species and strains specified by pharmacopeia (United States Pharmacopeia (USP), European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP).

[b] No. 7 to 12: Human-derived microorganisms that can be contaminated from operators working in-process.

[c] No. 13 to 18: Microorganisms that can be contaminated from environment such as soil and water.

[d] No. 19 to 23: Typical pathogenic microorganisms.

Table G.1 *(continued)*

No.[a,b,c,d]	Species name	Strains	Type	More potential sources
6	*Staphylococcus aureus*	ATCC 6538, CIP 4.83, NBRC 13276, NCIMB 9518, NCTC 10788	Aerobic and facultative bacteria	Process specific/skin
7	*Cutibacterium acnes*	ATCC 11827 NBRC 107605	Anaerobic bacteria	Process specific/skin
8	*Escherichia coli*	ATCC 8739, CIP 53.126, DSM 1576, NBRC 3972, NCIMB 8545	Aerobic and facultative bacteria	Process specific/ intestines
9	*Micrococcus luteus*	ATCC 10240, NBRC 13867	Aerobic and facultative bacteria	Environmental/air, process specific/skin
10	*Kocuria rhizophila*	ATCC 9341, NCIMB 8553, NBRC 3232, NBRC 12708	Aerobic and facultative bacteria	Environmental/air, process specific/skin
11	*Staphylococcus epidermidis*	ATCC 12228, CIP 68.21, DSM 1798, NBRC 12993	Aerobic and facultative bacteria	Process specific/skin
12	*Streptococcus pneumoniae*	ATCC 33400, CIP 102911, DSM 20566, NBRC 102642, NCTC 7465	Aerobic and facultative bacteria	Process specific/ respiratory system
13	*Streptococcus pyogenes*	ATCC 19615, CIP 1042.26, NCIMB 13285	Aerobic and facultative bacteria	Process specific/ pharynx
14	*Acinetobacter baumannii*	ATCC 19606, CIP 70.34, DSM 30007, JCM 6841, NBRC 109757, NCTC 12156	Aerobic and facultative bacteria	Environmental/water, process specific/urine

[a] No. 1 to 6: Microbial species and strains specified by pharmacopeia (United States Pharmacopeia (USP), European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP).

[b] No. 7 to 12: Human-derived microorganisms that can be contaminated from operators working in-process.

[c] No. 13 to 18: Microorganisms that can be contaminated from environment such as soil and water.

[d] No. 19 to 23: Typical pathogenic microorganisms.

Table G.1 *(continued)*

No.[a,b,c,d]	Species name	Strains	Type	More potential sources
15	*Acinetobacter calcoaceticus*	ATCC 23055	Aerobic and facultative bacteria	Environmental/water
16	*Brevundimonas diminuta*	ATCC 19146, DSM 1635, NBRC 14213, NCIMB 11091	Aerobic and facultative bacteria	Environmental/water
17	*Methylobacterium extorquens*	ATCC BAA-2500, NBRC 15911	Aerobic and facultative bacteria	Environmental/water
18	*Pseudomonas protegens*	ATCC 17386, NBRC 15842	Aerobic and facultative bacteria	Environmental/water
19	*Penicillium citrinum*	ATCC 9849, CBS 342.61, IMI 61272, NBRC 6352, NRRL 756, QM 1226	Yeast and mould	Environmental/air
20	*Aeromonas hydrophila*	ATCC 7966, CIP 76.14, DSM 30187, JCM 1027, NCTC 8049	Aerobic and facultative bacteria	Environmental/water
21	*Legionella pneumophila*	ATCC 33152, CIP 103854, DSM 7513, JCM 7571, NCTC 11192	Aerobic and facultative bacteria	Environmental/water
22	*Listeria monocytogenes*	ATCC 15313, CIP 82.110, DSM 20600, NCTC 10357	Aerobic and facultative bacteria	Process specific/animal
23	*Salmonella enterica* subsp. *enterica* serovar Abony	ATCC BAA-2162, CIP 80.39, DSM 4224, NBRC 100797, NCTC 6017	Aerobic and facultative bacteria	Process specific/ intestines

[a] No. 1 to 6: Microbial species and strains specified by pharmacopeia (United States Pharmacopeia (USP), European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP).

[b] No. 7 to 12: Human-derived microorganisms that can be contaminated from operators working in-process.

[c] No. 13 to 18: Microorganisms that can be contaminated from environment such as soil and water.

[d] No. 19 to 23: Typical pathogenic microorganisms.

Table G.1 *(continued)*

No.[a,b,c,d]	Species name	Strains	Type	More potential sources
24	*Salmonella enterica* subsp. *enterica* serovar Typhimurium	ATCC 13311, CIP 58.58, JCM 1652, NCTC 74 ATCC 14028 NBRC 105726	Aerobic and facultative bacteria	Process specific/ intestines
25	*Mycoplasmoides pneumoniae*	ATCC 15531 NBRC 14401	Aerobic and facultative bacteria	Environmental/skin
26	*Mycoplasmoides genitalium*	ATCC 33530 DSM 19775	Aerobic and facultative bacteria	Environmental/skin
27	*Metamycoplasma hominis*	ATCC 27545 NBRC 14850	Aerobic and facultative bacteria	Environmental/skin
28	*Metamycoplasma orale*	ATCC 23714 NBRC 14477 NCTC 10112	Aerobic and facultative bacteria	Environmental/skin
29	*Mesomycoplasma hyorhinis*	ATCC 17981 NBRC 14858 NCTC 10130	Aerobic and facultative bacteria	Environmental/skin

[a] No. 1 to 6: Microbial species and strains specified by pharmacopeia (United States Pharmacopeia (USP), European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP).

[b] No. 7 to 12: Human-derived microorganisms that can be contaminated from operators working in-process.

[c] No. 13 to 18: Microorganisms that can be contaminated from environment such as soil and water.

[d] No. 19 to 23: Typical pathogenic microorganisms.

Annex H
(informative)

Methods for rapid microbial testing

H.1 Sequence-based methods

Molecular (nucleic acid-based) methods are platforms for rapid microbial testing which allow for the detection of fastidious pathogens and non-culturable agents, the discovery of new microorganism types, and genotypic analysis of contaminants. Such techniques are increasingly indicated for rapid detection of microorganisms, which cannot be grown *in vitro* or under common culture conditions. PCR-based assays are specific for microorganisms of interest. Amplicon-based sequencing assays can detect and identify thousands of microorganisms without any prior knowledge of which species can be present in a sample. Even more broadly, metagenomics is a promising molecular approach, which utilizes next generation sequencing (NGS) technologies for the untargeted detection of any/all microorganisms in a sample. Despite the benefits, NGS-based methods are relatively more expensive and slower than nucleic acid-based assays, and typically require expert analysis and software. A major limitation of nucleic acid-based assays in general is that such methods can provide a positive result for both viable and non-viable microorganisms. The detection of microbial DNA does not necessarily equate to a viable infectious agent, which puts a patient at risk of infection.

H.2 Solid-phase cytometry

In solid-phase cytometry, the sample is filtered through a membrane. Retained cells are labelled with a fluorophore and the membrane is scanned by a laser to identify and enumerate the fluorescing cells.[27]

Size-exclusion chromatography (SEC) is a separation based on size and shape with detection via ultraviolet (UV) fluorescence or light-scattering detectors. SEC is potentially an online method with 5 min to 10 min turnaround, but samples need to be purified before analysis.

H.3 Slanted nano-arrays

Slanted nano-arrays (SNAs) are a chip-based technology that uses angled nanofilters for size-based separations. Detection is via fluorescence. SNAs operate continuously and have very low detection limits.[28] This emerging technology has not yet been validated for RMTM purposes.

H.4 Spectral analysis and partial least squares

The combination of spectral analysis and the partial least squares (PLS) chemometrics technique has been demonstrated to be promising for quantification of protein impurities. By combining diode arrays for detection and PLS for analysis, co-eluting proteins can be analysed online.[29]

H.5 Bioluminescence-based tests

Some microbial tests use bioluminescence-based techniques. Typically, these techniques are based on detection of ATP. This simple and relatively straightforward assay uses ATP as an indicator of viable microbiological contamination. It measures the amount of light emitted when the enzyme luciferase converts luciferin into oxyluciferin in the presence of molecular ATP, and is designed to be proportional to the amount of ATP present. This technique is very sensitive and rapid (within 4 h). Amplified ATP methods have also been developed to further reduce the time to result. ATP-based methods are sufficiently flexible in that virtually any combination of parameters can be used, provided that neither

the product nor the media possess significant levels of ATP or otherwise interfere with the enzymatic reaction, and that the selected conditions satisfy compendial requirements. Microbial testing via ATP-based methods is currently used in food and beverage production; therefore, these testing methods can potentially be adapted quickly for regenerative medicine therapies. Tests based on this technology have been developed for commercial use.

H.6 Mass spectrometry techniques

Mass spectrometry (MS), an analytical technique that measures the mass-to-charge ratio of ions to identify an analyte, is one major platform. MS-based tests are becoming increasingly more powerful due to technical advances including the availability of various ion sources to ionize the material, analysers to separate ions and detector capabilities. MS has also become more accessible and affordable in recent years. MS tends to have a good to excellent throughput both in terms of the number of samples and the speed of testing. Common challenges of MS approaches include the requirement for a brief culture step and the need for an established library for robust identification. MS is often coupled with a separation technique, such as liquid chromatography (LC) to physically isolate specific components in the sample mixture. LC-MS can provide clear identification of contaminating microorganisms, but requires extensive sample preparation and analysis. Shortening the sample preparation as well as automating the data processing are recent trends in LC-MS method development. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) is another MS approach that uses laser energy absorbing samples in a matrix to create ions from larger molecules with minimal fragmentation. MALDI-TOF instrumentation can be particularly suitable for use in high-throughput industrial QC settings or as in-process testing, as it is automated, uses small sample volumes and has a high throughput.

H.7 Flow cytometry

Flow cytometry is another promising platform for rapid microbial testing. In flow cytometry, suspended cells in a sample ideally pass one cell at a time through a laser beam, and either light scattering from the cell or emission from fluorescent molecules, or both, on or in the cell is detected. A single-parameter flow cytometer has proven applicable to the rapid detection of low numbers of microbial contaminants in finished products. It is also possible to do rapid cell sorting via flow cytometry to isolate microbial contaminants for further analysis. Flow-based methods such as these require expert sample preparation and analysis. Currently, most flow cytometry tests require a one to two-day incubation period for contaminant enrichment prior to analysis. Enrichment for contaminants can only be successful when the microbial contaminants are able to divide under these conditions; otherwise, these can remain undetected. The high correlation between flow cytometry results and product quality, shelf life and expiration date can allow for the establishment of realistic QC criteria for rapid positive release of product. The reproducibility of the results and the proven correlation with the standard plate count method obtained in industrial conditions make flow cytometry a promising predictive method for product and process QC.

H.8 LC-MS using post translational modification

Liquid chromatography mass spectrometry (LC-MS) is a hyphenated technology to first separate digested proteins (LC) then analyse the peptides (MS). LC-MS provides quantitative information but requires extensive sample preparation and analysis. Shortening the procedure as well as automating the data processing are the recent trends for LC-MS method development. Currently few other techniques are able to produce a similar quality of data. A top-down and middle-down LC-MS method by direct analysis of protein biopharmaceuticals with minimized sample preparation can represent a future trend, which will provide direct information related to the proteoforms rather than extracting information from peptide fragments from the proteins.[30]

H.9 Binding affinity assays

Another RMTM is based on an enzyme-linked immunosorbent assay (ELISA), which is a binding affinity assay detecting specific antibody-antigen interactions. ELISA is typically performed using