TECHNICAL SPECIFICATION

ISO/TS 23758

IDF/RM 251

First edition
2021-08

Guidelines for the validation of qualitative screening methods for the detection of residues of veterinary drugs in milk and milk products

Lignes directrices pour la validation des méthodes qualitatives de dépistage des résidus de médicaments vétérinaires dans le lait et les produits laitiers

Reference numbers
ISO/TS 23758:2021(E)
IDF /RM 251:2021(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11

Email: copyright@iso.org
Website: www.iso.org

International Dairy Federation
Silver Building • Bd Auguste Reyers 70/B
B-1030 Brussels
Phone: +32 2 325 67 40
Fax: +32 2 325 67 41
Email: info@fil-idf.org
Website: www.fil-idf.org

Published in Switzerland

Contents

Page

Forewords ..... **iv**

1 Scope **1**

2 Normative references **1**

3 Terms and definitions **1**

4 Principle **4**

5 General requirements for the test/kit **5**

6 Reagents **5**
6.1 Standard blank matrix 5
6.2 Antibiotics 6
6.3 Standard stock solution 6
6.4 Working stock solutions 6
6.5 Spiked sample 6

7 Apparatus **7**

8 Sample Preparation **7**
8.1 Stock solution preparation 7
8.2 Working stock solution preparation 8
8.3 Blank matrix sample selection 8
8.4 Spiked sample creation 8

9 Procedure **8**
9.1 Validation 8
9.1.1 General 8
9.1.2 Detection capability (CCβ) 9
9.1.3 Test selectivity/specificity 13
9.1.4 Robustness testing 14
9.1.5 Reader and test repeatability 18
9.1.6 Participation in a(n) (inter)national ring trial 20
9.2 Verification testing of a transferred screening method 20
9.2.1 General 20
9.2.2 Detection capability 21
9.2.3 Test selectivity/specificity 21
9.2.4 Robustness testing 21
9.2.5 Reader and test repeatability 21
9.2.6 Participation in a(n) (inter)national ring trial 23

Annex A (informative) **European legislation on veterinary drugs in cow milk** **24**

Annex B (informative) **USA legislation on animal drug residues in milk** **28**

Annex C (informative) **List of problematic compounds in the preparation of stock solutions** **29**

Annex D (informative) **Summary of the stability of antibiotics in solution and in matrix** **30**

Bibliography **33**

Forewords

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 302, *Milk and milk products — Methods of sampling and analysis,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement). It is being published jointly by ISO and IDF.

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.

IDF (the International Dairy Federation) is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/food control authorities.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. IDF shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

This document was prepared by the IDF *Standing Committee on Analytical Methods for Additives and Contaminants* and ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 302, *Milk and milk products — Methods of sampling and analysis,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement). It is being published jointly by ISO and IDF.

This IDF Reviewed method is equal to an ISO Publicly Available Specification (ISO/PAS) or an ISO Technical Specification (ISO/TS) and is therefore published jointly under ISO conditions.

The work was carried out by the IDF-ISO Action Team on A10 of the *Standing Committee on Analytical Methods for Additives and Contaminants* under the aegis of its project leader Dr W. Reybroeck (BE).

Guidelines for the validation of qualitative screening methods for the detection of residues of veterinary drugs in milk and milk products

1 Scope

This document describes general workflows and protocols for the validation and the verification of qualitative screening tests for the detection of residues of veterinary drugs in liquid milk (raw, pasteurized, UHT and reconstituted milk powders and whey protein extracts) including biological methods. This guideline does not cover the validation of residue analysis by HPLC, UHPLC or LC-MS/MS.

This document is intended to be useful for manufacturers of screening test kits, laboratories validating screening methods or tests, competent authorities and dairies or end users of reagents or tests for the detection of veterinary drug residues in milk products. This document facilitates and improves the validation and verification of screening methods. The goals of this document are a harmonization in validation of methods or test kits in order for all stakeholders to have full trust in the result of residue screening and to limit the overlap and multiplication of validation work in different laboratories by sharing the validation results generated by an independent laboratory. Furthermore, a harmonized validation and verification procedure allows for comparison of the performance of different screening methods.

This document does not imply that all end users are bound to perform all verification work proposed.

The verification of the correct use of reagents/kits for the detection of antimicrobials is not part of the scope of 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 terminological 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
biological method
method that is used to detect cellular responses to analytes

EXAMPLE Inhibition of bacterial growth, immunological test, and receptor test.

3.2
qualitative method
method that gives a yes/no response, with no indication of the concentration of the putative analyte

EXAMPLE 1 Bacterial growth inhibition tests which give a result of either “no zone” or “zone of inhibition”.

EXAMPLE 2 Inhibition tests which give a colour change of growth medium.

EXAMPLE 3 Immunochemical/ligand binding tests, where a response is considered as “above” or “below” a cut-off level; or where analytes with different cross-reactivities are included within the method scope.

EXAMPLE 4 Biosensors.

3.3
matrix
non-analyte portion of the sample

Note 1 to entry: Matrices are included in the scope.

3.4
detection capability
CCβ
smallest content of the analyte that can be detected, identified and/or quantified in a sample with an error probability of β

Note 1 to entry: The β error is the probability that the tested sample is truly non-conformant even though a conformant measurement has been obtained.

3.5
cut-off level
response or signal from a screening test which indicates that a sample contains an analyte at or above the screening target concentration

3.6
blank matrix sample
negative control sample
sample from animals with known history of treatment which have not been exposed to the substance in question

Note 1 to entry: If samples from such animals are not available, samples which have been previously confirmed as conformant and not containing residues of the substance of interest by suitably sensitive physicochemical tests can also be acceptable.

Note 2 to entry: See Table 1.

3.7
positive control sample
control sample that is spiked with the test analyte at the screening target concentration

Note 1 to entry: This can, however also be an incurred-positive sample (i.e. sample taken from animals which have been treated with the substance in question) or Certified Reference Material.

3.8
screening target concentration
concentration at which a screening test categorizes the sample as “screen positive” (potentially non-conformant)

Note 1 to entry: This should always be lower than the regulatory limit.

3.9
validation
confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application, such as a test or measurement method, have been fulfilled

EXAMPLE Procedure applied in the originator laboratory (manufacturer’s laboratory) or in an independent laboratory.

Note 1 to entry: Validation often determines the fitness for purpose of a method.

3.10
verification
procedure applied to a method which has been previously validated in the case of a transfer validation

Note 1 to entry: The verification procedure is applied by a receptor laboratory for the same matrix as initially validated, to demonstrate that the method will work reliably in that laboratory with locally sourced milk and is fit for purpose.

3.11
originator laboratory
laboratory that has performed the complete validation of the method

Note 1 to entry: This is by preference an ISO/IEC 17025 accredited independent laboratory and preferably not the laboratory that developed the method. The laboratory should have experience in residue testing and in validation of screening tests for the detection of residues of veterinary drugs in milk.

3.12
receptor laboratory
laboratory that will perform the verification of the method

Note 1 to entry: This could be any laboratory interested in using the method.

3.13
spectrum
range of substances that a test can detect

Note 1 to entry: Some tests detect several classes of antibiotics and a large number of substances, whereas others are more specific.

3.14
regulatory limit
level defined by food legislation for residues in food

Note 1 to entry: Regulatory limits can be MRL (see 3.15), MRPL (see 3.16), RPA (see 3.17).

3.15
maximum residue limit for veterinary drugs
MRL
maximum concentration of residue resulting from the use of veterinary drugs that is recommended by the Codex Alimentarius Commission to be legally permitted or recognized as acceptable in food

Note 1 to entry: Antibiotics are used to treat and prevent diseases in animal husbandry and as a result, low residues of antibiotics can be present in food. MRLs are set for pharmacologically active substances used or intended to be used in veterinary medicinal products placed on the market. In the EU the MRLs are set by EMA (European Medicines Agency).

3.16
minimum required performance limit
MRPL
minimum content of an analyte in a sample, which at least has to be detected and confirmed

Note 1 to entry: MRPL is intended to harmonize the analytical performance of methods for substances for which no permitted limit has been established.

3.17
reference point for action
RPA
level of a residue of a pharmacologically active substance established for control reasons in the case of certain substances for which a maximum residue limit has not been laid down following certain EU regulations

Note 1 to entry: EU Regulation 470/2009 is applicable for maximum residue limits.

Note 2 to entry: RPAs are currently based on analytical considerations (i.e. the lowest concentration that can be quantified using a validated analytical method). The aim is "to define an analytical concentration for a non-allowed pharmacologically active substance that can be determined by official control laboratories and that is low enough to adequately protect the consumers of food commodities which contain that substance"[19].

3.18
positive / negative result
result of the test after interpretation of the reading of the test taking into account the (pre-set) cut-off level

Note 1 to entry: Positive result: presence of antimicrobial residues (microbial inhibitor test) or presence of residues of veterinary drugs.

Note 2 to entry: Negative result: absence of antimicrobial residues (microbial inhibitor test) or absence of residues of veterinary drugs. Since only screening tests are involved, no judgement about 'conformant' or 'non-conformant' can be made.

3.19
repeatability limit
value less than or equal to which the absolute difference between two measurement results obtained under repeatability conditions is expected with a probability of 95 %

3.20
probability of detection
POD
proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration

Note 1 to entry: POD is concentration dependent (AOAC, 2014[7]).

4 Principle

Samples of matrix spiked with known levels of analyte are run on the test under validation or verification to determine the detection capability, sensitivity and robustness of the test. Evaluation of the test results determines the tests' suitability for routine use in screening milk for the presence of veterinary residues.

NOTE Annex B provides information on FDA tolerances and/or safe levels of animal drug residues in milk.

The key requirement for a screening method is its ability to reliably detect the analyte in question at the chosen screening target concentration. The screening target concentration should be chosen to avoid false-negative results, i.e. low enough to ensure that if the analyte in question is present in the sample at the Regulatory Limit, the sample will be classified as 'Screened Positive'.

Both validation and verification should provide the objective evidence that this key requirement is met. Validation should cover the entire matrix/species/analyte combinations claimed within the scope of the method standard operating procedure (SOP). Validation should be as broad as possible to cover the scope of all end users.

Verification should cover the matrix/species/analyte combinations included in the scope of the implementing (receptor) laboratory. The extent of validation required is variable, depending on whether it is a validation or a verification of a transferred method.

The verification does not need to cover the entire spectrum if the implementing laboratory is to be applicable to only a limited scope (e.g. some species and not others, some residues more relevant than others, raw but not UHT [Ultra-High temperature] milk, etc.).

If a receptor laboratory wants to use the method for screening in a different matrix (IDF 2014) not tested by the originator laboratory, the receptor laboratory should test all necessary validation parameters to prove that the method functions for that specific matrix.

5 General requirements for the test/kit

The developer or the manufacturer should provide information regarding methodology, test reagents, additional chemicals not necessarily included in the kit, operating requirements (information about the reading system, cut-off level), test specifications and documentation (extracted from ISO 18330 and ISO 13969). Additionally, the target country(ies) and its/their specific regulatory limits should be known, in order for the test to be evaluated against the appropriate regulatory limits.

Elements of information to be provided by the manufacturer/distributor/lab manager (in case of an in-house developed method) before starting the validation are as follows:

— Test principle, principle of reading and interpretation of the test (including cut-off level or calculation of cut-off level).

— Test formats, if relevant (e.g. ampoules/plates).

— Scope of the test:

  — Matrices suitable to be tested: matrices in the scope of the document (see Clause 1).

  — Animal species producing the milk.

  — Matrices with potential impact (interference) on the result.

— Potential impact of the use of sample preservatives.

— Spectrum of the test: list of veterinary drugs and expected detection capabilities (so far known).

— List with the current regulatory limits (RL) for the detectable veterinary drugs in the matrix(ces) of concern in the country(ies) of concern.

— Detailed protocol in a language understood by laboratory staff: if minor modifications need to be made to the method according to the matrix/species, they should be announced in the test protocol (kit manual).

6 Reagents

6.1 Standard blank matrix

— The raw milk used is commingled milk coming from at least 4 animals not treated with veterinary drugs within the last 2 months, in mid lactation, and delivering milk with a low to moderate number of somatic cells (e.g. < 150 000 ml^{-1} for bovine milk). The raw milk is collected in sterile containers and kept below 4 °C. The maximum period for the cold storage of the fresh raw milk should be in line with the definition of fresh raw milk as fixed locally.

— The milk used should be in line with the normal milk produced in the country or area of concern. This means that the composition and quality of the milk should approach the average composition of the milk of the country/region.

— Table 1 gives examples of parameters to consider for 'normal' milk. Actual figures are likely to vary depending on country and region.

— Milk of at least 4 animals is commingled and is considered as a sample of standard blank matrix. At least four such samples should be used for the determination of the detection capability when testing 20 replicates. If 40 or 60 replicates need to be tested to determine the detection capability, eight or twelve different blank milk samples should be used, respectively. At least four different commingled milks should be sourced and used in the verification work (20 replicates).

— The use of thawed or reconstituted lyophilized milk could also be authorized, but strictly on condition. The pre-requisite condition to work with these alternative solutions, is to demonstrate

previously the equivalence of results between raw milk and thawed or reconstituted lyophilized milk, after the analysis of negative and positive milk samples.

Table 1 — Examples of reference data for the composition and quality of normal milk of different animal species

Species		SCC[a] cells per ml	TBC[b] cfu[c] per ml	FC[d] g/l	PC[e] g/l	pH	Antibiotics	Lactating period
Cow	**Target value**	< 150 000	< 30 000	40	33	6,7 to 6,8	Absence	Between 60 and 200 days after calving
	Acceptable range	< 400 000	< 100 000	35 to 45	30 to 36	6,6 to 6,9		
Goat	**Target value**	< 2 000 000	< 60 000	38	34	6,7 to 6,8	Absence	Between 20 and 150 days after kidding
	Acceptable range			30 to 50	28 to 40	6,6 to 6,9		
Ewe	**Target value**	< 2 000 000	< 60 000	70	55	6,7 to 6,8	Absence	Between 20 and 150 days after lambing
	Acceptable range			50 to 90	40 to 70	6,6 to 6,9		

[a] Somatic cell count.
[b] Total bacterial count.
[c] Colony forming units.
[d] Fat content.
[e] Protein content.

6.2 Antibiotics

Only use analytical grade or certified reference material for validation or verification purposes.

6.3 Standard stock solution

— Standard stock solutions of the antibiotic at 100 mg/l are made in water or a suitable solvent and kept below 4 °C (refer to 8.1)[14]. The shelf life depends on the stability of the molecule.

— In the preparation of the stock solution, correction for impurity and water content is performed.

— For each substance a single stock solution is prepared, but by preference for certain problematic compounds (for example solubility problem, stability), at least two stock solutions should be prepared to determine the detection capability. A list of problematic compounds is given in Annex C.

— If only one stock solution is used it should be either prepared from certified material or verified with an independent physicochemical method.

— Some compounds like tetracyclines are light sensitive and need to be kept protected from light. Other compounds can require specific requirements for the glassware used.

6.4 Working stock solutions

Dilutions of 10 mg/l to 0,1 mg/l are freshly prepared on a daily basis.

6.5 Spiked sample

For the preparation of end concentration, the final spiking is performed in the standard blank matrix.

The blank milk will be spiked with each analyte.

The added volume of working stock solution should be below 5 % of the final volume of the milk sample to be tested.

7 Apparatus

Any apparatus specified in the test kits procedure that is not provided by the test kit manufacturer.

7.1 Test kit, in which reagents of at least two and by preference three different production lots are used.

Production lots should be determined randomly and be representative of the production level of the product.

7.2 Incubator or water-bath, capable of maintaining the appropriate incubation temperature for the test.

If the manufacturer has an incubator that is designed for the test, this incubator should be supplied by the manufacturer for use in the validation or verification.

7.3 Automated readers. If the manufacturer provides an apparatus for evaluating the results from the test, this apparatus should be supplied by the manufacturer for use and evaluation in the validation or verification.

Calibration procedures for the automated reader shall be made available and all readings shall be taken from only calibrated equipment.

7.4 Micropipettes, capable of delivering the appropriate amount of sample required for use in the test.

If the test kit supplies micropipettes or other liquid transfer equipment these should be by preference evaluated as part of the validation or verification.

8 Sample Preparation

8.1 Stock solution preparation

A 100 mg/l solution of each antibiotic is prepared by first calculating the amount of reference material needed to give 10 mg ± 0,1 mg of active compound. This calculation is done using Formula (1) and the information (purity, water content) from the certificate of analysis of the antibiotic.

$$m_m = m_a \times \left(\frac{100}{P}\right) \times \left(\frac{100}{100-W}\right) \quad (1)$$

where

m_m is the mass of the material required, in mg;

m_a is the mass of the analyte required, in mg;

P is the purity, in %;

W is the water content, in %.

Weigh this amount directly in a weight boat and transfer into a 100 ml volumetric flask and make up to 100 ml using the appropriate solvent.

The samples are vortexed or sonicated until dissolved. These can be kept at 4 °C for up to 4 weeks or at −18 °C or lower for up to 12 months. Stability of different antimicrobials in solution and in matrix at different storage temperatures are given in Table D.1 and Table D.2.

8.2 Working stock solution preparation

If necessary, intermediate stocks of 100 000 µg/l, 10 000 µg/l, 1 000 µg/l or 100 µg/l, respectively (or other appropriate concentrations) are made up in distilled water and stored at 4 °C to 6 °C for maximum one day.

8.3 Blank matrix sample selection

For each antibiotic to be tested, the number of different samples of standard blank matrix from different sources to be tested depends on the closeness of the predicted detection capability (CCβ) to the regulatory limit:

— If CCβ is below or equal to half MRL: at least two different batches of milk for 20 replicates.

— If CCβ is between 50 % and 90 % of MRL: at least four different batches of milk for 40 replicates.

— If CCβ is near MRL (≥90 % to 100 % of MRL): at least six different batches of milk for 60 replicates.

— If CCβ is above MRL: at least two different batches of milk for 20 replicates.

The sample should be of sufficient size to provide multiple test portions of the size specified by the test protocol. When selecting the source of the milk, samples covering the range of "normally" produced milk should be selected. This could cover factors like breed of animal, feed source, geographic region, etc.

The samples should be checked to ensure the absence of beta-lactamase or para-aminobenzoic acid by adding a small but detectable quantity of penicillin or sulfonamide to the milk, respectively and incubate and test. If a negative result is obtained it indicates the presence of beta-lactamase or para-aminobenzoic acid (PABA), respectively. This check is only needed if beta-lactams or sulfonamides are included in the validation, respectively. The pH of the sample should be recorded and a subsample should be kept and stored at −25 °C ± 5 °C. In case of questionable results during validation or verification (false-positive results, CCβ lower than the CCβ announced by the manufacturer or obtained with different milk), the laboratory thaws the sample for screening or confirmatory analyses.

8.4 Spiked sample creation

The appropriate working stock solution should be added to the blank matrix sample to produce a sample at the required level of antibiotic. The added volume of working stock solution should be below 5 % of the final volume.

Furthermore, the last working solution can be done in milk, except for tetracyclines.

NOTE For tetracyclines, it is recommended that only the added volume of working stock solution be below 1 % or 2 % of the final volume. It is recommended to prepare all the dilutions of working solutions in water and only the final dilution in milk to avoid binding to milk proteins and to calcium.

9 Procedure

9.1 Validation

9.1.1 General

The validation is a procedure applied to characterize the performances of a test, in the originator laboratory (manufacturer's laboratory) or in an independent laboratory. The validation demonstrates

that the method is fit for purpose. The originator laboratory could be the laboratory (or a group of laboratories) which developed the new analytical method or the first laboratory (or group of laboratories) performing a full validation study. The originator laboratory performing the validation should by preference be an independent laboratory with experience in the field and a quality control system (e.g. ISO/IEC 17025) in place and accredited for analogue methods for the same matrix.

Laboratory staff should have access to all required equipment (incubator and reader system when applicable) and should be fully trained to run the test.

The validation of a test covers:

— the detection capability;

— test selectivity/specificity;

— test robustness; and

— reader and test repeatability.

All factors should be present in the final validation report.

During the validation, the detection capabilities are determined as precisely as possible.

9.1.2 Detection capability (CCβ)

9.1.2.1 General

The detection capability should be determined in the specific matrix/species for which the test was developed, which in most cases will be raw cow milk. If the test is claimed to be appropriate for use in testing an alternative matrix/species then the validation is required to cover this matrix as well. In such a case two approaches are possible:

Option 1: The CCβ is determined in the main matrix as such (for example in raw cow milk) then other matrices/species are studied as part of the applicability and/or robustness testing. In the applicability and/or robustness testing, the samples may be spiked up to levels of CCβ + 20 %. For as far as positive results are obtained, the CCβ determined in the main matrix is also valid in the new matrix/species. If negative results are obtained, the new matrix/species should be fully validated to determine the CCβ, or the conclusion is that the method is not applicable to the new matrix/species with the same CCβ.

Option 2: The detection capabilities are in the same study determined directly for the different matrices with an equal number of replicates for each different matrix (e.g. cows' milk, goats' milk and ewes' milk; UHT milk, sterilized milk and reconstituted milk powder).

Reconstituted milk powder should not be mixed with raw milk when testing microbial inhibitor tests since they require a different incubation time. Only use similar matrices with equal incubation times in the same run.

This last procedure can increase the CCβ if the CCβ is different for each matrix. The highest CCβ is the final value. The same phenomenon can be observed when different lots of test reagents are used in combination for the determination of CCβ.

9.1.2.2 Compounds involved in the study

All substances relevant for end users in their routine application (whether defined by regulation, registration or actual use) should be validated.

The compounds to be tested for detection capability are determined by the type of test to be validated.

For bacterial growth inhibition assays that cover a wide range of compounds:

— the marker residues of all pharmacologically active substances of the involved group(s) of veterinary drugs (e.g. in the EU the pharmacologically active substances mentioned in Table 1 of the Annex of Council Regulation (EU) N° 37/2010 [see Table A.1]);

or:

— the marker residues of all pharmacologically active substances occurring in brands/trade names registered for use in dairy cattle in the country of interest and belonging to the involved group(s) of veterinary drugs (e.g. in the EU as mentioned in Table 1 of the Annex of Council Regulation (EU) N° 37/2010 [see Table A.1]).

There are many different sulfonamides. The number of sulfonamides to be validated will be at least three sulfonamides. The choice of which three should be made taking into account the registered sulfonamides for use in lactating cows, which can vary among countries.

For immunological or receptor assay tests:

— the marker residues of all pharmacologically active substances of the group(s) of veterinary drugs indicated to be able to be detected (e.g. if the test is designed to detect beta-lactams then detection ability for all beta-lactams shall be determined);

or:

— the marker residues of all pharmacologically active substances occurring in brands/trade names registered for use in dairy cattle in the country of interest and belonging to the group(s) of veterinary drugs indicated to be able to be detected (e.g. if the test is designed to detect beta-lactams then detection ability for all beta-lactams registered for use in the country of concern shall be determined).

If a test manufacturer is only claiming the detection of one or a limited number of substances, the other substances belonging to the same antibiotic group (e.g. within the EU as mentioned in Table 1 of the Annex of Council Regulation (EU) N° 37/2010) should be tested as part of test selectivity/specificity testing. If any cross-reactivity is noticed, the substance(s) should be added to list of substances for detection capability testing.

9.1.2.3 Determination of concentrations of substances to be involved in the study

For each compound at least two concentrations around the initial test concentration should be tested. For commercial kits, the results obtained by the test manufacturer can be used as a starting point. For the compounds with missing information, the regulatory limit in milk can be used.

The aim is to find the lowest concentration of compound that returns a positive test in 95 % of the cases.

Two options for the choice of increment between the concentrations are proposed:

1) the increment between the different concentrations is dependent on the concentration level, as indicated in Table 2.

Table 2 — Increment between the concentrations tested

Concentration µg/kg	Increment µg/kg
1 to 10	1
11 to 20	2
21 to 50	5
51 to 100	10
101 to 250	25

Table 2 *(continued)*

Concentration µg/kg	Increment µg/kg
251 to 500	50
501 to 1 000	100
1 001 to 5 000	500

In case of testing of concentrations below 0,5 × RL (regulatory limit) or above RL, the increment can be doubled. The increments suggested are starting points and can be further modified to identify the true CCβ;

or

2) the concentrations tested can be based on factors of the RL;

— RL;

— $^{3}/_{4}$ RL;

— $^{1}/_{2}$ RL;

— $^{1}/_{4}$ RL;

— $^{1}/_{10}$ RL.

This approach is most appropriate for broad-spectrum tests with a lot of substances involved.

9.1.2.4 Number of replicates required

The number of replicates tested at each concentration is based on the closeness of the predicted detection capability (CCβ) to the regulatory limit and is shown in Table 3. Each concentration will be tested 20, 40, or 60 times, in a time period of at least three days, with at least two operators and by using at least two and by preference three test kit lots.

Table 3 — Number of replicates based on closeness of the predicted detection capability (CCβ) to the regulatory limit (RL)

Closeness to Regulatory Limit	Number of replicates
≤ 0,5 RL	20
> 0,5 RL and < 0,9 RL	40
≥ 0,9 RL and ≤ RL	60
> RL	20

The detection capability is defined as the lowest concentration tested giving at least 95 % positive results, for example 19, 38, or 57 positive results, out of 20, 40, or 60 tests, respectively.

The analyses of 20, 40, or 60 samples may be performed sequentially (e.g. 10 samples first, if it is satisfactory, analyse a further 10 samples, etc.). When two, three or four negative results are obtained respectively, the assay at this target concentration may be stopped and a higher concentration should be tested.

9.1.2.5 Testing requirements

Every day the following daily standards are also tested:

— Negative control samples:

  — 2 × blank raw milk, free from antimicrobials, with a normal composition, quality and pH;

— Positive control samples:

  — 2 × a substance spiked in raw milk close to detection capability, for each group of veterinary drug agents (e.g. for a beta-lactam test: a penicillin and a cephalosporin).

  For multiresidue (non-group specific) tests like antimicrobial inhibitor tests, a minimum of 3 positive controls should be used (e.g. a beta-lactam, a tetracycline and a sulfonamide).

Multiple concentrations for the same compound (e.g. 3 ppb and 4 ppb of benzylpenicillin) can be tested at the beginning to help determine a suitable positive control level. Once decided upon, these concentrations may be used during the whole study.

A multi-compound standard for testing of group-specific multiplex tests (receptor assays, immunological assays) may be used provided that there is proof that the combination of compounds in the standard does not influence the testing of each compound.

All tests should be performed following the procedure (incubation temperature, incubation time, volume of milk, etc.) given by the kit manufacturer. For in-house methods, the final procedure on paper should be followed. All results should be recorded.

In the case of test kits with a reading system device provided by the manufacturer, the reader values are the results recorded.

Before starting, any electronic reader system should be calibrated.

Some tests have no reader system, and the results are interpreted visually.

If visual interpretation is required, this should be performed by at least 2 different technicians and the samples should be blind coded.

Where the kit uses a flexible cut-off level to determine the completion of the test run, it is very important that the instructions of the kit manufacturer are strictly followed and that the kit manufacturer specifies how to calculate the cut-off level (definition of type of milk to be used, etc.).

The validation report should contain the list of the known non-detected antibiotics at the target concentration in the target family.

9.1.2.6 Determination of a dose response curve (optional)

Where appropriate, it is suggested that information on the dose response curve and probability of detection is generated and provided in the validation report.

Information showing the percentage of positive results at 25 %, 50 % and 75 % of the determined detection capability (CCβ) and information identifying the highest concentration still giving 0 % positive results, can be provided and can be generated during the determination of the CCβ.

In this way, users of the test are able to know from which concentration on positive screening results are expected, and from which concentration uncertainty in the screening results can arise.

9.1.3 Test selectivity/specificity

9.1.3.1 General

This testing is designed to identify which (if any) substances not previously identified are detected by the test.

Table 4 presents, as an example, a list of antibiotics to be tested to determine the specificity of the test. This list can be modified depending on the test or on the veterinary practices in a country.

Table 4 — Families of veterinary drugs/agents and an example of a pharmacologically active substance for each family

Families of veterinary drugs agents	Example of a pharmacologically active substance
penicillins	benzylpenicillin
cephalosporins	cefalonium
tetracyclines	oxytetracycline
macrolides	erythromycin
aminoglycosides	neomycin B
quinolones	enrofloxacin
polymyxins	colistin
amphenicols	chloramphenicol
lincosamides	lincomycin
beta-lactamase inhibitors	clavulanic acid
sulfonamides	sulfadiazine
diamino pyrimidine derivates	trimethoprim
other chemotherapeutics	dapsone

9.1.3.2 Substance-specific tests

This includes testing of all other substances belonging to the same antibiotic group as mentioned in Commission Regulation (EC) N° 37/2010 (see Table A.1), spiked at a high concentration (100 × RL) in raw milk. Tested in duplicate.

This also includes testing of substances (minimum one per family) of all other families not claimed to be detected by the substance-specific test, spiked at a high concentration. 100 times the appropriate maximum permitted limit (regulatory limit) is used. Tested in duplicate. Examples are given in Table 4.

If any cross-reactivity is noticed, the substance(s) should be added to list of substances for detection capability testing.

9.1.3.3 Group-specific tests

This includes testing of substances (minimum one per family) of all other families not claimed to be detected by the group-specific test, spiked at a high concentration. 100 times the appropriate maximum allowable limit is used, tested in duplicate. Examples are given in Table 4.

If any cross-reactivity is noticed, the substance(s) shall be added to list of substances for detection capability testing.

9.1.3.4 Rate of positive results not caused by residues of veterinary drugs

This is required to show that the test will perform as expected on a range of samples that will be encountered as part of routine use of the test. This can be done in parallel with routine testing in a laboratory.

— Test:

  — 300 individual farm milk samples;

  — 300 tanker milk samples.

In case a positive result is obtained for a screening test, first the quality and composition of the milk (fat, protein, pH, somatic cells, etc.) should be determined. Other screening tests and finally a physicochemical confirmation of the milk sample shall be performed to indicate if the positive result is caused by an abnormal quality/composition/pH, by the presence of antimicrobial residues or by interfering substances (presence of residues of veterinary drugs which could not be confirmed). In case of the presence of residues of veterinary drugs, they should be identified and quantified. The time between screening and confirmatory analysis should be limited (less than 1 month) and in this period milk should be frozen to prevent degradation of the milk quality or degradation of the substance(s) possibly present. Other precautions may be taken to prevent degradation of the sample or compounds present. A minimum sample volume for collection of 40 ml to 50 ml is recommended to ensure uniformity and standardization during the testing.

9.1.4 Robustness testing

9.1.4.1 General

Robustness testing is designed to identify the range of samples in which the test will operate. These samples are typically designed to be at the extreme ends of the normal range of the sample received into a laboratory for routine testing.

Parameters include:

— influence of test protocol;

— influence of milk quality and composition;

— influence of type of milk or animal species (optional);

— influence of production batch and age of reagents;

— stability of readers and reagents.

To determine a baseline for the variations covered by the test, use:

— 4 to 10 different blank raw milk samples with a normal content, quality and pH;

— 4 to 10 different blank raw milk samples with a normal content, quality and pH and spiked with a substance (A) at or just above detection capability (maximum +20 %);

— 4 to 10 different raw milk samples with a normal content, quality and pH and spiked with a substance (B) at or just above detection capability (maximum +20 %).

Then for each parameter described in the following sections test, use:

— 4 to 10 different blank raw milk samples;

— 4 to 10 different raw milk samples spiked with a substance (A) around 20 % above detection capability;

— 4 to 10 different raw milk samples spiked with a substance (B) around 20 % above detection capability.

To limit the number of samples to be tested a differentiation can be made. For parameters like impact of temperature or time, the same milk samples are used in all conditions. For such parameters the number of replicates may be limited to four samples from same milk source, for example.

In case of impact of milk quality parameters like somatic cells, bacterial count is tested and more replicates (for example, 10) are minimally needed.

For a single residue-test or a single-family test, only one substance is integrated in the robustness testing.

For a multi-group test, at least one pharmacologically active substance of each group of veterinary drug residues is integrated in the robustness testing (e.g. for a beta-lactam test a penicillin and a cephalosporin). The representative substance can be the least sensitive detected or the most relevant compound.

9.1.4.2 Influences of test protocol (where applicable)

9.1.4.2.1 Influences

— Incubation temperature;

— incubation time;

— delay in reading;

— set up time;

— volume of test portion;

— temperature of test portion.

9.1.4.2.2 Incubation temperature (optional, conditions are met as per those set by the manufacturer)

The temperature range within which the test operates correctly should be determined. Requested temperature (= reference) versus a lower and a higher temperature shall be defined considering the recommendations of the manufacturer. Local environmental temperature ranges should be taken into account to ensure that the test operates in all possible local conditions.

If a test is performed at ambient temperature (without incubator) the testing should be performed at ambient temperature (= reference) and at a lower and a higher temperature in a temperature-controlled room. The temperatures should be defined, taking into account the recommendations of the manufacturer, as well as the abovementioned considerations regarding possible local conditions.

9.1.4.2.3 Incubation time (for each incubation step)

Requested incubation time (= reference) versus a shorter and longer time are taken into consideration.

For a multi-step test each incubation step is independently tested. This testing for a multi-step test may be extended by testing combinations (shorter-longer, longer-shorter, etc.).

9.1.4.2.4 Delay of reading

Direct reading is taken after last incubation when the assay is ended (= reference) versus delay of reading, after the assay is ended. Four different delay periods should be evaluated (e.g. 30 s, 1 min, 5 min and 15 min). The times given are examples and can be adapted to their relevance.

9.1.4.2.5 Set up time (keeping of extract before testing)

Direct incubation or testing after distribution of sample versus a delay in testing is taken into consideration. Four different delay periods should be evaluated (e.g. 1 min, 5 min, 15 min and 30 min).

9.1.4.2.6 Volume of milk

Correct volume (= reference) versus smaller (for example –10 %) and bigger volume (for example +10 %) are taken into consideration.

9.1.4.2.7 Temperature of milk

Cold milk (2 °C to 4 °C) (= reference) versus milk at 20 °C is taken into consideration. The time that milk is kept at 20 °C should be limited (e.g. max 1 h). In case of differences, a milk temperature of 10 °C and 15 °C should also be tested. Local conditions should be considered when determining the temperature of milk to test. If milk is normally delivered at 30 °C then this should be reflected in the test protocol.

9.1.4.3 Milk quality/composition influences

9.1.4.3.1 General

The influence of the composition parameters, somatic cell count and total bacterial count concerns only the analysis of raw or pasteurized milk.

Milk composition and factors with potential influence can be different all around the world. The limitations for considering the composition/quality of the milk as abnormal can be different from country to country. Therefore, the following values are given as examples and not as mandatory levels to be tested. Appropriate levels should be determined based on the normal composition/quality of the local milk supply. As a guide, high and low levels should be at least two standard deviations from the mean. Ensure that the ranges tested cover the expected variation in the local milk supply.

The following describes which veterinary substances at which level(s) and how many replicates should be tested in order to demonstrate the influence of the milk composition.

For each parameter described in 9.1.4.3, test:

— 10 different blank raw milk samples;

— 10 different raw milk samples spiked with a substance (A) around 20 % above detection capability;

— 10 different raw milk samples spiked with a substance (B) around 20 % above detection capability.

If an influence of one of the parameters listed in the following subclauses is observed to affect the detection capability, a confirmatory analysis should be performed to check if the antibiotic has been destroyed or the detection has been prevented.

9.1.4.3.2 High somatic cell count (SCC)

Milk with a normal composition/quality/pH (= reference) versus milk with a SCC > 10^6 per ml.

9.1.4.3.3 High total bacterial count (TBC)

Milk with a normal composition/quality/pH (= reference) versus milk with a TBC > 5×10^5 per ml. A high number of bacteria could affect the performance of the test, but beta-lactamase produced by certain bacteria could also influence the antibiotic content of the milk.

9.1.4.3.4 Low fat content (FC)

Milk with a normal composition/quality/pH (= reference) versus milk with a low fat content (e.g. FC < 2 g per 100 g).

9.1.4.3.5 High fat content (FC)

Milk with a normal composition/quality/pH (= reference) versus milk with a high fat content (e.g. FC > 6 g per 100 g).

9.1.4.3.6 Low protein content (PC)

Milk with a normal composition/quality/pH (= reference) versus milk with a low protein content (e.g. PC < 2,5 g per 100 g).

9.1.4.3.7 High protein content (PC)

Milk with a normal composition/quality/pH (= reference) versus milk with a high protein content (e.g. PC > 4 g per 100 g).

9.1.4.3.8 Low pH

Milk with a normal composition/quality/pH (= reference) versus milk with a pH = 6,0.

9.1.4.3.9 High pH

Milk with a normal composition/quality/pH (= reference) versus milk with a pH = 7,5.

9.1.4.3.10 Early lactation milk

Mid lactation milk (=reference) versus milk from early in the lactation cycle [e.g. the first 30 days after calving (parturition) but no colostrum milk].

9.1.4.3.11 Late lactation milk

Mid lactation milk (=reference) versus milk from late in the lactation cycle [e.g. > 270 days after calving (parturition)].

9.1.4.4 Influences by type of milk or animal species (optional)

9.1.4.4.1 General

In general, regulatory limits do not differ in the same matrix type (e.g. milk) between species. Nevertheless, if CCβ has been determined for one matrix (e.g. cow milk) during the initial validation and the method is to be applied to the same matrix in another species (e.g. ovine milk), an interfering matrix effect should be anticipated and it cannot be assumed that the same CCβ will apply to this new matrix. Therefore, CCβ should be checked for the analyte(s) in question in this new matrix. This should be performed for each analyte the laboratory is required to include in a residue analysis programme or, at least for a selected number of analytes which are representative for the analyte group in question.

9.1.4.4.2 UHT milk

Raw milk (= reference) versus UHT milk.

9.1.4.4.3 Sterilized milk

Raw milk (= reference) versus sterilized milk.

9.1.4.4.4 Thawed milk

Raw milk (= reference) versus thawed milk.

9.1.4.4.5 Reconstituted milk

Raw milk (= reference) versus reconstituted milk.

9.1.4.4.6 Other animal species' milk

Raw cows' milk (= reference) versus raw other animal species' milk. If other species' milk is to be validated, milk quality/composition influence robustness testing should also be carried out on the new species milk. Especially for goats' and ewes' milk, serious changes in milk composition can be expected depending on the state of lactation. By preference, milk from animals in mid-lactation should be used but the impact of the state of lactation should also be evaluated.

9.1.4.5 Influence of production and age of reagents

9.1.4.5.1 Batch differences

Testing with reagents of batch 1 versus the use of reagents of two other batches. Both tests are performed on the same milk samples on the same day with the two batches of reagents.

In the case that three different lots of reagents were used during the testing of the detection capability, there is no more need for extra testing of batch differences.

Most manufacturer instructions do not recommend mixing reagents from different batches. If the manufacturer has clearly indicated in their instructions or product insert that mixing reagents from different batches is not permitted, then testing for this is not required.

9.1.4.5.2 Age of reagents

Testing with reagents used shortly after production versus the use of reagents just before expiry date. Some of the reagents are stored till shortly before expiry date and then tested, using samples of 'normal' milk.

9.1.4.6 Stability of test kits and reagents

The positive and negative controls tested daily as detailed in 9.1.2.5 should be read via the automated reader if available. A record of this information should be kept in order to help determine the stability of the test kit over time.

For stability testing frozen raw milk, lyophilized samples or reconstituted skimmed milk powder may be used if necessary. In such a situation, in the case of an influence on stability, a confirmatory analysis should be performed to check if the antibiotic has been destroyed or the detection has been prevented.

9.1.5 Reader and test repeatability

9.1.5.1 Visual reading

Visual reading is allowed for qualitative methods. For tests with only visual reading, the reading should be performed by a minimum of 2 different trained technicians and the samples should be blind coded.

To determine the repeatability of the reading, test:

— minimum 20 blank milk samples;

— minimum 20 positive samples (low positive);

— minimum 20 positive samples (high positive).

Visual readings should be performed in duplicate, by the same technician. The second reading should be performed immediately after the previous reading. If possible, the devices/strips/plates are measured once they no longer change (dried, after addition of stop solution, etc.).

Calculate the percentage of agreement between both readings (- and + or -, + and ±).

9.1.5.2 Repeatability of the reader (instrumental reading)

To determine the repeatability of the readers, test:

— minimum 20 blank milk samples;

— minimum 20 positive samples (low positive);

— minimum 20 positive samples (high positive).

Take duplicate readings of the 20 tests for each parameter with the reader. Use the duplicate readings to calculate the reader repeatability.

The second reading should be performed immediately after the previous reading. If possible, the devices/strips/plates are measured once they no longer change (dried, after addition of stop solution, etc.).

Calculate the standard deviation of repeatability (s_r) using Formula (2):

$$s_r = \sqrt{\sum_{i=1}^{n} \frac{(R_1 - R_2)^2}{2n}} \tag{2}$$

where

R_1 is the result of the first reading;

R_2 is the results of the second reading;

n is the number of samples.

The repeatability limit of the reading is: $r = 2{,}83 \times s_r$

In some cases, duplicate readings are not possible. For example, when using an incubator/reader the repeatability can not be determined since duplicate readings are not possible.

9.1.5.3 Repeatability of the test

There is no need for extra testing since these data can be generated during the validation study (detection capability testing).

Take data from:

— minimum 10 blank milk samples.

— minimum 10 positive samples (low positive).

— minimum 10 positive samples (high positive).

Use duplicated sample results.

Calculate the standard deviation of repeatability (s_r) using Formula (3):

$$s_r = \sqrt{\sum_{i=1}^{n} \frac{(R_1 - R_2)^2}{2n}} \quad (3)$$

where

R_1 is the result of the first reading;

R_2 is the results of the second reading;

n is the number of samples.

The repeatability limit of the test is $r = 2{,}83 \times s_r$

If there is only an interpretation of results in categories (e.g. interpretation of the results in – and + or in -, ± and +), the percentage of classification in the same class can be calculated for the replicate results.

When an interlaboratory study is planned, the method precision can be calculated according to ISO/TS 16393.

9.1.6 Participation in a(n) (inter)national ring trial

Participation with the test under validation in a ring trial for the detection of antibiotics in milk with microbiological and rapid tests is strongly recommended.

The samples used in the ring trial should by preference contain residues in a concentration at or around the detection capability for the compound concerned.

9.2 Verification testing of a transferred screening method

9.2.1 General

Certain laboratories require a validation report (e.g. in the framework of accreditation). In such a case these laboratories can take over the results of the validation study and perform verification testing. A verification procedure is undertaken when transferring a method from the validation laboratory to another laboratory (receptor laboratory). The laboratory needs to demonstrate that they are able to achieve the same CCβ for the test on the scope of matrix/analytes relevant for their daily routine operations as in the validation report.

As such, the verification study is a significantly reduced version of the validation.

Other end users like dairies or farmers may still perform the screening test according to the manufacturer's rules even without performing verification testing. However, the use of certified reference material and participation in proficiency tests/ring trials is recommended.

Pertaining to conditions of transfer, the laboratory should have access to the complete test procedure and the complete validation report. The laboratory should perform the test in the same conditions as for the validation using the same type of equipment (reader type, incubator, etc.), using the same screening target concentrations and, if applicable (if no difference in cut-off levels between batches), the same cut-off level(s).

Before starting, the technicians should have access to the test procedure in a language understood (provided by the laboratory that performed the initial validation, or the manufacturer) and the required equipment. The verification is only started when the technicians are fully trained to run the test.

9.2.2 Detection capability

9.2.2.1 General

As the detection capability of the test has already been determined by the validation, this testing is to show that the detection capability of the test is the same in the receptor laboratory.

9.2.2.2 Compounds involved in the study

For each compound the concentration indicated as CCβ in the initial validation report should be tested. The aim is to discover if the transfer laboratory is also screening this concentration in 95 % of the cases as positive.

For each involved family of veterinary drugs, at least one representative pharmacologically active substance (by preference the most difficult compound to be determined or the most relevant compound) should be tested. For a broad-spectrum test, at least a penicillin, a cephalosporin, a tetracycline and a sulfonamide should be involved in the study.

The screening target concentration for verification should be the CCβ of the initial validation. When CCβ does not pass, the screening target concentration could be higher than the CCβ of the initial validation (+ 5 % to + 20 %) if the first CCβ was much lower than the regulatory limit (RL). The regulatory limit should not be surpassed.

If the first CCβ (initial validation) is very close to the RL, it is impossible to increase the screening target concentration because there is a risk that the CCβ of the verification could be higher than the RL, which is unsatisfactory.

9.2.2.3 Number of replicates required

Each concentration is tested 20 times (replicates), with at least two operators. The detection capability is defined as the lowest concentration tested giving at least 19 positive results out of the 20 tests.

The preparation of the standards and the spiking of milk samples may be performed by another laboratory, rather than by the receptor laboratory. The transfer laboratory should receive the samples in an appropriate condition (e.g. frozen) and repeatable cycles of freezing/thawing/freezing of the samples should be avoided.

Samples could be sent frozen if it was proved during the initial validation that freezing of the sample has no impact on the results.

9.2.3 Test selectivity/specificity

Since the same reagents are used, this testing does not need to be repeated.

9.2.4 Robustness testing

Provided that the same reagents and the same test procedure are used, robustness testing does not need to be repeated. If the transfer laboratory wishes to run samples with a composition or quality outside the range tested in the primary validation, then extra testing is required (for example, if initial validation covered fat from 2 % to 6,5 % but the local milk routinely has fat at 8 % then this higher range should be investigated to show it has no effect).

9.2.5 Reader and test repeatability

9.2.5.1 Visual reading

Visual reading is allowed for qualitative methods. If available, instrumental reading is recommended and should be checked in the verification study.

For tests with only visual reading, the reading should be performed by a minimum of 2 different trained technicians and the samples should be blind coded.

To determine the repeatability of the reading, test:

— minimum 20 blank milk samples;

— minimum 20 positive samples (low positive);

— minimum 20 positive samples (high positive).

Visual readings in duplicate, by the same technician. The second reading should be performed immediately after the previous reading. If possible, the devices/strips/plates are measured once they no longer change (dried, after addition of stop solution, etc.).

Calculate the percentage of agreement between both readings (- and + or -, + and ±).

9.2.5.2 Repeatability of the reader (instrumental reading)

To determine the repeatability of the readers, test:

— minimum 20 blank milk samples;

— minimum 20 positive samples (low positive);

— minimum 20 positive samples (high positive).

Take duplicate readings of the 20 tests for each parameter with the reader. Use the duplicate readings to calculate the reader repeatability.

The second reading should be performed immediately after the previous reading. If possible, the devices/strips/plates are measured once they no longer change (dried, after addition of stop solution, etc.).

There is no need for extra testing since these data have been generated during the validation study, detection capability testing.

Calculate the standard deviation of repeatability (s_r) using Formula (4):

$$s_r = \sqrt{\sum_{i=1}^{n} \frac{(R_1 - R_2)^2}{2n}} \tag{4}$$

where

R_1 is the result of the first reading;

R_2 is the results of the second reading;

n is the number of samples.

The repeatability of the reading is $r = 2{,}83 \times s_r$

In some cases, duplicate readings are not possible. For example, when using an incubator/reader the repeatability can not be determined since duplicate readings are not possible.

9.2.5.3 Repeatability of the test

There is no need for extra testing since these data can be generated during the verification study, detection capability testing.

Take data from:

— minimum 10 blank milk samples;

— minimum 10 positive samples (low positive);

— minimum 10 positive samples (high positive).

Duplicated sample results should be used.

Calculate the standard deviation of repeatability (s_r) using Formula (5):

$$s_r = \sqrt{\sum_{i=1}^{n} \frac{(R_1 - R_2)^2}{2n}} \quad (5)$$

where

R_1 is the result of the first reading;

R_2 is the results of the second reading;

n is the number of samples.

The repeatability of the test is $r = 2{,}83 \times s_r$.

9.2.6 Participation in a(n) (inter)national ring trial

Ongoing participation with the test under verification in a ring trial for the detection of antibiotics in milk with microbiological and rapid tests is strongly recommended.

The samples used in the ring trial should by preference contain residues in a concentration at or around the detection capability for the compound concerned.

Annex A
(informative)

European legislation on veterinary drugs in cow milk

A.1 General

Annex A provides information on the following European legislation on veterinary drugs in cow milk:

— Maximum Residue Limits (MRLs, as defined in Regulation (EC) No 470/2009, Commission Regulation (EU) No 37/2010 and amendments);

— Minimum Required Performance Limits (MRPLs, as defined in Commission Decision 2003/181/EC);

— Reference Points for Action (RPA, as defined in Regulation (EC) No 470/2009, Commission Regulation (EU) 2019/1871);

— Recommended concentrations (*Anon.,* 2007c).

A.2 Allowed substances

The current list of EU-MRLs can be checked on the EURLEX website under consolidated texts (https://eur-lex.europa.eu/advanced-search-form.html?qid=1571231722710&action=update), consolidated version of the following act; Year: 2010, Number: 37, Type: Regulation.

The list of MRLs in cow milk fixed for marker residues of pharmacological active substances (as of 2020/01/10) is given in Table A.1. The table also contains the compounds not allowed to be used in cows producing milk for human consumption.

Table A.1 — Maximum residue limits (MRLs, as defined in Regulation (EC) No 470/2009, Commission Regulation (EU) No 37/2010 and amendments) in cow milk (as of 2020/01/10)

Group (family)	Pharmacologically active substance	Marker residue	MRL µg/kg	Other provisions
sulfonamides	group	parent drug	100	the combined total residues should not exceed 100 µg/kg
diaminopyrimidine derivatives	baquiloprim	baquiloprim	30	
	trimethoprim	trimethoprim	50	
penicillins	benzylpenicillin	benzylpenicillin	4	
	ampicillin	ampicillin	4	
	amoxicillin	amoxicillin	4	
	oxacillin	oxacillin	30	
	cloxacillin	cloxacillin	30	
	dicloxacillin	dicloxacillin	30	
	nafcillin	nafcillin	30	for intra-mammary use only
	penethamate	benzylpenicillin	4	

Table A.1 *(continued)*

Group (family)	Pharmacologically active substance	Marker residue	MRL µg/kg	Other provisions
cephalosporins	ceftiofur	sum of all residues retaining the beta-lactam structure expressed as desfuroylceftiofur	100	
	cefquinome	cefquinome	20	
	cefazolin	cefazolin	50	
	cephapirin	sum of cephapirin and desacetylcephapirin	60	
	cefacetrile	cefacetrile	125	for intra-mammary use only
	cefoperazone	cefoperazone	50	for intra-mammary use in lactating cows only
	cefalexin	cefalexin	100	
	cefalonium	cefalonium	20	for intra-mammary use and eye treatment only

Group	Pharmacologically active substance	Marker residue	MRL µg/kg	Other provisions
quinolones	marbofloxacin	marbofloxacin	75	
	danofloxacin	danofloxacin	30	
	difloxacin	difloxacin	—[a]	not for use in animals from which milk is produced for human consumption
	enrofloxacin	sum of enrofloxacin and ciprofloxacin	100	
	flumequine	flumequine	50	
	oxolinic acid	oxolinic acid	—[a]	not for use in animals from which milk is produced for human consumption
macrolides	spiramycin	sum of spiramycin and neospiramycin	200	
	tylosin	tylosin A	50	
	erythromycin	erythromycin A	40	
	tilmicosin	tilmicosin	50	
	tulathromycin	tulathromycin equivalents [b]	—[a]	not for use in animals from which milk is produced for human consumption
	gamithromycin	gamithromycin	—[a]	not for use in animals from which milk is produced for human consumption
	tildipirosine	tildipirosine	—[a]	not for use in animals from which milk is produced for human consumption

[a] Use not allowed, zero tolerance.

[b] (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10,13-tetra-hydroxy-3,5,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-xylo-hexopyranosyl]oxy]-1-oxa-6-azacyclopent-decan-15-one expressed as tulathromycin equivalents.

Group	Pharmacologically active substance	Marker residue	MRL µg/kg	Other provisions
florfenicol and related compounds	florfenicol	sum of florfenicol and its metabolites measured as florfenicol-amine	—[a]	not for use in animals from which milk is produced for human consumption
	thiamphenicol	thiamfenicol	50	
tetracyclines	tetracycline	sum of parent drug and its 4-epimer	100	
	oxytetracycline	sum of parent drug and its 4-epimer	100	
	chlortetracycline	sum of parent drug and its 4-epimer	100	
	doxycycline	doxycycline	—[a]	not for use in animals from which milk is produced for human consumption
naftalene-ringed ansamycin	rifaximin	rifaximin	60	
lincosamides	lincomycin	lincomycin	150	
	pirlimycin	pirlimycin	100	
aminoglycosides	spectinomycin	spectinomycin	200	
	streptomycin	streptomycin	200	
	dihydrostreptomycin	dihydrostreptomycin	200	
	gentamicin	sum of gentamicin C1, C1a, C2 and C2a	100	
	neomycin (+framycetin)	neomycin B	1 500	
	kanamycin	kanamycin A	150	
	apramycin	apramycin	—[a]	not for use in animals from which milk is produced for human consumption
	paromomycin	paromomycin	—[a]	not for use in animals from which milk is produced for human consumption
other antibiotics	novobiocin	novobiocin	50	for intra-mammary use only
polypeptides	bacitracin	sum of bacitracin A, B and C	100	
beta-lactamase inhibitors	clavulanic acid	clavulanic acid	200	
polymyxins	colistin	colistin	50	
ionofors	monensin	monensin A	2	

[a] Use not allowed, zero tolerance.

[b] (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2-ethyl-3,4,10,13-tetra-hydroxy-3,5,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-xylo-hexopyranosyl]oxy]-1-oxa-6-azacyclopent-decan-15-one expressed as tulathromycin equivalents.

For authorized pharmacologically active substances (Substances included in Table 1 of the Annex to Regulation (EU) No 37/2010 or pharmacologically active substances that are authorized as a feed additive under Regulation (EC) No 1831/2003) for which no MRL has been set in milk produced within the EU, a cascade MRL, established under Commission Implementing Regulation (EU) 2018/470 for the concerned substance, can be applied.

A.3 Prohibited substances (MRL cannot be established)

The status of the legislation can be checked on the EURLEX website (https://eur-lex.europa.eu/collection/eu-law/legislation/recent.html) under Legal acts, Document reference, Year: 2003, Number: 181 and Decision:

Minimum Required Performance Limits (MRPLs) in Commission Decision 2003/181/EC and Reference Points for Action (RPAs) for chloramphenicol and nitrofuran metabolites in Commission Regulation (EU) 2019/1871.

The list of RPAs in cow milk is given below in Table A.2. The table also contains the Recommended concentrations (Anon., 2007[5]) for the marker residues of some nitro-imidazoles and for dapsone.

Table A.2 — Minimum required performance limits (MRPLs, Commission Decision 2003/181/EC), Recommended concentrations (*Anon.*, 2007c[5]) and Reference points for action (RPA, Commission Regulation (EU) 2019/1871) of veterinary drug agents in cow milk

Group	**Pharmaco-logically active substance**	**Marker residue**	**MRPL** µg/kg	**Recom-mended conc.** µg/kg	**RPA** µg/kg
florfenicol and related com-pounds	chloramphenicol	chloramphenicol	0,3	0,3 (MRPL)	0,15 [b]
nitrofurans	nitrofurans	metabolites AMOZ, AHD, SEM, AOZ, DNSH	1 [a]	1 (MRPL)	0,5 [b]
nitro-imidazoles	ronidazole	hydroxymetabolites		3	
	dimetridazole	hydroxymetabolites		3	
	metronidazole	hydroxymetabolites		3	
sulfones	dapsone	dapsone		5	

[a] MRPL set for poultry meat and aquaculture products (Commission Decision 2003/181/EC);

[b] Is intended to apply from 2022-11-28.