BS ISO 20391-2:2019

BSI Standards Publication

Biotechnology — Cell counting

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

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National foreword

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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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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.

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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]

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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.

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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.

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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.

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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.]

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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.

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[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
$\boldsymbol{DF}_{\textbf{measured}}$	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

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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 jth 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.

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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).

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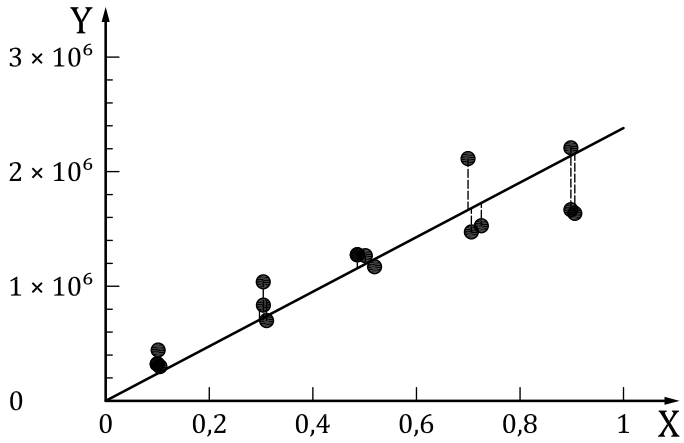

Key

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.

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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.

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