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Milk — Bacterial count — Protocol for the evaluation of alternative methods

*Lait — Dénombrement bactériologique — Protocole pour
l'évaluation de méthodes alternatives*

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Foreword

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). It is being published jointly by ISO and IDF.

This second edition cancels and replaces the first edition (ISO 16297 | IDF 161:2013), which has been technically revised with the following main changes:

- the number of samples and the calculation of the lower limit of quantification has been changed and aligned with ISO 16140-2;
- an example of carry-over effect given in [Figure 1](#) has been omitted;
- the requirements for the evaluation of the accuracy of the estimate and the accuracy profile have been clarified and aligned with ISO 16140-2;
- Annex A (informative) has been omitted.

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.

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This document was prepared by the IDF *Standing Committee on Statistics and Automation* and ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

The work was carried out by the IDF/ISO Action Team (S18) of the *Standing Committee on Statistics and Automation* under the aegis of its project leaders, Mrs V. Tzeneva (NL) and Mrs I. Andersson (SE).

Introduction

Any quantitative measurement in microbiology should consider that there is a requirement for the microbiological state in a sample to be regarded as one point within the coordinates of a multidimensional system, which is to be projected on to the one-dimensional scale of the method applied, i.e. plate count, flow cytometry. Aspects such as flora (types and numbers of microorganisms and their distribution), growth phase, sub-lethal damage, metabolic activity, and history, influence to a greater or lesser extent any parameter that is measured. It is evident that any projection of an n -dimensional situation onto a one-dimensional scale is bound to provide a picture of the real situation that is rather restricted. In this respect, one has to bow to the inevitable, regardless of which method of measurement is preferred.

The term “anchor method” in this document means a method internationally recognized by experts, used in legislation or by agreement between the parties. There are requirements for evaluation of an alternative method to refer to the anchor method and to be based on the examination of suitable samples for its intended use.

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Milk — Bacterial count — Protocol for the evaluation of alternative methods

1 Scope

This document specifies a protocol for the evaluation of instrumental alternative methods for total bacterial count in raw milk from animals of different species.

NOTE The document is complementary to ISO 16140-2 and ISO 8196 | IDF 128 (all parts).

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

ISO 8196 | IDF 128 (all parts), *Milk — Definition and evaluation of the overall accuracy of alternative methods of milk analysis*

ISO 16140-1, *Microbiology of the food chain — Method validation — Part 1: Vocabulary*

ISO 21187 | IDF 196, *Milk — Quantitative determination of bacteriological quality — Guidance for establishing and verifying a conversion relationship between results of an alternative method and anchor method results*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 5725-1, ISO 5725-2, ISO 8196-1 | IDF 128-1, ISO 8196-2 | IDF 128-2, ISO 8196-3 | IDF 128-3 and ISO 16140-1 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 <http://www.electropedia.org/>

4 Transformation of results

A prerequisite for statistics most common in the evaluation of measuring methods is the approximation of a normal distribution of the data. The exponential multiplication of microorganisms usually leads to a right-tailed distribution with quantitative microbiological parameters. Thus, in general, transformation of the raw data is necessary for approximation of normality. This is usually a common logarithmic transformation. The most appropriate transformation can be checked by comparing histograms. All statistics are then computed from the transformed data, unless specified otherwise, and only the final results are re-transformed to give a more expressive idea of the situation to the user (see Reference [2]).

5 Attributes of the alternative method

5.1 General

For each alternative method, only the relevant parameters outlined in this clause shall be evaluated. For example, the measuring range (see 5.3) of the plate loop method is determined by the loop(s) used and the determination of the parameter is not relevant.

5.2 Description of the method to be evaluated

5.2.1 Description

The description of the method under study shall be in line with the checklist in 5.2.2.

Most of the information is found in the specification of the method given by the responsible supplier or any other source (author) of the technique specified.

5.2.2 Checklist

- a) Principle of the method.
- b) Parameter or unit.
- c) Technical design of the measurement procedure.
- d) Field of application:
 - 1) purpose, e.g. research, screening, milk grading;
 - 2) matrix, e.g. raw milk from cows.
- e) Supplier(s) of instrument, reagents and standards.
- f) Specification of the method given by the producer or the author:
 - 1) prerequisites for sampling (often compared to the situation of fat analysis);
 - 2) possibilities for sample preservation [reagent(s), storage condition(s)];
 - 3) quantitative (units: method under study or anchor method) and qualitative (the kind of microorganisms covered) spectrum;
 - 4) precision (in units of the method under study or in anchor method units);
 - 5) accuracy of the estimate (in anchor method units);
 - 6) samples per hour;
 - 7) list of references.

5.3 Measuring range

5.3.1 Lower limit of quantification

The lower limit of quantification is defined on milk samples without or with a very low concentration of bacteria. The standard deviation of n -fold measurements is estimated; generally, $n = 20$. The standard

deviation s_0 is calculated in units of the alternative method without any transformation of the data using [Formula \(1\)](#):

$$s_0 = \sqrt{\frac{1}{n-1} \sum_{j=1}^n (y_j - \bar{y})^2} \quad (1)$$

where

- n is the total number of test portions used;
- y_j is the not transformed result of the test portion j ;
- $\bar{y}$ is the average not transformed result of all test portions.

The lower limit of quantification is calculated as $L_q = 10s_0$ (see also ISO 16140-2).

5.3.2 Upper limit of quantification

The upper limit of quantification is determined by the highest possible reading of the method or by methodological limitations, e.g. coincidence effects, inaccuracy in the upper measuring range, clogging of filters. Coincidence is when two or more elements of the measurand are detected simultaneously and identified as only one unit. For example, with flow cytometry, if two bacterial cells pass the detector simultaneously, they are detected as one. The coincidence effect is higher with higher concentrations of a measurand.

The upper limit of quantification is determined as the highest concentration where the instrument is still linear according to [5.3.3](#).

5.3.3 Linearity of the instrument signal

The relationship between the instrument readings and the expected values shall be linear within the concerned range of bacterial counts. Deviations from linearity may stem from non-specific signals and coincidence effects.

To evaluate linearity, use the raw data expressed in units of the alternative method without logarithmic or any other transformation.

A linearity check is at first performed visually using appropriate graphs to obtain an impression of the shape of the relationship. Whenever deviation from linearity appears evident, a quantitative parameter is calculated to indicate whether the observed trend is acceptable or not.

To achieve this, use a high bacterial count milk diluted serially with low bacterial count milk, resulting in a set of at least 10 samples covering the concentration range of interest.

Measure all samples at least four times and calculate the average result for each sample. This gives the measured value per sample. Use the measured values for the high-count milk and the low count milk to calculate values for the intermediate samples from the applied mixing ratios. This results in an expected value for each sample. Then, apply linear regression with the expected values per sample, C_e , on the x-axis and the measured values per sample, C_{meas} , on the y-axis.

Calculate, for each sample, the residuals $\Delta C_{1i} = C_{meas,i} - (a \times C_{e,i} + b)$ from the regression.

where

ΔC_{1i} is the calculated residual for the i th sample;

$C_{\text{meas},i}$ is the measured value for the i th sample;

$C_{e,i}$ is the expected value for the i th sample;

a is the slope from the linear regression;

b is the intercept from the linear regression.

Plot the residuals ΔC_{1i} on the y -axis versus the expected values, C_e , on the x -axis. A visual inspection of the data points usually yields sufficient information about the linearity of the signal. Any outlying residual should lead to deletion of the related result and to renewal of the calculation.

The curving can be expressed by the ratio, r_L , using [Formula \(2\)](#) and expressing it as a percentage:

$$r_L = \frac{(\Delta C_{\text{max}} - \Delta C_{\text{min}})}{(C_{\text{meas,max}} - C_{\text{meas,min}})} \times 100 \quad (2)$$

where

ΔC_{max} is the value of the maximum residual from the regression;

ΔC_{min} is the value of the minimum residual from the regression;

$C_{\text{meas,max}}$ is the measured value for the high count milk;

$C_{\text{meas,min}}$ is the measured value for the low count milk.

The ratio, r_L , shall be less than 5 %.

5.4 Carry-over

Carry-over effects can occur in analytical systems that operate continuously. It derives from the transfer of a certain portion of sample material from one test sample to the next or further sample(s).

This effect can be tested by analysing consecutively milk with high bacterial count and blank samples. Carry-over causes an increase of blank sample values compared to the target range of blank sample values (value of blank sample analysed after another blank sample).

The carry-over can be expressed as percentage of the corresponding preceding milk sample. To evaluate carry-over, use the raw data expressed in units of the alternative method without logarithmic or any other transformation.

For evaluation of carry-over, the number of samples and the bacterial count of the milk samples should be high enough to estimate the carry-over with sufficient certainty.

The samples should be representative of the routine samples, especially regarding the storage time (longer storage time leading to higher milk viscosity and potentially higher carry-over). One way of setting up the test is described in the example below. For detailed and theoretical aspects and alternative setups of carry-over estimation, refer to ISO 8196-3 | IDF 128-3.

As an example, one way to estimate the carry-over effect is to analyse at least 10 sets of samples, each set containing one milk sample with very high bacterial count followed by two blank samples. Blank samples can be milk with negligible bacterial count and the high sample can be milk with a bacterial count of approximately 2×10^6 cfu/ml (where cfu/ml is colony forming units per millilitre milk sample).

Due to the design of the mechanical process of analysis, sometimes not only the next sample but also samples in a later position can be influenced by samples with high bacterial count. This can happen, for

instance, with instruments with a periodic circulation of the incubation wheel. To estimate the carry-over effect that can occur at each well, two turns of the incubation wheel should be measured. In the second run, it should be ensured that a “blank milk” sample will be incubated in a well where, in the previous run, a milk sample with high bacterial count was incubated.

The sample sets to be measured could have the following sequence:

(milk, blank₁, blank₂)₁, (milk, blank₁, blank₂)₂ ... (milk, blank₁, blank₂)_n

The averaged carry-over, c , can be calculated from the carry-over for each sample set, c_i , using [Formula \(3\)](#) and [\(4\)](#) and expressing it as a percentage:

$$c_i = \frac{C_{b1i} - C_{b2i}}{C_{si}} \times 100 \quad (3)$$

$$c = \frac{\sum_i^n C_i}{n} \quad (4)$$

where

c_i is the carry-over in the i th sample set;

c is the averaged carry-over;

C_{b1i} is the result of the first blank sample in the i th sample set;

C_{b2i} is the result of the second blank sample in the i th sample set;

C_{si} is the result of the milk sample in the i th sample set;

n is the number of sample sets.

Even a very low carry-over effect can be relevant if the corresponding preceding sample has a very high level in comparison to the next one. It can even cause the result of the next sample to exceed a given limit. Carry-over shall be below 1%.

5.5 Stability

It is essential to evaluate the stability of the instrument throughout the working day with suitable samples.

For many microbiological methods, reference materials are not available or their widespread application under field conditions is not possible due to short shelf life and thus restricted transportability.

Compensate for this deficiency by a reference material substitute or a ring test procedure. The relevant characteristics of a reference material substitute should be as similar as possible to the nature of the components and the matrix in which the measurement takes place.

When reference material substitutes with longer shelf life are available, the stability of instrumental methods shall be evaluated throughout the working day and also during the period between instrument standardization operations (quality control in the laboratory). Use a control chart in accordance with ISO 8196-2 | IDF 128-2.

Start-up checks on the performance of the instrument shall be executed at the beginning of each working day. The start-up checks should be performed according to manufacturer's instructions.

Stability throughout the working day should be evaluated as described in ISO 8196-3 | IDF 128-3.

5.6 Precision

5.6.1 General

For guidance on the determination of precision, repeatability and reproducibility, see ISO 5725-1, ISO 5725-2, ISO 8196-1 | IDF 128-1 and ISO 16140-1.

5.6.2 Repeatability

The repeatability can be estimated from a large number ($n = 50 \dots 100$) of duplicate measurements made on samples covering the whole measuring range. If the repeatability is dependent on the level, it shall be specified as a function of the level, otherwise an average value can be used.

For total bacterial count in raw milk, the acceptability limits for the repeatability standard deviation, s_r , are:

- a) units of $0,09 \log_{10}$ units for contamination levels $\geq 2 \times 10^4$ cfu/ml;
- b) units of $0,12 \log_{10}$ units for contamination levels $< 2 \times 10^4$ cfu/ml.

5.6.3 Reproducibility

Estimate the reproducibility through an interlaboratory study in accordance with ISO 5725-2 from duplicate measurements in representative samples at the lower, medium and upper levels in the measuring range, preferably obtained from at least eight collaborators.

If no relationship exists between repeatability and the level, this can also be assumed to be true for the reproducibility. If there is a relationship between the reproducibility and the level, it shall be specified.

For total bacterial count in raw milk, the acceptability limit for the reproducibility standard deviation, s_R , is $0,16 \log_{10}$ units.

5.6.4 Evaluation of factors affecting the results

All non-bacteriological factors associated with the properties of the raw milk sample that could disturb the measurements by the alternative method shall be evaluated. Examples of factors are somatic cell count, composition of milk, history of milk, sampling of milk, preservation of milk, species and breed of animals.

Carefully consider which effects different factors could cause, and design experiments taking these into account.

If, for example, linearity is expected to be affected by a certain factor (e.g. fat content), the linearity test should be repeated using samples with a low and high content of this affecting factor. If repeatability is expected to be affected, the repeatability test should be repeated using samples with high and low content. Certain preservatives can affect the level of the counts. To check for this, analyse a series of samples with and without the addition of a preservative.

6 Alternative method as an estimate of the anchor method

6.1 General

This clause addresses the analysis of the interrelations of the results of the alternative method and the anchor method. For the establishment and verification of a conversion relationship, see ISO 21187 | IDF 196.

The analysis of the relation between two methods is based on the examination of test materials with both methods, covering the field of application and its spectrum of samples to be analysed with the method under study.

6.2 Evaluation of factors affecting the estimation

All factors associated with the properties of the raw milk sample that can affect the relationship between anchor and alternative method results shall be considered in order to make sure that samples chosen to evaluate the relationship are representative for the normal routine samples.

NOTE Factors influencing the relation can be bacteriological or non-bacteriological, e.g. type of bacteria, growth phase, storage condition, sample preservation, geographic differences, seasonal variations, species and breed of the animals from which the raw milk originates, method of milking, disinfection, feeding methods or individual supplier.

6.3 Measurement protocol

The evaluation of the alternative method as an estimate of the anchor method requires a large amount of different samples (typically 100 per $\log_{10}$ step). A minimum number of samples can be calculated in accordance with ISO 21187 | IDF 196:—¹⁾, Annex A.

Samples shall:

- a) be natural raw milk samples;
- b) uniformly cover the whole range of interest;
- c) be representative of the routine samples to be analysed, especially taking into account the above mentioned factors.

Samples shall be analysed in duplicate with both the alternative method and the anchor method at the same time or close to it. When necessary, the time between alternative and anchor method analysis may vary up to 2 h, but preferably less than 30 min (see ISO 21187 | IDF 196), whereby samples are kept at 0 °C to 4 °C.

6.4 Calculations

6.4.1 General

Before further evaluations, the alternative method results shall be converted into units of the anchor method by the conversion function. Logarithmic transformation of anchor method results as well as of alternative method results generally provides the required approximation of normality, see [Clause 4](#).

6.4.2 Visual check of a scatter diagram

Before any calculation is made, a scatter diagram shall be checked visually to obtain a first impression of the relationship and to determine whether the expected relationship between the methods is approximated. Plot the anchor method results and alternative method results (converted into units of the anchor method). See [Figure 1](#).

1) Under preparation. Stage at time of publication: ISO/DIS 21187:2019.

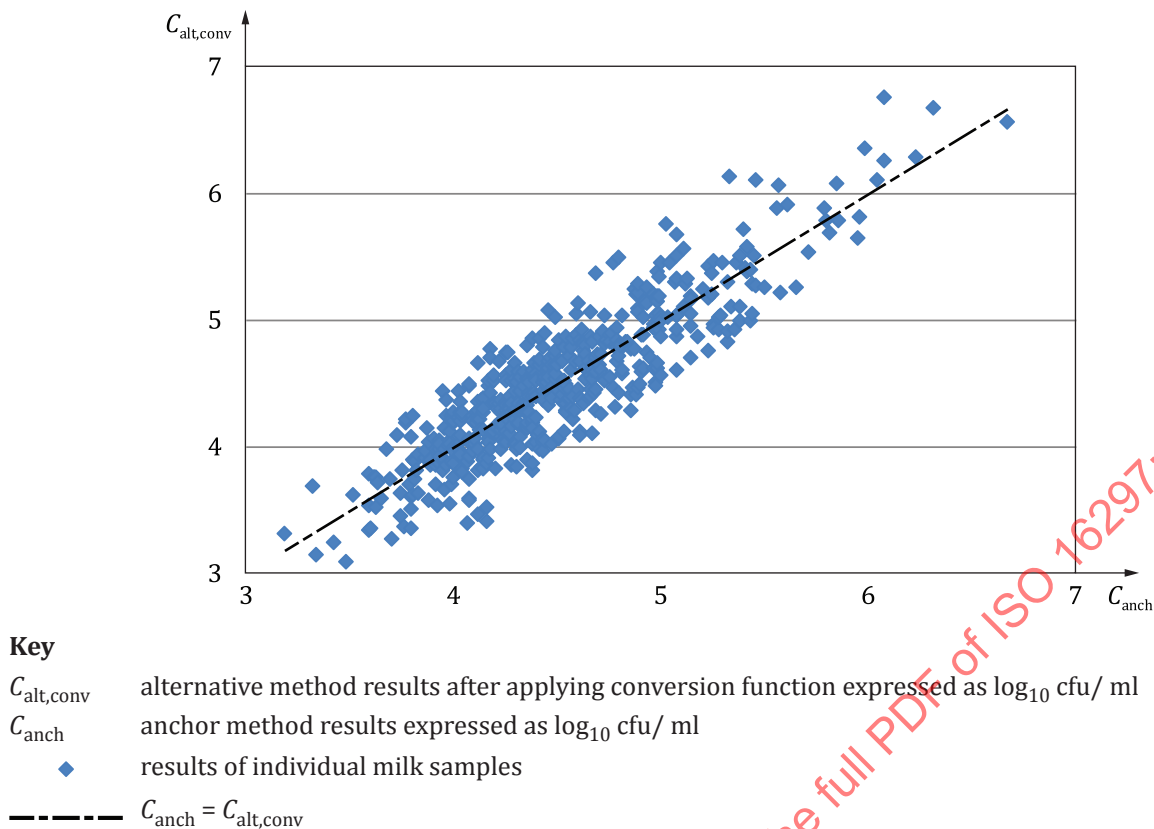


Figure 1 — Example relation between the results of an alternative method and of the corresponding anchor method for total bacterial count in raw milk

6.4.3 Outliers

Outliers shall be carefully scrutinized. No data shall be discarded unless there is a sound microbiological reason to do so. For outlier evaluation, use ISO 21187 | IDF 196.

6.4.4 Accuracy of the estimate and accuracy profile

The accuracy of the estimate is a measure of the reliability of the estimation of the value with one method from the measured value of another method. It can be described by the standard deviation of the regression, s_{yx} , of the individual results obtained with the alternative and the anchor method (see [Figure 1](#)), and by the mean and standard deviation of differences between alternative method results and anchor method results at different levels throughout the measuring range and illustrated by an accuracy profile.

For an accuracy profile, calculate, for each sample, the logarithmic difference between performed methods, using [Formula \(5\)](#):

$$\Delta C_{2i} = C_{alt,i} - C_{anch,i} \quad (5)$$

where

ΔC_{2i} is the difference between the results with the performed methods for the i th sample (logarithmic values);

$C_{alt,i}$ is the result with the alternative method for the i th sample (logarithmic values);

$C_{anch,i}$ is the result with the anchor method for the i th sample (logarithmic values).