Raad voor Accreditatie (Dutch Accreditation Council RvA)

Explanatory document on microbiology

Document code:	**RvA-T2**

November 2006

A RvA-Explanatory note describes the policy and/or the procedures of the RvA concerning a specific field of accreditation. In case the policy and/or procedures for a specific field of accreditation as described in a RvA Explanatory note, is documented by EA, ILAC or IAF, the RvA will bring its policy en procedures in line with the EA, ILAC or IAF-document.

A current version of the Explanatory notes is available through the website of the RvA. (www.rva.nl).

Contents

1 Introduction _____ 2

2 Validation of microbiological methods _____ 2

3 Measurement uncertainty _____ 3

4 Application of quality controls _____ 3

5 Application of the terms ‘Own method’, ‘Conforming to’ and ‘ Equivalent to’. 5

6 Scope of a testing laboratory _____ 5

7 Test report _____ 6

8 Modification compared to previous version _____ 7

9 Bibliography _____ 7

1 Introduction

This document is prepared by the Dutch RvA expert group on microbiological testing. It provides guidance on items that are of main importance in the assessment of microbiological analyses and whereby an explanation seems necessary.

These items are:
- validation and measurement uncertainty of microbiological methods;
- application of internal and external quality controls;
- application of the terms “conform”, “ equivalent to” and “in-house method”;
- scope and test report

2 Validation of microbiological methods

2.1 Introduction

Analytical methods submitted for accreditation should be validated. For the approach of method validation the following standard documents are available;

- microbiology of water – Guidance on validation of microbiological method (ISO/TR 13843)
- microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods (ISO 16140).

Specific standards for method validation and verification in the field of microbiological analyses of food, animal feeding stuff and environmental samples are under development.

In this chapter some essential aspects of method validation are discussed.

The term “validation” is used for the process demonstrating that a particular method is suitable for the intended purpose, i.e. to detect or quantify a specified microbe or microbial group with adequate precision and accuracy. The relevant performance characteristics such as trueness, detection limit, repeatability, reproducibility and measurement uncertainty must be determined, depending on the status and kind of method.

Microbiological methods are divided in:
- *qualitative methods*. Method of analysis whose response is either the presence or absence of the analyte detected either directly or indirectly in a certain amount in the sample (e.g. Salmonella in 25 g).
- *quantitative methods*. Method of analysis whose response is the amount of the analyte measured either directly (e.g. enumeration in a mass or volume), or indirectly (e.g. color absorbance, impedance, etc.) in a certain amount of sample.

2.2 Application of reference method (conform; see RvA-T1)

Reference method is an internationally recognised method and widely accepted.

Reference methods are prepared by national (Dutch NEN), European (EN) or international (ISO, IDF) bodies. At the moment six standards in the field of food microbiology are validated, The performance characteristics are added to these standard as an Annex.

If the performance of the microbiological test is claimed conform to be a particular reference method the laboratory must be able to provide the Dutch RvA with the following performance characteristics of the method, determined in the own laboratory situation:
- qualitative methods: limit of detection
- quantitative methods: trueness, repeatablity, reproducibility and measurement uncertainty.

In annex 1 guidance is presented for the determination of the following performance characteristics: trueness, repeatability, reproducibility and detection of limit.

2.3 Application of modified reference method (equivalent; see RvA-T1)

In case changes are applied to a reference method as meant in 2.2 this can result in the need of a revalidation of the method. The revalidation should deal with the particular performance characteristics which are affected by the changes in the method.

2.4 Application of an in-house method (own method; see RvA-T1)

The classification “in-house method” is applicable to the use of non-reference methods or the use of a standard method on a matrix that is not specified in the standard method.. For an in-house method a full validation is required, unless the method is already validated by an independent organization.
- Qualitative methods: detection limit, selectivity, specificity and robustness.
- Quantitative methods: trueness, repeatability, reproducibility , selectivity, specificity, robustness and measurement uncertainty.

If the method is already validated by an independent organisation and the performance charateristic of the method are available (see also RvA T-1) the user can follow paragraph 2.2.

2.5 Application of an alternative (rapid) method

The user of an alternative method that is validated according to ISO 16140 by an certification body such as MicroVal, AFNOR Certification, NordVal and AOAC can follow the procedure as described in paragraph 2.2

3 Measurement uncertainty

Guidelines for the estimation of measurement uncertainty for microbiological methods are presented in ISO/TS 19036. This standard is applicable to quantitative analysis. The estimation of the microbiological measurement uncertainty is based on a standard deviation of reproducibility of the final result of the measurement process. This is an approach based on experimental results with duplication of the same analysis in the laboratory under reproducibility conditions.

Within the microbiology there are present no information or protocols available for the determination of the measurement uncertainty of qualitative methods.

4 Application of quality controls

In ISO/IEC 17025 is stated that the laboratory should ensure the quality of the results by using and assessing different quality controls.

In the field of microbiology the use of the correct quality controls is of particular importance, because of the translation of the performance characteristics to the microbiological analysis is not always possible, also depending on the matrix and microorganism.

The terms trueness and precision for example are more difficult t define (and to determine) for microbiological analyses than in the case of analytical-chemical investigations. However, for microbiological methods it is possible to show the technical control of the method on basis of control samples. However, microbiological reference samples are not yet available for all types of micro organisms.

Quality controls can be implemented in different ways: Hereafter some internal and external quality controls are described as first, second and third line controls:

- *first line control* (internal serial control): internal control of serial analyses assessed by the technicians.

- *second line control* (internal process control): internal process control conducted by the technicians, but assessed by staff, e.g. the quality officer.
- *third line control* (external quality control): participation in external organized proficiency testing schemes

Information on the use of the quality controls can be found in the new ISO 7218. In addition additional information is given in paragraph 5.9 on assuring the quality of test and calibration results in ISO/EC 17025.

Besides the internal serial quality control the laboratory should have implemented at least an internal process control or external quality control. Wherever possible the laboratory should participate in proficiency testing or other interlaboratory comparisons.

4.1 First line control

In the first line control the following characteristics can be included:

Blanks. A blank sample, such as a sterile test sample, gives information on the sterility of the media and materials used, and aseptic handling of all manipulations. In general no growth may occur. However, depending on the purpose of the investigation sometimes the growth f a few bacteria is permitted, for which criteria must be specified.

Positive control. A positive control is of importance to demonstrate that analysis have been performed correctly. Using a quantitative method the laboratory (where possible) should employ quantitative control samples and the results should be introduced into control charts. In the case of qualitative methods the contamination level of the control sample should be representative for the limit of detection. All observations of positive control must be recorded and where relevant statistically analyzed.

Negative control. The use of negative controls is especially of importance for the quality control (or entrance control) of culture media with regard to selectivity and incubation temperature. When a negative reference strain for checking the media selectivity is used (after preparation) it is allwed to omit the negative quality control during the analysis itself.

It is important to use a positive and negative reference strain for a good interpretation of thr biochemical identification and confirmation tests.

Multi observations. Depending on the technique to be used standards often specify the use of one (membrane filtration) two (poured plates) or three (surface plates) agar plates. For the allowed deviation between plates specific criteria should be established.

4.2 Second line control

If no third line control is available for an organism the staff of the laboratory must develop another suitable control. This control should be independent of the technician. Where possible and relevant the quality check should be quantitative using statistical analysis. This can be conducted, for example by using microbiological reference material, spiked samples, splitting up samples, reinvestigation of samples and independent observation of incubated media by other personnel.

4.3 Third line control

This external control applies to the participation in proficiency schemes. The aim is to check the performance of the laboratory with respect to other laboratories. It is considered additional to the internal quality controls and can be noted as a level check (see Annex 1, trueness).

In the field of microbiology it is possible to participate in several national and internal interlaboratory proficiency schemes, for example in the field of drinking water, feed, food products, dairy products and environmental samples. By selecting such an organisation It is not only important to look to accreditation of the scheme, but to consider also the number of positive and negative samples, the representatively of the detection limit and the correct matrix.

5 Application of the terms 'Own method', 'Conforming to' and ' Equivalent to'.

Within the framework of an unambiguous use of the terms " conform" (in accordance with), " equivalent to" and "in-house" method (own method) in the scope list of accredited test methods (see RvA-T1) the following examples are formulated for the field of microbiology.
Of course this list is not complete but it serves as a guidance for the preparation of the list of microbiological test methods.
The RvA documents and the literature listed in the bibliography are of relevance with regard to the proven state of validation of non standard test methods.

Reference methods are usually prepared for specific food or water matrices. Laboratories cannot claim conformity or equivalence for matrices which are not specified in the standard. It is the responsibility of the trade itself to develop reference methods or to extend reference methods with other matrices.

"conform " reference is applicable to:
- non essential deviation in the composition and/or preparation of culture media regarding the instruction in the standard.

" Equivalent to" is applicable (after validation) to:
- use of alternative or rapid confirmation techniques (diagnostic kits);
- use of other counting range of colonies than stated in the relevant standard;
- use of a different method of counting colonies or method of calculation and expression of results than stated in the relevant standard;
- use of an different initial (primary dilution) suspension;
- use of an alternative technique, e.g. spiral plate technique;
- plating out into one Petri dish where two Petri dishes are specified in the relevant standard;
- use of alternative (rapid) methods validated according to the standard ISO 16140.

"In-house method" is applicable to:
- everything that cannot be claimed as conform or equivalent to;
- use of one enrichment and/ or isolation medium, while the standard requires two media;
- use of a different incubation cycle, period or temperature;
- use of deviating selection of colonies for confirmation (e.g. less than five);
- use of the standard method in an other matrix;
- use of a different amount of test portion than stated in the relevant standard.

6 Scope of a testing laboratory

Guidance on the specification of the scope is presented in the Explanatory document Rva-T25. The description the analytical method shall include the title of the activity (detection/ enumeration of the organism), followed by the method (presence/absence, colony-count, poured plate, surface plate, MPN, membrane), and technique or measuring principle used (e.g. Real-time PCR, immunology, ATP). For microbiological analyses additional information about the incubation temperature and isolation medium can be of relevance for the interpretation of the test results.

In general these information is also given in the title of microbiological ISO and CEN standards.

Nr.	**Material or product**	**Parameter / Analytical method**	**Internal reference number**
Microbiological analyses			
1	Poultry faeces	Detection of Salmonella; presence/absence method; MSRV	SOP 14 In accordance with PVE-branche method
2	Food products	Enumeration of Enterobacteriaceae at	SOP 10

		30°C; colony-count method, VRBG	Equivalent to NEN-ISO 21528-2
3	Animal feedingstuffs	Detection of Salmonella; presence/absence method; Real-time PCR	SOP 21 Equivalent to NEN-EN-ISO 6579
4	Tap and construction materials	Enumeration of culturable micro-organisms at 22°C; surface plate, R_2A-agar	LMB 010 In-house method
5	Food products	Enumeration of coagulase-positive staphylococci at 37°C; colony-count method, RPF	A-RSV 5 In accordance with NEN-EN-ISO 6888-2

7 Test report

7.1 Confirmation of reference methods

In microbiological standards it is stated that suspected colonies which are isolated from a selective medium should be confirmed by a biochemical and/or serological method. In not all cases the customer is interested in such a confirmation. For example, if the results are below a microbiological criteria or the analysis is carried in the frame of a stability test, confirmation gives the customer no more information.

In that case the test report shall include the information that the confirmation has been omitted and the numerical value shall be reported.

For example:
Microbiological criteria for Enterobacteriaceae : < 100 cfu/g. The test result without confirmation is 75 cfu/g.
Test report: < 100 cfu/g or 75 cfu/g.* *(* without confirmation*)

7.2 Confirmation of alternative methods

There are alternative methods such as certain immune techniques, where the confirmation of positive results with an other test is an integral part of method. For other methods such as PCR, an additional confirmation test is not always required. AFNOR CERTIFICATION supports the opinion that in the case of some pathogenic micro-bacteria an extra confirmation needs to be performed. This confirmation can be a classic confirmation test from the standard or any other confirmation method based on alternative methods. That is why it is important that the performing laboratory reports clearly the used alternative test method.

In accordance with ISO/IEC 17025 the test report shall include;
- the principle of the alternative method (immunological, real-time PCR, etc.).
- the confirmation technique (biochemical, serological, standard, DNA-probe, diagnostic kit, etc.)

The above-mentioned way of reporting gives the possibility to delete such a confirmation test. Depending the aim of the investigation the extra security is not always needed for the customer. If the additional confirmation test is a part of the validation protocol and this test is omitted, these deviation from the test method should be mentioned in the test report.

For example:
Salmonella detected in 25 gram using real-time PCR. Without biochemical or serological confirmation
or
Salmonella detected in 25 gram using real-time PCR. Confirmation according to ISO 6579.

8 Modification compared to previous version

Compared to the previous version of the regulation the following modifications are included:
- The addition of a reference to ISO/IEC 17025:2005;
- The addition of references to ISO/TR 13843 and ISO 16140 (paragraph 2.1);
- The addition of the estimation of measurement uncertainty for quantitative determinations (chapter 3).
- Remarks regarding the choice of a proficiency scheme (paragraph 4.3);
- The addition of examples to part 6 “Scope of a testing laboratory”
- The addition of part 7 “ Test reports”

9 Bibliography

RvA-T1, *Application of the terms “Own methods”, “Conforming to” and “Equivalent to”; Explanatory documents of Dutch Accreditation Council RvA, January 2006.*

RvA-T25, *Scopes for Testing Laboratory, Explanatory documents of Dutch Accreditation Council RvA, November 2006.*

ISO 7218, *Microbiology of food and animal feeding stuffs – General rules for microbiological examinations.*

ISO 8199, *Water quality- General guidance on the enumeration of micro-organisms by culture.*

ISO/TR 13843, *Water quality – Guidance on validation of microbiological methods.*

ISO 16140, *Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods.*

ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories*

ISO 17994, *Water quality – Criteria for establishing equivalence between microbiological methods*

ISO/TS 19036, *Microbiology of food and animal feeding stuffs – Guidelines for the estimation of measurement uncertainty for quantitative determinations.*

EA-04/10*, Accreditation for Microbiological Laboratories*

Annex 1 Guidance on determination of performance characteristics

Trueness

The trueness of a microbiological method can never be exactly determined. The trueness can be more closely estimated by conducting analytical tests within several laboratories and determining the mean (median) of their results as a group.

Dealing with a particular situation of a specific laboratory, the trueness can be determined by:

- Use of microbiological (certified) reference material;
- Use of artificially contaminated samples (recovery)
- Use of own data from an interlaboratory proficiency trial.

Repeatability

The repeatability can be determined by investigating identical samples within one run or duplicate-analyses, under the same conditions

At the assessment of a method, information about repeatability is only of relevance in those cases where samples are normally processed in plural.

In some cases, reference methods contain criteria for the maximum permitted variation. It should be noted that the variation depends on the number of bacteria and the nature of the matrix.

Reproducibility

The intra-laboratory reproducibility of a method for a certain matrix can be determined by a procedure as described in paragraph 5 of ISO/TS 19036.

Limit of detection

The limit of detection is difficult to determine for qualitative methods. The presence or absence of one colony forming unit in a fixed amount test sample can be investigated and proven; for example the presence or absence of *Salmonella* in 25 g, but this is only theory. As result of the presence of other microbial flora and matrix effects, practice is different. Taken these effects into consideration, reference material or artificial contaminated samples with 5 to cfu for each sample should be used for the determination of the limit of detection.