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Milk and milk products — Guidelines for the standardized description of immunoassays or receptor assays for the detection of antimicrobial residues

Laits et produits laitiers — Lignes directrices pour la description normalisée des essais immunologiques et des essais récepteur pour la détection des résidus antimicrobiens

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Foreword

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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.

ISO 18330 | IDF 188 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 AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO and AOAC International in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the National Committees casting a vote.

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All work was carried out by the Joint ISO/IDF/AOAC Action Team *Antimicrobials and other veterinary medical residues*, of the Standing Committee *Analytical methods for additives and contaminants*, under the aegis of its project leader, Mr E. Märtlbauer (DE)

Introduction

Because of the method of detection, the tests described in this International Standard may have limitations so that they cannot be used for quantification. For example, receptor assays have group-specific detection and not chemical-specific detection. Consequently, positive results cannot be subject to quantitation without knowledge of the identity of the specific contaminant. Moreover, assays based on a visual evaluation of colour development may not measure the degree of colour and thus may not provide a quantitative value.

Within an integrated system for antimicrobial residue detection, immunoassays and receptor assays may be used as primary-screening methods (e.g. for screening of compounds which can not be detected at regulatory levels by microbiological inhibition assays). These methods may also be used as post-screening methods for preliminary identification and quantification of compounds in samples with a positive result in a microbiological inhibition assay.

Depending on whether a certain test complies with the specifications given, immunoassays and receptor assays may be used for routine quality control, especially if the absence/presence of a certain compound in concentrations exceeding a certain level [e.g. maximum residue limit (MRL)] has to be determined. Substances which are not approved or for which no MRLs have been fixed, may require specific consideration. For legal purposes in many countries, positive results obtained by immunoassays or receptor assays require confirmation by an accepted physico-chemical method.

Milk and milk products — Guidelines for the standardized description of immunoassays or receptor assays for the detection of antimicrobial residues

1 Scope

This International Standard gives guidelines for the standardized description of immunoassays or receptor assays for the detection of antimicrobial residues in milk and milk products.

It is intended to provide a framework and basis for the evaluation/validation of tests based on the binding of an antimicrobial compound to its specific antibody or to other types of detecting molecules.

In addition to immunoassays [e.g. enzyme-immunoassay (EIA) and radio-immunoassay (RIA)], there are several quantitative, semi-quantitative and qualitative test formats based on the binding of antimicrobial compounds to microbial receptors or to receptor proteins. Enzymatic assays and particle-based assays based on receptor proteins are referred to as receptor assays in this International Standard.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 13969, *Milk and milk products — Guidance for a standardized description of microbial inhibitor tests*

3 Terms and definitions

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

3.1
false positives
percentage of positive results when testing negative samples

3.2
false negatives
percentage of negative results at the claimed detection level(s)

3.3
specificity
extent to which the presence of substances with chemical structures similar to that of the target analyte will result in a positive result (cross-reaction)

3.4
limit of detection for qualitative tests
concentration level at which a defined percentage of samples is detected, e.g. 95 % together with the respective confidence level.

3.5
limit of detection for quantitative tests
concentration level which gives a final result that is statistically different from that of negative milk

4 Information needed from the developer/manufacturer

4.1 Methodology

The developer or manufacturer of the test should provide information regarding methodology by mentioning the following:

a) description of the method (e.g. sample preparation and test performance);

b) principle of the method (e.g. competitive direct enzyme-immunoassay);

c) evaluation of test results (e.g. visual or instrumental reading, criteria for positive or negative result);

d) capacity (e.g. sample throughput);

e) special requirements for sampling, preservation and testing;

f) procedure for the purpose of quality assurance, including the use of positive/negative control samples;

g) field of application concerning

- the intended test use [e.g. screening for milk quality payment or for regulatory purposes (detection of banned substances)],
- the substrate or matrix (e.g. raw tanker bulk milk or heat-treated milk), and
- the limitations with respect to sample composition (e.g. cell count and bacteriological quality).

4.2 Test kit reagents

The following information should be given regarding test kit reagents.

a) In the case of immunoassays:

1) type of antibodies (e.g. monoclonal or polyclonal, sheep, rabbit or egg);

2) type of antigen used in the test (e.g. antigen-enzyme conjugate, solid-phase antigen);

3) type of marker for signal production (e.g. enzyme: peroxidase, alkaline phosphatase; radiochemical: H^3, C^{14}, I^{125}; inert bead: colour latex bead, gold);

4) type of enzyme substrate and type of substance used for transformation of enzyme activity to measured signal (e.g. hydrogen peroxide/tetramethylbenzidine), if applicable.

b) In the case of microbial receptor assays:

1) type of label (e.g. radioactive isotope),

2) amount of radioactivity and safety requirements, and

3) type of receptor (e.g. non-viable microbial cells or antibody).

c) In the case of receptor protein assays:

   1) type of receptor protein used (e.g. enzyme-receptor conjugate),

   2) type of reagent competing for receptor protein binding sites (e.g. analyte-enzyme conjugate, solid-phase analyte),

   3) type of enzyme substrate and type of substance used for transformation of enzyme activity to measured signal, and

   4) enzymatic reaction(s) used to produce signal.

4.3 Additional chemicals not necessarily included in the test kit

The following should be mentioned regarding additional chemicals not necessarily included in the test kit:

a) purity and quality of chemicals required;

b) composition and preparation of solutions;

c) storage conditions and stability of solutions;

d) water quality required.

4.4 Operating requirements

The following information should be given regarding operating requirements:

a) requirements for user experience and training;

b) requirements for laboratory equipment:

   1) microtitre plate reader,

   2) fluorometer, scintillation counter, computer hard- and software,

   3) incubators and precision pipettes;

c) requirements for safety (e.g. for handling and disposal of radioactive waste);

d) requirements for quality control by developer/manufacturer and/or user.

4.5 Test specifications

The following information should be given regarding test definitions:

a) false positives: see 3.1;

b) false negatives: see 3.2;

c) specificity: see 3.3;

d) limit of detection: see 3.4 and 3.5 respectively;

e) precision: figures for repeatability and reproducibility obtained from the results of collaborative studies, if carried out and available.

4.6 Documentation

The following information should be given regarding documentation:

a) user manual, including a trouble-shooting guide;

b) suppliers of instruments, reagents, standards, technical services and customer support;

c) status of official recognition in specified countries (if available);

d) availability of reference material;

e) availability of internationally recognized and/or validated references from ISO, IDF and AOAC International or others;

f) availability of, for example, literature and practical experiences.

5 Evaluation of the attributes of the enzyme-immuno or receptor assay

5.1 Prerequisites (see ISO 13969)

5.1.1 Milk free from antimicrobials ("negative milk")

The cows from which milk is collected in order to serve as "negative milk" shall meet the following requirements. If, however, a test is applied for milk of an animal species other than cows, the requirements with respect to the status of that animal should be adjusted accordingly.

a) The clinical and sub-clinical health status shall be good, with special emphasis on udder health (less than 150 000 somatic cells per millilitre).

b) The treatment or feeding with antimicrobial substances shall be prohibited for at least 8 weeks before milk collection. In the case of dry cow treatment, the milk shall not be collected earlier than 60 days after calving provided the dry cow period was at least 4 weeks.

c) The cows shall be mid-lactation: more than 60 days and less than 200 days after calving, producing more than 5 kg milk per day.

d) The milking of at least five to seven cows shall be combined to overcome individual variations in milk composition.

e) The total viable count shall be less than 10^4 colony-forming units (CFU) per millilitre before the preservation process (deep-freezing, lyophilisation). The possible presence of β-lactamase-producing microorganisms shall be kept in mind in the case of β-lactam antibiotic testing.

5.1.2 Test substances

The test substances which are used in the testing procedure should be obtained from a recognized developer/manufacturer, preferably with an analytical certificate with a guaranteed specification. The concentration required should be calculated based on the free acid or base forms of the drug corrected for purity. Special considerations should be given to substances with stability/potency problems.

Unless otherwise stated, it is preferable that the evaluation of detection limits (5.2.2) should be undertaken using those antimicrobials and/or concentrations that the developer/manufacturer claims the test will detect.

5.1.3 Solvents

If special solvents or other chemicals are required to dissolve the substances, it should have been ensured that these solvents or chemicals in the test samples have no influence on the test result.

5.1.4 Preparation of test samples

5.1.4.1 General

For large-scale evaluations (e.g. to obtain the data basis for a generalized description), all dilutions required should have been prepared in one batch to avoid day-to-day variations in weighing, diluting and differences in the status of the “negative milk”.

The preparation of test samples may cause problems and is a very laborious task for the test evaluation laboratories. Therefore, it might be appropriate to employ a centralized test sample preparation laboratory which agrees to supply interested laboratories with the test samples in stable form (e.g. lyophilized).

5.1.4.2 Selection of concentrations

The selection of concentrations for the determination of the detection limits is described in 5.2.2. For estimated purposes, if not otherwise stated, the concentration found to represent the detection limit should be tested together with one concentration level higher and two or three concentration steps lower than the claimed detection limit and the corresponding negative milk. These data may be used to estimate the 25 %, 50 %, 75 % and 95 % positive sample concentration. As an approximate guideline, it is recommended to divide the concentration range resulting in 50 % to 100 % positive samples into three or four equally distant levels (linear and logarithmic scales respectively) as demonstrated in Figure 1.

5.1.4.3 Dilution

The following precautions should be met when preparing dilution series of test substances.

a) The preparation of the dilution series should be carried out in such a way that only the final dilution step is prepared with milk in order to avoid protein binding.

b) The proportion of the added aqueous standard solution in the final milk dilution step should be the same for all test samples and less than 1 %.

5.1.4.4 Preservation

Preservation of test samples should preferably be done by lyophilization, if this is not deprecated by the developer/manufacturer of the test under study or the test principle. The following procedure for preservation has proved to be feasible.

a) Immediately after preparation of the various milk samples, all dilutions should be dispensed into test tubes with the desired volume and be frozen at –18 °C ± 2 °C in a sloping position.

b) Lyophilization should be carried out as soon as possible, and not later than one week after deep freezing. During the lyophilization process, the temperature should not exceed 25 °C.

c) Test tubes should be hermetically sealed immediately after lyophilization and stored in the dark at ⩽ 6 °C.

d) Test samples should be reconstituted with distilled water. The added volume of water should be 10 % less than the volume of sample that was lyophilized in order to compensate for the dry matter of milk.

e) Reconstituted test samples may be used on the day of reconstitution only. They should be kept in a refrigerator between uses and discarded at the end of the day.

f) Test samples may also be preserved by deep freezing and by certain chemical preservatives (see 5.2.5.3).

5.1.5 Experimental design

5.1.5.1 Number of replicates

The experimental design should, preferably, be a blind coded study and should follow the developer/manufacturer's instructions with regard to the number of replicates and test conditions, as closely as possible. The result of a qualitative assay should be expressed as the positive results, in percent, out of the total number of replicates within each evaluated concentration step. Calculation of percentages generally requires at least 10 to 20 replicates at each selected concentration. However, if a defined statistical confidence is required, the appropriate number of replicates should be calculated. As an example, for confirmation purposes, a minimum of 60 samples should be evaluated to determine a 90 % negative rate with 95 % confidence (e.g. two or less positives out of 60 tested).

Quantitative results should be evaluated by using the usual statistical methods for calculating repeatability. If using commercially available microtitre well test kits, at least duplicates for standards and samples should be used.

5.1.5.2 Evaluation of experimental data

The limit of detection is usually defined as the concentration that gives rise to a response which is significantly different from the response of negative samples. For statistical evaluation, it is imperative to have agreement on the confidence level of the method under study. It is common to apply a minimum confidence level of 95 %. However, to fulfil this, extensive testing and large numbers of replicates are required at each concentration. Depending on the intended use of the method, a more practical approach may be used.

In a qualitative assay, the response of a sample is usually compared to a screening point which may be based on results of negative or positive control standards. Screening points (e.g. a cut-off value or a reference standard/mark) may be provided by the kit manufacturer either as an internal test comparison or external reference calibrator. They are calculated or designed to distinguish a negative response from that of a positive one, including two or three standard deviations. For this purpose, test kit manufacturers sometimes provide a negative or a positive standard containing a relevant, defined concentration of the analyte(s) the test is claimed to detect. This concentration is usually chosen with respect to regulatory requirements (e.g. MRLs). Using reference samples, the response of the sample is compared with the cut-off or reference value to determine whether the sample contains concentrations similar to or exceeding those in the positive control.

For data analysis of a qualitative test, a graphical presentation is recommended, whereby the x-axis represents the concentration of the substance under study and the y-axis the percentage of positive results (dose/response curve). The choice of whether the x-axis is scaled linear or logarithmic depends on the range of concentrations tested. If the range covers more than 100-fold, a logarithmic scale is more appropriate than a linear one.

In a quantitative method, a standard curve is constructed and used to estimate the concentration of analyte in the sample. The x-axis represents the concentration of the substance under study and the y-axis represents a scale based on, for example, absorbance, fluorescence or radioactivity. The choice whether the x-axis is scaled linear or logarithmic depends on the range of concentrations tested. If the range covers more than 100-fold, a logarithmic scale is more appropriate than a linear one and a semi-logarithmic curve is constructed. Using this semi-logarithmic scale, the immunoassay standard curve shows a sigmoid shape that cannot be evaluated by using linear models. Although a variety of mathematical methods exist to describe immunoassay standard curves, the four-parameter-logistic model and the approximation of a cubic spline are most commonly used for enzyme immunoassays. Both methods give comparable results and at least one of them is implemented in most available programs for immunoassay data computing.

In a quantitative method, the precision of an estimate is to a large extent affected by the location of the observed absorbance value on the calibration curve. The measuring range for the standard curve is the approximately linear part of the sigmoid curve (concentration plotted in log scale).

5.2 Experimental parameters

5.2.1 False positives and false negatives

For qualitative methods, depending on the intended use of the method under study, it may be appropriate to evaluate the probability of false positive and false negative results. Testing for the probability of false positives includes n-fold examination of negative milk (see 5.1.1) or other negative test samples appropriate for the intended field of application. The number of replicates depends on the field of application of the test. As an example, a minimum of 60 samples should be evaluated to determine a 90 % negative rate with 95 % confidence.

The probability of false negatives at a specified level can be obtained from the dose/response curve for the different concentrations tested. If confidences are required, the evaluation should follow the procedure as described for false positives for the concentration concerned (e.g. the detection limit or the MRL).

5.2.2 Detection limit

The choice of antimicrobial compounds employed to determine the limit of detection depends on the claims of the developer/manufacturer of the test. The principle of preparation of the test samples is described in 5.1.4.

The selection of concentrations to be tested should be as follows.

a) At least four different concentrations estimated to correspond to 25 % positive, 50 % positive, 75 % positive and 95 % positive should be examined between the negative control and the concentration that is expected to be positive.

b) Additionally, one sample fortified with the antimicrobial at a concentration at least 50 % above the concentration expected to give 100 % positives should be included.

c) The selected concentrations should include the claimed detection limit and if, appropriate, the concentrations that correspond to any existing regulations (e.g. the MRL value).

For qualitative tests, as outlined in 5.1.5.1, at least 10 to 20 replicates per concentration are necessary to calculate the percentage of positive test results. If there are no defined requirements for statistical confidence, a more practical approach to define the detection limit may be applied. One such definition might be that the limit of detection is the concentration resulting in 95 % of the test results being interpreted as positive.

Using this definition, the detection limit may be expressed in two ways (Figure 1):

— the two concentrations tested surrounding the “95 % positive results” values (> µg/kg < µg/kg);

— the concentration corresponding to the intersection of the dose/response curve with the line representing “95 % positive results”.

Parameters of high practical importance are the percentages of positive results obtained either at the claimed detection limit or at the level of regulatory requirements (e.g. the MRLs) (see also 5.2.1). If statistical confidences are required, it is necessary to calculate the appropriate number of replicates needed.

For quantitative assays, the limit of detection is generally defined as the concentration which gives rise to a result significantly different from the result of negative samples; i.e. usually the concentration corresponding to the mean value of negative samples plus and minus three standard deviations, respectively.

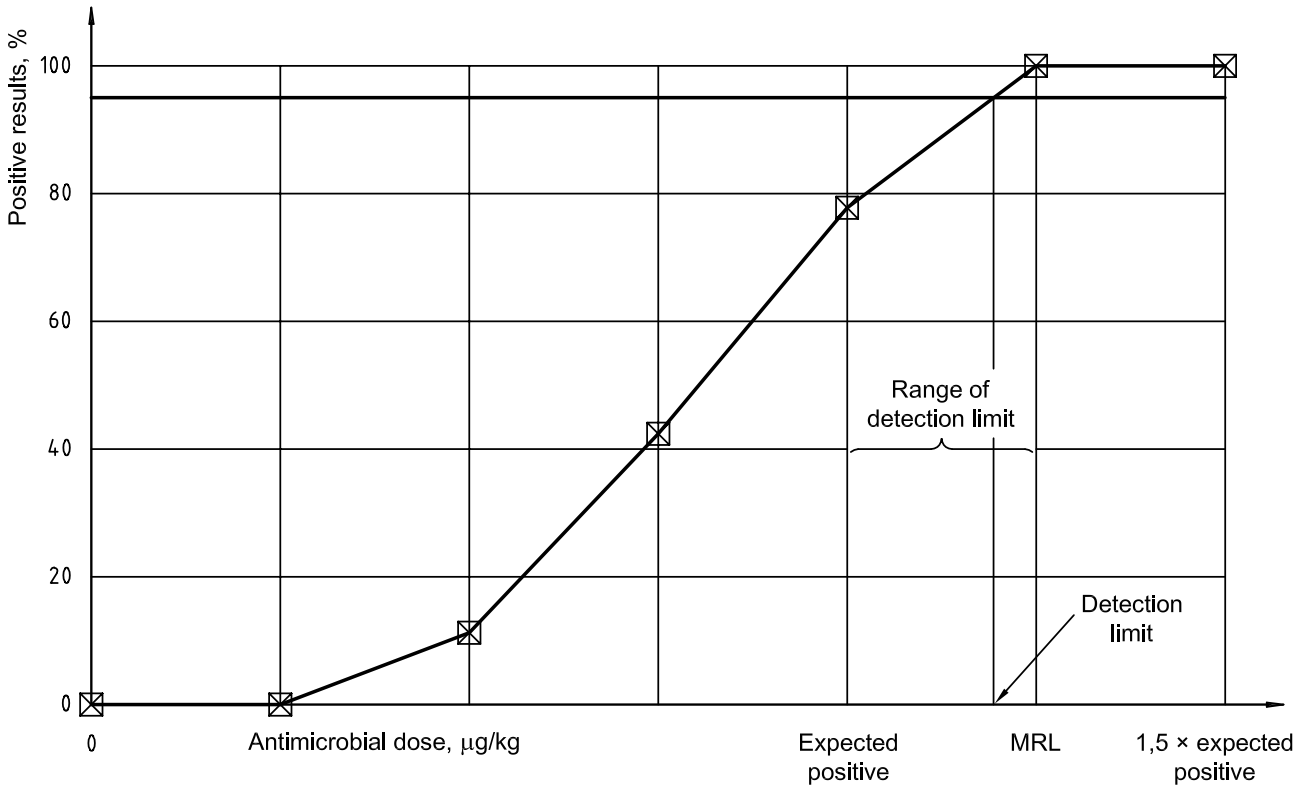

Figure 1 — Model of dose/response curve for the estimation of detection limits

5.2.3 Specificity

Test specificity describes the extent to which the presence of substances with a chemical structure or structural parts similar to that of the original analyte in a sample will also result in binding and thus give a positive result in the test system (cross-reaction).

As an example, metabolites of the original analyte may react with antibody produced against the parent compound. Test specificity is a result of a specific antigen-antibody or analyte-receptor reaction and should not be commingled with non-specific interference (5.2.5). To determine the cross-reactivity, C_r, of a certain substance, the substance should be tested at a concentration approximately 100 times the detection limit of the target substances(s).

For quantitative tests, specificity is calculated after determination of the concentrations of the target substance, T_s, and the cross-reacting substance, C_s, required for 50 % reduction of the test signal compared to that of the negative control standard using the following equation:

$$C_r = \frac{T_s}{C_s} \times 100\ \%$$

where

C_r is the relative cross-reactivity of a certain substance, expressed as percentage;

T_s is the numerical value of the concentration of the target substance;

C_s is the numerical value of the concentration of the cross-reacting substance.

All significant ($\geqslant$ 1 %) cross-reactions of an immunoassay should be indicated. In case of doubt, substances should be tested for cross-reactivity at a concentration corresponding to the highest concentration level of the standard curve. Tests with significant cross-reactions cannot be used for quantification purposes.

5.2.4 Shelf life of test kits

For the examination of the variation of test response throughout the period of claimed shelf life, the choice of test substances, the number of replicates and the evaluation follow the procedure described in 5.1. The examinations are carried out at least three times (at the beginning, at the 50 % level, or every 6 months from the date of developement/manufacture, and at the end of the shelf life). The storage conditions (e.g. temperature, duration, etc.), should be in accordance with the instructions of the test kit developer/manufacturer.

5.2.5 Susceptibility to interference (ruggedness)

5.2.5.1 Test procedure

To investigate potential influencing factors within the test procedure (e.g. variations in reagents, incubation conditions and sample volume), test samples with substance/concentration combinations selected according to 5.1 should be prepared. The experimental protocol, including some defined deviations from the correct test procedure to be examined, depends on the experience of the evaluating panel.

5.2.5.2 Sample composition/properties

The results of immuno- and receptor-assays might be influenced by a number of factors associated with the composition/properties of the sample. These factors are, for instance, the bacteriological quality, the somatic cell content, the fat content and its quality, the pH value, the species and lactation status of the animal (e.g. late lactation).

In this examination, taking into account the limitations described by the developer/manufacturer, the following questions should be considered.

a) Can such factors lead to “false” positive results (e.g. by an elevated somatic cell content)?

b) Can such factors lead to “false” negative results (e.g. by an masking effect due to β-lactamase producing microorganisms)?

c) Can such factors lead to “false” negative/positive results (e.g. by any effect due to the quantity or quality of the milk fat)?

5.2.5.3 Sample preservation

Test samples to be tested for antibiotic residues may be preserved either by addition of chemicals (e.g. boric acid) or by deep freezing.

Unless disclaimed by the developer/manufacturer, the influence of chemical preservatives and the risk for “false” positive/false negative results should be examined at the prescribed concentration and also at lower and higher concentrations. The test samples should be analysed before preservation, and then after preservation and storage. As the addition of preservatives might change the level of the results, the control samples should be preserved correspondingly.

5.2.6 Detection of incurred substance

If available, reference material from recognized institutions (e.g. the BCR[1)]), should be used. From milk samples containing a validated concentration, test samples with concentrations according to 5.1.4.2 should be prepared by proper dilutions with negative milk. A more practical approach is to use milk from treatment trials with several cows, using drugs marketed for farm use. For comparison, quantification of the administered compound in the test samples by a validated quantitative physico-chemical method is necessary.

5.2.7 Recovery

If a sample extraction and clean-up of the extract is included in the test procedure, the efficiency of this procedure should be indicated (recovery).

5.2.8 Collaborative studies

For collaborative studies, the test samples should be prepared in one laboratory according to 5.1.4. The test samples should be shipped with the test kits, under appropriate conditions, to at least 8 participating laboratories in the case of quantitative assays, and to at least 15 laboratories in the case of qualitative tests.

The selection of suitable substance or concentration combinations should follow the procedure in 5.1. The test samples should be coded and each substance or concentration combination should be analysed in each participating laboratory at least 10 to 20 times (visual test reading) or 3 to 5 times (test with measuring scale) for each test kit batch. A strict experimental protocol is compulsory.

6 Rating of the measured parameters

6.1 Applicability for the intended use

The expert's opinion on the applicability for the intended use is the following.

a) The measured parameters of the microbial inhibitor test under examination, which might be derived from different investigations carried out on various occasions, should as far as possible be collected in the form of tables.

b) The information given in that part (tables) forms the basis for a second part of information that will consists of an expert's opinion rating and evaluating the information given, with special regard to the claimed/proposed field of application of the test.

c) Considering the fact that the elaboration of the different attributes takes more or less time especially if storage periods have to be studied (e.g. the determination of the shelf life), the report which comprises the rating of the elaborated attributes should be given in sections within a certain time schedule.

Experts should constantly evaluate new data or information becoming available on the matter.

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1) BCR is the abbreviation for Bureau Communitaire de Référence of the European Commission. The reference materials are available from the Institute for Reference Materials and Measurements (IRMM), Management of Reference Materials (MRM) Unit, Retiesweg, B 2440 Geel, Belgium.

This information is given for the convenience of the user and does not constitute an endorsement by either ISO or IDF of the product.

6.2 Reporting recommendations

For consecutive reporting, the following order of sections is recommended:

a) Section 1:

- recovery (5.2.7);
- limit of detection (5.2.2);
- probability of false positives/false negatives (5.2.1);
- specificity (5.2.3).

b) Section 2:

- susceptibility to interference (ruggedness) (5.2.5);
- detection of incurred substance (5.2.6).

c) Section 3:

- shelf life of test kits (5.2.4);
- collaborative studies and precision (5.2.8).

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