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Biotechnology — Cell counting —

Part 2: Experimental design and statistical analysis to quantify counting method performance

Biotechnologie — Dénombrement des cellules —

Partie 2: Conception expérimentale et analyse statistique pour quantifier les performances de la méthode de dénombrement

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 276, *Biotechnology.*

A list of all parts in the ISO 20391 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Cell counting impacts many aspects of biotechnology, from biomanufacturing to medical diagnosis and advanced therapy. The cell count can serve as an in-process quality control or be used in decision-making. Cell count is also an important parameter in many cell-based assays, including activity and potency, which are often normalized to the cell count to allow data comparison.

Cell count is generally expressed as a concentration and can reflect the total cell count of a cell population (total cell count) or subpopulation (differential cell count). Advances in instrumentation have resulted in a wide range of cell counting techniques/instruments for total and/or differential cell counts. In the absence of a readily available reference material or ground truth, the accuracy of a measurement method has been difficult to ascertain. This has been confounded by the complexity of the biological preparation (e.g. cell type, sources, preparation, etc.). Several standards that address sector/application-specific cell counting or the use of a specific measurement system exist (See ISO 20391-1 and Reference [16] for further information). Some of these methods use a comparability approach whereby the result from a newer cell counting test method is traced to the results obtained from a more established cell counting method. While the comparability approach allows the data from the second instrument to be benchmarked against those obtained from a primary (more established) instrument, it does not address the quality of either measurement process[17]. There remains a need to develop strategies to provide assurance for the quality of a cell counting measurement process in the absence of a reference material or reference method[17].

This document provides a method for evaluating aspects of the quality of a cell counting measurement process through the use of a dilution series experimental design. From this experimental design, a set of quality indicators are derived to assess the performance of a cell counting measurement process. Specifically, the quality indicators assess precision and proportionality of cell counting measurement processes. This approach is particularly useful when accuracy cannot be determined (i.e. in the absence of a traceable reference method or traceable reference material) and is also relevant in aspects of validating and monitoring the quality of cell counting measurement processes in general[17].

Information in this document is intended to provide confidence in the data produced by a chosen cell counting measurement process. This approach can be useful for selecting or optimizing a measurement process for a given cell preparation. This approach can also provide supporting performance parameters that can be utilized during performance qualification of a particular cell counting measurement process.

Biotechnology — Cell counting —

Part 2: Experimental design and statistical analysis to quantify counting method performance

1 Scope

This document provides a method for evaluating aspects of the quality of a cell counting measurement process for a specific cell preparation through a set of quality indicators derived from a dilution series experimental design and statistical analysis. The quality indicators are based on repeatability of the measurement and the degree to which the results conform to an ideal proportional response to dilution. This method is applicable to total, differential, direct and indirect cell counting measurement processes, provided that the measurement process meets the criteria of the experimental design (e.g. cells are suspended in a solution).

This method is most suitable during cell counting method development, optimization, validation, evaluation and/or verification of cell counting measurement processes.

This method is especially applicable in cases where an appropriate reference material to assess accuracy is not readily available. This method does not directly provide the accuracy of the cell count.

This method is primarily applicable to eukaryotic cells.

NOTE Several sector/application specific international and national standards for cell counting exist. Where applicable, consulting existing standards when operating within their scope can be helpful.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 20391-1, *Biotechnology — Cell counting — Part 1: General guidance on cell counting methods*

3 Terms, definitions, symbols and abbreviated terms

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1 Terms and definitions

3.1.1
accuracy
<measurement> closeness of agreement between a measured quantity value and a true quantity value of a measurand

[SOURCE: ISO/IEC Guide 99:2007, 2.13, modified — Notes deleted]

3.1.2
bias
<measurement> estimate of a systematic measurement error

[SOURCE: ISO/IEC Guide 99:2007, 2.18]

Note 1 to entry: Systematic measurement error is a component of measurement error that in replicate measurements remains constant or varies in a predictable manner. A reference quantity value for a systematic measurement error is a true quantity value, or a measured quantity value of a measurement standard of negligible measurement uncertainty, or a conventional quantity value.

Note 2 to entry: Also defined as the difference between the expectation of the test results and an accepted reference value (ISO 3534-1).

3.1.3
cell concentration
cell count per volume

Note 1 to entry: Typically used for cells in suspension (e.g. cell number per ml).

Note 2 to entry: Cell concentration can refer to the total cell count or the count of a specific subset of cells within the volume (e.g. viable cell number per ml).

3.1.4
cell count
discrete number of measured cells

Note 1 to entry: Cell count for cells in suspension is typically expressed as cell concentration.

3.1.5
cell counting
measurement process to determine the cell count

3.1.6
cell suspension
single cells or aggregates of cells dispersed in a liquid matrix

3.1.7
debris
<cell suspensions> fragments of cells and/or particles of biological or non-biological origin

3.1.8
differential cell count
cell count of a subset of cells, which have been distinguished from other cell subpopulations by at least one distinct cell attribute identified in the measurement

Note 1 to entry: The concentrations derived from a differential cell count can be expressed in absolute concentration or as a relative measure (i.e. percentage) with respect to the total cell number or another predefined population.

3.1.9
dilution fraction
ratio by which the concentration of solute in a solution has been reduced from an original concentration

Note 1 to entry: Dilution fraction can range from 0 to 1.

Note 2 to entry: Dilution fraction is also sometimes referred to as “dilution ratio” or “dilution factor”.

EXAMPLE The ratio by which the concentration of cells (solute) in a cell suspension (solution) has been reduced from a starting concentration of cells in suspension.

3.1.10
dilution series
group of solutions that have increasing or decreasing concentrations of the same substance

Note 1 to entry: A dilution series can be generated by serial dilution or by independent dilution.

Note 2 to entry: For a cell suspension, a dilution series is a group of suspensions that have increasing or decreasing concentrations of cells.

3.1.11
experimental design
process of planning a study to meet specified objectives

Note 1 to entry: Plan for assigning experimental conditions to participants and the statistical analysis associated with the plan. Typically, this includes a specification of the independent variables, dependent variables, number of participants and sampling strategy, procedure for assigning participants to experimental conditions, and order in which test tasks are given.

3.1.12
independent dilution
dilution series where each dilution is conducted independently of other dilutions

Note 1 to entry: Generally independent dilution series are generated directly from a common stock solution at a pre-specified (or target) dilution fraction.

3.1.13
intermediate precision
condition of measurement, out of a set of conditions that includes the same measurement procedure, same location, and replicate measurements on the same or similar objects over an extended period of time, but may include other conditions involving changes

Note 1 to entry: The changes can include new calibrations, calibrators, operators, and measuring.

Note 2 to entry: Operator bias refers specifically to error introduced by human operator experience.

[SOURCE: ISO/IEC Guide 99:2007, 2.22, modified — Note 3 deleted]

3.1.14
limit of quantitation
LOQ
<cell counting> lowest cell count in a sample that can be quantitatively determined with a suitable precision and accuracy using a specific analytical method

Note 1 to entry: The limit of quantification describes quantitative assay for low levels of cells in sample matrices.

3.1.15
linearity
within a given range, ability of an analytical procedure to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample

[SOURCE: Reference [14], modified.]

Note 1 to entry: In cell counting the concentration of analyte refers to the concentration of cells (total or differential) in the sample.

Note 2 to entry: When a set of measurements exhibits linearity over a range of a given input (while all other inputs and measurement conditions are held constant), the expected value of the measurand can be expressed as the sum of a constant bias term and the input parameter multiplied by a fixed constant.

3.1.16
measurand
quantity intended to be measured

[SOURCE: ISO/IEC Guide 99:2007, 2.3, modified — Notes and examples deleted.]

3.17
measured dilution fraction
dilution fraction verified by a traceable measurement

Note 1 to entry: For example, the volume of liquid can be verified by measuring the mass of the liquid (taking into density) using a calibrated and traceable scale with appropriate sensitivity.

3.1.18
measurement process
<cell counting> entire process for obtaining a cell count

Note 1 to entry: A measurement process can include sample preparation procedures, the measuring system, its settings (e.g. aperture choice, cell size gating, magnification, light exposure time etc.), and data analysis.

3.1.19
measurement precision
closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions

Note 1 to entry: Measurement precision is usually expressed numerically by measures of imprecision, such as standard deviation, variance, or coefficient of variation (CV) under the specified conditions of measurement.

Note 2 to entry: The 'specified conditions' can be, for example, repeatability conditions of measurement, intermediate precision conditions of measurement, or reproducibility conditions of measurement (see ISO 5725-1).

[SOURCE: ISO/IEC Guide 99:2007, 2.15, modified — Notes 3 and 4 deleted.]

3.1.20
proportionality
ability of an analytical procedure, irrespective of range, to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample

Note 1 to entry: In cell counting the concentration of analyte refers to the concentration of cells (total or differential) in the sample.

Note 2 to entry: A collection of measurements exhibit proportionality with respect to a given input parameter when the ratio of the expected value of the measurement to the value of the input parameter at which the measurements were taken remains constant as the value of the input parameter changes (while all other inputs and measurement conditions are held constant).

Note 3 to entry: When a set of measurements exhibits proportionality over a range of a given input, then, $Y = cX$ where Y, the expected value of the measurements is expressed as the input parameter (X) multiplied by a fixed constant (c), with no bias term.

3.1.21
proportionality constant
constant multiplier that directly relates the measurand to an input parameter

3.1.22
proportionality index
<cell counting> measure of deviation from proportionality for a dilution series experimental design

Note 1 to entry: The proportionality index (PI) is specific to the cell preparation and cell counting measurement process being evaluated.

3.1.23
***p*-value**
output of a statistical hypothesis test

Note 1 to entry: The *p*-value is obtained in the following manner: The distribution of the test statistic under the assumption that the null hypothesis is true, called the null distribution, is determined. The *p*-value is computed from the null distribution as the probability of observing a test statistic that is as or more extreme than the test statistic obtained from the actual data.

3.1.24
quantity
property of a phenomenon, body, or substance, where the property has a magnitude that can be expressed as a number and a reference

[SOURCE: ISO/IEC Guide 99:2007, 1.1, modified — Notes and example deleted.]

3.1.25
range
quantity interval bounded by rounded or approximate extreme indications

3.1.26
reference material
reference standard
material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in measurement or in examination of nominal properties

[SOURCE: ISO/IEC Guide 99:2007, 5.13, modified — Notes and examples deleted.]

3.1.27
reference method
reference measurement procedure
measurement procedure accepted as providing measurement results fit for their intended use in assessing measurement trueness of measured quantity values obtained from other measurement procedures for quantities of the same kind, in calibration, or in characterizing reference materials

[SOURCE: ISO/IEC Guide 99:2007, 2.7]

3.1.28
repeatability
precision of the results of measurement under defined conditions of measurement

Note 1 to entry: Repeatability can also be considered as the closeness of the agreement between results of successive measurements of the same measurand carried out under the same conditions of the measurement[17].

3.1.29
residual
<numerical analysis> numerical difference between the observed value of a dependent variable and the predicted value

3.1.30
sample
one or more parts taken from a system and intended to provide information on the system

Note 1 to entry: Often the sample serves as a basis for decision on the system or its production.

Note 2 to entry: For example, a smaller volume or aliquot of cell suspension taken from a larger volume of cell suspension[17].

[SOURCE: ISO 15198:2004, 3.22, modified — “population” replaced by “system”, Notes added.]

3.1.31
serial dilution
stepwise dilution of a substance in solution where the reduction of concentration is cumulative, lessening with each subsequent dilution

Note 1 to entry: In a serial dilution series, all dilutions except for the first are dependent on the preceding dilution.

3.1.32
stock cell solution
sufficiently stable (over time) cell suspension at sufficiently high concentration to allow dilution into working concentrations during experimentation

3.1.33
systematic error
component of measurement error that in replicate measurements remains constant or varies in a predictable manner

Note 1 to entry: A reference quantity value for a systematic measurement error is a true quantity value, or a measured quantity value of a measurement standard of negligible measurement uncertainty, or a conventional quantity value.

Note 2 to entry: Systematic measurement error, and its causes, can be known or unknown. A correction can be applied to compensate for a known systematic measurement error.

Note 3 to entry: Systematic measurement error equals measurement error minus random measurement error.

[SOURCE: ISO/IEC Guide 99:2007, 2.17]

3.1.34
target dilution fraction
dilution fraction that is trying to be achieved by diluting with a specified volume of solution

3.1.35
test sample
small aliquot of the sample that is prepared for measurement in the method of interest

Note 1 to entry: Generally, test samples are representative of the sample they are prepared from and are sometimes referred to as "representative test sample(s)".

3.1.36
total cell count
cell count of all cells, independent of the attribute(s) of the cell

3.1.37
true count
true quantity value
quantity value consistent with the definition of a quantity

Note 1 to entry: In the error approach to describing measurement, a true quantity value is considered unique and, in practice, unknowable. The uncertainty approach is to recognize that, owing to the inherently incomplete amount of detail in the definition of a quantity, there is not a single true quantity value but rather a set of true quantity values consistent with the definition. However, this set of values is, in principle and in practice, unknowable. Other approaches dispense altogether with the concept of true quantity value and rely on the concept of metrological compatibility of measurement results for assessing their validity.

Note 2 to entry: In the special case of a fundamental constant, the quantity is considered to have a single true quantity value.

Note 3 to entry: When the definitional uncertainty associated with the measurand is considered to be negligible compared to the other components of the measurement uncertainty, the measurand can be considered to have an "essentially unique" true quantity value. This is the approach taken by the ISO/IEC Guide 98-3 and associated documents, where the word "true" is considered to be redundant.

[SOURCE: ISO/IEC Guide 99:2007, 2.11, modified — "GUM" replaced by "ISO/IEC Guide 98-3".]

3.1.38
validation
confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled

[SOURCE: ISO 9000:2015, 3.8.13, modified — Notes deleted.]

3.1.39
variability
quantification of probability distribution function for variable, parameter, or condition

[SOURCE: ISO 16732-1:2012, 3.29]

3.2 List of abbreviated terms and symbols

List of abbreviations in order of citation.

Abbreviated term or symbol	Description
β	proportionality constant that can differ from k_{ideal}
β_1	scalar coefficient estimated from the proportional model fitting
CV	coefficient of variation
CV_{ij}	coefficient of variation for a set of K_{ij} repeated observations of representative test sample j, at target dilution fraction df_i
$\%\overline{CV}_{df_i}$	mean percent CV for a set of n_i representative test samples, with target dilution fractions df_i
c_{ideal}	ideal proportionality constant
tc_j	theoretical/true count of sample j
DF	dilution fraction
DF_j	dilution fraction of sample j controlled by the measurement process or determined experimentally
tDF	theoretical/true dilution fraction
$\boldsymbol{DF}$	set of unique target dilution fractions
$\boldsymbol{DF}_{\mathbf{measured}}$	set of measured dilution fractions
df_i	targeted dilution fraction
df_{ij}	measured dilution fraction
e	residual between data and modelled fit
$e^{smoothed}$	smoothed residual between processed cell count and proportional model fit
$e_i^{smoothed}$	smoothed residual when target dilution fraction is used in the analysis of proportionality (smoothed residual at each target DF)
$e_{ij}^{smoothed}$	smoothed residual when measured dilution fraction is used in the analysis of proportionality (smoothed residual for each representative test sample)
$E(oc_j)$	expected value of observed counts
i	index for target dilution fraction
j	index for replicate representative test sample
k	index for replicate measurement made on a representative test sample
K_{ij}	number of repeated measurements of the representative test sample
I	number of target dilution fractions
n_i	number of replicate representative test samples at the target dilution fraction

Abbreviated term or symbol	Description
PI	proportionality index
R^2	coefficient of determination
Y_{ijk}	observed value from measurement k of representative test sample j at target dilution fraction i
$\bar{Y}_{df_i}$	mean cell count for a set of n_i representative test samples, with target dilution fractions (df_i)
$\bar{Y}_{ij\cdot}$	mean over the set of K_{ij} repeated observations for the j^{th} representative test sample of df_i
$\bar{Y}_{\cdots}$	mean of $\bar{Y}_{ij\cdot}$ over independent representative test samples j for a set of n_i replicate representative test samples
$\lambda_{DF_k}^{\text{proportional}}$	estimated cell count at DF_k using β_1 obtained from proportional model fit $\lambda_i^{\text{proportional}}$
$\lambda_{ij}^{\text{proportional}}$	proportional model fit to $\bar{Y}_{ij}$ versus df_{ij}

4 Principle

4.1 General

Achieving high confidence in cell counting implies that the measurement is both accurate and precise[15]. For a well-controlled dilution fraction series, the concept of proportionality may be used as an internal reference and deviation from proportionality can serve as an alternative to the direct evaluation of accuracy[16]. Specifically, using experimental design and statistical analysis, quality indicators that describe deviation from proportionality and coefficient of variation (CV) can be evaluated to assess aspects of the quality of a cell counting measurement process.

The quality indicators evaluate the overall quality of a cell counting measurement process, where the measurement process includes sample preparation and handling, data acquisition, and data processing/correction.

Accuracy is ideally evaluated using a reference method and/or reference material with a known "true" value (see ISO 5725-1 and ISO 5725-2 for further information). In the absence of an appropriate reference material or reference method, the quality of a cell counting measurement can be indirectly assessed through its adherence to or deviation from the fundamental principle of proportionality, which implies that the measured cell count shall be proportional to the dilution fraction (DF) under ideal experimental conditions. Deviation from proportionality would indicate that a systematic measurement error has occurred to reduce the overall measurement confidence. This approach however does not directly provide the accuracy of the cell count.

The precision of a cell counting measurement indicates the closeness of agreement between cell counts obtained by replicate measurements on the same or similar cell preparation under specified conditions. Experimental data with low precision but with average cell counts fitting well to proportionality would reduce the quality of the measurement process. Importantly, low measurement precision (i.e. large random measurement error) can mask deviations from proportionality.

4.2 Proportionality

The theoretical true counts of samples extracted from a common, ideally homogenized, stock solution are related by their respective dilution fractions in accordance with the expression shown in Formula (1):

$$tc_j = c_{\text{ideal}} \times tDF_j \tag{1}$$

where

tc_j is the theoretical true count for the sample j;

c_{ideal} is an unknown proportionality constant equal to the theoretical true count for an undiluted sample;

tDF_j is the true dilution fraction for sample j.

By rigorously controlling the dilution fraction, the theoretical tDF_j may be approximated by DF_j. See Formula (2):

$$tDF_j \cong DF_j \tag{2}$$

where DF_j is the dilution fraction of sample j controlled by the measurement process or determined experimentally.

An uncalibrated, but otherwise ideal, measurement process would exhibit a proportional relationship between the expected value of observed counts, $E(oc_j)$, and the dilution fraction. That is, in the absence of systematic measurement errors, $E(oc_j)$ is given by Formula (3):

$$E\left(oc_j\right) = \beta \times DF_j \tag{3}$$

where β is a proportionality constant that can differ from c_{ideal}.

Combining Formula (1) and Formula (3) provides the basis for directly relating tc_j to $E(oc_j)$ through a constant; see Formula (4):

$$tc_j = \left(\frac{c_{\text{ideal}}}{\beta}\right) \times E\left(oc_j\right) \tag{4}$$

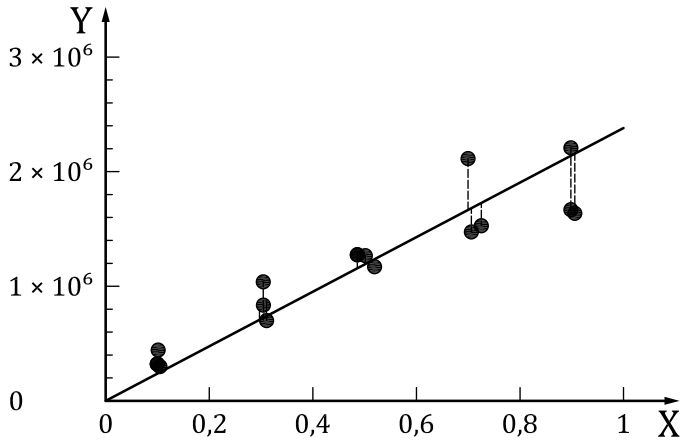
If β is known (e.g. through the use of a reference material) and $\beta \cong c_{\text{ideal}}$, then $E(oc_j) \cong tc_j$ (i.e. the accuracy of the observed counts could be established).

If β is not known, the closeness in agreement between the expected proportional relationship [Formula (3)] and the measured relationship may be used to assess the quality of a cell counting measurement process, since any deviation from the proportionality is indicative of the presence of measurement errors.

NOTE Measurement errors that scale proportionally with dilution will not result in significant changes to proportionality and therefore will not be detected in an analysis of deviation from proportionality.

4.3 Deviation from proportionality

Deviation from proportionality is assessed by summarizing the deviation of processed cell count data from a proportional model fit (see Figure 1).



Key

X dilution fraction

Y cell concentration (cells/ml)

● average cell count data across replicate observations

——— proportional model fit to the data

------- deviations or residuals (e) between the cell count data and the proportional fit

NOTE This is a schematic representation of a hypothetical study with five target dilution fractions and three test samples at each target dilution fraction.

Figure 1 — Schematic of a hypothetical cell counting results from a dilution series experimental design

Residuals (e) can occur as the result of systematic errors, random errors, or a combination thereof in the measurement process. As such, functions of residuals are sensitive to changes in both bias and precision.

A proportionality index will be calculated based on an analysis of smoothed residuals ($e_{smoothed}$) from the proportional model fit (see 6.4).

NOTE Evaluating deviation from proportionality via a hypothesis test (producing a p-value) in which the null hypothesis presumes the behaviour of expected counts to be proportional to dilution fraction and the alternative hypothesis is a more flexible (e.g. linear or quadratic) model p-value evaluation, is not considered here because high random measurement error will reduce the ability to detect statistically significant deviations from proportionality.

5 Experimental design

5.1 General

Cell counting method selection, considerations for performing a cell counting measurement, possible sources of uncertainty, as well as instrument qualification, method validation and reporting are described in ISO 20391-1.

The cell counting method selection, method validation and reporting shall be carried out in accordance with ISO 20391-1.

5.2 Considerations for the cell counting measurement process

The experimental design and statistical analysis methods described in this document may be used to evaluate the quality of cell counting measurement processes in which the test sample is in a suspension format.

This method may be applied to total, differential, direct and indirect cell counting measurement processes.

NOTE 1 If significant cell processing is required prior to counting, for example in the case of cells grown in aggregates that are individualized prior to counting, the processing steps to individualize cells can be considered a part of the cell counting measurement process. In this case the stock cell solution will contain the cell aggregates which are then diluted into the independent test samples. Each independent test sample can then undergo the processing steps to individualize cells prior to measurement. Challenges in this case include maintaining dilution integrity when samples contain aggregates.

NOTE 2 In the case that cells are embedded in a matrix or adhered to a surface, the process to bring the cells into a suspension format is not considered a part of the cell counting measurement process for the purpose of this document.

In the case of a measurement process where rare cell events or low numbers of cells will be counted relative to large background populations of cells, additional considerations beyond the experimental design and statistical analysis described in this document may apply.

5.3 Preparation of samples for the experimental design

5.3.1 General

The test material for the experimental design shall be cells in suspension. Cells can be in conditions that reflect the behaviour of the cells (i.e. single cells, cell aggregates, or cell agglomerates).

Sample preparation and handling procedures used to generate representative test samples for the cell counting measurement process should be optimized to maintain the properties of the test samples for counting.

Sample preparation procedures should avoid damaging cells in ways that change their ability to be counted or in ways that introduce/reduce debris that can interfere with or artificially improve the cell counting measurement process.

NOTE 1 References [18], [19], [20], [21] provide further guidance on sample preparation procedures for particular cell types and cell samples.

Sample preparation procedures should be conducted in an amount of time and under conditions that maintain the stability of the cells sample with respect to properties that can affect the cell counting measurement process. See ISO 20391-1 for further information.

NOTE 2 Samples containing live cells are dynamic and therefore can be unstable with regards to properties that can affect a cell count. Interactions such as cell-cell interactions and cell-material interactions can cause changes in the cell sample that can affect a cell count.

Samples for cell counting measurement processes resulting in a differential cell count (e.g. concentration of viable cells) will have some level of heterogeneity that can be affected by the dilution process. Sample preparation procedures should aim to generate test samples that are representative of the heterogeneity of the stock cell solution.

NOTE 3 For heterogenous cell samples, some cells can be more affected by conditions of the culture/sample environment than others, thus affecting representativeness of test samples.

5.3.2 Stock cell solution

A single stock cell solution should be used to generate all representative test samples. Additional stock cell solutions may be used if the concentration and composition of the stock cell solutions are nominally equivalent.

The cell concentration of the stock cell solution should be estimated using the same measurement process under evaluation.

In the use case where more than one measurement process is under evaluation (see Annex D), a single measurement process should be chosen to estimate the starting cell concentration in the stock cell solution and the measurement process used should be noted.

The concentration of the stock cell solution should be chosen to facilitate the generation of replicate representative test samples over the concentration range of intended use of the cell counting measurement process.

The quality indicators evaluated following the methods described in this document, are specific to the cell preparation (i.e. stock cell solution) investigated in the experimental design. The stock cell solution and representative test samples should therefore be similar to the test samples intended for the measurement process.

Similarity should be maintained with respect to attributes of the cell preparation that can affect the cell counting measurement process. For the purpose of evaluating the quality of a cell counting measurement process, attributes that should be similar between the representative test samples of this experimental design and the test samples intended for the measurement process can include the composition of the suspension medium, presence of debris, the degree of cell aggregation, the heterogeneity of the cell population, the optical properties of the cells, the morphology of the cells etc. (See Bibliography).

5.3.3 Dilution fraction experimental design

A generalized dilution fraction experimental design is illustrated in Figure 2. See Annex C and Annex D for more specific examples of dilution fraction experimental designs.

Let Y_{ijk} denote the value observed in the k^{th} replicate observation of the j^{th} replicate representative test sample for the i^{th} target dilution fraction from the common stock cell solution. That is, let I denote the number of unique target dilution fractions (i = 1, ..., I; each index referring to a unique target DF value between 0 and 1); let n_i denote the number of replicate representative test samples prepared for target dilution fraction i; and let K_{ij} denote the number of repeated measurements for the j^{th} replicate representative test sample for the i^{th} target dilution fraction.

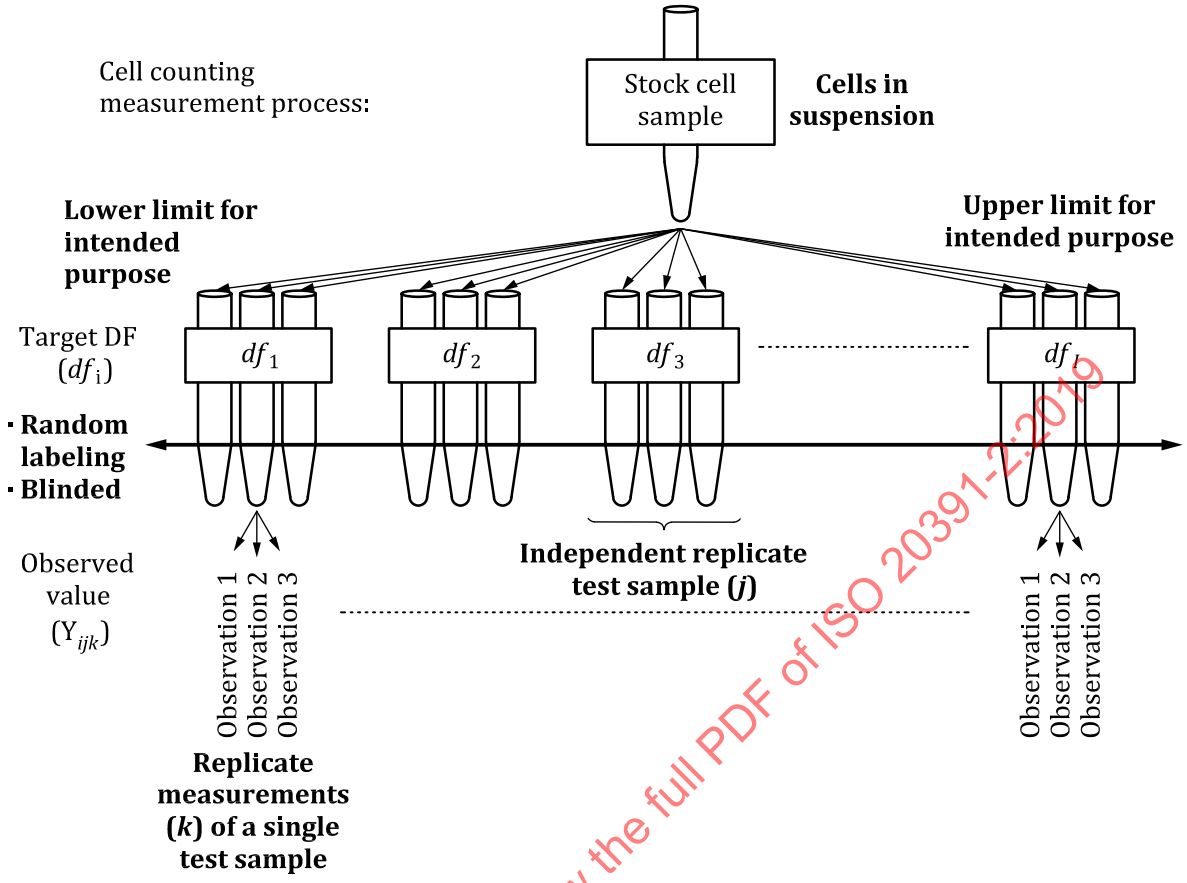
Representative test samples shall be generated over a set of unique target dilution fractions, denoted as, $\boldsymbol{DF} = \{df_i\}_{i=1}^{I}$ (e.g. $\boldsymbol{DF}$ = {0,1; 0,3; 0,5; 0,5; 0,7; 0,9}) from a stock cell solution such that the concentration of cells within the representative test samples fall within the concentration range of intended use of the cell counting measurement process. In general, a minimum of four unique df_i should be generated. df_i should be evenly spaced on a linear scale and represent the range of intended use. If another scale is selected for the spacing of dilution fractions, additional statistical considerations are necessary.

Replicate representative test samples shall be generated. In general, a minimum of three replicate representative test samples should be generated at each df_i (i.e. $n_i \geq 3$).

Replicate measurements shall be made. In general, a minimum of three replicate measurements should be made on each representative test sample (i.e. $K_{ij} \geq 3$) using the repeatability condition of measurement (i.e. the same measurement process, in the same laboratory, by the same operator using the same equipment and within short intervals of time.)

Selection of appropriate design (e.g. number of DFs, number of replicate representative test samples, number of measurements) should be made based on the stated purpose and considering the availability of the sample and stability of the representative test samples over the duration of the experimental time. An analysis of power (or power analysis) (i.e. a study's ability to detect the alternative hypothesis when it is true) can facilitate selection of an appropriate design[22]. An analysis of measurement uncertainty can also facilitate selection of an appropriate experimental design.

If sample availability and/or stability is a limiting factor, the experimental design should, as much as possible, firstly maintain the number of dilution fractions, then the number of replicate representative test samples, and finally the number of replicate observations.



NOTE This schematic depicts an experimental design starting from a single stock solution, where three replicate representative test samples (n_i = 3) are prepared for a set of I target dilution fractions (df_1, df_2, ..., to df_I) via independent dilution. Three replicate observations are to be made on each representative test sample (K_{ij} = 3).

Figure 2 — Schematic diagram depicting an example of a dilution fraction experimental design for cell counting measurement process

5.3.4 Considerations for generating dilution fractions

Diluent should be selected to maintain the stability of the representative test sample with respect to properties that can affect the cell counting measurement process over the course of the dilution series study.

Precautions should be taken to ensure dilution fractions are generated via a repeatable process.

The stock cell solution should be well mixed to generate homogeneous cell suspensions before preparing subsequent dilutions. Dilution of the cells should be carried out in a way that minimizes variability due to the dilution process.

NOTE 1 Reducing the number of operations for dilution can minimize the variability due to the dilution process.

The dilution process should avoid generating debris and/or damaging cells.

Each dilution fraction should be generated independently from the stock solution to avoid compounding errors associated with serial dilution. Serial dilution may be used if the serial dilution process is well understood and the dilution fractions are within pre-determined specifications or can be corrected through appropriate modelling.

Ideally, dilution fraction is known and is equivalent to df_i. In practice, the true dilution fraction can deviate from the target dilution fraction in random or systematic ways, compromising dilution integrity. Dilution integrity can be compromised due to pipetting error, inhomogeneity in the cell suspension, loss of cells to the vessel/pipette tip, instability of the cells over time, and compounding error in serial dilution. The process for generating dilution fractions should take into consideration the effects on dilution integrity.

NOTE 2 Further general guidance on use of pipettes can be obtained from literature see Reference [10] and ISO 8655-1, ISO 8655-2, and ISO 8655-6.

Contributions of pipetting error to dilution integrity shall be addressed.

NOTE 3 In the context of this document, pipetting error specifically refers to error introduced by inaccurate assumptions about the volumes of suspension pipetted in the preparation of a representative test sample. Errors due to improper mixing or sample handling are not included in pipetting error.

Contributions of pipetting error to dilution integrity should be assessed through an independent study prior to execution of the experimental design (pre-evaluation). Example procedures to pre-evaluate pipetting error contribution to dilution integrity are available in A.2.

When pipetting error contribution to dilution integrity is assessed through pre-evaluation, the user shall define acceptance criteria for pipetting error contribution to dilution integrity and demonstrate that the acceptance criteria have been met (see A.2). When user-defined acceptance criteria for pipetting error is met, then the target DF (df_i) should be used in the calculation of *PI* following statistical analysis methods described in 6.6.

NOTE 4 In some cases, it can be desirable to account for pipetting error contribution to DF for each test sample as they are generated during execution of the dilution series study. Examples include if there is limited test sample available for pre-evaluation, or if pipetting error is expected to be higher than desirable for generating dilution fractions.

When user-defined acceptance criteria for pipetting error contribution to dilution integrity cannot be achieved, or when the user chooses not to pre-evaluate pipetting error, the pipetting error contribution to dilution integrity shall be accounted for by obtaining a measured DF for each independent representative test sample as test samples are generated (see A.3). In this case, the set of measured dilution fractions, denoted as $\boldsymbol{DF}_{\mathbf{measured}} = \{df_{ij}\}_{ij}$ (e.g. $\boldsymbol{DF}_{\mathbf{measured}}$ = {0,104; 0,995; 0,102; 0,310; 0,294; 0,301; etc.} is used in the calculation of *PI* following modified statistical analysis methods. (See Annex B and Annex C).

5.4 Test sample labelling

Representative test samples shall be labelled with sufficient information to ensure traceability to the stock solution and the dilution fraction of the test sample.

In the case where the cell counting measurement process involves manual methods for obtaining cell counts (e.g. manual cell counting using a haemocytometer) and/or manual methods for identifying cells/cell populations (e.g. manual gating in flow cytometry methods), representative test samples should be labelled in a way to ensure that operators are blinded to the sample dilution and concentration during measurement and/or analysis to reduce the influence of operator bias.

5.5 Measurement of the test sample

Measurement of the representative test samples should be conducted in an amount of time and under conditions that maintain the stability of the representative test sample with respect to properties that can affect the cell counting measurement process over the course of the dilution series study.

Measurement samples should be prepared from each representative test sample according to the procedures established for the cell counting measurement process under evaluation. See ISO 20391-1 for considerations in preparing samples for measurement.

Representative test samples should be well mixed before the preparation of each measurement sample.

Measurements should be conducted following procedures established for the cell counting measurement process under evaluation. The cell counting measurement process includes sample preparation (e.g. staining, lysing, disaggregation), sample handling (e.g. pipetting, mixing), data acquisition (e.g. instrument settings, sample loading) and data processing/correction (e.g. coincidence correction, gating, image analysis parameter settings). See ISO 20391-1 for further information.

Measurements shall be conducted in a way to reduce systematic temporal effects.

NOTE Randomizing the order in which samples are measured can reduce the sensitivity of *PI* and other metrics of the cell counting measurement process quality, to systematic temporal effects.

The time elapsed between preparation of the final representative test sample and the time at which each observation was made should be documented to monitor for unexpected temporal effects during the course of the dilution series study.

6 Statistical methods

6.1 General

A generalized data analysis flow diagram is illustrated in Figure 3.

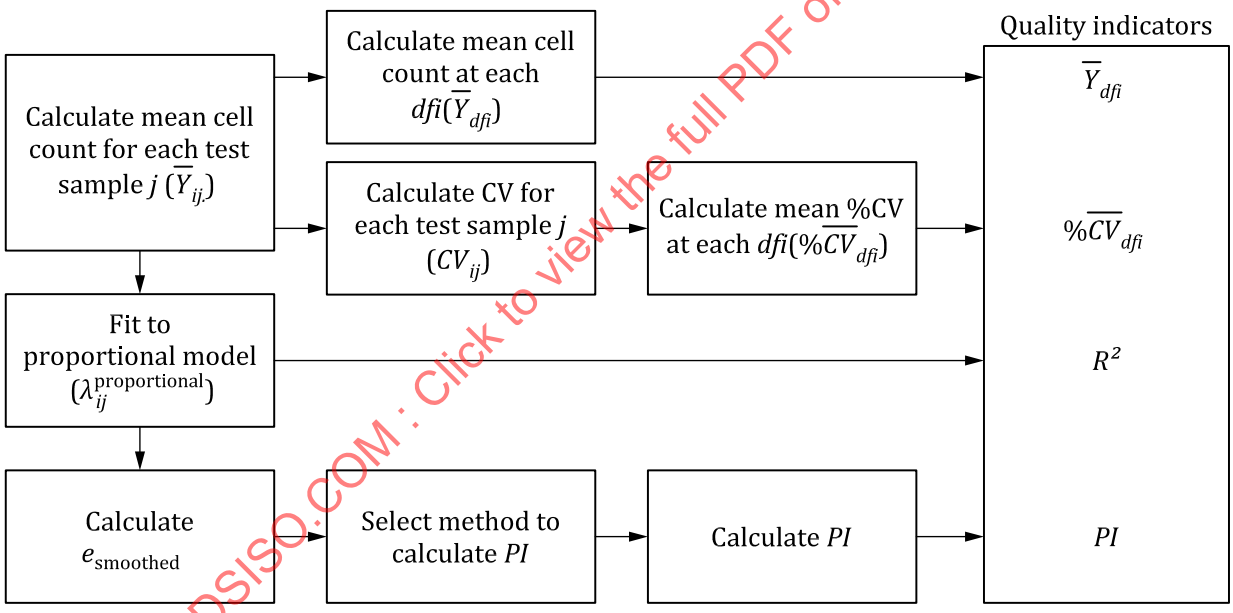


Figure 3 — Diagram illustrating analysis process for calculating quality indicators based on the dilution series experimental design

6.2 Mean cell count

Mean cell count ($\overline{Y}_{df_i}$) for a set of representative test samples (indexed by j = 1, 2, ..., n_i) with target dilution fraction df_i is given by Formula (5):

$$\overline{Y}_{df_i} = \frac{\Sigma_{j-1}^{n} \overline{Y}_{ij.}}{n_i} \tag{5}$$

where $\overline{Y}_{ij.}$ denotes the mean over the set of K_{ij} repeated observations for the j^{th} representative test sample of df_i and is given by Formula (6):

$$\overline{Y}_{ij.} = \frac{\Sigma_{k=1}^{K_{ij}} Y_{ijk}}{K_{ij}} \tag{6}$$

6.3 Measurement precision

Measurement precision shall be evaluated using coefficient of variation (CV) of repeatability over replicate observations, expressed as a percentage ($\%CV$).

For a set of K_{ij} repeated observations of representative test sample j, at target dilution fraction df_i the coefficient of variation (CV) is given by Formula (7):

$$CV_{ij} = \frac{\text{standard deviation}}{\text{mean}} = \frac{\sqrt{\frac{1}{K_{ij}-1}\sum_{k=1}^{K_{ij}}\left(Y_{ijk}-\overline{Y}_{ij.}\right)^2}}{\overline{Y}_{ij.}} \tag{7}$$

Mean percent $CV\left(\%\overline{CV}_{df_i}\right)$ for a set of n_i representative test samples, with target dilution fractions df_i is given by Formula (8):

$$\%CV_{df_i} = 100 \times \frac{\Sigma_{j=1}^{n_i} CV_{ij}}{n_i} \tag{8}$$

where CV_{ij} is given by Formula (7) and denotes the coefficient of variation of representative test sample j with target dilution fraction df_i.

6.4 Proportional model fit

A proportional model shall be fit to $\overline{Y}_{ij}$ versus df_{ij} using Formula (9):

$$\overline{Y}_{ij} = \beta_1 \times df_{ij} + \in_{ij} \tag{9}$$

where

β_1 is a scalar coefficient estimated from the model fitting;

$\in_{ij}$ represents the deviation of $\overline{Y}_{ij}$ from the proportional trend.

The dilution fraction df_{ij} is assumed to be the target dilution fraction df_i if dilution integrity was pre-evaluated and fell within user defined specifications for dilution integrity (see A.1.), otherwise a

measured dilution fraction (df_{ij}) for each representative test sample j at each target dilution fraction i is used in the proportional model fit.

An appropriate assumption for modelling the mean-to-variance relationship for the proportional model fit shall be selected.

The proportionality of observed counts to dilution fraction shall be assessed using a weighted least squares modelling approach with the assumed mean-variance relationship.

NOTE Cell counts would ideally be modelled using a Poisson assumption for the mean-variance relationship (variance is equal to the mean) instead of a constant mean-variance relationship (ordinary least squares). However, in biological studies, count data are often "over dispersed. In these cases, assumption of a Poisson distribution can produce models that underestimate the variability present in the data. Other relationships between the expected average count and the expected variance of counts can be considered. For example, a quasi-Poisson mean-variance relationship (i.e. the variability of repeated observations at a fixed dilution fraction is proportional (rather than equal) to the expected observation at a given dilution fraction) or a concave mean-variance relationship (i.e. the variability of repeated observations at a fixed dilution fraction is larger at low and high dilutions and smaller at intermediate dilutions) can be considered. A goodness-of-fit test can be used to support the choice of a specific mean to variance relationship.

For each sample, the predicted cell count from the proportional model shall be computed as Formula (10):

$$\lambda_{ij}^{\text{proportional}} = \beta_1 \times df_{ij} \tag{10}$$

6.5 Coefficient of determination

Coefficient of determination (R^2) is a number which is widely used in the field of data analysis and experiment design as an indicator that describes how well a model fits a set of observations.

R^2 value shall be calculated for the proportional model fit.

R^2 for the proportional model fit is given by Formula (11):

$$R^2 = 1 - \frac{\Sigma_i \Sigma_j \left(\overline{Y}_{ij} - \lambda_{ij}^{\text{proportional}}\right)^2}{\Sigma_i \Sigma_j \left(\overline{Y}_{ij} - \overline{Y}_{...}\right)^2} \tag{11}$$

where $\overline{Y}_{...}$ is the mean of $\overline{Y}_{ij}$ over independent representative test samples j for a set of n_i replicate representative test samples across a set of target dilution fractions (***DF***) given by Formula (12):

$$\overline{Y}_{...} = \frac{\Sigma_i \Sigma_j Y_{ij.}}{\Sigma_i n_i} \tag{12}$$

6.6 Proportionality index (*PI*)

6.6.1 General

A proportionality index (*PI*) shall be calculated based on an analysis of smoothed residuals (e_{smoothed}) from the proportional model fit.

NOTE 1 Evaluations based on non-smoothed residuals can be an appropriate indicator of how close individual cell count measurements are to their respective ideal value as opposed to systematic error in deviation from proportionality. Even with a "smoothing" step, deviations from the ideal measurement behaviour result from an unknown mixture of random variability and systematic error; however, the evaluation of smoothed residuals, rather than individual residuals, reduces the influence of random error, providing a clearer view of the systematic error in deviation from proportionality.

NOTE 2 *PI* is a value that is specific to the cell counting measurement process in question, including the cell preparation, measuring system and the selected settings and data corrections, data processing parameter, and sampling and measurement procedures, as well as the experimental design and statistical analysis method used in the derivation of *PI* (e.g. number of observations and the dilution fractions investigated).

NOTE 3 *PI* calculations rely heavily on an accurate dilution fraction [assumption stated in Formula (2)]. Unaccounted random or systematic error in dilution fractions will increase the variability in *PI*.

6.6.2 Calculation of the smoothed residual (e_{smoothed})

When a user-defined criterion for dilution integrity is met, the mean cell count ($\overline{Y}_{df_i}$) over the set of n_i representative test samples with target dilution fractions df_i is used to calculate e_i^{smoothed} at each df_i as Formula (13):

$$e_i^{\text{smoothed}} = Y_{df_i} - \lambda_{df_i}^{\text{proportional}} \tag{13}$$

where $\lambda_{df_i}^{\text{proportional}}$ is the estimated cell quantity at df_i and is calculated using β_1 from Formula (10) as described in Formula (14):

$$\lambda_{df_i}^{\text{proportional}} = \beta_1 \times df_i \tag{14}$$

In the case where user-defined criteria for dilution integrity cannot be met by improving pipetting technique, a measured dilution fraction may be obtained at the time of independent replicate sample preparation. The measured dilution fraction (df_{ij}) can then be used to calculate a smoothed residual at each representative test sample (e_{ij}^{smoothed}) according to Annex B.

6.6.3 Calculation of proportionality index (*PI*)

The user shall calculate a proportionality index (*PI*) based on smoothed residuals (either e_i^{smoothed} or e_{ij}^{smoothed}).

The user shall select an approach for calculating *PI* based on the intended use of the cell counting measurement.

Table 1 provides examples of approaches for calculating *PI*.

NOTE *PI* can be computed following a number of approaches, with each approach placing a different emphasis on the influence of each smoothed residual.

Table 1 — Approaches for evaluating the proportionality index (*PI*) using a smoothed residual based method[a]

Approach for calculating *PI*	Description of effect on proportionality index (*PI*)
R^2	R^2 (or the coefficient of determination), a residual based method to assess how closely predicted values from a statistical model follows the observed values can be an appropriate method for calculating *PI* when variance is equal across measurements. (*PI* based on R^2 ranges from 0 to 1).
Scaled	Penalizes on a percentage scale rather than absolute scale. This approach will evenly weight the contributions to *PI* across the dilution fractions

[a] This is not an exhaustive list; other methods to calculate *PI* based on smoothed residuals are also be applicable.

Table 1 *(continued)*

Approach for calculating *PI*	Description of effect on proportionality index (*PI*)
Unscaled	Penalizes on an absolute scale rather than a percentage scale. In this approach, *PI* will be most influenced by observations from large (i.e. highly concentrated) dilution fractions.
Absolute value	Total length of absolute differences (either scaled or unscaled) between the proportional and flexible model. Following scaling, residual fragments count equally.
Sum of squares	Total of squared differences (either scaled or unscaled) between the proportional and flexible models. Squaring the model residuals assigns greater influence to the larger residuals.
[a] This is not an exhaustive list; other methods to calculate *PI* based on smoothed residuals are also be applicable.	

A combination of approaches can be applied to select a *PI* calculation that is fit for the intended application.

NOTE As an example, the sum of the absolute value of each smoothed residual scaled by the corresponding predicted count from the proportional model [i.e. sum of the absolute value of scaled smoothed residuals (PI_{AbsSSR}, see C.1) can be used to more evenly weigh the contributions of the residual summary statistic across dilution fractions. Otherwise, the behaviour at nearly undiluted samples tends to dominate the *PI* characterization. In addition, the use of the "absolute value of residuals" as opposed to the "squared value of residuals" reduces the influence of outliers and weighs each fragment of a residual equally in the summary statistics (i.e. if one scaled residual is twice as large as another, its contribution to *PI* will be twice as much).

See Annex C for examples of formulae that can be used to calculate *PI*.

6.7 Additional statistical analysis and quality metrics

Additional statistical analysis approaches that are fit for the intended purpose may be applied to the cell counting data collected from the experimental design and used to generate additional metrics that are useful in evaluating the quality of the cell counting measurement process (e.g. linearity, intermediate precision, %*CV* across replicate representative test samples, etc.). When additional metrics are reported, these shall be defined and the methods used to generate them shall be reported.

6.8 Data interpretation

6.8.1 General

The quality indicators derived from the experimental design and statistical analysis described in this document evaluate the entire cell counting measurement process including the procedures used in sample preparation, sample handling, data acquisition, and data processing/correction. Quality indicators are specific to the measurement process and cell sample investigated.

6.8.2 Interpretation of %*CV*

Lower %*CV* values indicate a more precise cell counting measurement.

A *p*-value or confidence interval can be used to compare *CV* between different cell count measurement processes.

6.8.3 Interpretation of R^2

R^2 ranges from 0 to 1 and larger R^2 means a better fit of the model to the observations. Larger R^2 indicates that the predicted values from the model capture a greater proportion of the variation in the observed data. Evaluation of R^2 is dependent on the random variability of the data. Therefore, the R^2 value does not distinguish the source of deviations from the proportional model as arising from random variability (imprecision in the data) or systematic disproportionality. Evaluation of R^2 on raw residuals (i.e. not smoothed) characterizes performance based on how close individual measurements are to their respective ideal value as opposed to systematic error in deviation from proportionality.

Requirements should be specified for R^2 based on the intended use of the measurement.

NOTE The general interpretation that $R^2 > 0{,}95$ is a meaningful requirement is not relevant for this analysis.

6.8.4 Interpretation of *PI* values

PI values indicate the deviation from proportionality of a cell counting measurement process. *PI* may be used to assess, in part, the quality of a cell counting measurement process, since any deviation from the proportionality is indicative of the presence of measurement errors, random and/or systematic. For example, poor sensitivity of the cell counting measurement process or using the cell counting measurement process beyond the limits of quantitation can result in measurement errors that reduce *PI*.

Requirements should be specified for *PI* based on the intended use of the measurement.

PI is most sensitive to measurement errors that do not scale with dilution. Measurement errors that scale proportionally with dilution will not result in significant changes to *PI* and should be evaluated separately.

Based on the method used to calculate *PI*, a higher *PI* value can indicate more or less deviation from proportionality. For example, in the case of using *PI* based on R^2 of smoothed residuals (i.e. PI_{R^2SR}) a value closer to 0 indicates greater deviation from proportionality, and less measurement quality.

NOTE *PI* values are not appropriate for evaluating the general performance of a cell counting device.

6.8.5 Comparison of *PI* values

Comparison of *PI* values is valid in the case that the same dilution series experimental design (including nominally similar stock cell concentration) and the same statistical analysis method is used.

PI values can be compared to evaluate differences in deviation from proportionality between cell counting measurement processes, between cell types, and over time.

Three or more evaluations of *PI* under similar dilution series experimental conditions can be conducted to generate an average *PI* with confidence intervals to facilitate comparison of *PI* values across counting measurement processes.

Alternatively, to assess the significance of the differences between *PI* values across cell counting measurement processes when a single evaluation of *PI* is performed, a non-parametric bootstrap analysis method may be applied to generate confidence intervals for the *PI* values.

If a non-parametric bootstrap analysis is performed, the precondition of analysis should be clarified, including for example the number of bootstrap iterations, and confidence level, before performing the analysis and conditions of the non-parametric bootstrap simulation should be reported (e.g. number of bootstrap iterations and confidence level).

NOTE 1 Non-parametric bootstrap analysis is applicable when the precondition of simulation and the executed analysis are consistent.

NOTE 2 A large number of *PI* values determined from repeated *PI* measurements can be used to identify trends and/or outliers for the cell counting measurement process.

7 Reporting

7.1 Reporting of quality indicators

Cell count shall be expressed in terms of cell concentration as cells/ml.

Additional expressions of cell count (e.g. cells/cm^2 or cells/mg microcarrier) may be reported depending on the intended use of the cell count measurement.

The report shall contain the following quality indicators:

a) $\bar{Y}_{df_i}$ for each df_i;

b) $\%\overline{CV}_{df_i}$ for each df_i;

c) R^2 for the proportional model fit;

d) *PI.*

7.2 Documentation of experimental design parameters and statistical analysis method

7.2.1 The report shall contain information on the experimental design and statistical analysis to enable independent assessment by entities who review the data, but were not participating in the experimental design and/or statistical analysis, including the following.

a) Description of the cell counting measurement system(s) investigated including:

   1) cell type(s) used in the investigation;

   2) cell counting method(s) evaluated;

   3) concentration range(s) investigated;

b) dilution fraction experimental design elements including:

   1) the set of unique target dilution fractions ($\boldsymbol{DF} = \{df_i\}_{i=1}^{I}$);

   2) number of replicate representative test samples (n_i);

   3) number of replicate observations (K_{ij});

c) method for evaluating pipetting error contributions to dilution integrity (e.g. pre-evaluation as described in A.1., or gravimetrically obtained measured dilution fraction during the evaluation of *PI* as described in A.2); when the pre-evaluation of pipetting error contribution to dilution integrity is conducted, the user-defined acceptance criteria for pipetting error contributions to dilution integrity shall be reported;

d) the assumed mean-variance relationship used in the proportional model fit;

e) formula for the proportional model fit [Formula (10)];

f) method for calculating *PI.*

7.2.2 Additional reporting elements on the experimental design and statistical analysis may also include information on the following items:

a) stock cell solution – stock cell concentration and method for estimating the stock cell concentration;

b) experimental design – process used to obtain dilutions (i.e. mixing steps, diluent, independent or serial dilution) including time elapsed during representative test sample preparation;

c) time each cell counting measurement was made and/or time elapsed between sample preparation and measurement;

d) statistical analysis procedures – additional metrics, definition of additional metrics, justification for modelling choices, bootstrap simulation parameters;

e) unexpected observations made during the execution of the experimental design and statistical analysis.

7.3 Additional reporting elements on the cell counting measurement process

Additional reporting elements on the cell counting measurement process may include information on the following items:

a) cells – type, passage number or population doubling, lot number, source;

b) reagents – name, source, lot number, country of origin, animal or human derived;

c) measurement process for the cell counting method(s) – including instrument, instrument settings, sample preparation, data manipulation.

See Annex D and Annex E for use case examples for evaluating the quality of cell counting measurement process and associated reporting elements.

Annex A
(informative)

Method to assess pipetting error contributions to dilution integrity

A.1 General

In this annex, dilution integrity specifically refers to the contribution of pipetting error to dilution integrity and does not address other aspects of dilution integrity. This annex provides example procedures for evaluating dilution integrity (with respect to pipetting error) specifically for this document.

A.2 Example procedure for pre-evaluating pipetting error contributions to dilution integrity using a calibrated scale to obtain accurate volume estimates upon pipetting

A.2.1 The example procedure outlined for pre-evaluating dilution integrity can also be used as a tool to improve dilution integrity such that the final pipetting procedure selected for execution of the experimental design described in this document meets user-defined specifications. Additionally, the procedure outlined for pre-evaluating dilution integrity can also be used as a training tool for operators prior to executing procedures specified in this document.

An example procedure for pre-evaluating pipetting error contributions to dilution integrity is as follows.

A.2.2 Follow the dilution scheme that will be used in the experimental design to generate independent representative test samples at each target dilution fraction (df_i) for the calculation of *PI* (including replication).

Dilution should be conducted by the same operator, using the same pipettes/type of pipette tips, and using similar pipetting procedures that will be used during the evaluation of *PI*.

The sample to be diluted can be one of the following:

a) a cell suspension similar to the cell suspension that is intended to be evaluated for *PI*;

b) a suspension medium similar to that in the cell suspension intended to be evaluated for *PI.*

It is not recommended to use water for the sample to be diluted, as pipetting error associated with water is generally lower than pipetting error associated with pipetting cell suspensions.

The diluent should be similar to the diluent that will be used during the evaluation of *PI.*

During the dilution, measure the mass of the suspensions being pipetted, $m_{1_{ij}}$ and $m_{2_{ij}}$, where $m_{1_{ij}}$ is the mass of the sample to be diluted and $m_{2_{ij}}$ is the mass of the diluent being pipetted to generate the test sample for target dilution fraction df_i.

A calibrated scale with sufficient sensitivity to measure the mass of the pipetted suspension should be selected. If an appropriate scale is not available, an alternative method may be used to obtain verified sample volumes.

A calibrated scale with sensitivity of 0,000 1g can for example be used to obtain mass measurements for sample volumes as low as 0,01 ml to be accurately measured (see ISO 8655-2 for additional information).

Assuming the density of sample to be diluted and the diluting medium is approximately equivalent, pre-evaluated *DF* ($df_{ij}^{\text{pre-evaluated}}$) for each representative test sample can be calculated as Formula (A.1):

$$df_{ij}^{\text{pre-evaluated}} = \frac{m_{1_{ij}}}{m_{1_{ij}} + m_{2_{ij}}} \quad \text{(A.1)}$$

In some cases, it will not be reasonable to assume that the sample to be diluted and the diluting medium have approximately equivalent densities. In these instances, the respective densities of each solution should be considered in the calculation of $df_{ij}^{\text{pre-evaluated}}$.

A.2.3 Calculate the pre-evaluated integrity of dilution.

Plot $df_{ij}^{\text{pre-evaluated}}$ vs df_i and fit a line constrained to pass through the origin at (0,0), i.e. a proportional model fit [Formula (A.2)]:

$$df_{ij}^{\text{pre-evaluated}} = \beta^{\text{pipetting}} df_i + \in_{ij} \quad \text{(A.2)}$$

where

$\beta^{\text{pipetting}}$ is a constant;

$\in_{ij}$ represents the deviation of $df_{ij}^{\text{pre-evaluated}}$ from the proportional trend.

In an ideal case $\beta^{\text{pipetting}}$ would be equal to 1.

Calculate a coefficient of determination for pipetting error contribution to dilution integrity (R^2_{Dilution}) (see Table A.1).

If R^2_{Dilution} is greater than or equal to a pre-specified user-defined criterion (based on the intended use), then dilution integrity is sufficient, and df_i may be used in the evaluation of *PI*.

Generally, criterion for acceptable pipetting error contribution to dilution integrity should be at least $R^2_{\text{Dilution}} \geq 0{,}98$.

To maintain dilution integrity at low dilution fraction, multi-step dilution processes may be utilized. For example, a larger volume sample from the stock cell solution can be obtained then serially diluted to reach the desired target dilution fraction. Error introduced by each dilution step should be taken into account when calculating dilution integrity.

The specification of R^2 is dependent on the intended use of the cell counting measurement. Allowing lower R^2 for dilution integrity will result in reduced sensitivity for detecting deviation from proportionality in the cell counting measurement process.

If R^2_{Dilution} is less than a pre-specified user-defined criterion, then dilution integrity is not sufficient. In this case, liquid handling procedures should be improved and re-evaluated. In the case that liquid handling procedures cannot be further improved, and the user-defined criteria are still not met, a measured DF (df_{ij}) can be obtained during the evaluation of *PI* and used in the calculation of *PI*, to satisfy the requirements for dilution integrity (see A.2).

Table A.1 — Example table for data collection and evaluation of dilution integrity

Target DF	Target cell suspension volume ml	Target diluent volume ml	Replicate sample	Mass of pipetted sample m_1 g	Mass of pipetted diluent m_2 g	$df_{ij}^{\text{pre-evaluated}}$	$\beta_{\text{pipetting}}$	Dilution integrity R^2_{Dilution}
0,3	0,6	1,4	1	0,586	1,444	0,289		
0,3	0,6	1,4	2	0,589	1,444	0,290		
0,3	0,6	1,4	3	0,586	1,452	0,287		
0,5	1	1	1	0,966	1,029	0,484		
0,5	1	1	2	0,985	1,035	0,488	0,975 4	0,999 4
0,5	1	1	3	0,963	1,037	0,482		
0,7	1,4	0,6	1	1,351	0,613	0,688		
0,7	1,4	0,6	2	1,356	0,615	0,688		
0,7	1,4	0,6	3	1,335	0,616	0,684		

A.3 Example procedure for obtaining a measured DF during the evaluation of *PI* using a calibrated scale to obtain accurate volume estimates

A.3.1 The mass of the pipetted cell suspension and the mass of the diluting medium used to obtain the target dilution fraction can be measured. When it can be reasonably assumed that the densities of these solutions are nominally equivalent, the ratio of the mass of the solutions provides a measured dilution fraction (df_{ij}).

A.3.2 Follow the dilution scheme described in the experimental design to generate independent representative test samples at each target DF (df_i) for the calculation of *PI.*

During the dilution to obtain each independent representative test sample, *j*, at target dilution fraction *i* measure the mass of the cell suspension ($m_{1_{ij}}$) being pipetted and the mass of the diluent being pipetted ($m_{2_{ij}}$).

A calibrated scale with appropriate sensitivity to measure the mass of the pipetted cell suspension and diluent should be used. If an appropriate scale is not available, an alternative method should be used to obtain verified sample volumes.

A calibrated scale with sensitivity of 0,000 1g can for example, be used to obtain mass measurements for sample volumes as low as 0,01 ml to be accurately measured (see ISO 8655-2 for additional information).

Assuming the density of the cell suspension and the diluting medium are approximately equivalent, measured DF for each representative test sample (df_{ij}) can be calculated as Formula (A.3):

$$df_{ij} = \frac{m_{1_{ij}}}{m_{1_{ij}} + m_{2_{ij}}} \tag{A.3}$$

In some cases, it will not be reasonable to assume that the cell suspension and diluting medium have approximately equivalent densities. In these instances, the respective densities of each solution should be considered in the calculation of df_{ij} (see Table A.2).

Table A.2 — Example table for data collection and evaluation of measured DF for each representative test sample

Target DF	Target cell suspension volume ml	Target diluent volume ml	Replicate test sample	Mass of pipetted cell suspension $m_{1_{ij}}$ g	Mass of pipetted diluent $m_{2_{ij}}$ g	df_{ij}
0,3	0,6	1,4	1	0,582	1,407	0,293
0,3	0,6	1,4	2	0,599	1,415	0,298
0,3	0,6	1,4	3	0,597	1,426	0,295
0,5	1	1	1	1,013	1,012	0,500
0,5	1	1	2	1,009	1,019	0,498
0,5	1	1	3	0,959	1,021	0,484
0,7	1,4	0,6	1	1,397	0,604	0,698
0,7	1,4	0,6	2	1,399	0,604	0,698
0,7	1,4	0,6	3	1,395	0,596	0,701

Annex B
(normative)

Method to calculate smoothed residual ($e_{smoothed}$) when a set of measured dilution fractions ($DF_{measured}$) is obtained

When a measured dilution fraction (df_{ij}) is obtained during the evaluation of the cell counting measurement process, $e_{smoothed}$ shall be calculated based on a modelled cell count for each representative test sample ($\lambda_{df_{ij}}^{flexible}$) as described in this annex (see Figure B.1).

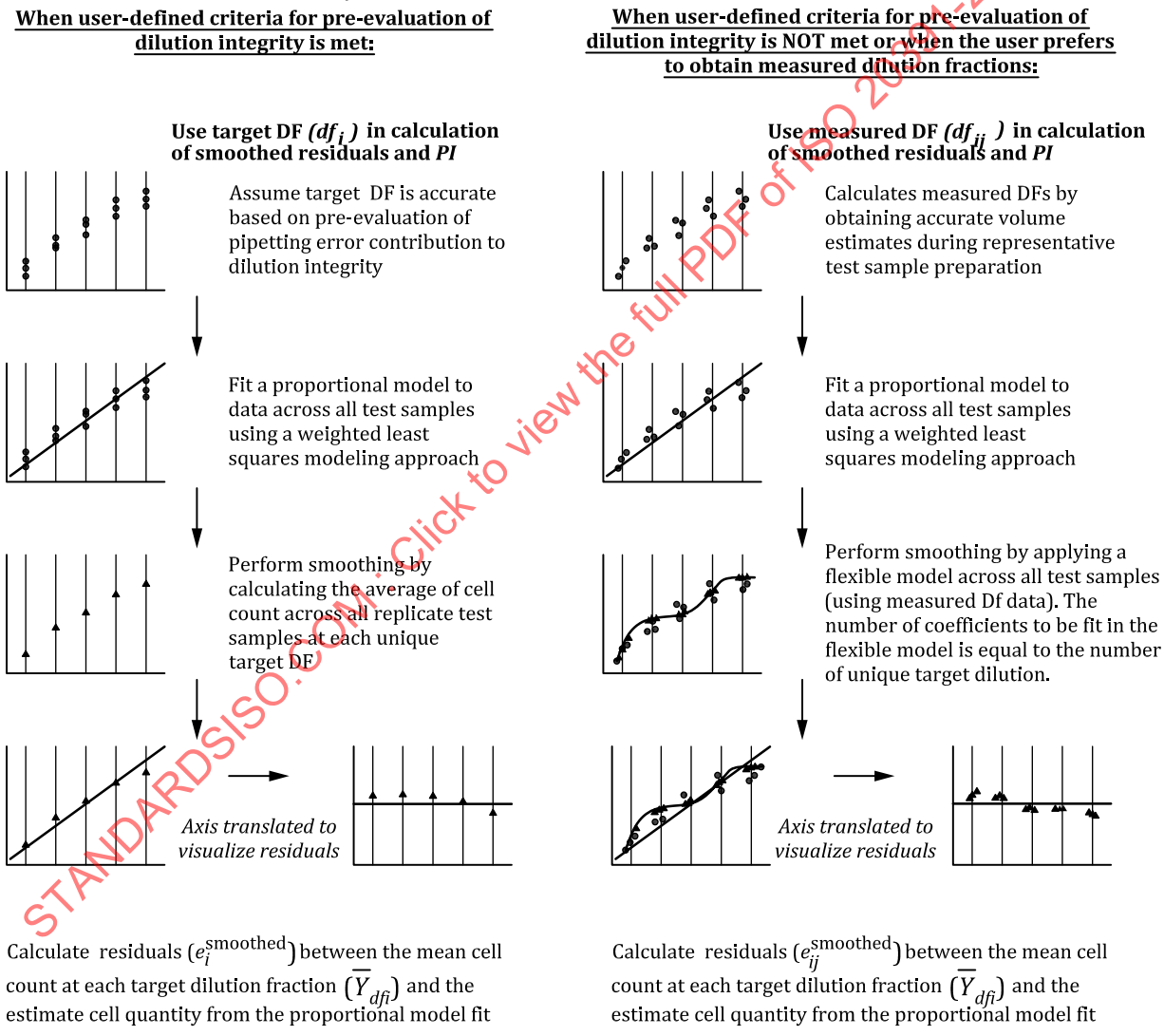


Figure B.1 — Graphical representation of different approaches to calculate smoothed residuals based on target and measured dilution fractions (i.e. method used to obtain a dilution fraction)

To calculate smoothed residuals when a measured DF is obtained (e_{ij}^{smoothed}), a modelled cell quantity value ($\lambda_{df_{ij}}^{\text{flexible}}$) for each representative test sample is estimated by generating a weighted least squares model for $\bar{Y}_{ij.}$ versus df_{ij} following a flexible model constraint.

The total number of coefficients to be fit in the flexible model, including the intercept, is equal to the number of unique target dilution fractions I.

For example, in the case of an experimental design with three target dilution fractions ($I = 3$), a quadratic model is applied following the quasi-Poisson distribution assumption [Formula (B.1)]:

$$\lambda_{df_{ij}}^{\text{flexible}} = \gamma_0 + \left(\gamma_1 df_{ij}\right) + \left(\gamma_2 df_{ij}^2\right) \tag{B.1}$$

where γ_0, γ_1, γ_2 are scalar coefficients estimated during model fitting.

e_{ij}^{smoothed} for representative test sample j at target dilution fraction i is calculated as Formula (B.2):

$$e_{ij}^{\text{smoothed}} = \lambda_{df_{ij}}^{\text{flexible}} - \lambda_{df_{ij}}^{\text{proportional}} \tag{B.2}$$

where $\lambda_{df_{ij}}^{\text{proportional}}$ is the estimated cell quantity at df_{ij}, and is calculated using β_1 from Formula (10) as described by Formula (B.3):

$$\lambda_{df_{ij}}^{\text{proportional}} = \beta_1 \times df_{ij} \tag{B.3}$$

Annex C
(informative)

Example formulae for calculating *PI*

C.1 Detailed example of the calculation of *PI* based on sum of the absolute value of scaled smoothed residuals

PI calculated based on sum of the absolute value of scaled smoothed residuals (PI_{AbsSSR}):

PI_{AbsSSR} when using e_i^{smoothed} [Formula (13)] is given by Formula (C.1):

$$PI_{\text{AbsSSR}} = \sum_i \left| \frac{e_i^{\text{smoothed}}}{\lambda_{df_i}^{\text{proportional}}} \right| \tag{C.1}$$

where $\lambda_{df_i}^{\text{proportional}}$ is the estimated cell quantity at df_i given by Formula (14).

PI_{AbsSSR} when using e_{ij}^{smoothed} (Formula (B.2)] is given by Formula (C.2):

$$PI_{\text{AbsSSR}} = \sum_i \sum_j \left| \frac{e_{ij}^{\text{smoothed}}}{\lambda_{df_{ij}}^{\text{proportional}}} \right| \tag{C.2}$$

where $\lambda_{df_{ij}}^{\text{proportional}}$ is the estimated cell quantity at df_{ij} and is given by Formula (B.3).

C.2 Detailed example of the calculation of *PI* based on R^2 of smoothed residuals

PI calculated based on R^2 of smoothed residuals (PI_{R^2SR}):

PI_{R^2SR} when using e_i^{smoothed} [Formula (13)] is given by Formula (C.3):

$$PI_{R^2SR} = 1 - \frac{\sum_i \left(e_i^{\text{smoothed}} \right)^2}{\sum_i \left(\bar{Y}_{df_i} - \bar{\bar{Y}}_{\cdots} \right)^2} \tag{C.3}$$

where

$\bar{Y}_{df_i}$ is given by Formula (5);

$\bar{\bar{Y}}_{\cdots}$ is the mean of the averaged cell counts at each df_i across a set of I target dilution fractions given by Formula (C.4):

$$\bar{\bar{Y}}_{...} = \frac{\sum_i \bar{Y}_{df_i}}{I} \tag{C.4}$$

PI_{R^2SR} when using e_i^{smoothed} [Formula (B.2)] is given by Formula (C.5):

$$PI_{R^2SR} = 1 - \frac{\sum_i \sum_j \left(e_{ij}^{\text{smoothed}}\right)^2}{\sum_i \sum_j \left(\lambda_{df_{ij}}^{\text{flexible}} - \bar{\lambda}^{\text{flexible}}\right)^2} \tag{C.5}$$

where

$\lambda_{df_{ij}}^{\text{flexible}}$ is given by Formula (B.1) for each replicate representative test sample j at target dilution fraction df_i;

$\bar{\lambda}^{\text{flexible}}$ is the mean of the flexible modelled cell quantity values $\left(\lambda_{df_{ij}}^{\text{flexible}}\right)$ across all replicate representative test samples across all target dilution fractions given by Formula (C.6):

$$\bar{\lambda}^{\text{flexible}} = \frac{\sum_i \sum_j \lambda_{df_{ij}}^{\text{flexible}}}{\sum_i n_i} \tag{C.6}$$

PI_{R^2SR} typically ranges from 0 to 1. PI_{R^2SR} closer to 1 describes a measurement process with greater proportionality.

C.3 Additional examples of the calculation of *PI* when measured dilution fraction is utilized

PI can be calculated based on the sum of squared smoothed residuals (PI_{SqSR}) as Formula (C.7):

$$PI_{\text{SqSR}} = \sum_i \sum_j \left(e_{ij}^{\text{smoothed}}\right)^2 \tag{C.7}$$

PI can be calculated based on the sum of absolute smoothed residuals (PI_{AbsSSR}) as Formula (C.8):

$$PI_{\text{AbsSR}} = \sum_i \sum_j \left| e_{ij}^{\text{smoothed}} \right| \tag{C.8}$$

PI can be calculated based on the sum of squared scaled smoothed residuals (PI_{SqSR}) as Formula (C.9):

$$PI_{\text{SqSSR}} = \sum_i \sum_j \left(\frac{e_{ij}^{\text{smoothed}}}{\lambda_{df_{ij}}^{\text{proportional}}} \right)^2 \tag{C.9}$$

In these cases, *PI* closer to 0 describes a measurement process with greater proportionality.

Annex D
(informative)

Use case 1 — Evaluating the quality of a single cell counting measurement process

D.1 General

The simplest case is the evaluation of the quality of a single cell counting method. In this use case, it is useful to evaluate the parameters described in this document (%*CV*, R^2 and *PI*) for a method and evaluate if the quality of that method meets pre-defined specifications.

D.2 Description of experimental design and statistical analysis

In this case study, a total of five target dilution fractions were investigated with three independent replicate representative test samples per dilution fraction. Three measurements of total cell count were made using Method 2 for cell type A for each replicate representative test sample. Sample labels were randomly assigned and representative test samples were measured in the order of their numerical sample label. Sample identity was blinded to the analyst conducting the total cell count measurement. A measured dilution fraction was obtained while preparing the independent replicate representative test samples. Measured dilution fraction was then used in the analysis of *PI* as outlined in A.2 and Annex B. See Figure D.1.

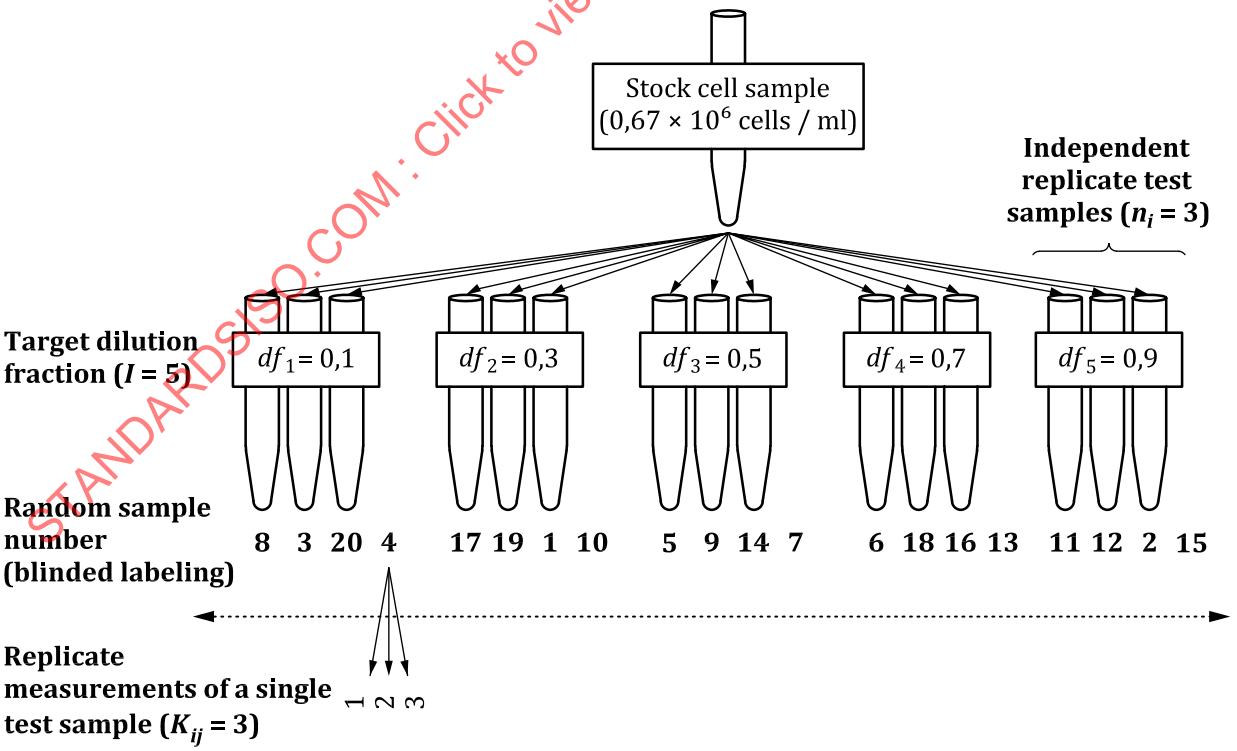


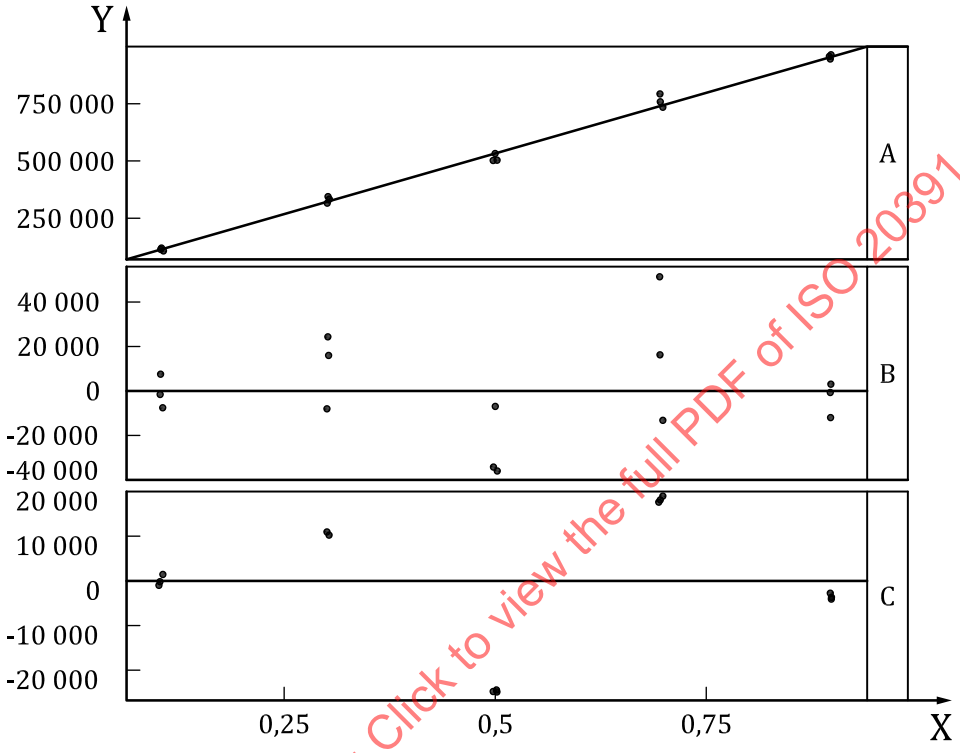
Figure D.1 — Schematic diagram of the experimental design for use case 1 with cell counting Method 2 for a total cell count of cell type A

Total cell count data was modelled using a Quasi-Poisson assumption (mean proportional to the variance) for the mean-variance relationship. 95 % confidence intervals (CI) were estimated using a non-parametric bootstrap with 200 iterations.

D.3 Raw data and data analysis for use case 1

The raw data spreadsheet for use case 1 can be found at: https://standards.iso.org/iso/20391/-2/ed-1/en.

An overview of data and residuals from proportional model fit are shown in Figure D.2.



Key

X dilution fraction

Y total cell concentration (cells/ml) of cell type A

A proportional model fit to data after averaging across replicate observations

B raw residuals from proportional model fit

C smoothed residuals $\left(e_{ij}^{\text{smoothed}}\right)$ from proportional model fit.

Figure D.2 — Proportional model fit to dilution series data using a measured dilution fraction

D.4 Example report for use case 1

D.4.1 Quality indicators (reporting elements from 7.1):

a) The mean cell concentration ($\bar{Y}_{df_i}$) for each df_i. [see 7.1, a)] is given in Table D.1.

Table D.1 — $\bar{Y}_{df_i}$ for each target dilution fraction df_i

Counting method	Target dilution fraction df_i	$\bar{Y}_{df_i}$ cells/ml	Standard deviation for $\bar{Y}_{df_i}$ cells/ml
Method 2	0,1	108 333	6 300
Method 2	0,3	329 983	17 536
Method 2	0,5	504 705	16 297
Method 2	0,7	757 462	30 249
Method 2	0,9	949 571	7 874

b) The mean percent CV ($\%\overline{CV}_{df_i}$) for each df_i, [see 7.1, b)] is given in Table D.2.

Table D.2 — $\%\overline{CV}_{df_i}$ for each df_i

Counting method	Target dilution fraction df_i	$\%\overline{CV}_{df_i}$	Standard deviation for $\%\overline{CV}_{df_i}$
Method 2	0,1	5,6 %	4,6 %
Method 2	0,3	7,7 %	5,2 %
Method 2	0,5	4,9 %	3,9 %
Method 2	0,7	3,8 %	1,0 %
Method 2	0,9	3,9 %	0,5 %

c) R^2 for the proportional model fit [see 7.1, c)] is given in Table D.3.

Table D.3 — R^2 for the proportional model fit

Counting method	R^2	Lower 95 % CI (cells/ml) based on non-parametric bootstrap	Upper 95 % CI (cells/ml) based on non-parametric bootstrap analysis
Method 2	0,998 4	0,996 7	0,998 9

d) The proportionality index (PI) [see 7.1, d)] representing systematic deviation from the proportional model fit is given in Table D.4.

Table D.4 — Proportionality index (PI)

Counting method	PI_{AbsSSR}	Lower 95 % CI (based on non-parametric bootstrap analysis)	Upper 95 % CI (based on non-parametric bootstrap analysis)
Method 2	0,346 5	0,210 0	0,687 5

D.4.2 Documentation of experimental design parameters and statistical analysis method (reporting elements for 7.2):

a) description of the cell counting measurement system(s) investigated:

1) cell type(s) used in the investigation: cell type A;

2) cell counting method(s) evaluated: Method 2 (total cell concentration);

3) concentration range(s) investigated: approximately 108 000 cells/ml to 950 000 cells/ml as evaluated by Method 2;

b) description of dilution fraction experimental design elements:

1) set of unique target dilution fractions ($\boldsymbol{DF} = \{df_i\}_{i=1}^{I}$): $\boldsymbol{DF}$ = {0,1; 0,3; 0,5; 0,7; 0,9};

2) number (n_i) of replicate test samples (j) at each df_i (see Table D.5);

3) number (K_{ij}) of replicate observations (k) for each replicate test sample (j) at each df_i (see Table D.6);

Table D.5 — Number (n_i) of replicate test samples (j) at each df_i

n_i	Method 2
n_1	3
n_2	3
n_3	3
n_4	3
n_5	3

Table D.6 — Number (K_{ij}) of replicate observations (k) for each replicate test sample (j) at each df_i

K_{ij}	Method 2
K_{11}	3
K_{12}	3
K_{13}	3
K_{21}	3
K_{22}	3
K_{23}	3
K_{31}	3
K_{32}	3
K_{33}	3
K_{41}	3
K_{42}	3
K_{43}	3
K_{51}	3
K_{52}	3
K_{53}	3

c) description of the method for evaluating pipetting error contributions to dilution integrity:

1) a measured dilution fraction (df_{ij}) was obtained for each test sample that was generated following A.2. (see Table D.7);

2) a calibrated scale with sensitivity of 0,000 1g was used to obtain mass measurements;

3) PI was calculated based on df_{ij} and e_{ij}^{smoothed} following Annex B and C.1;

Table D.7 — Measured dilution fraction for each test sample

Replicate test Sample	Target dilution fraction df_i	Measured dilution fraction
1	0,1	0,102 0
2	0,1	0,101 3
3	0,1	0,104 7
1	0,3	0,301 3
2	0,3	0,302 2
3	0,3	0,300 3
1	0,5	0,502 4
2	0,5	0,498 6
3	0,5	0,500 5
1	0,7	0,700 3
2	0,7	0,696 3
3	0,7	0,696 9
1	0,9	0,900 4
2	0,9	0,899 1
3	0,9	0,899 3

d) description of the assumption used to model the mean-variance relationship in the proportional model fit: assumption for mean-variance relationship: variance is proportional to the mean;

e) description of formula for the proportional model fit: ($\lambda_{ij}^{\text{proportional}} = \beta_1 \times df_{ij}$) with coefficents described in Table D.8;

Table D.8 — Formula for the proportional model fit

Counting method	Cell type	Concentration type	Proportionality constant β_1 (cells/ml)	Lower 95 % CI (cells/ml) based on non-parametric bootstrap analysis	Upper 95 % CI (cells/ml) based on non-parametric bootstrap analysis
Method 2	A	Total cell Concentration	1 059 214	1 040 052	1 080 296

f) description of the method used for calculating *PI*:

1) primary approach for calculating *PI*: sum of the absolute value of the scaled smoothed residual (PI_{AbsSSR});

2) smoothing approach: smoothing based on measured dilution fraction (e_{ij}^{smoothed});

3) formula for calculating PI_{AbsSSR}: $PI_{\text{AbsSSR}} = \sum_i \sum_j \left| \frac{e_{ij}^{\text{smoothed}}}{\lambda_{df_{ij}}^{\text{proportional}}} \right|$.

D.4.3 Additional reporting elements from 7.2 and 7.3 on the experimental design, statistical analysis and the cell counting measurement process:

a) description of stock cell solution:

   1) stock solution: stock solution 1;

   2) suspending medium: complete MEM with 10 % FBS;

   3) stock solution concentration (cells/ml): approximately 923 700;

   4) method used to estimate stock cell concentration: Method 2;

b) description of dilution method:

   1) diluent: complete MEM with 10 % FBS;

   2) independent dilutions in randomized stock extraction order;

   3) measured dilution fractions obtained at the time of sample preparation;

c) description of cell type and concentration type:

   1) cell type: cell type A, passage 3;

   2) counting method: Method 2, total cell concentration;

d) description of test sample generation and measurement order:

   1) stock extraction order (generation order): 2; 1; 3; 6; 4; 5; 8; 9; 7; 11; 12; 10; 14; 15; 13;

   2) random sample number (measurement order): 3; 6; 15; 1; 12; 14; 7; 10; 4; 13; 11; 5; 9; 2; 8;

e) description of non-parametric bootstrap analysis settings:

   1) number of bootstrap iterations conducted: 200;

   2) confidence level for boot strap analysis: 0,95;

f) description of additional metrics to evaluate proportionality are given in Table D.9.

Table D.9 — Additional metrics to evaluate proportionality

PI approach	*PI* (for Method 2, cell type A)	Lower 95 % CI (based on non-parametric bootstrap analysis)	Upper 95 % CI (based on non-parametric bootstrap analysis)
$PI_{R^2\_SR}$	0,999 3	0,997 7	0,999 8
PI_{AbsSR}	1,592 1	0,772 2	3,177 9
PI_{SqSSR}	0,011 6	0,004 1	0,041 9

D.5 Interpretation

Method 2, for cell type A, within the concentration range of approximately 108 000 cells/ml to 950 000 cells/ml has a *PI* (based on the sum of the absolute value of scaled smoothed residuals) of 0,346 5 with CI [0,210 0, 0,687 5]. It is expected that with repeated measures of *PI* for this method and cell type within this concentration range, the *PI* value should have a CI that overlaps with this CI. If *PI* deviates from this range over time or across for example instruments, operators, locations etc., it is an indication

that there has been a change in the systematic behaviour of the overall measurement process that should be investigated. R^2 and $\%\overline{CV}_{df_i}$ can be similarly interpreted.

Annex E
(informative)

Use case 2 — Comparing the quality of several cell counting measurement processes

E.1 General

In this use case four cell counting measurement processes are compared in parallel. In the experimental design, cell suspensions from each representative test sample are evaluated in triplicate by four different cell counting measurement processes. In this use case, it is useful to compare the parameters described in the standard ($\%\overline{CV}_{df_i}$, $\overline{Y}_{df_i}$, R^2 and PI) between measurement processes. These comparisons can facilitate selection of a cell counting measurement process that is fit-for-purpose.

E.2 Description of experimental design and statistical analysis

In this case study, four cell counting measurement processes were simulated in silico and modelled to exhibit proportional/non-proportional responses as well as precise/non-precise responses. A total of five target dilution fractions were simulated with three independent replicate representative test samples per dilution fraction. Three simulated observations of total cell count were collected using simulated methods, Method 5, Method 6, Method 7, and Method 8 for a simulated cell type for each replicate representative test sample. Pipetting error and pre-evaluation dilution fraction were simulated. Pre-evaluation dilution fraction met user-specified criteria, and target dilution fraction was used in the calculation of quality metrics.

Total cell count data was modelled using a quasi-Poisson assumption (mean proportional to the variance) for the mean-variance relationship. 95 % confidence intervals were estimated using a non-parametric bootstrap with 200 iterations. See Figure E.1.

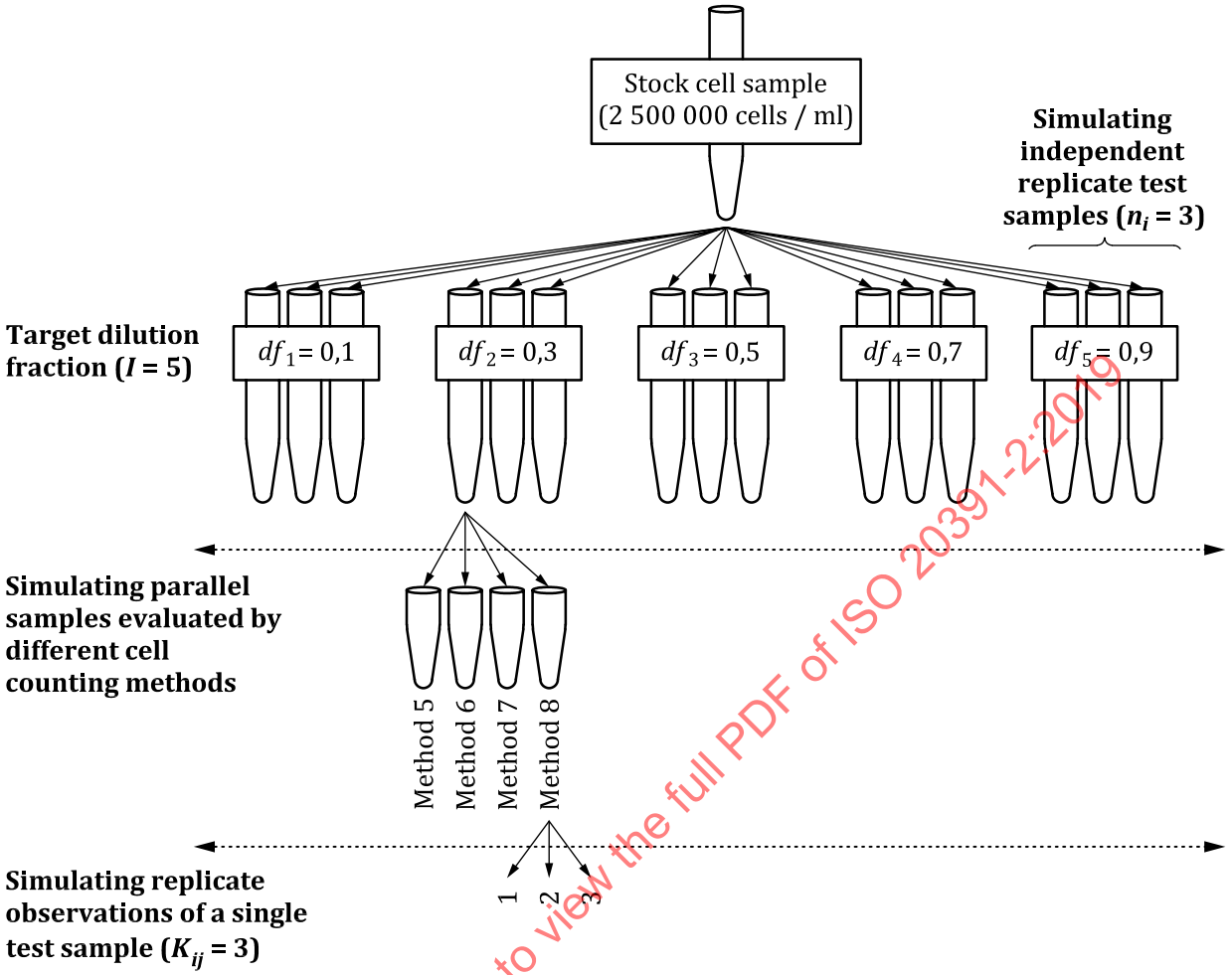


Figure E.1 — Schematic diagram of the experimental design for use case 2 comparing simulated cell counting methods for total cell count for a simulated cell type

E.3 Raw data and data analysis for use case 2

The raw data sheet for use case 2 can be found at: https://standards.iso.org/iso/20391/-2/ed-1/en.

Pre-evaluation of pipetting error contributions to dilution integrity was conducted according to A.1 (see Table E.1). User-defined criteria for dilution integrity was set to $R^2_{\text{Dilution}} \geq 0,980$.

Table E.1 — Raw data for pre-evaluation of pipetting error contributions to dilution integrity with proportional model fit and coefficient of determination for dilution integrity

Target dilution fraction	Pre-evaluated dilution fraction
df_i	$df_{ij}^{\text{pre-evaluated}}$
0,1	0,100 4
0,1	0,098 9
0,1	0,101 8
0,3	0,303 6
0,3	0,31
0,3	0,304 2

Table E.1 *(continued)*

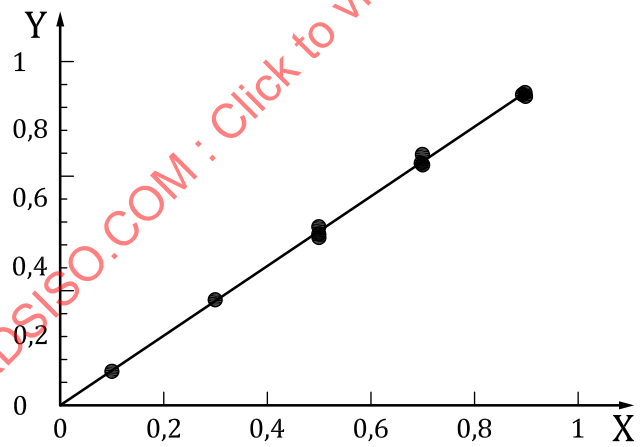
Target dilution fraction	Pre-evaluated dilution fraction
df_i	$df_{ij}^{\text{pre-evaluated}}$
0,5	0,487 4
0,5	0,497 9
0,5	0,519 3
0,7	0,725 3
0,7	0,708
0,7	0,700 2
0,9	0,906 9
0,9	0,899 2
0,9	0,900 6

A plot was generated for $df_{ij}^{\text{pre-evaluated}}$ vs df_i and a line constrained to pass through the origin at (0,0), (i.e. a proportional model fit) was fit to the data (see Figure E.2):

$$df_{ij}^{\text{pre-evaluated}} = \beta^{\text{pipetting}} df_i + \epsilon_i$$

where $\beta^{\text{pipetting}} = 1{,}008$;

with $R^2_{\text{Dilution}} = 0{,}999\ 1$.



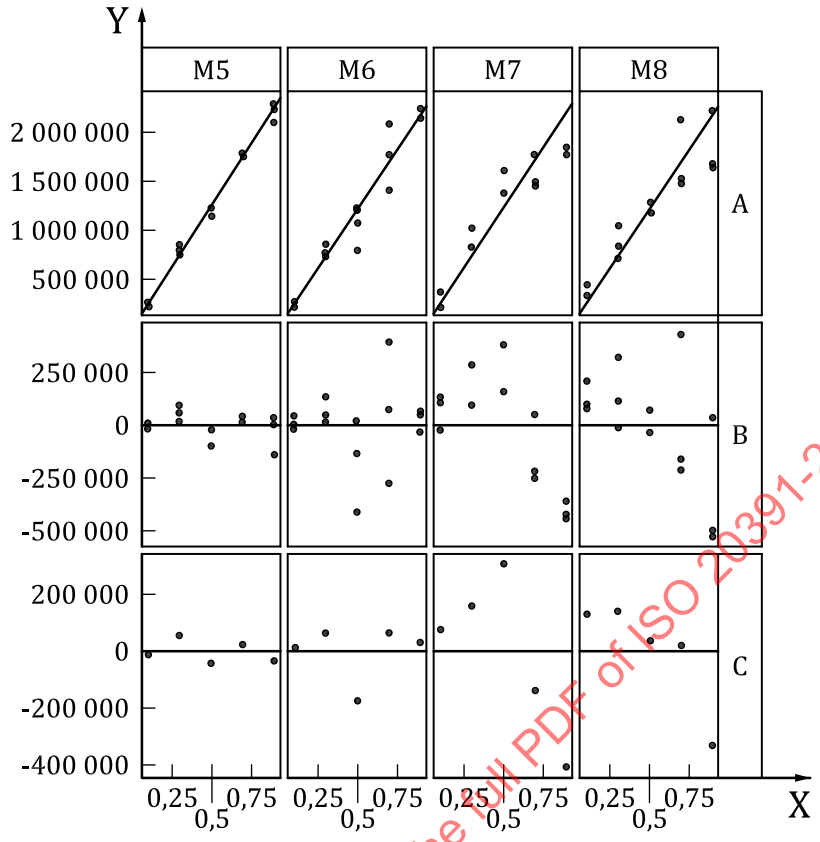
Key

X $df_{\text{pre-evaluated}}$

Y target dilution fraction (df_i)

Figure E.2 — Plot of $df_{\text{pre-evaluated}}$ versus target dilution fraction (df_i) with proportional model fit to evaluate pipetting error contributions to dilution integrity

Overview of data and residuals from proportional model fit is seen in Figure E.3.



Key

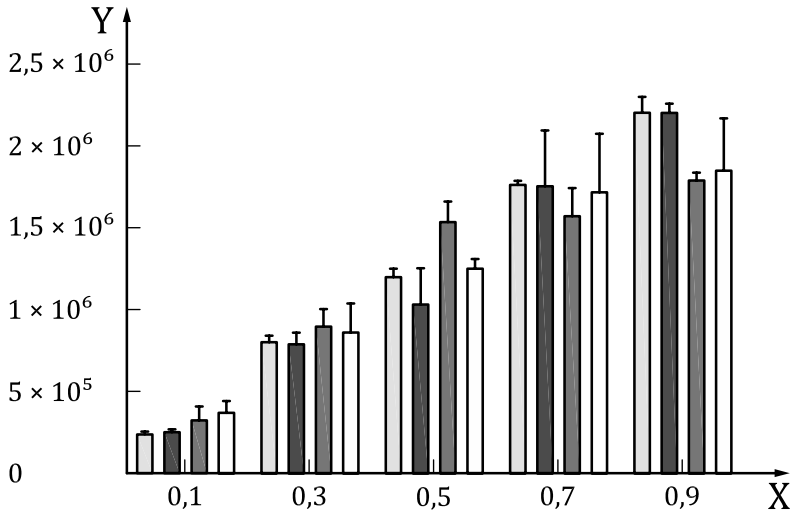
X dilution fraction
Y cell concentration (cells/ml)
A proportional model fit to data after averaging across replicate observations (in cells/ml)
B raw residuals from proportional model fit (in cells/ml)
C smoothed residuals from proportional model fit (in cells/ml)

Figure E.3 — Proportional model fit to dilution series data for each method in Use Case 2

E.4 Example report for use case 2

E.4.1 Quality indicators (reporting elements from 7.1):

a) the mean cell concentration ($\bar{Y}_{df_i}$) for each df_i [see 7.1a)] is given in Figure E.4 and Table E.2:



Key

X	target dilution factor (df_i)		Method 6
Y	mean cell concentration ($\bar{Y}_{df_i}$) (cells/ml) with error bars representing the standard deviation		Method 7
			Method 8
	Method 5		

Figure E.4 — Mean cell concentration ($\bar{Y}_{df_i}$) of a simulated cell type across replicate representative test samples at each target dilution fraction (df_i) for each counting method

Table E.2 — $\bar{Y}_{df_i}$ for each target dilution fraction df_i for each method

Counting method	Target dilution fraction df_i	$\bar{Y}_{df_i}$ cells/ml	Standard deviation for $\bar{Y}_{df_i}$ cells/ml
Method 5	0,1	244 498	12 612
Method 5	0,3	804 353	39 527
Method 5	0,5	1 203 474	46 994
Method 5	0,7	1 769 138	13 062
Method 5	0,9	2 209 022	92 759
Method 6	0,1	252 670	32 013
Method 6	0,3	790 678	65 099
Method 6	0,5	1 033 595	217 992
Method 6	0,7	1 755 531	335 989
Method 6	0,9	2 205 380	52 234
Method 7	0,1	321 385	87 008
Method 7	0,3	894 437	111 825
Method 7	0,5	1 531 075	127 905
Method 7	0,7	1 574 980	166 916
Method 7	0,9	1 796 086	40 930
Method 8	0,1	372 990	68 611
Method 8	0,3	869 230	167 854