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Water quality — The variability of test results and the uncertainty of measurement of microbiological enumeration methods

Qualité de l'eau - Variabilité des résultats d'essais et incertitude de mesure des méthodes d'énumération microbienne

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Foreword

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Introduction

Testing laboratories are required to apply procedures for estimating uncertainty of measurement (see ISO/IEC 17025[5]). Without such an indication, measurement results cannot be compared, either among themselves or with reference values (see ISO/IEC Guide 98-3:2008[7]).

General guidelines for the evaluation and expression of uncertainty in measurement have been elaborated by experts in physical and chemical metrology, and published by ISO and IEC in ISO/IEC Guide 98-3:2008.[7] However, ISO/IEC Guide 98-3:2008[7] does not address measurements in which the observed values are counts.

The emphasis in ISO/IEC Guide 98-3:2008[7] is on the “law of propagation of uncertainty” principle, whereby combined estimates of the uncertainty of the final result are built up from separate components evaluated by whatever means are practical. This principle is referred to as the “component approach” in this International Standard. It is also known as the “bottom-up” or “step-by-step” approach.

It has been suggested that the factors that influence the uncertainty of microbiological enumerations are not well enough understood for the application of the component approach (see ISO/TS 19036:2006[6]). It is possible that this approach underestimates the uncertainty because some significant uncertainty contributions are missed. Reference [19] shows, however, that the concepts of ISO/IEC Guide 98-3:2008[7] are adaptable and applicable to count data as well.

Another principle, a “black-box” approach known as the “top-down” or “global” approach, is based on statistical analysis of series of repeated observations of the final result (see ISO/TS 19036:2006[6]). In the global approach it is not necessary to quantify or even know exactly what the causes of uncertainty in the black box are.

According to the global philosophy, once evaluated for a given method applied in a particular laboratory, the uncertainty estimate may be reliably applied to subsequent results obtained by the method in the same laboratory, provided that this is justified by the relevant quality control data (EURACHEM/CITAC CG 4[10]). Every analytical result produced by a given method thus should have the same predictable uncertainty. This statement is understandable against its background of chemical analysis. In chemical analyses the uncertainty of the analytical procedure and the uncertainty of the final result of analysis are usually the same. The global principle dismisses the possibility that there might be something unique about the uncertainty of a particular analysis.

The uncontrollable “variation without a cause” that always accompanies counts alters the situation for microbiological enumerations. The full uncertainty of a test result can be estimated only after the final result has been secured. This applies to both the global and the component approaches.

The unpredictable variation that accompanies counts increases rapidly when counts get low. The original global design is therefore not suitable for low counts, and therefore also not applicable to most probable number (MPN) methods and other low-count applications, such as confirmed counts.

It is often necessary, and always useful, to distinguish between two precision parameters: the uncertainty of the technical measuring procedure (operational variability), which is more or less predictable, and the unpredictable variation that is due to the distribution of particles. A modification of the global principle that takes into account these two sources of uncertainty is free from the low-count restriction. This is the global model detailed in this International Standard.

In theory, the two quantitative approaches to uncertainty should give the same result. A choice of two approaches is presented in this International Standard. Offering two approaches is appropriate not only because some parties might prefer one approach to the other. Depending on circumstances one approach may be more efficient or more practical than the other.

Neither of the main strategies is, however, able to produce unequivocal estimates of uncertainty. Something always has to be taken for granted without the possibility of checking its validity in a given situation. The estimate of uncertainty is based on prior empirical results (experimental standard uncertainties) and/or reasonable general assumptions.

Water quality — The variability of test results and the uncertainty of measurement of microbiological enumeration methods

1 Scope

This International Standard gives guidelines for the evaluation of uncertainty in quantitative microbiological analyses based on enumeration of microbial particles by culture. It covers all variants of colony count methods and most probable number estimates.

Two approaches, the component (also known as bottom-up or step-by-step) and a modified global (top-down) approach are included.

The aim is to specify how values of intralaboratory operational variability and combined uncertainty for final test results can be obtained.

The procedures are not applicable to methods other than enumeration methods.

NOTE 1 Most annexes are normative. However, only the annexes relevant to each case are to be applied. If the choice is the global approach, then all normative annexes that belong to the component approach can be skipped and vice versa.

NOTE 2 Pre-analytical sampling variance at the source is outside the scope of this International Standard, but needs to be addressed in sampling designs and monitoring programmes.

NOTE 3 The doubt or uncertainty of decisions based on the use of analytical results whose uncertainty has been estimated is outside the scope of this International Standard.

NOTE 4 The extra-analytical variations observed in proficiency tests and intercalibration schemes are also not detailed in this International Standard, but it is necessary to take them into consideration in analytical control. The use of intercalibration data in uncertainty estimation offers the possibility for the bias between laboratories to be included (Nordtest Report TR 537[12]).

2 Key concepts

2.1 Uncertainty of measurement

Uncertainty of measurement according to ISO/IEC Guide 98-3:2008[7] is defined as a “parameter, associated with the result of measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand”. It is a measure of imprecision. The parameter is expressed as a standard uncertainty or relative standard uncertainty.

2.2 Estimation of the uncertainty of measurement

According to ISO/IEC Guide 98-3:2008,[7] the parameter can be evaluated by statistical analysis of series of observations. This is termed type A estimation of uncertainty.

Any other type of procedure is called type B estimation of uncertainty. The most common type B estimates in microbiological analysis are those based on assumed statistical distributions in the component approach.

Types A and B may refer to the uncertainty of individual components of uncertainty as well as to the combined uncertainty of the final result.

Type A evaluations of standard uncertainty are not necessarily more reliable than type B evaluations. In many practical measurement situations where the number of observations is limited, the components obtained from type B evaluations can be better known than the components obtained from type A evaluations (ISO/IEC Guide 98-3:2008[7]).

2.3 Intralaboratory reproducibility

A somewhat abstract expression of uncertainty, **intralaboratory reproducibility**, is frequently considered the most appropriate parameter of the uncertainty of measurement, see ISO/TS 19036:2006.[6] It is also known as intermediate reproducibility or intermediate precision, e.g. [time + equipment + operator]-different intermediate precision standard uncertainty as defined by ISO 5725-3.[2] The idea is to evaluate how much the analytical result might have varied if the analysis had been made by another person in the same laboratory using different equipment and batches of material and different analytical and incubation conditions than those actually employed. The value of intermediate precision estimated never belongs to any actual analytical result, but is assumed to give a general estimate of reasonable uncertainty for the application of a method in one particular laboratory.

Intralaboratory reproducibility is estimated either by combining separate components of uncertainty determined under intralaboratory reproducibility conditions (component approach) or by special experiments in which the analytical conditions are varied by design (global approach).

2.4 Combined standard uncertainty

2.4.1 General

The final test results of microbiological analyses are calculated from intermediate **observed values**. The main intermediate observation is the count. Most of the other observed values are connected with volume measurements.

Combined standard uncertainty, as defined in ISO/IEC Guide 98-3:2008,[7] is the “standard uncertainty of the result of a measurement when that result is obtained from the values of a number of other quantities, equal to the positive square root of a sum of terms, the terms being variances or covariances of these other quantities weighted according to how the measurement result varies with changes in these quantities”.

NOTE 1 Observation of covariances is only necessary if significant correlations occur between components of uncertainty. Otherwise a simple root sum of variances is sufficient (see 2.4.2 and 2.5).

NOTE 2 In cases of microbiological enumeration, it can be assumed that all components of uncertainty are independent, i.e. statistically uncorrelated. In such instances, the combined standard uncertainty is the positive square root of the sum of component variances, i.e. the root sum of squares (Annex B). (ISO/IEC Guide 98-3:2008.[7])

2.4.2 Significant property of combined uncertainties

According to EURACHEM/CITAC CG 4[10], “Unless there is a large number of them, components (standard uncertainties) that are less than one-third of the largest need not be evaluated in detail”. This statement implies that in borderline cases, even a single component might provide an adequate estimate of the combined uncertainty. To decide when a component is unimportant, its approximate size should be known in relation to other components. Generally at least two, usually more, components are significant and should be included.

EXAMPLE The combined uncertainty of two components, one three times the other, is calculated as $u_c(y) = \sqrt{3^2 + 1^2} = \sqrt{10} = 3{,}16$.

Without the smaller component, the estimate would be 3,00. Ignoring the smaller component underestimates the combined uncertainty in this case by about 5 %. For the sake of caution, setting a four-fold difference as the limit might be recommended.

2.5 Relative standard uncertainty

2.5.1 General

The formula for the final results of microbiological analyses involves only multiplication and division. Under such conditions, the combined standard uncertainty should be calculated from components expressed as relative standard uncertainties (ISO/IEC Guide 98-3:2008[7])(see Annex B).

With both type A and type B estimates, the symbol chosen to represent the **relative standard uncertainty** is u_{rel}.

NOTE 1 Relative standard uncertainty is often expressed as a percentage. The term commonly used for this expression is coefficient of variation (CV), C_V.

NOTE 2 When it is important to stress that the standard uncertainty has been calculated by the type A process, the symbol used is s.

NOTE 3 Any systematic or random variation that takes place in the process before the final suspension, such as subsampling, matrix, and dilution effects, influence the target concentration in the final suspension proportionally. Relative variances of these components are therefore additive. Such effects after inoculation as incubation, and reading, can be more complicated statistically and are not well enough known. Proportionality can still be the best simple approximation. Systematic errors in these influences are usually treated as if they were random effects.

2.5.2 Logarithms and relative standard uncertainty

“Global” estimates of experimental standard uncertainty are traditionally made by calculation with common logarithms. When using such estimates in further calculations together with other estimates, it is necessary to express all components of uncertainty on the same scale of measurement, either by converting relative standard uncertainties into logarithms or logarithms into relative standard uncertainties.

In most cases, absolute standard uncertainty calculated in natural logarithmic scale and the relative standard uncertainty in interval scale can be assumed to be numerically equal. Values calculated in common logarithms can be converted to natural logarithms and vice versa by use of appropriate coefficients. The mathematical relationships between relative standard uncertainty and standard uncertainty on different logarithmic scales are shown in B.9.

2.6 Relative variance

The square of the relative standard uncertainty is called the relative variance (ISO/IEC Guide 98-3:2008).[7]

2.7 Expanded uncertainty and expanded relative uncertainty

Especially when the test result is used for assessing limits concerned with public health or safety, it is pertinent to give an uncertainty value that encompasses a large fraction of the expected range of the observed values. The parameter is termed the **expanded uncertainty**, for which the symbol is U.

The value of U is obtained by multiplying the combined uncertainty with a **coverage factor** k:

$$U = ku_{\mathrm{c}}\left(y\right)$$

The value of k is typically in the range 2 to 3. On the relative scale

$$U_{\mathrm{rel}} = ku_{\mathrm{c,rel}}\left(y\right)$$

For normal distributions, about 95 % of the results are covered by the expanded uncertainty interval $\mu \pm U$, where μ is the mean, when the coverage factor k = 2 is chosen. When k = 3, coverage corresponds to about 99 %.

Microbiological test results almost never fit a normal distribution perfectly. Distributions are often markedly asymmetrical (skewed). When there are sufficient reasons for assuming distributions to be other than normal (e.g. Poisson or negative binomial or log–normal distributions) and plausible estimates of the relevant parameters are available, upper and lower 95 % boundaries can be based on these distributions. Annex N gives more details about estimation and use of expanded uncertainty.

3 Microbiological methods

3.1 Common basis

Microbiological enumeration methods based on culture are technical variants of the same basic principle. The analysis often begins with the mixing of a measured portion of the laboratory sample into a suitable liquid medium to produce a homogenate called the **initial suspension**. It may have to be diluted further to produce a **final suspension** of appropriate density for detection and enumeration of the target microorganism. In water analysis, the water sample is the initial suspension and, when dilution is unnecessary, also directly serves as the final suspension.

3.2 Quantitative instruments

Measured portions of the final suspension are transferred into a detection instrument for quantitative evaluation.

The detection instruments in microbiological analyses vary from a single Petri dish to systems of many parallel plates in different dilutions and to most probable number (MPN) systems of diverse complexity.

3.3 Uncertainty structure

A complete microbiological analytical procedure consists of five or six successive steps:

a) subsampling and mixing;

b) dilution;

c) delivery of test portions(s) into the detection system of nutrient media;

d) development during incubation;

e) counting and possibly confirming the (presumptive) target organisms.

The operational variability consists of the effects of these technical steps. They are individually estimated for use in the component approach. When estimating the uncertainty of the final result, the uncertainty due to random distribution of particles in suspension is additionally taken into account (5.2). In the traditional global approach all operational components and the random distribution of particles are estimated together.

3.4 Expression of combined uncertainty

3.4.1 Two-component model

For many practical and illustrative purposes it is sufficient to consider the uncertainty of microbiological test results to consist of two groups of components.

Combined uncertainty of measurement is obtained by combining the operational variability and the intrinsic variability (distribution uncertainty).

In microbiological contexts both variances are to be expressed as relative (or logarithmic) variances. The symbols used in this connection in this International Standard are:

$$u_{\mathrm{c,rel}}(y) = \sqrt{u_{\mathrm{o,rel}}^2 + u_{\mathrm{d,rel}}^2} \tag{1}$$

where

$u_{c,rel}(y)$ is the combined relative standard uncertainty;

$u_{o,rel}$ is the relative operational variability (experimental relative standard uncertainty);

$u_{d,rel}$ is the relative intrinsic variability (relative distribution uncertainty).

Equation (1) is applied in both the modified global and the component approaches to construct the combined relative uncertainty of measurement of the final result.

NOTE Subscripts can be used to indicate the experimental conditions or level of uncertainty (r for repeatability, R' for intermediate or intralaboratory repeatability and R for interlaboratory repeatability).

3.4.2 Operational variability (technical uncertainty)

Operational variability is the combination of all the uncertainties associated with the technical steps of the analytical procedure. It includes the variability of the subsampling, mixing, and dilution of the laboratory sample to prepare the final test suspension. It also includes the possible effects of incubation and the uncertainty of reading the result. Bias components are involved but form parts of random variation.

3.4.3 Intrinsic variability (distributional uncertainty)

Intrinsic variability is the unavoidable variation without a cause that is associated with the distribution of particles in the final suspension and in the detection instrument. In microbiological suspensions it is usually believed to follow the Poisson distribution. When partial confirmation is practised or the MPN principle is used, the intrinsic variation increases considerably and no longer follows the Poisson distribution (Annexes D and E).

NOTE The intrinsic variability can be decreased by using replicate plates and for MPN estimates by increasing the number of parallel tubes.

4 Choices of approach

4.1 General

The tradition of evaluation, presentation and use of measurement uncertainty is short in microbiology. Different parties still have different interpretations and understanding of the meaning and use of measurement uncertainty. Because of this fluid state, there is no unique right way of determining, expressing and using the uncertainty of measurement.

This International Standard is primarily intended to provide guidelines for laboratories on how to get started with establishing the practices of evaluating the uncertainty of measurement. Basic global and component approaches are described. While the recommendations presented do give valid approaches to the evaluation of measurement uncertainty for many purposes, there exist other uncertainty evaluation systems, both wider and narrower in scope than the present protocol. They can provide solutions to specific demands or different quality control situations. Some of them are briefly characterized in the remainder of this subclause.

In addition to the two basic approaches in this International Standard, there exist other approaches to the analysis and expression of the uncertainty of measurement. They have gained favour particularly in those parts of the world where they have been developed. Five examples are given below. Their common feature is that they are mainly or completely based on data generated in connection with internal and external quality assurance activity. They address the technical aspects of method validation and analytical competence of laboratories, and the associated uncertainties, rather than the measurement uncertainty of test results. Also the statistics may differ. For instance, robust statistics instead of standard statistics may be employed.

The methods in the Nordtest Report TR 537[12] and NMKL Procedure No. 8[11] are based on stable reference samples which permit some control of the bias components within and between laboratories. The connection and applicability to microbial populations of real natural samples necessarily remain somewhat obscure.

NMKL Procedure No. 8[11] for the uncertainty in quantitative microbiological examinations is widely accepted among food analysts in the Nordic countries. It uses internal control data as well as results of validation

data from collaborative studies for estimating the measurement uncertainty at the participating laboratories. Nordtest Report TR 537[12] deals for example with intralaboratory bias.

The most comprehensive of the systems is, at the time of publication, under preparation by AFNOR (see Reference [9]). It is reported to address the evaluation of different levels of uncertainty (repeatability, intermediate and interlaboratory reproducibility) from internal and external quality control data and to employ Bayesian statistics in confidence interval (CI) estimation.

BS 8496[8] is designed to detect the presence of overdispersion (termed “uncertainty of measurement”) between duplicate counts of natural drinking water samples. A value for the uncertainty of measurement is not determined.

A system in use in New Zealand (Reference [16]) is based on special experiments with natural water samples. The design is an extension of the basic global design. Data by three technicians analysing several water samples in quintuplicate are used to estimate general measurement uncertainty values. Operational and intrinsic components are not separated. As a consequence, low and “normal” counts (limit set at 20 colonies) require separate assessment.

4.2 Choices of evaluation approach

The uncertainty of measurement established under intralaboratory reproducibility conditions (the intermediate precision) is the focus of this International Standard. Under these conditions, the components of uncertainty can be identified and both the global and component approach basically apply. Experiments based on natural samples are considered important.

Both main approaches to uncertainty of measurement described in this International Standard should, in principle, give the same results. There are few objective reasons for choosing one approach rather than the other. Subjective preferences or requests by a customer or an accreditation authority may be equally valid reasons. Neither of these approaches might be the one to choose, if one of the approaches outlined in 4.1 is more fitting to the quality control system and quality control data possessed by the laboratory.

If a laboratory already has a good quality control system for monitoring details of the analytical procedure, it probably has most of the necessary data available to calculate a component uncertainty estimate. If not, then the global approach would seem to provide the fastest way to get started with estimation of the uncertainty of measurement.

According to recent observations, two components of operational uncertainty are expected to be larger than others. They are the subsampling variance (matrix effect) with solid materials, and the incubation effect with many methods. Subsampling variance often exceeds the particle distribution effect in solid samples. With difficult microbial populations and poor selective methods, the incubation effect can become as important as the particle distribution effect, whereas with good selective methods, simple microbial populations, and easily interpreted colony morphology, the incubation effect is insignificant.

The incubation effect is evaluated by observing the possible overdispersion of parallel counts of final suspensions. Such tests belong to the quality control arsenal of all laboratories irrespective of which evaluation approach they prefer. Evaluation of the incubation-effects component is therefore usually possible without any special arrangements.

With water samples, the subsampling variance is not expected to exceed the Poisson distribution variance significantly. Other liquid samples and finely powdered materials might be in the same category. In laboratories where the quality control is based on details of procedure, the estimate of measurement uncertainty can be constructed from the normally available quality control data. A global approach in such cases would be superfluous.

With solid samples the situation is different. Significant overdispersion between subsamples is the rule. In these cases, either the global approach should be chosen or the subsampling variance component should be evaluated by a dedicated experiment. The dedicated experiment for subsampling variance (Annex H) is a statistically somewhat more complex design than the entire global experiment (Annex F). The question is which is considered a more useful parameter to evaluate, the global operational uncertainty or the subsampling variance. The choice depends on subjective preference.

Evaluation of the operational variance by the global approach is based on subtraction. The smaller the operational component is in comparison with the distribution uncertainty, the greater its relative imprecision. The global approach is less efficient with low counts than the component approach. This is a typical situation

with water samples. With increasing heterogeneity of samples, the efficiency of the global approach improves progressively. As soon as the operational variance is expected to become larger than the distribution variance, the global approach is a reasonable choice. This is a likely situation with solid samples.

When the estimate of uncertainty is to include interlaboratory biases, the evaluation is based on intercalibration data using the same reference samples for all laboratories. In such cases, the analysis can only be based on the global approach. The components of uncertainty cannot even be identified. Such evaluations are not within the scope of this International Standard. Those interested in the approach are advised to consult relevant protocols (e.g. Nordtest Report TR 537[12]).

4.3 Choices of expression and use of measurement uncertainty

Customers, accreditors and the laboratory may have different expectations and uses of the measurement uncertainty information. Observation of these requirements determines whether the uncertainty should be given as operational uncertainty, combined uncertainty, expanded uncertainty or an interval based on expanded uncertainty of measurement, and in which specific form or scale of measurement. Both the use and the expressions relevant to various uses are presented in Annex N.

5 The component approach to the evaluation of operational uncertainty

5.1 General

In the component estimation, individual contributions to the uncertainty of measurement (subsampling, dilution, inoculation, incubation, and reading) evaluated separately are mathematically combined using the law of propagation of uncertainty (ISO/IEC Guide 98-3:2008[7]). Computationally, it means forming the root sum of squares of the component uncertainties. The combined estimate produced can be called the intralaboratory reproducibility when the components are determined under reproducibility conditions within one laboratory.

5.2 Identification of the components of uncertainty

Statistically thinking the uncertainty structure in microbiological enumerations consists of three layers: a) *before*; 2) *within*; and 3) *after* the final suspension. For a more detailed list, see 3.3.

Uncertainty *before* the final suspension consists of the subsampling and matrix variation, as well as dilution. Influence quantities before the final suspension affect the combined uncertainty proportionally to the mean concentration. Whatever additional variation occurs in subsampling or during dilution is transported to the mean of the final suspension proportionally.

Uncertainty *within* the final suspension consists of the random distribution of particles in suspension. Together with the distribution of colonies on the plate, and the possible contribution of the uncertainty of partial confirmation, they constitute the intrinsic variation. Intrinsic variation does not contribute to the operational uncertainty.

Variation *after* the final suspension includes the uncertainties connected with the reading of the results and influences of the incubation environment and time on the apparent observed result. The uncertainties may include both additive (e.g. contamination) and proportional elements (e.g. uncertainty of counting).

Experiments and examples for the quantitative estimation of the components of uncertainty are detailed in the annexes. The variance components for subsampling and incubation effects require special experiments. The other three operational components are available from quality control procedures.

5.3 Evaluation

When components are independent (statistically uncorrelated) and the influence quantities are multiplicative, the combined relative operational uncertainty is calculated as the positive square root of the sum of relative variances.

The combined operational relative variance is obtained as the sum of the relative variances of the components.

$$u_{\mathrm{o,rel}}^2 = u_{\mathrm{rel,M}}^2 + u_{\mathrm{rel},F}^2 + u_{\mathrm{rel},V}^2 + u_{\mathrm{rel,I}}^2 + u_{\mathrm{rel},L}^2 \quad (2)$$

The meaning of the symbols is given in Table 1. Detailed instructions for the practical evaluation of the components are found in the annexes listed in Table 1.

Table 1 — Meaning of symbols

Component	Symbol	Determination
Matrix and subsampling	$u_{\mathrm{rel,M}}$	Annex H
Dilution factor	$u_{\mathrm{rel},F}$	Annex K
Test portion	$u_{\mathrm{rel},V}$, $u_{\mathrm{rel},\Sigma V}$	Annexes I, J
Incubation	$u_{\mathrm{rel,I}}$	Annex M
Counting	$u_{\mathrm{rel},L}$	Annex L

The uncertainty of the first three analytical steps is largely independent of the methods and the operator, but may depend on equipment and material. Once determined, the values can be used repeatedly and for several microbiological methods.

In the fourth step, the uncertainty depends on the incubation conditions (temperature, atmosphere, and time) and the target organism, but is independent of the operator. It can depend on the nutrient medium. It is method specific.

In the fifth step, the uncertainty depends on the operator or equipment used for detection or enumeration.

6 The global approach to the determination of the operational uncertainty

6.1 General

If all of the quantities on which the result of a measurement depends are varied, its uncertainty can be evaluated by statistical means (ISO/IEC Guide 98-3:2008[7]). This is the theoretical basis of the global approach. Planning a global experiment requires not only a good vision of the important influence quantities, but also a plan to vary them in a plausible and realistic way.

The global approach presented in this International Standard is based on experiments identical in design with the model given in ISO/TS 19036:2006.[6] The idea is to duplicate the whole analytical process from preparation of the initial suspension to the final count. Natural samples shall be studied whenever possible. The modification in this International Standard concerns only calculations.

The precision of the average estimate depends on the number of samples examined. According to the original source, 10 samples might provide a sufficient database. In this International Standard at least 30, but preferably more, samples are recommended.

The global estimate of uncertainty should be evaluated separately for every procedure, type of matrix, and every target microorganism. To make the estimate realistically representative of the intralaboratory reproducibility, the factors that are expected to be important should be varied during the experiment. The list presented in 3.3 is helpful.

It is implied in ISO/TS 19036:2006[6] that the generally validated estimate of uncertainty is determined under conditions in which the distribution (intrinsic) uncertainty is negligible. These conditions are not met when the counts are low, or even with high counts when the subsampling variation is low. For this reason, the original global approach is not well suited for water, MPN methods, and low count applications in general. A modification to the original approach is proposed.

The suggested modification in the calculations is to estimate the more stable part, the operational variance, by subtraction (6.2). This should correct the low count restriction. Low counts and partial confirmation should nevertheless be avoided, if possible, in the global experiment, because they increase the imprecision of the

estimate of operational uncertainty. If this cannot be avoided, the effect should be compensated by increasing the number of samples studied.

6.2 Evaluation

The parameter initially determined is the combined standard uncertainty. It can be called the intralaboratory reproducibility standard uncertainty $s_{R'}$ (common logarithmic scale) when the experiments are carried out under intralaboratory reproducibility conditions. Planning and analysing such experiments is detailed in Annex F.

After conversion of $s_{R'}$ to a natural logarithmic scale by $u_{R',\text{rel}} = 2{,}303 s_{R'}$ an estimate of the relative operational variance, $u^2_{\text{o,rel}}$ is obtained by subtracting the intrinsic variance (distribution uncertainty), $u^2_{\text{d,rel}}$, from the intralaboratory reproducibility variance, $u^2_{R',\text{rel}}$ (B.9).

$$u^2_{\text{o,rel}} = u^2_{R',\text{rel}} - u^2_{\text{d,rel}} \tag{3}$$

where

$u_{\text{d,rel}}$ is an estimate of the relative distribution standard uncertainty (intrinsic variability);

$u_{R',\text{rel}}$ is the relative intralaboratory reproducibility standard uncertainty.

With colony counts it is usually assumed that the Poisson distribution is a plausible model for the intrinsic variation of the final count.

NOTE 1 It also has been suggested that reproducibility standard uncertainties generated in interlaboratory method performance trials and proficiency tests could serve a similar function to s_R (see ISO/TS 19036:2006[6]). The parameter evaluated is the interlaboratory reproducibility standard uncertainty, s_R, which is not linked with any given analytical result in one laboratory. Those interested in the use of interlaboratory trial data in uncertainty evaluation are advised to consult, for instance, Nordtest Report TR 537.[12]

In keeping with ISO/TS 19036:2006,[6] the intralaboratory reproducibility is initially calculated using common logarithms. An estimate denoted $s^2_{R'}$ is computed. Conversion to natural logarithms enables the calculations as above: $u^2_{R',\text{rel}} = 5{,}3019 \times s^2_{R'}$. See also Annex B.

Calculations without taking logarithms are also possible (see Annex I).

In principle, the global approach is valid for any type of microbiological enumeration. However, it is not always clear what the best estimate of distribution uncertainty is. It is therefore advisable to avoid situations in which the distribution uncertainty might form the main source of uncertainty in global determinations. Low counts in general, MPN counts, and especially partial confirmation are such situations. Application of the subtraction approach is detailed in Annex F.

NOTE 2 If a considerable number of replicate series of analyses covering the whole practical counting range of colony numbers within one dilution are available a regression approach can be applied (see ISO/TR 13843[4]).

An estimate of the relative operational variance is obtained as the slope of the regression line fitted to a plot of the variance to mean ratio, K, vs. the mean number of colonies, $\bar{n}_\text{C}$

$$K = a + u^2_{\text{o,rel}} \bar{n}_\text{C} \tag{4}$$

where $u^2_{\text{o,rel}}$ is the estimate of the relative operational variance.

The advantage of the regression approach is that it is not upset if the distribution of the final numbers of colonies does not fit the Poisson distribution after incubation. The estimate of $u^2_{\text{o,rel}}$ is possibly more reliable than that obtained by the subtraction approach.

7 Combined uncertainty of the test result

7.1 Basic principle

The variability (combined uncertainty) of the test result, $u_{c,rel}(y)$, is obtained by combining the operational variability estimate with the intrinsic variability that corresponds to the current observed result of enumeration (count, confirmed count or MPN estimate).

$$u_{c,rel}(y) = \sqrt{u_{o,rel}^2 + u_{d,rel}^2} \tag{5}$$

where

$u_{o,rel}$ is the relative operational standard uncertainty under intermediate reproducibility conditions;

$u_{d,rel}$ is the relative intrinsic standard uncertainty of the test result.

The principle is the same for global and component approaches. Also the intrinsic variation is the same in both cases. The difference is that the operational variability is determined differently in the two approaches. Another difference is that common (base 10) logarithms are likely to be the favoured scale of measurement in the global approach while natural logarithms or estimates of relative standard uncertainty in interval scale are the choice in the component approach.

7.2 Operational variability

With global estimates, there is only one operational uncertainty estimate per matrix and target microbe combination. Once determined for a given method, the value is believed to be valid for the sample type in question until the next major change in equipment, operators or procedure. The experiments for the determination of global estimates of operational uncertainty are detailed in Annex F.

Details of the procedures for the determination of individual components in the component approach are described in Annexes H to M and the combined estimate of operational uncertainty in Annex G.

7.3 Intrinsic variability

Detailed descriptions for determination of the intrinsic component can be found in Annex C for “normal” colony counts, Annex E for confirmed colony counts, and Annex D for MPN systems.

7.4 Combined uncertainty

The components, the combined and expanded uncertainty, and the uses of measurement uncertainty, are presented in Annex N.

7.5 Borderline cases

When one component dominates the combined uncertainty, it is possible to omit the smaller component (2.4.2). This is particularly advantageous for situations in which the operational variability is the insignificant component. Then the intrinsic variability can be considered representative of the combined uncertainty. No experimental work is required. The estimate is obtained from the test result itself assuming Poisson or possibly other *a priori* distributions (e.g. MPN, partial confirmation).

Analyses of indicator organisms directly from water samples by either colony methods or MPN systems traditionally have been considered such borderline cases, see ISO 8199.[3] Some liquid and powdery materials undoubtedly belong to the same category, especially when the counts of colonies are low.

Annex A
(informative)

Symbols and definitions

μ	mean (arithmetic mean)
F	dilution factor of a dilution series
f	dilution factor of one dilution step
K	variance to mean ratio (Lexis ratio), s^2/μ
k	coverage factor
L	counted number
n	number of repeated observations, number of objects
n_+	number of positive tubes
n_c	number of colonies
n_q	total number of sectors
n_s	selected number of sectors
n_z	a count, the number of discrete entities (cells, colonies, positive tubes, etc.)
s	experimental standard uncertainty based on a series of observations
s_R	experimental reproducibility standard uncertainty
$s_{R'}$	experimental intralaboratory reproducibility standard uncertainty
T_0	lower limit of interval estimator
T_1	upper limit of interval estimator
U	expanded uncertainty, $U = ku_c(y)$
U_{rel}	expanded relative standard uncertainty, $U_{rel} = ku_{c,rel}(y)$
u	standard uncertainty
u_c	combined standard uncertainty
$u_{c,rel}(y)$	combined relative standard uncertainty
$u_{d,rel}$	relative distribution uncertainty
$u_{o,rel}$	relative standard uncertainty of operational components, overdispersion
u_{rel}	relative standard uncertainty, $u_{rel} = s / \bar{x} = u / \mu$
u_{rel}^2	relative variance, the square of relative standard uncertainty

$u_{R'\text{,rel}}$	intralaboratory relative reproducibility standard uncertainty
V	volume
x	an input quantity, a measured value, confirmed colony count
y	an output quantity, the final test result

Annex B
(normative)

General principles for combining components of uncertainty

B.1 General

The complete *law of propagation of uncertainty* as expressed in the ISO/IEC Guide 98-3:2008[7] is complicated because of the covariances involved. Whenever the components of uncertainty are independent (orthogonal, statistically uncorrelated) the covariances vanish and calculations become simpler. Most of the components of uncertainty in microbiological methods can be assumed independent or so weakly correlated that the simplification is acceptable. The combined uncertainty of independent components is calculated from the component uncertainties as the positive square root of the sum of the variances. A quantity of that nature is generally called the root sum of squares.

The uncertainties of sums and differences are added together in the interval scale and those of products and quotients in the relative (or natural logarithmic) scale of measurement.

Estimation of the uncertainty sometimes involves both sums and products. In order to minimize the possible confusion caused by moving between two scales of measurement, different symbols, u and u_{rel}, are used for the uncertainty in the two scales. The relation between the absolute, u, and relative, u_{rel}, uncertainty is $u_X = u_{\text{rel},X}X$, where X is the measured value (or mean) of the quantity. Additionally, the symbol s is used to denote an experimental standard uncertainty based on a series of results.

To know which scale(s) of measurement to apply, the mathematical relation of the test result with the input quantities should be expressed in the form of a mathematical equation.

B.2 Basic rules for combining two independent components of uncertainty

Assume the values of two independent quantities A and B and their standard uncertainties u_A and u_B or relative standard uncertainties $u_{\text{rel},A}$ and $u_{\text{rel},B}$ are known.

The combined uncertainties of the quantities derived by the basic algebraic operations, $A + B$, $A - B$, AB, and A/B are detailed below.

B.3 Standard uncertainty of a sum, $A + B$

$$u_{A+B} = \sqrt{u_A^2 + u_B^2} \tag{B.1}$$

The relative standard uncertainty of a sum is

$$u_{\text{rel},A+B} = \frac{u_{A+B}}{A+B} \tag{B.2}$$

B.4 Standard uncertainty of a difference, $A - B$

The standard uncertainty of a difference is the same as that of a sum

$$u_{A-B} = \sqrt{u_A^2 + u_B^2} \tag{B.3}$$

but the relative standard uncertainty is different:

$$u_{\mathrm{rel},A-B} = \frac{u_{A-B}}{A - B} \tag{B.4}$$

B.5 Standard uncertainty of a product, AB

$$u_{AB} = AB\sqrt{\frac{u_A^2}{A^2} + \frac{u_B^2}{B^2}} = AB\sqrt{u_{\mathrm{rel},A}^2 + u_{\mathrm{rel},B}^2} \tag{B.5}$$

The relative standard uncertainty

$$u_{\mathrm{rel},AB} = \frac{u_{AB}}{AB} = \sqrt{u_{\mathrm{rel},A}^2 + u_{\mathrm{rel},B}^2} \tag{B.6}$$

B.6 Standard uncertainty of a quotient, A/B

$$u_{A/B} = \frac{A}{B}\sqrt{\frac{u_A^2}{A^2} + \frac{u_B^2}{B^2}} = \frac{A}{B}\sqrt{u_{\mathrm{rel},A}^2 + u_{\mathrm{rel},B}^2} \tag{B.7}$$

The relative standard uncertainty of a quotient is the same as that of a product

$$u_{\mathrm{rel},A/B} = \frac{u_{A/B}}{A / B} = \sqrt{u_{\mathrm{rel},A}^2 + u_{\mathrm{rel},B}^2} \tag{B.8}$$

B.7 Extension to more than two components

The uncertainty of a sum or difference of more than two components follows from B.3 and B.4. For example, for the sum $y = A + B - C$ the combined uncertainty is

$$u_{\mathrm{c}}(y) = \sqrt{u_A^2 + u_B^2 + u_C^2} \tag{B.9}$$

For products and quotients, the corresponding rule follows from B.5 and B.6. The individual components are expressed as relative uncertainties. For example, the combined relative uncertainty of the product $y = AB/C$ is

$$u_{\mathrm{c,rel}} = \sqrt{u_{\mathrm{rel},A}^2 + u_{\mathrm{rel},B}^2 + u_{\mathrm{rel},C}^2} \tag{B.10}$$

When the equation involves both sums and products, take care to use the right scale of measurement in each elementary operation.

B.8 Equations for dependent variables

Whenever the two variables are correlated, the value of the combined uncertainty is different from independent variables. A positive correlation increases and a negative correlation decreases the combined uncertainty according to the general Equation (B.11):

$$u_{A+B} = \sqrt{u_A^2 + u_B^2 + 2r_{cc}u_A u_B} \tag{B.11}$$

where u_A and u_B are the respective uncertainties of A and B and r_{cc} is the correlation coefficient between the uncertainties.

It is not often that information is available about the correlation (or covariance) of two influence quantities in microbiological test results.

B.9 Conversions

It is a common practice in microbiology to convert test results or counts to logarithms before mathematical calculations. A considerable part of the scientific information on the precision of microbiological test results is reported on the common (base 10) logarithmic scale. In water microbiology, logarithms are no longer commonly used. Standard uncertainties are expressed in interval scale or in relative scale, possibly as percentages.

The relative uncertainty of a quantity is approximately equal to the absolute uncertainty of its natural logarithm. Taking the natural logarithm before computing the standard uncertainty is thus one way of converting results to the relative scale of measurement: $u_{rel}(y) \approx u(\ln y)$. About the same result is obtained from $u_{rel}(y) = u(y)/y$. Although estimates calculated in the interval scale are usually not exactly the same as those calculated using natural logarithms, they do not differ markedly.

Conversion from one scale to another is often necessary when computing and combining components of uncertainty. Conversion from common logarithms to natural (base e) logarithms is achieved by multiplying by the modulus between the two systems. The value of the modulus is 2,302 59; for all practical purposes 2,303 or 2,3 are adequate approximations. The coefficient for converting natural to common logarithms is 1/2,302 59 = 0,434 3.

To convert variances from a logarithmic scale to another, the square of the conversion factor is used. Common to natural logarithms: $(2{,}302\ 59)^2 = 5{,}301\ 9$. Natural to common logarithms: $(0{,}434\ 3)^2 = 0{,}188\ 6$.

B.10 Calculation of the relative variance

A frequent mathematical operation in uncertainty estimations in this International Standard is to calculate the relative variance between two values (x_1 and x_2). Values calculated in different scales are presented below.

The standard equation is

$$s^2 = \frac{\sum (x_i - \bar{x})^2}{n-1}$$

When $n = 2$, the variance becomes

$$s^2 = \frac{(x_1 - x_2)^2}{2}$$

and relative variance is

$$u_{\text{rel}}^2 = \frac{s^2}{\bar{x}^2}$$

The mean being $(x_1 + x_2)/2$, the relative variance calculated in interval scale is:

$$u_{\text{rel}}^2 = 2 \times \left(\frac{x_1 - x_2}{x_1 + x_2} \right)^2 \tag{B.12}$$

Taking logarithms means, in effect, transformation to relative scale. Hence, relative variance calculated using natural logarithms:

$$u_{\text{rel}}^2 = \frac{(\ln x_1 - \ln x_2)^2}{2} \tag{B.13}$$

in common logarithms:

$$s^2 = \frac{(\lg x_1 - \lg x_2)^2}{2} \tag{B.14}$$

The symbol s was chosen in keeping with ISO 3534-1:2006,[1] Annex A and ISO/TS 19036:2006.[6]

Interconversion of s^2 and u_{rel}^2 is possible by the use of the relation:

$$u_{\text{rel}}^2 = (2{,}303)^2 \, s^2 = 5{,}302 \, s^2$$

B.11 Example

When there are counts from more than one plate, the microbial concentration of the final suspension is calculated by dividing the sum of colony counts by the sum of test portion volumes.

$$y = \frac{\sum n_{\text{c}}}{\sum V} \tag{B.15}$$

In order to estimate the uncertainty of the result y, the standard uncertainties of the two sums Σn_{c} and ΣV are needed. They are computed as indicated in Equation (B.1).

The estimate of y is the result of division. Its uncertainty is computed from the relative standard uncertainties of Σn_{c} and ΣV as indicated in B.6.

Assume the results $n_{\text{c}1}$ = 45 and $n_{\text{c}2}$ = 35 from parallel plates inoculated with 0,1 ml of the final suspension. Calibration experiments had given the result that the relative standard uncertainty of measuring 0,1 ml was 3 %.

Assuming a Poisson distribution in the final suspension, every colony count is simultaneously an estimate of the mean and variance of the population. Accordingly, the standard uncertainty of the sum is

$$u_{\Sigma n_\mathrm{c}} = \sqrt{n_\mathrm{c1} + n_\mathrm{c2}} = \sqrt{\sum n_\mathrm{c}} = \sqrt{45 + 35} = \sqrt{80} \tag{B.16}$$

The relative uncertainty of the sum is

$$u_{\mathrm{rel},\Sigma n_\mathrm{c}} = \frac{\sqrt{\sum n_\mathrm{c}}}{\sum n_\mathrm{c}} = \frac{1}{\sqrt{\sum n_\mathrm{c}}} = \frac{1}{\sqrt{80}} = 0{,}111\ 8 \approx 11{,}2\ \% \tag{B.17}$$

In order to calculate the standard uncertainty of the sum of test portions, the uncertainty of 0,1 ml should be expressed in millilitres. The value of 3 % of 0,1 ml is 0,003 ml. The uncertainty of 0,2 ml (ΣV) is

$$u_{\Sigma V} = \sqrt{0{,}003^2 + 0{,}003^2} = 0{,}004\ 2\ \mathrm{ml} \tag{B.18}$$

and the relative uncertainty of ΣV is therefore $u_{\mathrm{rel},\Sigma V}$ = 0,004 2 ml/0,2 ml = 0,021 (2,1 %).

Having available the relative standard uncertainty of the sum of counts (0,112) and the relative standard uncertainty of the sum of test portion volumes (0,021), the relative standard uncertainty of their quotient can be calculated according to B.6

$$u_{\mathrm{rel},y} = \sqrt{0{,}112^2 + 0{,}021^2} = 0{,}114 = 11{,}4\ \% \tag{B.19}$$

Annex C
(normative)

Intrinsic variability — Relative distribution uncertainty of colony counts

C.1 General

Final suspensions can be considered well enough mixed such that the Poisson distribution governs the numbers of particles in subsamples. The unavoidable intrinsic variability, which also can be called distribution uncertainty, depends on the mean number of particles or colonies counted in the test portions cultivated. In the following discussion, it is assumed that n_c living particles (colony-forming units) deposited in a plate of growth medium result in n_c observable colonies in the plate.

C.2 Relative uncertainty of a single colony count

With perfectly mixed ideal suspensions, the mean and variance of particle numbers are the same. This applies to distributions when the test portion is a small fraction of the total volume. Due to the equality of the mean and variance of an "infinite Poisson" distribution the relative variance of a single colony count, n_c, is

$$u^2_{d,rel} = \frac{1}{n_c} \tag{C.1}$$

Conversion to the common logarithmic scale:

$$u^2_{d(lg)} = 0{,}188\ 6 \times u^2_{d,rel} = \frac{0{,}188\ 6}{n_c}$$

EXAMPLE Assume n_c = 36 colonies were observed on a single plate.

According to Equation (C.1), the relative distribution variance is

$$u^2_{d,rel} = \frac{1}{36} = 0{,}027\ 8 \tag{C.2}$$

Its common logarithmic equivalent is 0,188 6 × 0,027 8 = 0,005 2.

If the test portion is a large fraction (more than 5 % or 10 %) of the laboratory sample (not the portion of the final dilution), the relative distribution variance should be multiplied by a finite sample correction factor (Reference [15]):

$$u^2_{d,rel} = \frac{1}{n_c}\left(\frac{V - V_{tp}}{V}\right) \tag{C.3}$$

where

n_c is the observed number of colonies;

V_{tp} is the test portion volume in terms of the laboratory sample;

V is the laboratory sample volume.

Large subsampling proportions are common with water samples and membrane filtration methods.

NOTE Sometimes the whole laboratory sample is consumed in the test ($V_{tp} = V$). In such cases, the finite sample correction factor becomes zero and there theoretically is no distribution variance.

C.3 Relative uncertainty of a sum of counts

When several test portions (e.g. parallel plates) are derived from the same final suspension, the quantitative estimate of bacterial density is calculated by dividing the sum of all counts with the sum of test portion volumes.

Because of the additivity of the Poisson distribution the intrinsic variability is inversely proportional to the sum of counts. The relative variance is computed from

$$u^2_{\mathrm{d,rel}} = \frac{1}{\sum n_{\mathrm{c}\,i}} \tag{C.4}$$

where $\Sigma n_{\mathrm{c}i}$ is the total number of colonies observed.

The sum of the volumes of the test portions can constitute a large proportion of the laboratory sample. The finite sample correction should be applied to $u^2_{\mathrm{d,rel}}$ (Reference [14]). In this case the correction factor is

$$u^2_{\mathrm{d,rel}} = \frac{1}{\sum n_{\mathrm{c}i}}\left(\frac{V - \sum V_{\mathrm{tp}}}{V}\right) \tag{C.5}$$

where ΣV_{tp} is the sum of test portion volumes expressed in terms of the laboratory sample.

EXAMPLE The following counts were observed in four plates that form a multiple-plate detection instrument involving two parallel plates in two successive dilutions. The test portions inoculated on each plate were of volume 1 ml. The total volume of the final dilution was 100 ml and the finite sample correction was unnecessary.

Dilution	Counts	Sum
10^{-4}	185, 156	**341**
10^{-5}	17, 22	**39**
	Total n_{c} =	**380**

The relative Poisson distribution variance is $u^2_{\mathrm{d,rel}} = \frac{1}{380} = 0{,}002\,6$.

Its common logarithmic equivalent is 0,188 6 × 0,002 6 = 0,000 49.

Annex D
(normative)

Intrinsic variability of most probable number estimates

D.1 General

Even though the Poisson distribution is assumed to prevail in all suspensions of the MPN instrument, the intrinsic variability of the MPN value is increased because of an additional element of binomial probability.

Common tables do not provide an estimate of the uncertainty of the MPN value directly. They usually give estimates of the lower and upper 95 % confidence limits from which the uncertainty can be calculated. Some computer programs do provide, in addition to the 95 % CI, the standard uncertainty of the common logarithmic value of the MPN. It can be converted to an estimate of the relative standard uncertainty, u_{rel}, by multiplying by 2,303 (Annex B).

For single-dilution MPN instruments, it is possible to calculate the uncertainty without the assistance of a computer by an equation from Reference [17].

D.2 Calculation from confidence limits

The value of the relative distribution uncertainty can be computed from the upper, T_1, and lower, T_0, confidence limits by Equation (D.1):

$$u_{\text{d,rel}} = \frac{\ln T_1 - \ln T_0}{2 \times 1{,}96} = \frac{\ln T_1 - \ln T_0}{3{,}92} \tag{D.1}$$

The standard uncertainty expressed in common logarithmic units is

$$u_{\text{d(lg)}} = \frac{\lg T_1 - \lg T_0}{3{,}92} \tag{D.2}$$

where

$u_{\text{d,rel}}$, $u_{\text{d(lg)}}$ are the square roots of the respective variances sought, $u^2_{\text{d,rel}}$, $u^2_{\text{d(lg)}}$;

T_0 is the lower 95 % confidence limit;

T_1 is the upper 95 % confidence limit.

D.3 Calculation by equation in the single-dilution case

An equation adapted from Reference [17] for the calculation of the standard uncertainty of the logarithm of an MPN estimate is

$$u\left(\ln M\right) = \frac{1 - \exp\left(-MV\right)}{MV\sqrt{n_+ \exp\left(-MV\right)}} \quad \text{(D.3)}$$

where

M is the MPN value per millilitre;

V is the volume of sample per tube, in millilitres;

n_+ is the number of positive tubes.

Calculations become easier if the symbol for the most probable number is replaced with the formula for the calculation of the most probable number

$$u(\ln M) = \frac{n_+}{\ln\left[n / \left(n - n_+\right)\right]\sqrt{nn_+(n - n_+)}} \quad \text{(D.4)}$$

where n is the total number of tubes.

The relative distribution uncertainty $u_{d,rel} = u(\ln M)$

If the uncertainty on the common logarithmic scale is needed, it can be obtained from

$$u_{d(lg)} = \frac{u\left(\ln M\right)}{2{,}303} = 0{,}434\,3\, u\left(\ln M\right)$$

D.4 An example

D.4.1 Introduction

Assume an MPN system with 50 parallel wells. A sample of 100 ml was run and 23 of the wells were found positive after incubation. The manufacturer of the 50-well MPN system provided the following information.

Positive wells	MPN/100ml	95 % CI lower	95 % CI upper
23	31	20	47

D.4.2 Calculation by formula

For the application of Equation (D.4) the necessary input data are: n = 50; n_+ = 23.

$$u(\ln M) = \frac{23}{\ln\left[50 / \left(50 - 23\right)\right]\sqrt{50 \times 23(50 - 23)}} = 0{,}2118 \approx 0{,}21 \quad \text{(D.5)}$$

D.4.3 Calculation from confidence limits

The estimate of relative distribution uncertainty calculated from the confidence limits is

$$u_{d,rel} = \frac{\ln 47 - \ln 20}{3{,}92} = 0{,}218\,0 \approx 0{,}22$$

The output from a computer program (Reference [18]) in the same case is

Positive wells	MPN/100 ml	95 % CI lower	95 % CI upper	Standard deviation of lg(MPN)
23	30,8	20,3	46,7	0,091 99

From these numbers

$$u_{\mathrm{d,rel}} = \frac{\ln 46{,}7 - \ln 20{,}3}{3{,}92} = 0{,}212\,5 \approx 0{,}21$$

Conversion of the standard uncertainty of the common logarithm of the MPN to natural logarithms gives practically the same value: 2,303 × 0,091 99 = 0,211 9 ≈ 0,21.

NOTE The theoretical relative variance of a Poisson distribution with the mean 30,8 is 1/30,8 = 0,032 5. Its square root is 0,18. Comparison with the values 0,21…0,22 obtained in D.4.2 and D.4.3 indicates that the Poisson distribution is not always an adequate model of distribution uncertainty for MPN values.

Annex E
(normative)

Intrinsic variability (standard uncertainty) of confirmed counts

E.1 General

Some selective methods are not as specific as is desirable. When the specificity (confirmation fraction) is suspected to be unsatisfactory (less than about 80 %) the presumptive results should be confirmed by additional tests. In method performance studies and comparative trials, “total confirmation”, i.e. confirmation of every presumptive colony, may be imperative. In daily routine work, laboratories no doubt prefer “partial confirmation” which means taking a random pick of a small number of presumptive colonies for confirmatory tests.

Suppose n_z out of a total of n_C presumptive colonies are tested and n_k of them are found to be positive in the confirmation test. The relative number of success n_k/n_z is used as the multiplier to convert the presumptive count n_C into the confirmed count.

E.2 Total confirmation

In total confirmation, the confirmed count is obtained directly

$$x = \frac{n_k}{n_C} n_C = n_k$$

In this situation, the intrinsic variation (distribution uncertainty) of the confirmed count can be calculated as is usual for colony counts that follow the Poisson distribution for infinite populations.

$$u_{\mathrm{rel},x} = \sqrt{\frac{1}{n_k}} \tag{E.1}$$

If the test portion forms a significant proportion of the laboratory sample, the correction for finite populations can be taken into account as described in Annex C.

E.3 Partial confirmation

E.3.1 General

There are different practices and recommendations for the random selection of the subset n_z out of n_C ($n_z < n_C$) colonies when there are too many presumptive colonies for total confirmation. With MPN methods, partial confirmation should be avoided if possible.

E.3.2 Selection of a constant or proportional number

The most common partial confirmation practice is to pick at random a preselected number n_z of colonies for testing ($n_z < n_C$). The practice of selecting a proportion or the square root of the presumptive count cannot be recommended.

The final result, the estimate of the confirmed count of the target organism per plate, x, is calculated from Equation (E.2):

$$x = \frac{n_k}{n_z} n_c \tag{E.2}$$

where

n_c is the number of presumptive colonies;

n_z is the number picked for confirmation;

n_k is the number confirmed positive.

The number n_c of presumptive colonies is related to the presumptive concentration in the laboratory sample, with uncertainty governed by the Poisson distribution. The confirmation fraction n_k/n_z provides an estimate of the proportion of positives in it, with uncertainty according to the binomial or hypergeometric distribution. The two uncertainties combined provide the uncertainty of the confirmed count of target organisms.

Equation (E.3) shows how the relative Poisson distribution variance of the presumptive count and the relative binomial variance of the confirmation fraction are combined to yield an approximate relative standard uncertainty of the confirmed count.

$$u_{\mathrm{rel},x} = \sqrt{\frac{1}{n_c} + \frac{n_z - n_k}{n_k n_z}} \tag{E.3}$$

NOTE More sophisticated statistical theory of the finite confirmation situation gives an improved equation for the relative variance of the binomial proportion n_k/n_z (References [13][15]). With this formula the estimate of the relative standard uncertainty of the confirmed count becomes in most cases somewhat smaller than with the simple approximation in Equation (E.3).

$$u_{\mathrm{rel},x} = \sqrt{\frac{1}{n_c} + \frac{(n_k + 0{,}5)(n_z - n_k + 0{,}5) n_z^2}{(n_z + 1)^2 (n_z + 2) n_k^2}} \tag{E.4}$$

E.3.3 Random sector approach

In this alternative confirmation work is reduced by dividing the surface of the plate into n_q equal sectors. All colonies of one or more randomly selected sectors are subjected to confirmatory tests. The number of sectors, n_S, is chosen such that the mean number of presumptive colonies in the n_S sectors together is expected to be reasonable. It is not necessary to make an actual count of the total number of presumptive colonies in the plate. This omission, however, increases the uncertainty of the confirmed count to some extent.

The random sector alternative represents “total confirmation” of colonies in a test portion that is the fraction n_S/n_q of the original test portion. The total number picked (n_z) varies and cannot be decided beforehand. It is a random sample from the final suspension in the same way as n_c, but its origin is a test portion that is n_S/n_q times smaller than with n_c.

The result, an estimate of the confirmed count of the target organism per plate, x, is calculated from

$$x = \frac{n_k}{(n_S V / n_q)} = \frac{n_q n_k}{n_S V} \tag{E.5}$$

where

n_q is the number of sectors the surface is divided into;

n_S is the number of sectors selected;

n_k is the sum of the confirmed numbers of colonies in the n_S sectors;

V is the test portion volume in millilitres.

The equation for the final result contains neither the original number of presumptive colonies, n_C, nor the number, n_z, isolated for confirmation. All the information concerning the distribution uncertainty of the estimate, is contained in the number of confirmed colonies, n_k. The relative distribution uncertainty is calculated in the same way as for any "totally confirmed" count

$$u_{\text{rel},x} = \sqrt{\frac{1}{n_k}} \tag{E.6}$$

where n_k is the number of confirmed colonies.

If the technician takes the additional trouble of also noting the total number n_C and the sampled number n_z of presumptive colonies, the random sector approach becomes another case of the general random sample approach (E.3.1). In that case, it is appropriate to calculate the confirmed count using all the information in the normal way, $x = n_k n_C / n_z$. The additional information lowers the estimate of uncertainty. Equations (E.3) or (E.4) apply.

E.3.4 Finite sample correction

Should the test portion form more than about 10 % of the laboratory sample, the estimate of $u^2_{\text{rel},x}$ may be multiplied by the finite sample correction factor (see C.2).

E.4 Example

Five colonies, n_z, were randomly selected from a total of n_C = 50 presumptive colonies observed. Four of the colonies, n_k, were confirmed positive. The estimated confirmed count is x = 50(4/5) = 40.

The relative distribution uncertainty of the confirmed count is

$$u_{\text{rel},x} = \sqrt{\frac{1}{50} + \frac{5-4}{5\times 4}} = \sqrt{\frac{1}{50} + \frac{1}{20}} = \sqrt{0{,}02 + 0{,}05} = 0{,}26 \tag{E.7}$$

Using the advanced formula, the relative distribution uncertainty of the confirmed count is

$$u_{\text{rel},x} = \sqrt{\frac{1}{50} + \frac{(4+0{,}5)(5-4+0{,}5)\times 5^2}{(5+1)^2(5+2)\times 4^2}} = \sqrt{\frac{1}{50} + \frac{168{,}75}{403\,2}} = \sqrt{0{,}02 + 0{,}0419} = 0{,}25 \tag{E.8}$$

A marginally smaller value than above.

Most of the uncertainty of 26 % was due to the uncertainty of confirmation. If all colonies had been confirmed with the result k = 40, the uncertainty of confirmation would be zero and the relative uncertainty of the confirmed count $\sqrt{(1/40)} = 0{,}16$.

Annex F
(normative)

Global approach for determining the operational and combined uncertainties

F.1 General

The leading idea of the original global approach according to ISO/TS 19036:2006[6] is to estimate the combined uncertainty of the final test results by an experiment based on duplication of the whole analytical process. Several samples of the same kind are studied. To obtain a reliable mean, results of at least 30 samples should be generated over time. The first tentative estimate of the operational uncertainty can be calculated after 10 samples have been studied. The test protocol and the calculations are suitable for a gradual build-up of data.

By appropriate selection of different technicians, equipment, and incubation conditions in the basic experiment the estimate can be made to represent the intralaboratory [equipment + time + operator]-different intermediate reproducibility standard uncertainty, see ISO 5725-3.[2] Once determined, the uncertainty value is assumed to be a valid estimate for all analyses of the target microorganism in the given type of matrix by the same method. A reassessment is required only following changes to any of the influence factors.

The original global approach runs into difficulties in microbiological analyses whenever the distribution uncertainty is a non-negligible component of variance. As a consequence, the original global design is unsuitable for low counts in general and especially for MPN methods and partial confirmation.

The difficulty with low counts can be overcome by a modification of the original approach. The more stable operational uncertainty component is “extracted” from the reproducibility standard uncertainty of each sample in the course of the basic experiment. The average (root mean square) operational uncertainty calculated from several specimens of the same type of sample is expected to be stable enough also to represent future analyses. Because the evaluation is based on subtraction, the estimate is imprecise. As a consequence, the values generated when the counts are low can be imprecise to the extreme. Therefore it is advisable to avoid low counts and MPN methods, and not to use partially confirmed counts. This recommendation refers to the *exploratory experiment only*. Once an estimate of operational uncertainty is available, low counts and MPN methods are not a problem. There is no difference in the daily use of the estimates in the global and component approaches. Both can deal with partial confirmation.

When future use of an estimate of the standard uncertainty of a test result is required, the operational uncertainty is reunited with the relevant distribution uncertainty, u_{d}, to form a combined uncertainty for any given test result, y.

$$u_{\mathrm{c}}^2(y) = u_{\mathrm{o}}^2 + u_{\mathrm{d}}^2 \tag{F.1}$$

where

u_{o} is the standard operational uncertainty;

u_{d} is the assumed distribution uncertainty.

It is the task of a global experiment to provide a plausible general estimate for the operational uncertainty.

F.2 Experimental protocol

The design for determining the reproducibility standard uncertainty of the final result in microbiological analyses by the global approach appears in ISO/TS 19036:2006.[6] The model is the same as corresponding models in chemistry. In microbiology it is a tradition to make the calculations in common logarithms.

For practical reasons and simplicity of calculations, the recommended experimental unit consists of only two independent analyses of every sample. A large number of similar samples should be studied. A challenge is to create experimental conditions such that the estimate can legitimately be considered an intralaboratory reproducibility standard uncertainty.

According to ISO/TS 19036:2006,[6] the conditions in the two replicate analyses should be as different as possible and should ideally include as many variations as may be encountered from one day to another within the laboratory, in terms of technicians, batches of culture media and reagents, equipment and time of analysis. “As different as possible” should be interpreted to mean “as few of the technical influence quantities the same” as possible. For each sample, two different operators take one test portion (a subsample from the same laboratory sample), and prepare from it one initial suspension, and analyse it once. It is not necessary that the same two operators study all samples. The two operators should use different equipment and materials for the test.

With at least five operational uncertainty components to consider it is not easy to design an experiment based on two replicates in which all components are varied in a statistically balanced manner.

Variation of influence quantities, such as equipment for homogenization of the initial suspension, position and time in the incubator, and batches of nutrient media and reagents, can only be approximated by arranging a random choice. Even then it is not certain that truly representative variation is achieved. Ideas for randomization of such details can be found in Annex M.

F.3 Calculations

F.3.1 The combined reproducibility standard uncertainty

In this annex, the calculations are made in the common logarithmic scale. For each sample, two analytical results are obtained, one for each operator. An estimate of intralaboratory reproducibility variance can be calculated from the results. Calculation in logarithmic scale ensures that the value of the parameter is not sensitive to contamination level (dilution). Therefore, it can be calculated from the original counts.

The common logarithmic scale is a relative scale of a kind. Nevertheless, in this annex the subscript “rel” is not applied in connection with uncertainty estimates. This is done in order to avoid confusion with other parts of this International Standard, where subscript “rel” indicates standard deviations in natural logarithmic scale. To change the end result in common logarithmic scale to real relative standard uncertainty, conversion to natural logarithms is possible. See examples F.5.1 and F.5.2

From the results of the basic global experiment, the reproducibility standard uncertainty is calculated for every sample.

$$u^2_{R,} = \frac{\left(\lg n_{c_1} - \lg n_{c_2}\right)^2}{2} \tag{F.2}$$

where

n_{c_1} is the number of colonies in the first replicate;

n_{c_2} is the number of colonies in the second replicate.

The parameter determined for each sample is accepted as an estimate of the relative reproducibility standard variance. For use in further calculations, as presented in the examples below, take care to express the relevant uncertainties (operational, distributional and combined) in the same logarithmic scale.

F.3.2 The distribution uncertainty

The intrinsic variability of the determination, in common logarithmic scale, due to particle distribution is estimated for each sample, i, by Equation (F.1)

$$u_{\mathrm{d}_i}^2 = \frac{2 \times 0{,}188\,6}{n_{\mathrm{c}_1 i} + n_{\mathrm{c}_2 i}} = \frac{0{,}1886}{\bar{n}_{\mathrm{c}i}} \tag{F.3}$$

where $\bar{n}_{\mathrm{c}i}$ is the mean count of colonies per plate in sample i.

The arithmetic mean of the sample distribution variances is

$$u_{\mathrm{d}}^2 = \frac{\sum_{i=1}^{n} u_{\mathrm{d}_i}^2}{n} \tag{F.4}$$

where n is the number of samples.

F.3.3 Mean operational uncertainty

Operational uncertainty is estimated by subtracting the mean distributional variance from the mean reproducibility variance, taking care that both are expressed in the common logarithmic scale.

$$u_{\mathrm{o}}^2 = u_{R'}^2 - u_{\mathrm{d}}^2 \tag{F.5}$$

The subtraction can be made individually in each sample, and the mean difference calculated as the last step. Another alternative is to calculate the mean values of $u_{R'}^2$ and u_{d}^2 first and make the subtraction last. Both alternatives are illustrated in the example below.

NOTE When the global approach is applied with an MPN method, the average distribution variance is calculated as the mean of the relative variances of the two MPN estimates. See Annex D.

F.4 Combined standard uncertainty of the test result

Once a global estimate of the operational uncertainty for a given sample type is available, it can be used for construction of a combined uncertainty estimate with any new test result by the same method. Combined relative uncertainty of the final test result is calculated according to

$$u_{\mathrm{c}}(y) = \sqrt{u_{\mathrm{o}}^2 + u_{\mathrm{d}}^2} \tag{F.6}$$

where

u_{o} is the mean relative operational uncertainty for the given sample type and parameter;

u_{d} is the relative distribution uncertainty (intrinsic variation) of the count.

It is necessary to express u_{o} and u_{d} on the same scale of measurement.

The distribution uncertainty is derived without experiments from assumed statistical distributions. In microbiological methods, the value of the parameter depends on the observed count and is different for colony methods and MPN methods (Annex C and Annex D).

NOTE Sometimes microbiological results are confirmed by testing a subset of presumptive colonies. This is termed “partial confirmation”. In such instances, the uncertainty of confirmation becomes a major additional component of intrinsic variation and considerably decreases the precision of the combined uncertainty estimate (Annex E).

F.5 Examples

F.5.1 Example 1: Colony count method — Calculation with common logarithms

Several samples were studied independently by two analysts. For each sample, each operator took one test portion (a subsample from the same laboratory sample), and prepared one initial suspension from it, which was analysed once.

Each analyst used dilution blanks and plates of medium from a batch selected randomly and the plates were placed in randomly selected positions in the incubator. The plates were removed for counting after randomly allocated times of incubation. Each analyst read their own plates. The results (colony counts) of six samples are shown in Table F.1.

Table F.1 — Calculation with common logarithms

Sample No	Dilution[a]	n_{c_1}	n_{c_2}	lg n_{c_1}	lg n_{c_2}	$u^2_{R'}$	u^2_d	u^2_o
1	−4	5	8	0,699 0	0,903 1	0,020 8	0,029 0	−0,008 2
2	−3	15	11	1,176 1	1,041 4	0,009 1	0,014 5	−0,005 4
3	−4	11	19	1,041 4	1,278 8	0,028 2	0,012 6	0,015 6
4	−6	21	39	1,322 2	1,591 1	0,036 1	0,006 3	0,029 8
5	−5	68	45	1,832 5	1,653 2	0,016 1	0,003 3	0,012 8
6	−4	151	203	2,179 0	2,307 5	0,008 3	0,001 1	0,007 2
					Mean	0,019 8	0,011 1	0,008 6

[a] Dilution needs not to be considered when working with logarithms.

NOTE Theoretically, variance should never be negative. However, when an estimate of variance is obtained by subtraction and the experimental variances are based on small numbers of replicates such things can happen.

The relative operational variance in common logarithmic scale is given by $u^2_o = u^2_{R'} - u^2_d$.

The final result, the estimate of the mean operational uncertainty, can be obtained in two ways.

a) From mean values: $u^2_o = u^2_{R'} - u^2_d = 0{,}019\,8 - 0{,}0111 = 0{,}008\,7$ (with this calculation the last column of the table is unnecessary.)

b) From the mean of the individual values (last column): mean $u^2_o = 0{,}008\,6$.

The small difference between the two estimates could be due to the effects of rounding in hand calculations.

Common logarithms are not likely to be utilized in the component approach. Uncertainty estimates are expressed as relative standard uncertainties or percentages. If comparison with uncertainty estimates expressed as relative or ln values is desired, the result of the global analysis, u^2_o, can be converted to the relative scale by multiplying by 5,302. In this example $u^2_{o,rel} = 5{,}302 \times 0{,}008\,6 = 0{,}045\,6$.

Hence, the average relative operational uncertainty from the set of six samples is $u_{o,rel} = \sqrt{0{,}045\,6} = 0{,}208 \approx 21\%$.

F.5.2 Example 2: Most probable number method — Calculation with common logarithms

Two analysts made independent analyses of coliforms in water samples using an MPN method. The trays were incubated in randomly allocated positions on the incubator shelves and in randomly chosen layers in stacks. The maximum height of stacks was 20. The MPN values, x_1 and x_2, and their confidence limits, T_0 and T_1, were obtained from tables provided by the manufacturer. Results of five samples are shown in Table F.2

The estimates of relative reproducibility variance are

$$u^2_{R'} = \frac{\left(\lg x_1 - \lg x_2\right)^2}{2} \tag{F.7}$$

where x_1 and x_2 are MPN estimates.

Table F.2 — Calculation with common logarithms

Sample	x_1	$T_{0,1}$	$T_{1,1}$	x_2	$T_{0,2}$	$T_{1,2}$	$u^2_{R'}$	u^2_{d1}	u^2_{d2}	u^2_d	u^2_o
1	42,9	29,7	62,5	53,1	37,5	76,2	0,004 3	0,006 8	0,006 2	0,006 5	-0,002 2
2	22,2	14,1	35,2	28,8	19,0	43,9	0,006 4	0,010 3	0,008 6	0,009 4	-0,003 1
3	25,4	16,5	39,4	27,1	17,7	41,6	0,000 4	0,009 3	0,009 0	0,009 1	-0,008 7
4	23,8	15,3	37,3	45,3	31,5	65,6	0,039 1	0,009 7	0,006 6	0,008 2	0,030 9
5	65,9	47,2	93,7	50,4	35,4	72,5	0,006 8	0,005 8	0,006 3	0,006 1	0,000 7
						Mean	0,011 4			0,007 9	0,003 5

$$u^2_{d1} = \left(\frac{\lg T_{0,1} - \lg T_{1,1}}{3,92}\right)^2 \quad ; \quad u^2_d = \frac{u^2_{d1} + u^2_{d2}}{2} \quad ; \quad u^2_o = u^2_R - u^2_d$$
$$u^2_{d2} = \left(\frac{\lg T_{0,2} - \lg T_{1,2}}{3,92}\right)^2 \tag{F.8}$$

The relative common logarithmic operational variance is the difference between the means of $u^2_{R'}$ and u^2_d: $u^2_o = 0{,}011\,4 - 0{,}007\,9 = 0{,}003\,5$. An alternative is to calculate the mean of the individual differences (last column). Its square root is the global estimate of the relative operational uncertainty $u_o = \sqrt{0{,}003\,5} = 0{,}059\,2$.

Conversion of the operational standard uncertainty to natural logarithmic scale makes it more expressive: 0,059 × 2,303 = 0,136 = 13,6 %.

F.6 Calculating the combined uncertainty of a new test result

For a colony count result, n_c, the combined relative standard uncertainty in common logarithmic scale is

$$u_c(y) = \sqrt{\frac{0{,}188\,6}{n_c} + u^2_o} \tag{F.9}$$

For an MPN result, with upper and lower 95 % CI given, T_1, T_0, the combined relative standard uncertainty in common logarithmic scale is

$$u_c(y) = \sqrt{\left(\frac{\lg T_1 - \lg T_0}{3,92}\right)^2 + u^2_o} \tag{F.10}$$

Annex G
(normative)

Component approach to evaluation of the combined relative uncertainty under intralaboratory reproducibility conditions

G.1 General

A component estimate of combined uncertainty is constructed from previously determined operational components that represent the analysis, added with the intrinsic distribution uncertainty connected to the observed count(s). It is normally sufficient to consider five operational components and one or two components of (intrinsic) distribution uncertainty. All components shall be expressed as relative standard uncertainties either in decimal form or percentages because the mathematical relation of the principal influence quantities (dilution, count, and volume) is a product.

Standard uncertainty values in natural logarithms are considered equivalent to relative standard uncertainties. If standard uncertainties are available in common logarithmic (base 10) scale, it is best to convert them to natural logarithms before further calculations.

G.2 The relative operational uncertainty

Three of the operational influence quantities associated with the analytical process are identified by stating the equation for the final test result, y.

$$y = F \frac{\sum n_c}{\sum V} \tag{G.1}$$

where

F is the dilution factor from sample to final dilution ($F \geq 1$);

Σn_c is the sum of colonies counted;

ΣV is the sum of the volumes of the test portions, in millilitres, of the final suspension.

Equation (G.1) shows that it is necessary to consider the relative operational uncertainties of the dilution factor, of counting the number of colonies, and of measuring the test portions. These components are derived from calibration experiments and other tests that should be part of the normal analytical quality control in a laboratory.

In addition to these, there may be “hidden” causes of uncertainty, which do not appear in Equation (G.1). The most important are: the variation of subsampling of the laboratory sample (the matrix effect) and the uncertainty generated during incubation. Special experiments are required for the evaluation of these components. Values once determined are assumed to be valid for future similar analyses (see Annexes H and M).

G.3 Combined relative uncertainty of the test result

Combined relative uncertainty of the final test result is calculated in the same way as in the global model according to

$$u_{\mathrm{c,rel}}(y) = \sqrt{u_{\mathrm{o,rel}}^2 + u_{\mathrm{d,rel}}^2} \tag{G.2}$$

where

$u_{\mathrm{o,rel}}$ is the combined operational uncertainty (relative standard uncertainty);

$u_{\mathrm{d,rel}}$ is the distribution uncertainty (relative intrinsic variation).

In the component procedure the operational uncertainty is obtained as the sum of five relative variances of influence quantities

$$u_{\mathrm{o,rel}}^2 = u_{\mathrm{rel,M}}^2 + u_{\mathrm{rel},F}^2 + u_{\mathrm{rel},V}^2 + u_{\mathrm{rel},L}^2 + u_{\mathrm{rel,I}}^2 \tag{G.3}$$

The meanings of the symbols are shown in Table G.1.

The distribution uncertainty is derived without experiments from assumed statistical distributions. In microbiological methods, the value of the parameter depends on the observed count and is different for colony methods and MPN methods (Annex C and Annex D). Possible partial confirmation should be taken into account in the distribution uncertainty (Annex E).

It is useful to set up a table for keeping track of the relevant components of uncertainty. A full list of components is given in the table below. If a component is absent or too small to be effective, the space can be left open.

Table G.1 — Meaning of symbols

Component	Symbol	Relative standard uncertainty	Determination
Matrix and subsampling	M	$u_{\mathrm{rel,M}}$	Annex H
Dilution factor	F	$u_{\mathrm{rel},F}$	Annex K
Test portion	V, ΣV	$u_{\mathrm{rel},V}$, $u_{\mathrm{rel},\Sigma V}$	Annex I, Annex J
Incubation	I	$u_{\mathrm{rel,I}}$	Annex M
Counting	L	$u_{\mathrm{rel},L}$	Annex L
Distribution and confirmation	d	$u_{\mathrm{d,rel}}$	Annex C, Annex D, Annex E

The combined uncertainty is obtained as the sum of all components of variance (squares of the relative uncertainties). It is informative to add up separately the operational and intrinsic components.

G.4 Examples

G.4.1 Example 1

G.4.1.1 General

The standard plate count of aerobic mesophilic flora was determined for a sample.

The essential features of the test protocol:

a) a subsample of 25 g was weighed into 225 g of diluent and homogenized in a mechanical mixer to produce the initial suspension (10^{-1});

b) further decimal dilutions were made (1 ml + 9 ml);

c) two parallel plates with 1 ml test portions from the 10^{-5} dilution gave countable numbers of colonies;

d) the numbers of colonies counted were 41 and 45.

The task was to report the estimated mesophilic count of the sample with the expanded uncertainty attached.

The final result $y = F\,\Sigma n_C/\Sigma V = 10^5 \times (41 + 45)/(1 + 1) = 4{,}3 \times 10^6\ g^{-1}$.

The combined relative uncertainty is obtained as the square root of the sum of relative variances (squares of relative uncertainties) of the relevant components. Table G.2 is helpful for keeping the calculations in order. (The values are taken mostly from the other annexes.)

Table G.2 — Order of calculations

Component	Relative uncertainty		Relative variance
	Symbol	Value	
Matrix and subsampling	$u_{rel,M}$	0,152	0,023 104
Dilution factor	$u_{rel,F}$	0,039	0,001 296
Test portion	$u_{rel,\Sigma V}$	0,011	0,000 121
Incubation	$u_{rel,I}$	0,237	0,056 169
Counting	$u_{rel,L}$	0,097	0,009 409
		Subtotal	0,090 099
Distribution	$u_{d,rel}$	0,108	0,011 628
Confirmation			
		Sum	0,101 727

G.4.1.2 Matrix

The uncertainty of subsampling for the test material was obtained in a special experiment of the kind described in Annex H. Its average value on the basis of 10 representative samples in this laboratory was $u_{rel,M} = 0{,}152$.

NOTE If a laboratory's own experimental data on subsampling are unavailable, information can sometimes be found in the literature. For instance, the tables annexed to the food analytical global uncertainty standard (see ISO/TS 19036:2006[6]) contain experimental standard uncertainties for the "initial suspension" (matrix and subsampling) for a great number of food types in common logarithms. Corresponding data for solid or semisolid materials of environmental sample types (soil, sludge, etc.) seem at the time of publication to be unavailable.

G.4.1.3 Dilution factor

The estimate is based on the principles and values presented in Annex K following basic data on volume and mass uncertainties. The uncertainty in weighing 25 g was 1 %, the relative uncertainty of the volume (actually mass) of 225 ml was 0,025 (2,5 %). The uncertainty due to the initial dilution is therefore 0,024 2. The four further dilution steps from the initial dilution (10^{-1}) had the same uncertainty calculated from $V_{1\ ml}$ = 1,6 %, $V_{9\ ml}$ = 0,5 %. Uncertainty of one dilution step $u_{rel,f} = 0{,}015$. The initial dilution followed by four dilution steps give the total $\sqrt{0{,}024\,2^2 + 4 \times 0{,}015^2} = u_{rel,F} = 0{,}038\,7$.

G.4.1.4 Test portion

The total volume of the test portions was 2 × 1 ml. The combined uncertainty was calculated according to J.2 from the information that the relative reproducibility standard uncertainty of 1 ml was $u_{rel,V} = 0{,}016$ (1,6 %). With two replicates of equal volume, the relative standard uncertainty of the sum is $u_{\Sigma V} = 0{,}016/\sqrt{2} = 0{,}011\,3$.

G.4.1.5 Incubation

The overdispersion attributed to incubation for the standard heterotrophic plate count was determined in a special experiment following the principles illustrated in Annex M. The value once determined was believed to be valid. The value obtained from the study of seven samples with six replicates was $u_{rel,I} = 0{,}237$.

G.4.1.6 Counting

The intralaboratory reproducibility of counting had been determined as part of the AQC previously (Annex L). The value estimated was $u_{\mathrm{rel},L}$ = 0,097.

G.4.1.7 Particle distribution

The relative distribution uncertainty is based on the sum of colonies counted. The uncertainty due to the Poisson particle distribution in the final suspension is thus $u_{\mathrm{d,rel}} = 1/\sqrt{\Sigma n_{\mathrm{c}}} = 1/\sqrt{41+45} = \sqrt{1/86} = 0{,}108$.

G.4.1.8 Estimation and application of combined uncertainty

The root sum of squares of the five operational components is $u_{\mathrm{o,rel}} = \sqrt{0{,}090\,099} = 0{,}300$. The small components due to volume measurements (dilution factor and test portion) could have been omitted as their effect on the total is negligible. Without these two, the operational uncertainty component would have been 0,298.

The combined relative uncertainty (variance) is obtained by adding together the operational and intrinsic variances $u^2_{\mathrm{c,rel}}(y) = 0{,}090\,099 + 0{,}011\,628 = 0{,}101\,727$. Accordingly, $u_{\mathrm{c,rel}}(y) = 0{,}319 = 31{,}9\ \%$.

Expanded relative uncertainty, twice the relative combined uncertainty, is accordingly U_{rel} = 0,638. To express the interval based on expanded uncertainty of the test result, the point estimate y should be multiplied and divided by exp(U) expressed in decimal form (Annex N).

In this example y = 4,3 × 10^6 and exp(U) = exp(0,638) = 1,893. The upper limit is 4,3 × 1,893 × 10^6 = 8,1 × 10^6, the lower limit is (4,3/1,893) × 10^6 = 2,3 × 10^6.

G.4.2 Example 2

The concentration of *Escherichia coli* in a water sample was estimated using a commercial MPN system. Corresponding to the number of positive wells, the tables supplied by the manufacturer gave the MPN value 8,7 (per 100 ml) with lower and upper 95 % confidence limits of 4,5 and 17,1, respectively.

With special experiments, the laboratory had previously estimated the relative reproducibility of counting for this method as 0,067 (6,7 %) and an incubation effect 0,10 (10 %).

The average relative reproducibility (of several technicians) of measuring a test portion of 100 ml was determined by calibration experiments to be about 0,05 (5 %).

With water samples, the subsampling variance, apart from the uncertainty of the test portion volume, can be assumed negligible. There was no dilution.

An estimate of the relative distribution uncertainty was calculated from the 95 % confidence limits:

$u_{\mathrm{d,rel}}$ = (ln 17,1 – ln 4,5)/3,92 = 0,34 = 34,0 %

NOTE Calculation with common logarithms: $u_{\mathrm{d(lg)}}$ = (lg 17,1 – lg 4,5)/3,92 = 0,148; on conversion to a natural logarithmic scale, $u_{\mathrm{d,rel}}$ = 2,3 × 0,148 = 0,34 = 34 %.

The combined uncertainty is obtained as the root sum of squares of the known components of uncertainty:

$$u_{\mathrm{c,rel}}(y) = \sqrt{0{,}067^2 + 0{,}10^2 + 0{,}05^2 + 0{,}34^2} = \sqrt{0{,}132\,589} = 0{,}364 \qquad \text{(G.4)}$$

Should it be preferable to report the combined uncertainty in common logarithmic scale, $u_{\mathrm{c,rel}}(y)$ should be transformed by dividing by the modulus: $u_{\mathrm{c}}(y)$ = 0,364/2,303 = 0,158.

Annex H
(normative)

Experimental evaluation of subsampling variance

H.1 General

The ordinary global design does not enable the evaluation of any components of uncertainty in detail. The variation of subsampling of the laboratory sample, also called the matrix effect, can be evaluated with an experiment in which duplicate analyses are made from two or more (preferably three or four) initial suspensions made from equal subsamples measured from a laboratory sample.

The statistical design is more complex than the global experiment for the evaluation of the uncertainty of the entire analytical process. It therefore requires some thought whether the quantitative information about subsampling is important enough to warrant the effort of evaluating this single parameter separately. Such information is invaluable when there is a need to analyse reasons for an observed or suspected high combined uncertainty.

Replication at subordinate (lower) levels is necessary to permit estimation of the variance component connected to subsampling of the laboratory sample. In water and other liquid matrices, the subsampling variance is likely to be unimportant and experimental evaluation of subsampling variance is unnecessary. The example presented in this annex shows the analysis of one sample. At least 10 specimens of the same type of sample material should be tested to obtain a sufficiently reliable mean value for the subsampling variance. It is permissible that different persons process different samples, but it is recommended that the analysis be made under repeatability conditions. Only one person should be involved in reading the results of any one sample.

H.2 Analysis of variance

An analysis of variance with replication of subsamples and dilution series is a standard method for investigating the precision (imprecision) of subsampling. The laboratory sample, mixed as well as possible under the circumstances, is randomly subsampled k times and n repeat analytical tests are carried out on each subsample. The minimum values for n and k are 2. An estimate of subsampling variance can be computed from such data.

Logarithmic transformation of the data is usually recommendable. The main reason is to eliminate the effect of variable contamination levels of the different samples. Also the normality of the data is likely to be improved. Natural logarithms are somewhat more convenient than common logarithms because the results can be directly interpreted as relative variances. With common logarithms the final result requires multiplication with a constant if conversion to relative or percentage expression is desired.

EXAMPLE A laboratory sample of solids containing material was mixed as well as possible. Six independent subsamples (k = 6) were measured and suspended in sterile diluent. They were carefully mixed. Two dilution series (n = 2) were made from each initial suspension and one plate was inoculated from the final suspension of each series. A count of target colonies was made after the specified incubation time. The results are presented in Table H.1.

Table H.1 — One-way analysis of variance, using natural logarithms, of the results of six subsamples of one laboratory sample for the extraction of the subsampling variance

Subsample	Dilution series	Observed count, n_c	ln n_c	$(\ln n_c)^2$	Subsample sum of ln n_c	Subsample sum of ln n_c squared
1	1	34	3,526 4	12,435		
	2	45	3,806 7	14,491	7,333 0	53,772 9
2	1	50	3,912 0	15,304		
	2	61	4,110 9	16,899	8,022 9	64,366 9
3	1	60	4,094 3	16,764		
	2	72	4,276 7	18,290	8,371 0	70,073 6
4	1	82	4,406 7	19,419		
	2	70	4,248 5	18,050	8,655 2	74,912 5
5	1	58	4,060 4	16,487		
	2	64	4,158 9	17,296	8,219 3	67,556 9
6	1	40	3,688 9	13,608		
	2	59	4,077 5	16,626	7,766 4	60,317 0
		Sum	48,367 9	195,669		391,000

Natural logarithms of the counts were taken and a one-way analysis of variance was computed. The results are shown in Table H.2. Calculations proceed as follows.

a) Calculate the sum of all natural logarithmic values (48,3679).

b) Calculate correction term (CT), t_{corr} = $(48,367\ 9)^2/nk$ = 2 339,453 8/12 = 194,954 5.

c) Calculate the sum of squared natural logarithmic values (195,669).

d) Add the parallel results of each subsample (3,526 4 + 3,806 7 = 7,333 0, 3,912 0 + 4,110 9 = 8,022 9, etc.).

e) Square the values and sum them up (391,000).

f) Obtain the total sum of squares (SS) from 195,669 – t_{corr} = 195,669 – 194,954 5 = 0,714 5.

g) Calculate between subsamples sum of squares (391,000/n) – t_{corr} = (391,0/2) – t_{corr} = 195,500 0 – 194,954 5 = 0,545 5.

h) Obtain within subsamples sum of squares from 0,714 5 – 0,545 5 = 0,169 0.

i) Calculate variances (mean squares, MS) by dividing sums of squares by appropriate degrees of freedom (DF).

Table H.2 — One-way analysis of variance

Source of variation	DF	SS	MS	Estimate	F_{stat}	P
Between subsamples	$(k - 1) = 5$	0,545 5	0,109 1	$s^2 + ns_B^2$	3,87	0,064
Within	$k(n - 1) = 6$	0,169 0	0,028 2	s^2		
Total	$(kn - 1) = 11$	0,714 5				

The results in Table H.2 were calculated by hand. If the data are entered in a computer program the results may come out slightly different because of greater precision in the calculations.

The column headed "estimate" shows how each mean square (MS) is structured. The within subsamples variance, s^2, is a measure of the average variation between the duplicate dilution series from one initial suspension, with distribution uncertainty and all other operational components of uncertainty except subsampling included.

It is of only passing interest to note that the between subsamples variance is not statistically significant (at the 5 % level) compared to the within subsamples variance (F_{stat} = 0,109 1/0,028 2 = 3,87, P = 0,064). Even if not statistically significant, it contains the necessary information for calculating the subsampling variance, s_B^2. An estimate of the subsampling variance can be obtained from the calculation:

$$s_B^2 = \frac{0{,}109\,1 - 0{,}028\,2}{2} = 0{,}040\,5$$

Because natural logarithms were used, s_B gives the estimate of relative uncertainty of subsampling,

$$u_{rel,M} = 0{,}20 = 20\ \% \qquad \text{(H.1)}$$

The result is an estimate of the subsampling uncertainty in the material tested, or actually in the one particular laboratory sample mixed using the equipment and practices of the laboratory.

One sample is not enough for a general subsampling uncertainty statement. Several samples of the same type should be examined and their subsampling variances averaged. The entire experiment from which this example was chosen consisted of 10 samples of the same type of material. The mean value from the experiment was 15,2 %.

NOTE The same analysis using common logarithms first gives the subsampling variance (0,020 6 − 0,005 3)/2 = 0,007 65, whose square root is 0,087 5. Conversion to natural logarithms yields $u_{rel,M}$ = 2,303 × 0,087 5 = 0,201 5 = 20 %, i.e. the same result as given by Equation (H.1).

Annex I
(normative)

Relative repeatability and intralaboratory reproducibility of volume measurements

I.1 Principle

Weighing is more accurate than the ordinary volume measurements in microbiological routine. The precision of volumetric instruments can be determined by weighing portions of distilled or deionized water. The estimates of mean and standard uncertainty are obtained by standard statistical equations from the series of measurements.

To calibrate a volumetric instrument (pipette, cylinder, etc.), a series of at least 20 observations should be obtained using the instrument in question. In order to simulate aseptic working techniques, a different pipette or tip should be picked for each measurement.

A series of results from one person provide a repeatability standard uncertainty. Analysing results from two or more persons together produces an uncertainty estimate that may be termed intralaboratory reproducibility. Possible biases between operators become included in the common uncertainty estimate.

Relative standard uncertainty is the most convenient parameter for further use. It is obtained by dividing the standard uncertainty by the mean or by doing the calculations using natural (or common) logarithms as specified in Annex B (see I.3, Note 2).

I.2 Personal repeatability standard uncertainty

The precision of measuring 0,1 ml test portions using a glass pipette was studied by weighing. Each of two technicians (A and B) made a series of 20 measurements. To illustrate the calculations, a small number of the data (six measurements per person) are shown in Table I.1.

Table I.1 — Results of calibrating 0,1 ml test portion volumes by weighing

NOTE Six independent volume measurements by two persons (A and B) are shown. The relative standard uncertainty was calculated in all three optional ways presented in Annex B (see I.3, Note 2).

	Interval scale		Natural logarithmic scale		Common logarithmic scale	
	V_A	V_B	ln V_A	ln V_B	lg V_A	lg V_B
	0,105	0,107	−2,253 8	−2,234 9	−0,978 8	−0,970 6
	0,113	0,116	−2,180 4	−2,154 2	−0,946 9	−0,935 5
	0,109	0,097	−2,216 4	−2,333 0	−0,962 6	−1,013 2
	0,103	0,097	−2,273 0	−2,333 0	−0,987 2	−1,013 2
	0,115	0,083	−2,162 8	−2,488 9	−0,939 3	−1,080 9
	0,123	0,085	−2,095 6	−2,465 1	−0,910 1	−1,070 6
Mean	0,111 3	0,097 5	—	—	—	—
Standard uncertainty	0,007 3	0,012 6	0,065	0,129	0,028 2	0,056 1
Relative standard uncertainty	0,066	0,129	0,065	0,129	0,065[a]	0,129[a]

[a] Obtained by multiplying the standard uncertainty by 2,303.

The personal standard uncertainties indicate the repeatability of volume measurements by the two operators separately. The laboratory would probably only use them for internal quality control purposes and personnel

training. A general reproducibility estimate for the laboratory would be preferred when calculating the combined uncertainty of a test result for a customer.

I.3 Relative intralaboratory reproducibility

An intralaboratory reproducibility standard uncertainty of volume measurements should include any possible biases between the operators. Bias is included in the estimate if the uncertainty is calculated by putting the results of different technicians in the same data file before calculating the mean and standard uncertainty. This was done with all 12 values in Table I.1 using all three methods of estimation as specified in Annex B (see Note 2). The results are as shown in Table I.2.

Table I.2 — Analysis of the variability of volume measurements

NOTE 1 Data of two technicians put together.

	V	ln V	lg V
N	12	12	12
Mean	0,104 4	—	—
Standard uncertainty	0,012 2	0,121 2	0,052 6
Relative value	0,116 9	0,121 2	0,121 1[a]
Percent value	11,7	12,1	12,1[a]
V is the volume N is the number of observations			
[a] Obtained by multiplying the standard uncertainty by 2,303.			

The arithmetic mean 0,104 4 does not differ significantly from the nominal volume 0,1 ml. It would be legitimate to calculate the relative standard uncertainty also from 0,012 2/0,1 = 0,122.

The intralaboratory reproducibility estimate should be used as the test portion uncertainty when computing the combined uncertainty.

NOTE 2 When studying the uncertainty of dilution blank volume, it is probably of no importance to employ several technicians in order to evaluate intralaboratory reproducibility. Observations on different autoclave batches and/or different dispensers should be made instead. Dilution blanks should be weighed after autoclaving.

Annex J
(normative)

Relative uncertainty of a sum of test portions

J.1 General

Uncertainty of a sum of test portions is needed in computations of the uncertainty of the concentration of the analyte in a final suspension when the detection system consists of a set of plates from one or more dilutions. The uncertainty of the sum is an intralaboratory reproducibility estimate when the uncertainty values of the component volumes have been estimated under reproducibility conditions (I.2).

J.2 One dilution, general case

J.2.1 General

When a series of plates is made from the same final suspension, the uncertainty of the total volume is obtained by direct application of the combined uncertainty rules. According to Annex B the uncertainty of the sum $\Sigma V = V_1 + V_2 + \ldots + V_n$ is calculated from

$$u_{\Sigma V} = \sqrt{u_1^2 + u_2^2 + \ldots + u_n^2} \tag{J.1}$$

where u_1, u_2, … are estimates of the standard uncertainty of V_1, V_2, … expressed in units of volume (millilitres).

For further use in uncertainty calculations, it is usually best to express the result as relative uncertainty $u_{\text{rel},\Sigma V} = u_{\Sigma V}/\Sigma V$.

J.2.2 Example

Four plates have been made from the same suspension: two plates with a 1 ml test portion and two plates with a 0,1 ml test portion. The total volume of the final suspension examined was therefore 1 ml + 1 ml + 0,1 ml + 0,1 ml = 2,2 ml. The uncertainty of the total volume should be calculated. The relative uncertainty (reproducibility) of the 0,1 ml pipetted volume had been previously estimated to be 8 % and that of 1 ml to be 2 %.

Before summation, the relative uncertainties shall be expressed in units of volume: $u_{1\text{ ml}}$ = 0,02 × 1 ml = 0,02 ml and $u_{0,1\text{ ml}}$ = 0,08 × 0,1 ml = 0,008 ml.

The uncertainty of the sum is

$$u_{\Sigma V} = \sqrt{0{,}02^2 + 0{,}02^2 + 0{,}008^2 + 0{,}008^2} = \sqrt{0{,}000\,928} = 0{,}030\,5 \text{ ml} \tag{J.2}$$

The relative uncertainty is: $u_{\text{rel}\Sigma V} = u_{\Sigma V}/\Sigma V$ = 0,030 5/2,2 = 0,014 = 1,4 %.

J.2.3 One dilution, equal test portions

Usually, in a series of parallel plates the test portions are equal. If the uncertainty of the volumetric instrument is given in relative scale, for instance as a percentage, the relative uncertainty of the sum, as a percentage, can

be obtained more directly than shown above. The relative uncertainty of the sum is the relative uncertainty of one measurement divided by the square root of the number of parallels.

$$u_{\mathrm{rel},\Sigma V} = \frac{u_{\mathrm{rel},V}}{\sqrt{n}} \tag{J.3}$$

where

n is the number of parallel plates;

$u_{\mathrm{rel},V}$ is the relative uncertainty of one test portion.

J.3 Two dilutions

J.3.1 General

Assume a final result based on the enumeration of colonies from n_0 plates of the final suspension and n_1 plates from a second dilution obtained by dilution of the final suspension one more step with a dilution factor f between dilutions.

The sum of test portion volumes in terms of the final suspension is:

$$\sum V = n_0 V_0 + \frac{1}{f} n_1 V_1 \tag{J.4}$$

where

n_0 is the number of parallel plates made from the final suspension;

V_0 is the volume of test portions measured from the final suspension;

n_1 is the number of parallel plates made from the second dilution;

f is the dilution factor;

V_1 is the volume of test portions measured from the second dilution.

Typically the numbers of replicates and inoculum volumes are the same in both dilutions $n_0 = n_1 = n$ and $V_0 = V_1 = V$. The equation for the relative uncertainty of the sum of test portion volumes is

$$u^2_{\mathrm{rel},\Sigma V} = \frac{1}{\left(f+1\right)^2}\left[\frac{u^2_{\mathrm{rel},V}}{n}\left(f^2+1\right) + u^2_{\mathrm{rel},f}\right] \tag{J.5}$$

where

f is the dilution factor between the two levels within the detection system;

n is the number of parallel plates in each dilution;

V is the volume of the inoculum per plate;

$u_{\mathrm{rel},V}$ is the relative uncertainty of the inoculum volume;

$u_{\mathrm{rel},f}$ is the relative uncertainty of the dilution factor (calculated by the formula in Annex K from the relative uncertainties of the transfer volume and dilution blank volume).

NOTE The contribution of the second dilution to the uncertainty of the volume sum is insignificant when the dilution factor f between the two dilutions is great (five or more). Uncertainty of the dilution and the uncertainty of test portion measurements in the second dilution can be ignored with impunity. A satisfactory estimate of the relative uncertainty is obtained by considering only the first dilution:

$$u^2_{\text{rel},\Sigma V} \approx \frac{u^2_{\text{rel},V}}{n}$$

(See J.3.2.)

Sometimes a plate may be missed in one dilution, or different inoculum volumes may be used. A general Equation (J.6) can then be applied. The relative variance of the total test portion volume is obtained from

$$u^2_{\text{rel},\Sigma V} = \frac{1}{\left(fn_0V_0 + n_1V_1\right)^2}\left[n_0 f^2 V_0^2 u^2_{V_0} + n_1^2 V_1^2\left(\frac{u^2_{\text{rel},V_1}}{n_1} + u^2_{\text{rel},f}\right)\right] \quad \text{(J.6)}$$

where

f is the dilution factor between the two dilutions;

n_0 is the number of parallel plates in the final suspension;

V_0 is the volume of suspension inoculated per plate in the final suspension;

n_1 is the number of parallel plates in the second dilution;

V_1 is the volume of suspension inoculated per plate in the second dilution;

u_{rel,V_0} is the relative uncertainty of volume V_0;

u_{rel,V_1} is the relative uncertainty of volume V_1;

$u_{\text{rel},f}$ is the relative uncertainty of the dilution factor f.

The equation is complicated because it permits different numbers of parallel plates (n_0 and n_1) in the two dilutions and different volumes of inocula (V_0 and V_1) in different dilutions.

J.3.2 Example

A sample was studied by making the initial suspension by homogenizing 25 g of sample with 225 ml diluent. This constitutes the first dilution (10^{-1}) and is also called the initial suspension. The initial suspension was further diluted in several steps of 1 ml + 9 ml. Two parallel plates were inoculated from each dilution with test portion volumes of 1 ml. The dilutions with suitable numbers of colonies for enumeration were found to be 10^{-4} and 10^{-5}.

The dilution history until the final suspension (the first countable dilution) is of no consequence for the task at hand.

The total test portion volume in units of the final suspension was ΣV = 1 ml + 1 ml + 0,1 ml + 0,1 ml = 2,2 ml.

To calculate the relative (percentage) uncertainty of the sum of test portions information about the uncertainty of different volume measurements is needed. This information is collected in Table J.1.

Table J.1 — The relative uncertainty of volume measurements

Measurement ml	Relative standard uncertainty $u_{\text{rel},V}$
1	0,005
9	0,02

The relative uncertainty of the dilution step was calculated as described in Annex K.

$$u_{\text{rel},f}^2 = \left(\frac{9^2}{10^2}\right)\left(0{,}02^2 + 0{,}005^2\right) = 0{,}000\ 344 \tag{J.7}$$

The information relevant to the calculation of the relative uncertainty of ΣV is shown below.

$$V = 1\text{ ml},\ f = 10,\ n = 2,\ u_{\text{rel},f}^2 = 0{,}000\ 344\,,\ u_{\text{rel},V}^2 = 0{,}000\ 025 \tag{J.8}$$

The values are inserted into Equation (J.6):

$$u_{\text{rel},\Sigma V}^2 = \frac{1}{(10+1)^2}\left[\frac{0{,}000\ 025}{2}(100+1) + 0{,}000\ 344\right] = 0{,}000\ 013 \tag{J.9}$$

The relative variance of the sum of test portions is 0,000 013. The estimate of relative uncertainty of the sum of volumes is therefore, $u_{\text{rel},\Sigma V} = \sqrt{0{,}000\ 013} = 0{,}003\ 6$.

Using the approximate solution presented in the note, the calculation simplifies to

$$u_{\text{rel},\Sigma V}^2 = \frac{u_{\text{rel},V}^2}{n} = \frac{0{,}000\ 025}{2} = 0{,}000\ 012\ 5$$

The square root of 0,000 012 5 gives the relative uncertainty 0,003 5. The difference between 0,003 6 and 0,003 5 is negligible.

J.4 Most probable number

Some MPN systems are inoculated by measuring one large test portion (e.g. 100 ml) which gets distributed into numerous wells. The uncertainty of the total volume is determined by calibrating the volumetric instrument as described for volume measurements in Annex I.

In some other MPN systems, test portions are measured into the reaction wells or tubes one at a time. The collective uncertainty is determined as described in J.2. The uncertainty of the total volume in these instances is usually negligible.

Annex K
(normative)

Relative uncertainty of dilution factor F

The dilution factor may consist of several successive steps

$$F = f_1 \, f_2 \, ... \, f_k \tag{K.1}$$

The relative uncertainty (variance) of each individual step is estimated from

$$u^2_{\text{rel},f} = \left(\frac{V_\text{b}}{V_\text{a} + V_\text{b}} \right)^2 \left(u^2_{\text{rel,a}} + u^2_{\text{rel,b}} \right) \tag{K.2}$$

where

V_a is the volume of microbial suspension transferred;

V_b is the volume of the dilution blank;

$u_{\text{rel,a}}$ is the relative uncertainty of the transfer volume;

$u_{\text{rel,b}}$ is the relative uncertainty of the dilution blank volume.

Relative variance of the total dilution factor is the sum of squares of the individual relative uncertainties

$$u^2_{\text{rel},F} = u^2_{\text{rel},f_1} + u^2_{\text{rel},f_2} + ... + u^2_{\text{rel},f_k} \tag{K.3}$$

EXAMPLE An initial suspension was made by homogenizing 25 g of sample with 225 ml diluent. This constitutes the first dilution (10^{-1}) and is also called the initial suspension. The initial suspension was diluted further in several steps of 1 ml + 9 ml. The dilutions with suitable numbers of colonies for enumeration were found to be 10^{-4} and 10^{-5}. The first dilution with countable colonies (10^{-4}) becomes the “final suspension”.

The task is to calculate the relative standard uncertainty of the dilution factor of the final dilution $F = 1/10^{-4}$. It represents the relative uncertainty of the true but unknown mean concentration of the analyte in the final suspension.

Data on the uncertainty of different measurements of mass and volume were needed. This information is listed in Table K.1. (The values ought to be available from observations in the quality assurance programme of the laboratory.)

Table K.1 — Data on the relative uncertainty of different measurements of mass and volume

Measurement	Relative uncertainty	Squared uncertainty
25 g	0,01	0,000 1
225 ml	0,025	0,000 625
1 ml	0,016	0,000 256
9 ml	0,005	0,000 025

The relative uncertainties of the different dilution steps were calculated as shown using Equation (K.2). Dilution steps f_2, f_3, and f_4 were identical. Therefore:

Initial dilution $u^2_{\text{rel},f_1} = \left(\frac{225}{250} \right)^2 \times \left(0{,}025^2 + 0{,}01^2 \right) = 0{,}000\,587$

Other dilution steps $u^2_{\text{rel},f_2} = \frac{9^2}{10^2} \times \left(0{,}016^2 + 0{,}005^2\right) = 0{,}000\,228$

Consequently, $u^2_{\text{rel},F} = 0{,}000\,587 + 0{,}000\,228 + 0{,}000\,228 + 0{,}000\,228 + 0{,}000\,228 = 0{,}001\,499$

and the relative uncertainty of the dilution factor is $u_{\text{rel},F} = \sqrt{0{,}001\,499} = 0{,}038\,7 \approx 3{,}9\ \%$

Annex L
(normative)

Repeatability and intralaboratory reproducibility of counting

L.1 General

The uncertainty of reading the number of colonies of a plate is often one of the significant components of uncertainty in quantitative microbiology. A person can usually repeat his/her own result of reading to a precision of a few per cent. The counts of different persons agree less well, resulting in a significant intralaboratory uncertainty of counting.

The values of the parameter are studied by obtaining readings of the same plates by different operators or repeated readings by the same operator. This should be done as part of the normal quality assurance programme of a laboratory. Normal routine samples should be studied. The plates should be picked randomly for second counting after the initial count has already been made. Problem cases should be included only to the extent that they get chosen randomly.

Personal differences are especially marked when target colonies should be distinguished by their outward appearance (shape, colour, size) from a background of other colonies. For this reason, the uncertainty is not only personal but also method-specific and possibly sample-type specific.

Both repeatability and reproducibility of counting are meaningful parameters. Repeatability may have to be taken into account in internal quality assurance work and reproducibility is needed for combined uncertainty values estimated by the component procedure.

Reading positive reactions in MPN series is more reproducible than counting colonies. However, the effects of even slight differences in reading are magnified in the MPN values. Recent observations show that also the reproducibility of reading MPN results may deserve attention.

L.2 Personal uncertainty of counting

L.2.1 General

An estimate of personal uncertainty of counting has some intrinsic value, but this information is also needed when components of uncertainty, such as incubation effects, are estimated by cultivation experiments. An evaluation should be made with every method and target organism separately.

Data can be cumulated over days and weeks until an adequate number (at least 30, preferably many more) of plates have been read. The only practical problem is how to avoid the first count influencing the second. The calculation presented in the example allows gradual build-up of data.

L.2.2 Example 1

A technician has noted down the results of her/his own repeated reading (L_1, L_2) of several plates. The plates were chosen at random during the daily routine work. A small part of the results is shown in Table L.1:

The relative variance of each pair was calculated using the equation

$$u^2_{\mathrm{rel},L} = 2\left(\frac{L_1 - L_2}{L_1 + L_2}\right)^2$$

Table L.1 — Results of repeated reading (L_1, L_2) of several plates

Plate	L_1	L_2	$L_1 - L_2$	$L_1 + L_2$	$u^2_{\text{rel},L}$
1	343	337	−6	680	0,000 156
2	40	39	1	79	0,000 320
3	57	62	−5	119	0,003 531
4	399	397	2	796	0,000 013
5	112	130	−18	242	0,011 064
6	349	325	24	674	0,002 536
7	85	84	1	169	0,000 070
8	129	122	7	251	0,001 556
9	16	17	−1	33	0,001 837
10	27	27	0	54	0,000 000
Sum	**1 557**	**1 540**			**0,021 083**

The average estimate of the personal relative variance of counting is the mean value of

$$u^2_{\text{rel},L} = \frac{0{,}021\,083}{10} = 0{,}002\,108\,3$$

The square root, 0,045 9, thus indicates a 4,6 % relative standard uncertainty of the repeatability of counting by this person.

L.3 Intralaboratory reproducibility of counting colonies

L.3.1 General

The intralaboratory uncertainty can be evaluated by involving all or several technicians of the laboratory in the reading of the same plates. Any systematic differences (biases) between persons are included in the uncertainty estimate and are viewed as random variation.

L.3.2 Example

Four technicians (A,B,C,D) involved in daily routine microbiological analyses read the same eight randomly selected plates independently. The results were the following:

Table L.2 — Randomly selected plates

Plate	L_A	L_B	L_C	L_D	$\bar{x}_i$	s_i	u_{rel,L_i}
1	21	23	24	26	23,5	2,08	0,089
2	38	38	42	40	39,5	1,91	0,048
3	27	29	34	30	30,0	2,94	0,098
4	16	22	19	21	19,5	2,65	0,136
5	33	25	33	38	32,3	5,38	0,167
6	67	65	74	66	68,0	4,08	0,060
7	160	166	176	174	169,0	7,39	0,044
8	89	81	94	92	89,0	5,72	0,064
Sum	451	449	496	487			

s_i is the standard uncertainty

u_{rel,L_i} is the relative standard uncertainty of counting of the ith plate

$\bar{x}_i$ is the mean

The sums at the bottom of Table L.2 indicate that the average results of counting might differ between the persons, A and B and C and D, forming two “schools” of interpretation. The data set is too small to draw firm conclusions. The possible systematic differences might be worth study, but are presently included in the standard uncertainty.

The sum of squared u_{rel,L_i} values (sum of relative variances) $0{,}089^2 + 0{,}048^2 + ... + 0{,}064^2 = 0{,}075\ 846$ and their mean is 0,009 480 75. It is the sought estimate of the average relative reproducibility variance of reading in the laboratory as a whole. Its square root 0,097 4 is the average relative intralaboratory uncertainty of counting with this method and group of operators (9,7 %).

The estimate strictly applies only to similar situations as in the experiment (same type of sample, same method, same group of operators).

L.4 Intralaboratory reproducibility due to uncertainty of reading most probable number results

L.4.1 General

The intralaboratory uncertainty of reading MPN results can be studied by allowing different operators to read the same routine MPN series. No special experiments are needed.

It may be of some interest to note the average differences and standard uncertainties of the primary positive wells or tubes, but the actual effect of the uncertainty is seen in the MPN estimates. The uncertainty calculations are made with the MPN values.

L.4.2 Example

Analyses of *E. coli* in water were made using a commercial MPN system. The results of many routine samples were blindly read by two technicians and the corresponding MPN values were obtained from tables. The beginning (seven samples) of the series of results is shown in Table L.3. The numbers are MPN values per 100 ml of water.

Table L.3 — Series of results

Sample	x_1	x_2	Relative variance	Common logarithm of the variance
1	3 450	3 500	0,000 1	0,000 02
2	4	4	0,000 0	0,000 00
3	12	14	0,011 9	0,002 24
4	126	130	0,000 5	0,000 09
5	14	14	0,000 0	0,000 00
6	13	17	0,036 0	0,006 79
7	3	4	0,041 4	0,007 80
		Mean	0,012 8	0,002 42

The relative variance was calculated according to

$$u_{\text{rel}}^2 = \frac{\left(\ln x_1 - \ln x_2\right)^2}{2} \tag{L.1}$$

The common logarithm of the variance is equal to

$$\frac{\left(\lg x_1 - \lg x_2\right)^2}{2} \tag{L.2}$$

The square root of the mean relative variance is the average value of the relative uncertainty of MPN due to uncertainty of reading by different operators. It can be called the intralaboratory reproducibility of reading. Its value on the basis of this limited set of data was $\sqrt{0{,}012\,8} = 0{,}113 = 11{,}3\ \%$.

Using common logarithms, the average uncertainty in common logarithmic scale is first obtained as the square root of 0,002 42. The value is 0,049 2. Conversion to natural logarithms gives u_{rel} = 2,303 × 0,049 2 = 0,113 3 ≈ 11,3 %.

The reliability of the mean improves with increasing numbers of samples.

L.5 Relative uncertainty of reading a sum of counts

The sum of colony counts from several plates is an element of calculations of the final results whenever multiple-plate instruments are in use. There are not enough experimental data to be able to decide whether the relative uncertainty of reading a sum depends on the number of parts the sum consists of. It is, however, known that the relative uncertainty of reading does not change much when the number of colonies in the same plate increases.

For the time being, it is recommended that the same relative uncertainty of reading should be applied to single counts and sums of counts alike. The value of uncertainty estimated in experiments of the kind described in L.2 and L.3 is applied to sums of counts as well.

Annex M
(normative)

Incubation effects — Uncertainty due to position and time

M.1 General

Things can happen during the incubation that change the number of colonies in a plate. Colonies can fail to develop. They can spread or merge with neighbouring colonies or acquire a strange appearance. Contamination can add colonies, and so on. As a consequence, the number of colonies observed after incubation can differ from the (unknown) number of viable colony-forming particles originally deposited on the plate. When incubation conditions are near the tolerance limits of some members of the target population, slight differences in conditions (temperature, humidity, atmosphere) may affect the count differently in different parts of the incubation space.

Even if the numbers of colony-forming particles in a series of plates from a suspension originally probably follow the Poisson law, the effect of the influences during incubation is to cause “overdispersion” of parallel counts. The effect arises during incubation. It can be called the incubation effect although the ultimate cause is probably less the incubation equipment than reactions and interactions between different members of the microbial population on the plate.

Method standards allow a time span during which the results should be read. Laboratories ought to test how much uncertainty in their results might result from the varying time of incubation in the limits that actually occur in the daily routine. This can be done most conveniently in connection with the experiments on positional effects by removing plates from the incubator for reading after randomly selected times within the time specified for the analysis in question.

Influences that inhibit growth because of medium failure affect the result proportionally and in all plates of the series. Other effects may be additive and limited to one plate. Contamination is the most obvious example. Antibiotic and synergistic effects between colonies are other examples. Some spurious errors can occur. For these reasons, there is rather little chance of correctly modelling the overdispersion due to incubation. It can be approached by experiments based on series of parallel plates, but the correct mathematical description remains arbitrary. Assuming the total effect of incubation conditions proportional to the mean assumes the negative binomial model. It is possibly the most realistic of the simple approximations.

M.2 The experimental design

The experiments are based on well mixed test suspensions. The only requirement is that the test suspensions represent typical target populations in a concentration fit for direct cultivation. Natural routine samples or their dilutions are the best. In order to minimize other than incubation effects, repeatability conditions should prevail during the preparation.

A set of, for instance, 6 to 10 parallel plates is made from each test suspension. The combined volume of test portions plated should not exceed 10 % of the volume of the suspension to avoid the complication of finite sample correction (see Annex C).

The plates should be placed in randomly selected positions in the incubator. After incubation, all plates should be read by the same operator. The total variance between the parallel plates is calculated and the intrinsic (Poisson) variance component is subtracted. If there is reason to believe that the uncertainty of counting and/or the uncertainty of test portion volume are significant, they should be subtracted as well. The remainder is interpreted as additional uncertainty due to incubation.

It is not easy to obtain a completely representative random arrangement of influence quantities in an incubation experiment. For the practical work, it is convenient to divide the incubation positions into shelves, areas on a shelf, and positions in a stack of plates, for instance:

a) shelves 1, 2, and 3 in the incubator;

b) six areas on each shelf (left front and back, middle front and back, right front and back);

c) layer in a stack.

Each plate of the parallel series is randomly allocated to a shelf, an area, and a layer by the help of any convenient means (dice, playing cards, tables of random numbers, etc.). The plates are placed in their positions among the normal routine plates. In order to facilitate the recovery of the plates after their incubation is completed it is recommendable to use clearly visible markings or Petri dishes of distinct colour.

If more than one incubator is used, the incubator in which to place all parallel plates can be selected by flipping a coin or rolling a die. Randomly selecting the incubator for each plate separately is theoretically a better alternative but hardly a practical one.

Table M.1 — An example of randomly selected positions and times of incubation for a set of six plates

Plate	Shelf	Area	Layer	Time
1	2	3	5	t_{min} + 3,5 h
2	2	3	3	t_{min} + 3,0 h
3	3	2	2	t_{min} + 4,0 h
4	2	5	2	t_{min} + 2,5 h
5	3	2	5	t_{min} + 1,0 h
6	1	4	4	t_{min} + 0,5 h
The shortest incubation time permitted is t_{min}. The times added to t_{min} were chosen randomly from 0 to 8 half hour periods. A similar random allocation should be made separately for the series of plates for every test suspension.				

M.3 Example 1 — A colony count method

Six 10 ml aliquots from a water sample of 1 000 ml were cultured by a membrane filter method. The incubation positions on three shelves, in six areas per shelf, and six layers in stacks were randomly allocated by the use of dice. The layers were numbered from one (bottom) to six (top). The plates were inserted among plates of routine samples. A similar series was made with another sample on another day. Tables M.2 and M.3 show the randomly allocated positions and the numbers of colonies observed, n_c.

Table M.2 — Sample 1

Plate	Shelf	Area	Layer	Time h	n_c
1	2	5	5	19,5	25
2	1	4	4	18,0	40
3	1	3	1	22,0	54
4	3	6	1	21,5	32
5	3	4	6	19,5	20
6	1	1	4	21,0	35

Table M.3 — Sample 2

Plate	Shelf	Area	Layer	Time h	n_c
1	1	3	2	21,0	114
2	3	2	2	22,0	162
3	1	4	6	21,0	61
4	3	4	1	21,0	142
5	2	6	5	20,0	105
6	1	6	3	20,5	155

In the first sample the mean was 34,333 3, standard uncertainty 11,977 8, and relative standard uncertainty 11,977 8/34,333 3 = 0,348 9 and relative variance $0{,}348\ 9^2 = 0{,}121\ 7$.

In the second sample the mean was 123,166 7, the standard uncertainty 37,828 1 and the relative standard uncertainty 0,307 1 and relative variance $0{,}307\ 1^2 = 0{,}094\ 3$.

The estimates of relative standard uncertainty include the Poisson uncertainty, the personal uncertainty of counting, and the uncertainty of measuring the test portions. The variances ought to be cleared of these components first. The sampling fraction 6 × 10 ml/1 000 ml = 0,060 was small enough to be ignored.

The mean of the first sample was 34,333 3. The relative variance of an “infinite Poisson distribution” (Annex C) with that mean is 1/34,333 3 = 0,029 1.

Assuming the relative uncertainty of counting and the uncertainty of measuring the test portion both to be about 5 % (values from the quality assurance program of the laboratory), the value $0{,}05^2 = 0{,}002\ 5$ was subtracted two times.

The cleared estimate of the relative variance of the incubation effect based on the first sample was accordingly $0{,}348\ 9^2 - (0{,}029\ 1 + 0{,}002\ 5 + 0{,}002\ 5) = 0{,}121\ 7 - 0{,}034\ 1 = 0{,}087\ 6$.

Corresponding calculations in the second sample gave the estimate $0{,}307\ 1^2 - (0{,}008\ 1 + 0{,}002\ 5 + 0{,}002\ 5) = 0{,}094\ 3 - 0{,}013\ 1 = 0{,}081\ 2$.

The mean of the two relative variances was (0,087 6 + 0,081 2)/2 = 0,084 4. Its square root: 0,29 (29 %) is an estimate of the additional relative uncertainty (overdispersion) due to effects of incubation.

M.4 Example 2 — A most probable number method

M.4.1 Experimental design

Five series of incubation effects experiments were carried out by making six parallel determinations on five different water samples. The method used was a commercial procedure for *E. coli* based on the MPN principle. The trays were incubated in random positions as described in the foregoing. Some of the data (results of two series) are presented in Table M.4, with the random positions indicated. Time effects were not studied. All trays were removed from the incubator at the same time (18 h).

Area codes are based on L = left, M = middle, R = right, F = front, B = back. Stacks of 10 trays were permitted.

Symbols used:

n_+ is the number of positive wells;

n_{MP} is the MPN value;

T_0 is the lower 95 % confidence limit;

T_1 is the upper 95 % confidence limit.

Relative distribution variance:

$$u^2_{d,rel} = \left(\frac{\ln T_0 - \ln T_1}{3{,}92} \right)^2$$

(See Annex D).

Table M.4 — Results of incubation effects experiments

Sample	Tray	Shelf	Area	Layer	n_+	n_{MP}	T_0	T_1	ln n_{MP}	$u^2_{d,rel}$
A	1	2	RB	6	36	62,4	44,6	88,8	4,133 6	0,030 9
	2	1	LF	3	41	83,1	59,9	118,3	4,420 0	0,030 1
	3	2	RF	3	35	59,1	42,0	84,4	4,079 2	0,031 7
	4	2	MF	7	37	65,9	47,2	93,7	4,188 1	0,030 6
	5	2	LF	1	36	62,4	44,6	88,8	4,133 6	0,030 9
	6	1	RB	1	42	88,5	63,9	126,2	4,483 0	0,030 1
								s(ln n_{MP})	0,168 9	
								Mean		0,030 7
B	1	3	MF	5	37	65,9	47,2	93,7	4,188 1	0,030 6
	2	2	LF	4	39	73,8	53,1	104,8	4,301 4	0,030 1
	3	2	LF	9	42	88,5	63,9	126,2	4,483 0	0,030 1
	4	1	RF	8	27	38,4	26,4	56,6	3,648 1	0,037 9
	5	3	RB	1	37	65,9	47,2	93,7	4,188 1	0,030 6
	6	3	RF	9	32	50,4	35,4	75,2	3,920 0	0,036 9
								s(ln n_{MP})	0,295 5	
								Mean		0,032 7
n_+	is the number of positive wells (out of 51);									
n_{MP}	is the corresponding MPN value.									

M.4.2 Calculations

Follow steps a) to f).

a) Relative standard uncertainty of the six parallel MPN values of a sample was directly obtained by calculating the standard uncertainty of the MPN values in natural logarithmic scale. Sample A: standard uncertainty of ln n_{MP} = s(ln n_{MP}) = u_{rel} = 0,168 9, Sample B: standard uncertainty of ln n_{MP} = s(ln n_{MP}) = u_{rel} = 0,295 5.

NOTE If common logarithms are preferred, the relative standard uncertainty is obtained from 2,303 times the standard uncertainty of lg n_{MP} values. Sample A: standard uncertainty of lg n_{MP} = 0,073 4, u_{rel} = 2,303 × 0,073 4 = 0,169 0, Sample B: standard uncertainty of lg n_{MP} = 0,128 3, u_{rel} = 2,303 × 0,128 3 = 0,295 5.

b) Relative variance is obtained as the square of the relative standard uncertainty.

Sample A: $0{,}1689^2$ = 0,0285. Sample B: $0{,}2955^2$ = 0,0873.

c) The intrinsic distribution variance, $u^2_{d,rel}$ was obtained separately for each MPN estimate from the 95 % CI limits by the method based on natural logarithms, as described in Annex D. The mean values of the relative intrinsic variance were: Sample A mean $u^2_{d,rel}$ = **0,030 7** , Sample B mean $u^2_{d,rel}$ = **0,032 7** .

d) Subtraction of the mean distribution variance from the relative variance of the series gives an estimate of the variance of incubation effects. Sample A: $u^2_{rel,I}$ = 0,028 5 – 0,030 7 = **–0,002 2** , Sample B: $u^2_{rel,I}$ = 0,087 3 – 0,032 7 = **0,054 6** .

NOTE Theoretically, variance can never be negative. However, when an estimate of variance is obtained by subtraction and the experimental variances are based on small numbers of replicates, such things can happen.

e) The mean of the incubation effect variances is the general estimate of incubation effects. With the two samples the mean is (–0,002 2 + 0,054 6)/2 = 0,026 2.

f) The square root of the mean variance is the estimate of the standard uncertainty of added variation due to incubation conditions. It can also be expressed as a percentage. The square root of 0,026 2 is 0,162 ≈ 0,16 = 16 %.

With the great variation observed between the two series of samples, it is obvious that considerably more samples should be studied before a reliable estimate can be obtained.

NOTE It would be appropriate for the same kind of random allocation of incubation position and time to be practised in connection with the experiments organized for determination of so-called global uncertainty estimates.

Annex N
(informative)

Expression and use of measurement uncertainty

N.1 General

It is a requirement of ISO/IEC 17025[5] that laboratories determine the measurement uncertainty of the analytical result. The customer needs an uncertainty estimate together with the result to make correct decisions. Accreditors may want to see a value and how it was derived. The laboratory can use the information to improve its own analytical practices.

When working according to this International Standard, the laboratory should be able to obtain an estimate of the operational uncertainty, u_{O}, for every relevant method and sample type combination under intralaboratory reproducibility (intermediate precision) conditions. In the component approach, the uncertainty of measurement is expressed as relative standard uncertainty. In the global approach the value is likely to be expressed in the common logarithmic scale.

The interests of the laboratory and of the accreditors may be sufficiently served by providing the values of the operational uncertainty, and/or its components when available. The combined measurement uncertainty may also be of interest. Another possibility is that a customer might request an estimate of the expanded measurement uncertainty in the form of approximate 95 % confidence limits or limits of some other interval estimator.

According to ISO/IEC Guide 98-3:2008,[7] it is not inconsistent with other concepts of uncertainty of measurement to consider an uncertainty estimate characterizing the range of values within which the true value of a measurand might lie. Whereas this International Standard is concerned with (im)precision of the observed result, this other concept addresses the estimation, by the application of Bayes's theorem, of the range of possible population means, given the present observation.

N.2 Combined uncertainty of measurement

N.2.1 General

When requested by customers or accreditors, an estimate of the combined uncertainty of measurement of a test result is constructed from the test result, n_z, and the relative operational uncertainty, $u_{\mathrm{O,rel}}$.

N.2.2 Combined uncertainty of colony counts

The scale of measurement is chosen according to the intended use of the combined uncertainty:

a) interval scale:

$$u_{\mathrm{c}} = \sqrt{n_z + u_{\mathrm{O,rel}}^2 n_z^2} \tag{N.1}$$

where

$u_{\mathrm{O,rel}}$ is the relative operational uncertainty component;

n_z is the number of colonies observed.

b) relative and natural logarithmic scale:

$$u_{\mathrm{c,rel}} = \sqrt{\frac{1}{n_z} + u_{\mathrm{O,rel}}^2} \tag{N.2}$$

c) common logarithmic scale:

$$u_{c(lg)} = \sqrt{\frac{0{,}188\,6}{n_z} + u_{o(lg)}^2} \tag{N.3}$$

where $u_{o(lg)}$ is the operational uncertainty in common logarithms.

See also Annex C.

N.2.3 Combined uncertainty of confirmed colony counts

Relative and natural logarithmic scale:

$$u_{c,rel} = \sqrt{u_{o,rel}^2 + \frac{1}{n_c} + \frac{n_z - n_k}{n_z n_k}} \tag{N.4}$$

or

$$u_{c,rel} = \sqrt{u_{o,rel}^2 + \frac{1}{n_c} + \frac{(n_k + 0{,}5)(n_z - n_k + 0{,}5)n_z^2}{(n_z + 1)^2 (n_z + 2)n_k^2}} \tag{N.5}$$

where

n_c is the total number of presumed target colonies counted;

n_z is the total number of presumed target colonies isolated for confirmation;

n_k is the number of colonies confirmed.

Conversion of these estimates into arithmetic or common logarithmic scale is best performed afterwards.

Interval scale: $u_c = n_c u_{c,rel}$

Common logarithmic scale: $u_{c(lg)} = 0{,}434\,3 u_{c,rel}$

See also Annex E.

N.2.4 Combined uncertainty of most probable number counts

$$\begin{aligned} u_{c,rel} &= \sqrt{u_{o,rel}^2 + \left(\frac{\ln T_1 - \ln T_0}{3{,}92}\right)^2} \\ u_{c(lg)} &= \sqrt{u_{o(lg)}^2 + \left(\frac{\lg T_1 - \lg T_0}{3{,}92}\right)^2} \end{aligned} \tag{N.6}$$

where

T_1 is the upper 95 % confidence limit of the MPN value;

T_0 is the lower 95 % confidence limit of the MPN value;

$u_{o,rel}$ is the relative operational uncertainty,

$u_{o(lg)}$ is the same in common logarithms.

See also Annex D.

N.2.5 Combined interlaboratory uncertainty of measurement

Estimation and use of interlaboratory reproducibility estimates is not included in the scope of this International Standard. When considered relevant, estimates of interlaboratory reproducibility can be derived from the results of collaborative proficiency test data. The parameter estimated, the interlaboratory reproducibility standard uncertainty, s_R, is obtained from the analysis of identical reference samples in different laboratories.

A document under preparation, at the time of publication, by AFNOR (see Reference [9]) is reported to present a detailed protocol for utilization of quality control data for the estimation of different levels of uncertainty of measurement (repeatability, intralaboratory and interlaboratory reproducibility).

N.2.6 Expanded combined uncertainty of measurement

When requested, an expanded combined uncertainty, U, can be calculated by multiplying the combined uncertainty estimate by a coverage factor k. The value k = 2 gives approximate half-width of the 95 % interval estimator and the value k = 3 that of the 99 % interval estimator.

N.3 Interval estimators

N.3.1 General

The statistical distribution of microbial counts, whether parallel counts from one suspension or from replicate analyses, are asymmetrical (skewed) to varying degrees. The asymmetry increases with increasing operational uncertainty. Determination of the “exact” bounds of the CI requires a plausible model of the probability distribution of counts and application of computer programs. This facility is not provided by this International Standard. Two approximations that can easily be computed are presented. They are compared with the exact limits and the limits based on Bayes’s theorem in worked examples.

N.3.2 Exact confidence intervals

While it is never certain that the negative binomial distribution is a perfect model of microbial counts, it probably is the best available simple approximation for the purpose. “Exact” confidence limits can be computed by inserting estimates of the parameters (mean and operational uncertainty) into the appropriate statistical probability distribution. For instance, the 95 % confidence limits are obtained by observing the 2,5 % and 97,5 % points of the probability density function. Application of computer programs is necessary.

N.3.3 Approximation by symmetrical limits in interval scale

The interval estimates can be calculated without the use of computers. The limits are symmetrical around the observed value in interval scale. The assumption is that the observed count is an unbiased estimate of the mean. The limits are obtained from (see N.2.2)

$$T_0 = n_z - 2\sqrt{n_z + u_{\text{o,rel}}^2\, n_z^2}$$

$$T_1 = n_z + 2\sqrt{n_z + u_{\text{o,rel}}^2\, n_z^2} \qquad \text{(N.7)}$$

where

T_0 is the lower limit of interval estimator;

T_1 is the upper limit of interval estimator;

n_z is the observed number of colonies;

$u_{\text{o,rel}}$ is the relative operational uncertainty.

NOTE If the observed number of colonies consists of counts from parallel plates of the final suspension or from plates representing different dilutions, the calculations are based on the sum of colony counts. The interval estimates are obtained from

$$T_0 = \sum n_z - 2\sqrt{\sum n_z + u_{\text{o,rel}}^2 \left(\sum n_z\right)^2}$$
$$T_1 = \sum n_z + 2\sqrt{\sum n_z + u_{\text{o,rel}}^2 \left(\sum n_z\right)^2} \quad \text{(N.8)}$$

where Σn_z is the sum of colony counts.

If the sample should have been diluted to produce the final suspension, the result is multiplied by the dilution factor, F .

If the operational uncertainty is zero, the distribution reduces to the Poisson model. The CI can be calculated using the traditional equations:

$$T_0 = n_z - 2\sqrt{n_z}$$
$$T_1 = n_z + 2\sqrt{n_z} \quad \text{(N.9)}$$

where n_z is the number of colonies observed in the test portion from the final suspension.

With operational uncertainty less than about 0,1 (0,04 lg units) the Poisson distribution is likely to be a suitable model. However, it gives a negative lower limit with counts less than four. The operational uncertainty in water samples is seldom greater than 0,1 and is not expected to exceed 0,25 under any circumstances. Symmetrical limits in interval scale can be expected to be good approximations in water analysis. (See examples in N.4.)

N.3.4 Approximation by relative limits

Relative interval estimates may be appropriate when the operational uncertainty is greater than 0,25, especially in combination with high colony counts (see examples in N.4).

With combined uncertainty expressed in relative or natural logarithmic scale (N.2.3) the limits are:

Approximate upper interval estimate (95 %):

$$T_1 = n_z \exp\left(2u_{\text{c,rel}}\right) \quad \text{(N.10)}$$

where n_z is the final colony count.

Approximate lower interval estimate (95 %):

$$T_0 = \frac{n_z}{\exp\left(2u_{\text{c,rel}}\right)} \quad \text{(N.11)}$$

When the uncertainty is expressed in common logarithms the confidence limits are calculated as follows:

Approximate upper interval estimate (95 %):

$$T_1 = n_z \, 10^{2u_{c(lg)}} \tag{N.12}$$

Approximate lower interval estimate (95 %):

$$T_0 = \frac{n_z}{10^{2u_{c(lg)}}} \tag{N.13}$$

N.3.5 Bayesian probability calculation

CIs according to Bayes's theorem are estimated by the *a posteriori* probability calculations given a model of the probability distribution. This thinking is reported to be applied in a document under preparation, at the time of publication, by AFNOR (see Reference [9]). Tables are provided from which 95 % and 99 % CIs for negative binomial distributions with given means and values of operational uncertainty can be obtained directly or by linear interpolation.

N.4 Comparison of four interval approaches

The approximate 95 % intervals (coverage factor k = 2) evaluated by four methods were compared. Two methods based on probability distributions ("exact" and "Bayes"), given values of the mean and operational uncertainty, were employed. The results were compared with the approximate symmetrical and relative intervals based on the combined uncertainty of measurement.

Table N.1 —Interval estimates calculated by four different methods for colony counts 4, 10, 30, and 100

Count n_z	$u_c = \sqrt{n_z + u_{o(rel)}^2 n_z^2}$	$u_{c,rel} = \frac{u_c}{n_z}$	Exact[a]	Symmetrical[b]	Relative[c]	Bayes
4	2,040	0,509 9	0 to 8	0 to 8	1 to 11	1 to 9
10	3,317	0,331 7	3 to 17	3 to 17	5 to 19	5 to 18
30	6,245	0,208 2	18 to 43	18 to 42	20 to 45	20 to 45
100	14,142	0,141 4	73 to 129	72 to 128	75 to 133	78 to 131

NOTE Relative operational uncertainty $u_{o,rel}$ = 0,1 (10 %); estimates rounded to the nearest whole number.

[a] Derived from probability density function of the negative binomial distribution.

[b] Derived from $n_z \pm 2u_c$.

[c] Derived from $n_z \,/\, \exp\left[2u_{c(rel)}\right]$ and $n_z \exp\left[2u_{c(rel)}\right]$.

Table N.2 — Interval estimates by four different methods for colony counts 4, 10, 30, and 100

Count	u_c	$u_{c,rel}$	Exact	Symmetrical	Relative	Bayes
4	2,236	0,559 0	0 to 9	0 to 8	1 to 12	1 to 11
10	4,031	0,403 1	2 to 19	2 to 18	4 to 22	4 to 23
30	9,287	0,309 6	13 to 50	11 to 49	16 to 56	16 to 59
100	26,926	0,269 3	53 to 159	46 to 154	58 to 171	59 to 184

NOTE Relative operational uncertainty $u_{o,rel}$ = 0,25 (25 %); estimates rounded to the nearest whole number.

Table N.3 — Effect of the relative operational uncertainty on estimated intervals by four methods

$u_{o,rel}$	u_c	$u_{c,rel}$	Exact	Symmetrical	Relative	Bayes
0,00	5,477 2	0,182 6	19 to 41	19 to 41	21 to 43	20 to 42
0,05	5,678 9	0,189 3	18 to 42	19 to 41	21 to 44	20 to 43
0,10	6,245 0	0,208 2	18 to 43	18 to 42	20 to 45	20 to 45
0,20	8,124 0	0,270 8	15 to 47	14 to 46	17 to 52	17 to 53
0,30	10,535 7	0,351 2	12 to 53	9 to 51	15 to 61	15 to 68
0,40	13,190 9	0,439 7	8 to 60	4 to 56	12 to 72	12 to 96
0,50	15,968 7	0,532 3	6 to 68	0[a] to 62	10 to 87	9 to 155
0,60	18,815	0,627 2	4 to 76	0[a] to 68	9 to 105	7 to 337
NOTE Relative operational uncertainties ($u_{o,rel}$) from 0 to 0,60 (60 %); results rounded to the nearest whole number; colony count assumed n_z = 30 in all cases.						
a Lower limit negative.						

From Tables N.1 to N.3, it seems possible to conclude that for the whole practical range of counts and operational uncertainty values in water analysis, the symmetrical interval estimates are a suitable approximation. Relative interval estimates are usually preferable for samples with high subsampling variation or other operational causes.

N.5 Uses of the uncertainty of measurement

N.5.1 Reporting uncertainty

The reporting of an uncertainty estimate depends on the intended use of the test result. For customer needs, it shall be clearly stated how the uncertainty estimate was calculated and in what form it is presented (combined uncertainty, expanded uncertainty or interval estimate). Its scale of measurement, interval, relative, common logarithm, natural logarithm or percentage, shall always be given.

N.5.2 Comparisons using uncertainty

Comparison of two test results x_1 and x_2, having estimates of expanded uncertainty U_1 and U_2, is often done graphically. The results with line segments of expanded uncertainty extended vertically above and below the value are plotted side by side. If the line segments overlap, the results are considered not to differ significantly. This corresponds computationally to the comparison whether the absolute difference of x_1 and x_2 is greater or smaller than the sum $U_1 + U_2$, a method not perfectly comparable with a statistical test.

Comparison of the result x against an allowable value can be done in the same way. If the allowable value falls within the range $x \pm U$, the difference is not significant.

A more “scientific” way of comparing x with a is by calculating the z score.

$$z = \frac{|x - a|}{u_c(x)} \tag{N.14}$$

where

a is the allowable value;

$u_c(x)$ is the combined uncertainty of the result x.

If the value of the quotient is greater than 2, x is considered significantly different from a.

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