RB

CERTIFICATION AND ACCREDITATION INDUSTRY

STANDARD OF THE PEOPLE'S REPUBLIC OF CHINA

ICS 03.120.00

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RB/T 033-2020

Guidance on verification and validation of microbiological testing methods

微生物检测方法确认与验证指南

Issued on: August 26, 2020 **Implemented on: December 01, 2020**

Issued by: Certification and Accreditation Administration

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Guidance on verification and validation of microbiological testing methods

1 Scope

This Standard provides laboratory guidance for method verification and validation of microbiological testing methods.

This Standard applies to method verification of methods developed by laboratories, standard methods used beyond their intended scope, and expanded and modified standard methods. It is also suitable for method validation before the official use of newly introduced standard methods in laboratories.

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 16140-2, *Microbiology of the food chain-method validation - Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method*

ISO/IEC 17025:2017, *General requirements for the competence of testing and calibration laboratories*

ISO/IEC Guide 99: 2007, *International vocabulary of metrology - Basic and general concepts and associate terms (VIM)*

3 Terms and definitions

For the purposes of this document, the terms and definitions defined in ISO/IEC Guide 99:2007 and ISO/IEC 17025:2017 as well as the followings apply.

3.1 method verification

Through verification or testing, the laboratory provides objective evidence to prove that the requirements specified in the testing method are met. That is to provide objective evidence to prove that the laboratory can correctly apply the method and ensure that the required method performance is achieved.

NOTE 1: Where applicable, measurement uncertainty shall be considered.

NOTE 2: It is adapted from ISO/IEC Guide 99:2007, Definition 2.44.

3.2 method validation

Through testing, the laboratory provides objective evidence that the method to be determined meets the intended use.

NOTE 1: Method validation shall identify the factors that affect method performance and their degree of influence, verify the performance characteristics of the method, and determine the matrix to which the method is applicable and the limitations of its use.

NOTE 2: Validation may include validation of sampling, handling and transportation procedures for test items.

NOTE 3: It is adapted from ISO/IEC Guide 99:2007, Definition 2.45.

3.3 reference method

Methods that are recognized and widely accepted by international, national, industry or organizations.

NOTE: The reference method gives priority to the methods recommended by the International Organization for Standardization (ISO), Chinese National Standards (GB), and the Association of Analysts International (AOAC). Other industry accepted methods may also serve as reference methods.

3.4 qualitative method

An analytical method that detects the presence of target microorganisms using direct or indirect methods.

3.5 quantitative method

An analytical method that measures the number of target microorganisms directly (such as counting a certain mass or volume of samples) or indirectly (such as absorbance value, impedance, etc.) on a certain number of samples.

3.6 confirmatory method

A method that can provide all or part of the information on the target, based on which information can be clearly identified and, if necessary, quantified at the concentration level of concern.

3.7 screening method

A method that has the ability to efficiently process large numbers of samples and is used to detect the presence of a substance or group of substances at a concentration level of interest.

NOTE: These methods are used to screen large numbers of samples for possible positive results and to avoid false negative results. The detection results obtained by such methods are usually qualitative results or semi-quantitative results.

3.8 sensitivity

The ability of a reference method or method to be validated to detect an analyte.

3.9 relative trueness; RT

The same sample is tested using the validation method and the reference method to see how consistent the results are. That is, the sum of the number of validated positives and the number of negative validated by both the method to be validated and the reference method is divided by the total number of samples used for validation.

3.10 level of detection; LOD_x

Using a microbial qualitative detection method, through a given detection step, the content of the measured analyte is obtained with a probability x of a positive detection result.

Example: LOD_{50} means the level of detection at which 50% of tests give positive results.

[ISO 16140-1:2016, Definition 2.35]

3.11 relative level of detection; RLOD

For the same sample, the ratio of the level of detection (LOD_x) of the validated method and the reference method.

3.12 estimated LOD_{50}

LOD_{50} (positive result detection probability of 50%) is determined based on the experimental design described in this Standard.

NOTE: Due to the very small number of test samples, the LOD_{50} cannot be determined accurately. Therefore, the term estimated LOD_{50} is used.

3.13 inclusivity study

Research on qualitative or quantitative detection of purified target bacteria using methods to be validated.

[ISO 16140-1:2016, Definition 2.31]

3.14 exclusivity study

Research on purified non-target bacteria using a method to be validated. These non-target strains may be cross-reactive, but will ultimately not be qualified or quantified

difference between a specified value of a quantitative result and the mean of repeated measurements.

[ISO 16140-1:2016, Definition 2.9]

3.20 estimated bias

Bias determined based on the experimental design described in this Standard.

NOTE: Due to the very small number of samples tested, bias cannot be accurately determined. Therefore, the term estimated bias is used.

3.21 precision

Measurement precision. The degree of consistency between the indicated values or measured quantities obtained by repeated measurements of the same or similar measured objects under specified conditions.

NOTE 1: Measurement precision is usually expressed in terms of standard deviation, variance or coefficient of variation under specified measurement conditions.

NOTE 2: "Specified conditions" may be, for example, repeatability measurement conditions, intermediate precision conditions or reproducibility measurement conditions (see ISO 5725-3).

NOTE 3: Measurement precision is used to define measurement repeatability, intermediate measurement accuracy and measurement reproducibility.

NOTE 4: Sometimes, "measurement precision" is mistakenly used to mean measurement accuracy.

[ISO 16140-1:2016, Definition 2.51]

4 Method validation

4.1 General

The laboratory shall validate its established methods, standard methods used beyond the intended scope, and expanded and modified standard methods. Validation shall be as comprehensive as possible to meet the needs of the intended use or application area. For validated methods, laboratories shall develop work instructions.

The laboratory shall first conduct in-laboratory method validation research tests according to the technical plan. Then, interlaboratory collaborative testing is performed by the collaborating laboratory using the same samples. The characteristic parameters of the method to be validated can be validated by comparing the method to be validated with the reference method.

sufficient number of naturally contaminated samples cannot be obtained, the use of artificially contaminated samples is allowed. The matrix of the test sample shall include samples with different contents of background microorganisms (high content, low content), samples with different processing methods, and original (unprocessed) samples.

Example 1: If the method to be validated is only applicable to a certain type of matrix, it only needs to be validated for that type of matrix. If the method to be validated is applicable to multiple matrices, such as food, select at least 5 food categories. For each type of food, no less than 3 different types of samples (such as unprocessed food, primary processed food, and deeply processed food, etc.) are selected for testing and research. There are 20 copies of each type of sample.

Example 2: For qualitative methods, the ideal situation is to have 10 positive samples and 10 negative samples of each type of sample. At least 50% of positive samples shall be set for each type of matrix. Sample levels that are all negative or positive are meaningless.

4.3.2 Inter-laboratory method validation

4.3.2.1 General principle

Inter-laboratory method validation is when different laboratories use the same sample for testing, compare the characteristic parameters of the method to be validated and the reference method, and compare these results with the limiting standards for acceptable differences between the method to be validated and the reference method. Inter-laboratory method validation is usually completed by 8 collaborating laboratories. Under special circumstances, it may be completed by at least 5 collaborative laboratories.

4.3.2.2 Test sample requirements

Uniform test samples are prepared by the organizing laboratory and a combination of artificial and naturally contaminated samples is used wherever possible. If there are no naturally contaminated samples, artificially contaminated samples can be used. See 4.3.1.2 for substrate selection.

For qualitative methods, each matrix includes at least three test levels of samples, including negative controls and two test levels of positive samples. Samples with at least one level are set to obtain partially positive results. Based on the probability distribution, theoretically, a test level of 1 colony forming unit (CFU)/sample shall yield 30% negative results. Each collaborative laboratory needs to conduct 8 replicate tests for each level, using two methods. That is, each collaborative laboratory provides at least 48 result data (3 levels × 2 methods × 8 repetitions).

For quantitative methods, each matrix includes at least 3 test levels and covers the test range of the method. Two methods are used, with 8 repeated tests for each level. Each

LOD_{alt} - the level of detection of the method to be validated;

LOD_{ref} - the level of detection of the reference method.

The acceptability limit (AL) of a paired study is 1.5, that is, the LOD_x of the method to be validated must not be higher than 1.5 times the LOD_x of the reference method. The AL for unpaired studies is set to 2.5, that is, the LOD_x of the method to be validated must not be higher than 2.5 times the LOD_x of the reference method. It is acceptable for the LOD_x value of the method to be validated to be smaller than the LOD_x value of the reference method. This indicates that the method to be validated can detect lower levels of contamination than the reference method.

When the calculated value of the result is higher than AL, it is validated that the result does not meet the requirements of AL. When AL is not met, root cause analysis shall be performed to explain the observed results. Based on the AL and attachment information, validate whether the method to be validated is suitable or not suitable for the matrix type or category being analyzed.

4.4.1.3 Inclusivity study and exclusivity study

4.4.1.3.1 General

Each strain used for inclusivity study and exclusivity study shall be biochemically and/or serologically and/or genetically characterized in sufficient detail. For quantitative methods, inclusivity study and exclusivity study tests are suitable for counting specific microorganisms (for example, Listeria). They are not suitable for methods that count the total number of all microorganisms, such as total bacterial colonies, mold and yeast counts, etc.

4.4.1.3.2 Inclusivity study

Only inclusivity study testing is required using the method to be validated. No sample matrix needs to be added during the test. For inclusivity study testing, at least 50 pure cultures of the (target) microorganism shall be tested. Pure cultures of test strains shall be cell populations in stationary phase cultured on non-selective medium under optimal growth conditions. The inoculum level shall be 10 to 100 times higher than the minimum level of detection of the validated method. When the inclusivity study result is negative or suspicious, the reference method shall be used for repeated testing at the same time to analyze whether the relevant strains can be detected using the reference method.

4.4.1.3.3 Exclusivity study

Only exclusivity study testing using the method to be validated is required. No sample matrix needs to be added during the test. For exclusivity study testing, pure cultures of at least 30 (non-target) microorganisms shall be tested. The pure culture of the test strain shall be a cell population cultured on a non-selective medium under optimal growth

conditions and in the stationary phase. If there is a selective enrichment step in the method to be validated, for the purpose of exclusivity study testing, a non-selective medium shall be used instead of the selective medium used in this step. When an exclusivity study result is positive or doubtful, the method to be validated shall be repeated. At the same time, the reference method is tested to analyze whether the reference method can detect relevant strains.

4.4.1.4 Relative trueness

A relative trueness study is a comparative study between the results of a reference method and the results of the method to be validated. It is performed using natural and/or artificial contamination of samples. Select at least 3 different types of samples for each matrix type, at least 5 samples of each type. The contamination levels of all samples shall cover the concentration range of microbial species.

The results obtained are analyzed using the Bland-Altman method. Calculate the average and difference between the method to be validated and the reference method. Bias can be estimated by using the mean D of the difference between the two methods. The variation of the mean value D is described by the standard deviation S_d of the difference. If the distribution of differences follows a normal distribution, the vast majority of differences shall lie within the 95% confidence interval of the bias. Therefore, the difference between the two measurements is acceptable if it lies within the limits of agreement.

4.4.1.5 Accuracy

An accuracy study is a comparative study between the results of a reference method and the results of the method to be validated. It is performed using artificial contamination of samples. There is a total of 6 test samples for each type of sample. Two of the samples show low concentration contamination levels, 2 are intermediate concentration pollution levels and 2 are high contamination levels. They cover the entire contamination range for the selected type of sample. Each test sample is tested in duplicate 5 times.

For each test sample, two methods are used to perform quantitative detection under repeated conditions. Convert count results to logarithms. If the logarithmic value (yi) of the result of the method to be validated is assumed to be normally distributed, calculate the β-expectation tolerance interval (β-ETI) for the value of yi.

The acceptability limit for accuracy was set to: AL = ±0.5 log units. Plot the accuracy distribution graph. The upper and lower limits of the tolerance interval are connected by straight lines to interpolate the limiting behavior between different levels of the validation sample. The horizontal line represents the reference value obtained with the reference method. The difference between the reference value and the mean value for each contamination level of the method to be validated is represented by a black dot. When there is no bias, these values lie on the horizontal reference line. In addition, the

$$\text{Specificity of reference method}: SP_{ref} = (1 - \frac{P_0}{N_-}) \times 100\% \quad \cdots\cdots (6)$$

$$\text{Specificity of method to be validated}: SP_{alt} = (1 - \frac{CP_0}{N_-}) \times 100\% \quad \cdots\cdots (7)$$

Where,

N_- -Number of negative results at L_0 level;

P_0 - The number of false positive results obtained from blank control samples before result identification;

CP_0 - The number of false positive results obtained for blank control samples.

For the results at L_1 and L_2 levels, calculate the sensitivity of the method to be validated (SE_{alt}) and the sensitivity of the reference method (SE_{ref}), the relative trueness (RT) and the false positive rate (FPR) of the method to be validated.

In paired studies, partial positive results shall be obtained for at least one of levels L_1 or L_2. Calculate the difference between the negative deviation (ND) and the positive deviation (PD) of the level (ND - PD) and the sum of the negative deviation (ND) and the positive deviation (PD) (ND+PD). The resulting values shall not be higher than the acceptability limits (ALs). The number and acceptability limits (ALs) of different collaborating laboratories can be found in Table A.2.

In unpaired studies, the difference between the negative deviation (ND) and the positive deviation (PD) at the level (L_1 or L_2) where partial positive results are obtained (ND-PD) is calculated. The value of (ND - PD) shall not be higher than AL. The AL value is defined as $[(ND - PD)_{max}]$. Its calculation can refer to ISO 16140-2.

4.4.2.2 Quantitative method

Calculate the accuracy of results. Plot the accuracy distribution graph. The upper and lower limits of the tolerance interval limits are connected by straight lines to interpolate the limiting behavior between different levels of the verification sample. The horizontal line represents the reference value obtained with the reference method. The difference between the reference value and the mean value for each contamination level of the method to be validated is represented by a black dot. When there is no bias, these values lie on the horizontal reference line. In addition, the acceptability limits are represented by two horizontal dashed lines and the β-ETI limits are represented by a broken line.

The acceptability limit is set to ±0.5 log units.

The method is considered equivalent to the reference method when the values of β-ETI lie within the acceptable limits for all contamination levels.

5.3 Qualitative method

Each matrix needs to be tested under at least three different inoculation levels: high, medium, low and no inoculation. Each case is tested twice, and a total of 8 test results are obtained. It includes 2 high levels: the inoculum is no more than ten times the expected LOD_{50}; 2 medium levels: the high-level inoculum is diluted 2 times to obtain the intermediate level inoculum; 2 low levels: the high-level inoculum is diluted 10 times to obtain low level inoculum; 2 non-inoculums are served as blank controls.

Only positive results can be obtained with high levels of vaccination ($10\times LOD_{50}$). If negative results are obtained, the experiment shall be repeated for all levels. Blank controls shall not yield positive results. If a positive result is obtained, the experiment shall be repeated for all levels.

The number of positive results obtained for each vaccination level is recorded. Use Table B.1 in Annex B to determine the estimated LOD_{50}. Compare to LOD_{50} from method validation study. If there is no validation data for the method, the estimated LOD_{50} shall be $\leq$3 CFU/test unit.

5.4 Quantitative method

5.4.1 Estimated bias

Select the type of matrix to be used for method validation. Validation tests for quantitative methods shall use naturally contaminated samples whenever possible. Samples can be from different batches (different products, different manufacturers, etc.) to cover the measurement range of the method. Three different batches are required for each validated matrix type. Each batch represents one contamination level. Three contamination levels shall cover the range of use of this method. Depending on the level of contamination and the target microbial species, if the expected natural contamination level of the sample test unit is below a certain limit, artificial contamination is recommended.

The laboratory uses the reference method (preferred) or the method currently used by the laboratory as a control method to perform tests simultaneously with the method to be verified. If the laboratory does not have a control method that can be performed simultaneously, the laboratory shall use non-selective medium to count the concentration of the culture.

For each contamination level, the difference between the count results of the method to

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