SLS ISO 20391 PART 1: 2022
(ISO 20391-1:2018)
UDC 602

BIOTECHNOLOGY – CELL COUNTING - PART 1: GENERAL GUIDANCE ON CELL COUNTING METHODS

SRI LANKA STANDARDS INSTITUTION

Sri Lanka Standard

BIOTECHNOLOGY - CELL COUNTING - PART 1: GENERAL GUIDANCE ON CELL COUNTING METHODS

SLS ISO 20391 PART 1: 2022
(ISO 20391-1:2018)

Gr. H

SRI LANKA STANDARDS INSTITUTION
17, Victoria Place
Elvitigala Mawatha
Colombo 08
Sri Lanka

Sri Lanka Standards are subject to periodical revision in order to accommodate the progress made by industry. Suggestions for improvement will be recorded and brought to the notice of the Committees to which the revisions are entrusted.

This Standard does not purport to include all the necessary provisions of a contract

Sri Lanka Standard
BIOTECHNOLOGY - CELL COUNTING - PART 1: GENERAL GUIDANCE ON CELL COUNTING METHODS

NATIONAL FOREWORD

This Sri Lanka Standard was approved by the Sectoral Committee on Food Products and was authorized for adoption and publication as a Sri Lanka Standard by the Council of the Sri Lanka Standards Institution on 2022-07-07

Cell counting (or cell enumeration) is a fundamental measurement that broadly impacts many aspects of biotechnology, from biomanufacturing to advanced therapy. The cell count (or discrete number of cells) is often expressed as cell concentration (i.e. cell count per volume) when in suspension and area density of cells (i.e. cell count per unit area) when adhered to a surface. Cell count is critical in evaluating potency and efficacy for cell-based therapy. The cell concentration within a bioreactor can serve as a quality assurance metric in cell-based manufacturing processes. Many cell-based bioassays need to be normalized to the respective cell count to allow data inter-comparability. This document (which is Part 1 of a multi-part standard on cell counting) defines terms and provides general guidance for the cell counting measurement process, including method selection, sample preparation, measurement, qualification and validation, and data analysis and reporting.

This Sri Lanka Standard is identical with **ISO 20391-1:2018** Biotechnology - Cell counting Part 1: General guidance on cell counting methods published by the International Organization for Standardization (ISO).

Terminology and conventions:

The text of the International Standard has been accepted as suitable for publication, without deviation, as a Sri Lanka Standard. However, certain terminology and conventions are not identical with those used in Sri Lanka Standards. Attention is therefore drawn to the following:

a) Wherever the words “International Standard” appear referring to this Standard, it should be interpreted as “Sri Lanka Standard”.

b) The comma has been used throughout as a decimal marker. In Sri Lanka Standards it is the current practice to use the full point on the base line as the decimal marker.

c) Wherever page numbers are quoted, they are ISO page numbers.

……..

INTERNATIONAL STANDARD

ISO 20391-1

First edition
2018-01

Biotechnology — Cell counting —

Part 1: General guidance on cell counting methods

Biotechnologie — Dénombrement des cellules —

Partie 1: Lignes directrices générales relatives aux méthodes de dénombrement des cellules

Reference number
ISO 20391-1:2018(E)

COPYRIGHT PROTECTED DOCUMENT

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

Published in Switzerland

Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received. www.iso.org/patents

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on 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 WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

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

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

Introduction

Cell counting (or cell enumeration) is a fundamental measurement that broadly impacts many aspects of biotechnology, from biomanufacturing to advanced therapy. The cell count (or discrete number of cells) is often expressed as cell concentration (i.e. cell count per volume) when in suspension and area density of cells (i.e. cell count per unit area) when adhered to a surface. Cell count is critical in evaluating potency and efficacy for cell-based therapy. The cell concentration within a bioreactor can serve as a quality assurance metric in cell-based manufacturing processes. Many cell-based bioassays need to be normalized to the respective cell count to allow data inter-comparability. This document (which is Part 1 of a multi-part standard on cell counting) defines terms and provides general guidance for the cell counting measurement process, including method selection, sample preparation, measurement, qualification and validation, and data analysis and reporting.

INTERNATIONAL STANDARD **ISO 20391-1:2018(E)**

Biotechnology — Cell counting —

Part 1:
General guidance on cell counting methods

1 Scope

This document defines terms related to cell counting for biotechnology. It describes counting of cells in suspension (generally cell concentration) and cells adhered to a substrate (generally area density of cells). It provides key considerations for general counting methods (including total and differential counting, and direct and indirect counting) as well as for method selection, measurement process, and data analysis and reporting.

This document is applicable to the counting of all cell types – mammalian and non-mammalian (e.g. bacteria, yeast) cells.

This document is not intended for counting of cells while in a tissue section or a biomaterial matrix.

Several sector/application-specific international and national standards for cell counting currently exist. When applicable, the user can consult existing standards when operating within their scope (specific measurement techniques and/or applications).

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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:

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

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

3.1
accuracy
closeness of agreement between a measured quantity value and a true quantity value of a measurand

Note 1 to entry: The concept of “measurement accuracy” is not a quantity and is not given a numerical quantity value. A measurement is said to be more accurate when it offers a smaller measurement error.

Note 2 to entry: “Measurement accuracy” is sometimes understood as closeness of agreement between measured quantity values that are being attributed to the measurand.

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

3.2
agglomerate
<cells> two or more cells clustered weakly together and detected as a larger object

Note 1 to entry: Agglomerates of cells can be separated into nominally single cells without causing significant damage to the cell.

3.3
aggregate
<cells> two or more cells clustered together (tightly or loosely) and detected as a larger object

Note 1 to entry: Aggregates of cells are generally more difficult to be separated into single cells.

3.4
area density
<cells> cell count of adherent cells on a surface, typically expressed as number of cells per unit area

3.5
attribute
physical, chemical, biological or microbiological property or characteristic

3.6
cell concentration
cell count per volume

Note 1 to entry: Typically used for cells in suspension.

3.7
cell count
discrete number of cells

Note 1 to entry: Cell count is typically expressed as *cell concentration* (3.6) or *area density* (3.4).

3.8
cell counting
measurement process to determine the cell count

3.9
cell suspension
cells dispersed in a liquid matrix

3.10
debris
<in cell suspensions> fragments of cells and/or particles of biological or non-biological origin

3.11
differential cell count
number 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.

3.12
direct cell counting
counting method in which one signal is (or several signals are) detected for each single event

Note 1 to entry: Each single event should represent a single cell in an idealized measurement.

3.13
indirect cell counting
counting method during which a signal (or a set of signals) is measured from a population of cells and that signal is then related to cell number based on a measurement-specific mathematical model (e.g. calibration curve)

3.14
limit of quantitation
LoQ
lowest amount of analyte 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 quantitation describes quantitative assay for low levels of cells in sample matrices.

3.15
linearity
ability to elicit test results that are directly, or indirectly by means of well-defined mathematical transformations, proportional to cell count within a given range

3.16
measurand
quantity intended to be measured

[SOURCE: ISO/IEC Guide 99:2007, 2.3, modified]

3.17
precision
measurement precision
closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions

[SOURCE: ISO/IEC Guide 99:2007, 2.15, modified]

3.18
proportionality
characteristic exhibited by a collection of measurements in which 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 1 to entry: When a set of measurements exhibits proportionality over a range of a given input, the expected value of the measurements can be expressed as the input parameter multiplied by a fixed constant, with no bias term.

3.19
reagent
substance used in chemical/biochemical analysis or other reactions

3.20
reference material
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

Note 1 to entry: Reference materials with or without assigned quantity values can be used for measurement precision control whereas only reference materials with assigned quantity values can be used for calibration or measurement trueness control.

[SOURCE: ISO/IEC Guide 99:2007, 5.13, modified]

3.21
reference method
thoroughly investigated measurement procedure shown to yield values having an uncertainty in measurement commensurate with its intended use, especially in assessing the trueness of other measurement procedures for the same quantity and in characterizing reference material

[SOURCE: ISO 17511:2003, 3.29, modified]

3.22
repeatability
<results of measurement> measurement precision under defined conditions of measurement

[SOURCE: ISO/IEC Guide 99:2007, 2.21, modified]

3.23
ruggedness
measure of a method's capacity to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage

[SOURCE: ICH Harmonised Tripartite Guideline, 1994]

3.24
selectivity
property of a measuring system, used with a specified measurement procedure, whereby it provides measured quantity values for one or more measurands such that the values of each measurand are independent of other measurands or other quantities in the phenomenon, body, or substance being investigated

[SOURCE: ISO/IEC Guide 99:2007, 4.13, modified]

3.25
total cell count
count of all cells, independent of the attribute(s) of the cell

3.26
uncertainty
<measurement> non-negative parameter characterizing the dispersion of values attributed to a measurand, based on the information used

[SOURCE: ISO/IEC Guide 99:2007, 2.26, modified]

3.27
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]

3.28
verification
confirmation, through the provision of objective evidence, that specified requirements have been fulfilled

[SOURCE: ISO 9000:2015, 3.8.12, modified]

3.29
viable cells
cells within a sample that have an attribute of being alive (e.g. metabolically active, capable of reproduction, possessed of intact cell membrane, or with the capacity to resume these functions) defined based on the intended use

4 General concepts of cell counting

4.1 General

Various cell counting methods (as described in Annex A) can be broadly categorized as total or differential cell counting, and direct or indirect cell counting.

4.2 Total cell counting

Total cell counting involves the measurement of all cells, independent of the attribute(s) of the cell.

Criteria should be applied to distinguish cells from debris (cellular and non-cellular in origin).

4.3 Differential cell counting

Differential cell counting involves the measurement of a subset of cells that have been distinguished from other cells by at least one distinct cell attribute.

EXAMPLE Differential cell counting includes viable cell counting, counting of cells that express a specific surface marker, or counting of cells that exhibit specific cell morphology.

4.4 Direct cell counting

Direct cell counting involves the recording of a signal or a set of signals from each cell (3.12). In this context, the signal(s) can be electrical (as in impedance), optical (as in fluorescent or colorimetric), or mechanical. The signal can be recorded manually by a user or automatically by an instrument. Due to the large number of cells in a typical sample, certain direct cell counting methods require dilution of samples. The cell count is then extrapolated based on a dilution factor.

4.5 Indirect cell counting

Indirect cell counting involves the recording of a signal or a set of signals from all cells or a subset of cells in the sample and then relating that signal to a cell count based on measurement specific mathematical model(s) (e.g. calibration curve) (3.13).

EXAMPLE Indirect cell counting includes measurement of total cell mass, total DNA, and metabolic activity.

NOTE Uncertainty in the cell counts derived from indirect cell counting can arise from the mathematical model(s) (e.g. calibration curve), in addition to other sources of measurement errors.

5 Considerations for cell counting measurements

5.1 Selection of a cell counting method

Many cell counting methods exist (see Annex A); these methods can be used to measure total or differential cell count via direct or indirect cell counting (Figure 1 and Annex B).

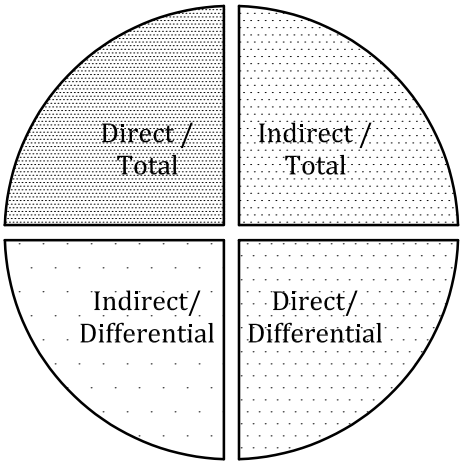


Figure 1 — Cell counting categories

Some methods can be employed for multiple categories based on the intended measurand for the stated purpose.

EXAMPLE 1 Automated microscopy can be used for *direct/total* cell counting if the measurand is the total number of objects/cells; it can be used for *direct/differential* cell counting if the measurand is the number of labelled objects/cells; it can also be used for *indirect/total* cell counting if the measurand is percent confluence.

Some instruments and/or methods can provide a cell count for more than one counting category simultaneously by detecting different measurands.

EXAMPLE 2 Total and viable cell count can be determined at the same time based on differences in optical properties, labels, morphology, etc.

Each method has inherent noise and bias that can affect accuracy and precision. The user shall consult available knowledge to select a method or methods suitable for the intended cell type, application, and/or sample preparation procedure (fit-for-purpose).

NOTE Requirements for cell counting can vary by intended use. Intended use can be, for example, product release or in-process cell counting.

Direct cell counting (both total and differential) requires well-dispersed cells for optimal performance. The presence of debris and aggregated or agglomerated cells can lead to over- or underestimated cell count. Whenever possible, a process should be established to prepare well-dispersed samples with minimized debris, aggregate, and agglomerate content.

Indirect cell counting methods use a surrogate measure to evaluate the cell count. The accuracy of these methods depends on the accuracy of the measurement as well as the accuracy of the calibration curve. For example, when total DNA quantity is used to estimate the cell count, the ability to accurately measure the total DNA within a sample and establish an accurate relationship between DNA and cell number is important. When possible, the calibration should be established using appropriate reference material(s).

5.2 Considerations for selecting a cell counting method

Selection of the cell counting method depends on the intended purpose as well as sample and processing factors. These can include:

— intended purpose for cell counting;

— counting category(ies);

— appropriate measurand(s);

— appropriateness of instrumentation to assess defined measurand(s), including the limit of quantitation (LoQ);

— sample characteristics, including cell attributes and potential effects of sample heterogeneity;

— potential impact on the measurement due to the presence of debris, aggregates, and/or agglomerates;

— potential impact on the measurement due to bioprocessing and pre-measurement processing: including storage, transfer, cryopreservation (including the freeze and thaw process);

— potential impact on the measurement due to ancillary materials and other components in the cell sample (e.g. media, beads).

5.3 Sampling of cells for counting

The cell count is often determined from one or several sample(s) taken from the larger whole.

Proper sampling procedures should be used to minimize sampling errors associated with measuring a cell sample rather than measuring the entire batch or lot (e.g. master cell bank, whole cell population).

Measurements from a small sample size/fraction can have a larger sampling error. In some instances, sampling errors can be reduced by taking a larger random sample size/fraction or multiple samples especially for measuring cells per area.

When taking an aliquot from cells in suspension, the suspension should be sufficiently homogeneous that the aliquot is representative of the suspension. Heterogeneity in the cell suspension can lead to aliquots that are not representative of the larger whole.

5.4 Preparation of cell samples for counting

Cell counting processes can require preparation (e.g. mixing, lysing, staining) of the cell sample prior to counting.

Aspects of a sample preparation process, such as environmental factors, procedures, and reagents can introduce variability in cell counting.

A sample preparation process can alter the cell sample in systematic or random ways, reducing its representativeness of the larger whole or altering the cell attribute associated with the counting measurand, leading to misinterpretation of measurement results.

The presence of debris can lead to an overestimation of the number of cells. The influence of debris on cell count measurements should be considered, and when possible, debris should be removed and/or accounted for before or during counting.

The presence of aggregates or agglomerates can lead to undercounting of cells. Sample preparation procedures should be established to prepare well-dispersed samples prior to taking an aliquot.

5.4.1 Environmental factors

Environmental factors that could change the sample in ways that affect cell counting should be minimized. Environmental factors can include temperature, humidity, light exposure, sterility conditions, and airflow.

EXAMPLE The temperature at which a cell sample is held can alter its attribute and needs to be selected accordingly.

5.4.2 Procedures

The effect of equipment and consumables on cell counting should be considered. Appropriate containers and transferring apparatus should be selected to minimize loss of cells associated with sample transfer. Transferring procedures (e.g. pipetting) should be suitable to an acceptable level of sample loss.

The mixing methods (e.g. mode, speed, duration) as well as wait/hold time in between processes can alter the cell attribute associated with the counting measurand. Cell mixing procedures should be designed to minimize the effect on the counting measurand.

Errors in measuring cell suspension or diluent volume should be minimized when diluting cells.

Procedures to stain, lyse, disaggregate, disperse, or otherwise manipulate the cells should be evaluated for their effects on the cell counting measurand. Potential effects on cell counting should be minimized.

EXAMPLE Excessive shear can rupture some cells.

5.4.3 Quality and stability of reagents

When possible, reagents used in sample preparation should be verified to ensure quality and consistency. The quality of the reagent should be verified using available methods or reference materials.

Some reagents (e.g. fluorescent dye, buffer) might not be stable over time or under certain environmental conditions. Cell counting measurements should be conducted within the accepted stability range of the reagents.

Formulation errors of some reagents can cause either overestimation or underestimation of the cell count. Acceptable reagent concentration ranges should be determined.

Some reagents (e.g. antibodies) might not be consistent from lot-to-lot or from different suppliers. The user should define acceptable specifications prior to using these reagents.

The binding efficiency of reagents (such as absorption of dyes) used in cell counting should be considered, and when appropriate, specifications should be established.

5.5 Performing a measurement

Cell counting shall be performed on properly maintained instruments.

The instrument should be calibrated or verified at appropriate intervals.

Cell counting shall be performed using validated procedures. Appropriate instrument settings should be established for the intended cell counting.

NOTE 1 Settings for one instrument might not be directly transferable to another instrument.

NOTE 2 Optimization of instrument settings might be required for each cell count measurement process (when the sample or the purpose has changed).

The measurand shall be within the qualified range of the method for the intended purpose. The lower limit for the validated range shall be greater than the limit of quantitation.

If signals for more than one measurand are detected at a time, interference and/or overlap should be minimized and/or compensated for (e.g. compensation correction in multi-channel flow cytometry measurements).

Cell counting measurements shall be performed within a specified time limit, where the stability of the cell sample has been verified. The stability of the sample during a measurement process should be considered. Loss of signal intensity or a change in cell attribute can affect the measurand.

Errors associated with operator bias and imprecision should be minimized. Training protocols, proficiency testing, implementation of automated systems, and randomization of samples can reduce the contributions of operator bias and imprecision to cell count uncertainty.

6 Qualification, validation, and verification

6.1 Instrument qualification

The measurement instrument shall be qualified per predefined specifications. Qualification protocols shall be documented prior to conducting instrument qualification and results shall be documented.

Installation qualification (IQ) and operational qualification (OQ) should be performed.

Instrument manufacturer-defined installation qualification (IQ) and operational qualification (OQ) may be used.

Routine performance qualification (PQ) should be conducted per documented procedures at predetermined intervals.

NOTE Instrument qualification can be a part of validation.

6.2 Method validation and verification

The cell counting measurement method shall be validated. Method performance parameters should be provided to give evidence that the method produces results that are suitable for the intended purpose. Method performance parameters may include specification for accuracy, precision, working range

(LoQ, linearity, etc.), selectivity/specificity, ruggedness, and intermediate precision, such as inter-operator, inter-device, and inter-day variability.

Validation of accuracy is ideally obtained by evaluating the difference between the mean value of analytical results and a reference value, obtained by certified reference material(s). A standard method, multiple experimental designs with statistical analysis, or another established method may also be used.

NOTE Inter-laboratory study and/or other benchmark activities can be used to evaluate repeatability and reproducibility.

ISO 20391-2 provides additional information on experimental design and statistical analysis.

A validation plan shall be documented and maintained. The validation plan shall include method performance parameters for the intended use. Validation results shall be documented.

Method verification may be conducted to ensure a validated method is performing within specifications. A reduced set of method performance parameters may be determined for this purpose. Verification plans and results shall be documented and maintained.

6.3 Reference materials

Reference materials should be used to ensure measurement traceability, enable comparison, and/or verify a measurement process. When available, an appropriate cell-based reference material should be used for its intended purpose.

6.3.1 Certified reference materials

Suitable certified reference materials should be used when available. A reference material should be used for its intended purpose based on its certified or reference value(s) (see Reference [1]).

6.3.2 In-house reference materials

In-house reference materials should be evaluated for their purpose in a counting process. When possible, reference values for in-house reference materials should be generated with associated uncertainties.

6.3.3 Uses of reference materials

Appropriate reference materials should be used for cell counting instrument qualification, validation, and verification.

EXAMPLE 1 Beads can serve as appropriate reference materials for instrument qualification or in installation qualification and operational qualification for direct counting instruments.

NOTE Beads generally do not recapitulate the properties of cells; results from beads might not be representative of expected results from a cell sample.

EXAMPLE 2 Solutions of known fluorophore concentration can be used to qualify fluorescence based cell counting methods.

An appropriate reference material may be used for the calibration of indirect cell counting methods.

Reference materials may also be used for training or proficiency testing.

7 Data processing, analysis, and reporting

7.1 Data processing and analysis

7.1.1 General

Sound data processing and analysis methods should be used for the selected cell counting method. Data processing considerations can include but are not limited to those described in 7.1.2 to 7.1.4.

7.1.2 Image processing and analysis

Digital image processing techniques are generally used to process, analyse, and present images obtained from a microscope. In cell counting, image analysis can be used to identify cellular objects and exclude debris from analysis. Image analysis can also be used to identify specific subsets of cells in the sample. Basic image processing can include correction of brightness and contrast of the image, and correction of illumination non-uniformities. Image analysis is used to derive cell count. Image analysis parameters and algorithms should be validated.

7.1.3 Gating

A gate is a set of value limits (boundaries) that serve to isolate a subset of cells from a large set or the total cell population, typically visualized in the form of a density plot or histogram. Gates can be defined by discrimination analysis, or can simply be drawn around a given set of data points manually. Gates can be drawn in a step-wise process (e.g. in flow cytometry measurements, cells can first be gated based on forward scatter to distinguish cells from debris by relative size, then gated based on fluorescence intensity for specific surface markers).

7.1.4 Coincidence correction

Coincidence in cell counting measurements is the temporal overlap of signals from cells in a flow-based measurement or spatial overlap in microscopy-based images, resulting in counting loss. Appropriate coincidence corrective methods should be used to avoid under counting of cells. The specific influence in flow-based measurements can be derived by dilution series experimental design and extrapolation to zero volume fraction of the cells in the measurement sample. Some flow-based instruments provide data analysis options to account for coincidence loss.

7.2 Reporting

The data report shall contain sufficient detail to allow independent assessment of the cell count results. Reporting elements may include:

a) sample — ID, cell descriptors (type, lot number, source);

b) reagents — name, source, lot number;

c) sample preparation procedures and conditions;

d) instrument used — including instrument settings;

e) qualification, validation, and verification plans;

f) measurement results with appropriate units and uncertainty;

g) data analysis procedure;

h) unexpected observations.

Annex A
(informative)

Description of common cell counting methods

A.1 General

This annex describes some commonly used cell counting methods.

A.2 Packed cell volume

Packed cell volume, also known as packed cell height, is the volume percentage (% by vol.) of cells for a given sample. A typical procedure involves centrifuging a capillary tube containing the sample; the height of the cells collected at the bottom is used to estimate the volume percentage of cells. This method is more appropriate for samples that do not contain components or debris that would settle with cells. This method is not appropriate for cell aggregates where packed volume is impacted by aggregate size, structure, void space, etc.

A.3 Cell mass

The cell number can be estimated by measuring the mass of dry or wet cells per volume. Cells are separated from broth/media and weighed while they are wet, or the cells may be thoroughly dried before weighing. The dry mass generally gives a more consistent result. Contributions of extra-cellular matrix should be considered for bacterial counting.

A.4 Manual counting chamber

A counting chamber, also known as a haemocytometer, is a microscope slide that is especially designed to enable direct microscopic cell counting. The slide has a depression at its middle and a special cover slip is placed over the area; the depression is marked with a grid. A drop of cell suspension is deposited into the depression such that it fills the space between the cover slip and the slide grid with a defined volume. The depth of the depression is predefined; thus, the volume and concentration of the counted cell suspension can be calculated. Samples generally need to be diluted to enable manual counting; each dilution step can contribute to inaccuracy to the measurement; therefore, it is important to get the cell concentration to a practical level to enable counting, but not too dilute to affect data accuracy. Common sources of error include inconsistencies in filling volume and operator bias. Non-adherent cells (including motile bacterial cells) or cells on multiple focal planes would make counting challenging using this method.

A.5 Plating and CFU counting

Cells plated in the presence of a growth medium are cultured until colonies form to enable counting. If the cell suspension is dilute and cells are separated and sparsely distributed on the plate, it can be generally assumed that each cell will give rise to a single colony or Colony Forming Unit (CFU). The colonies are counted, and based on the known volume of culture that was spread on the plate, the cell concentration can be calculated. Samples generally need to be diluted to enable well-dispersed plating; each dilution step could contribute to the inaccuracy of the measurement. The accuracy and utility of this procedure will be dependent on the cell type of interest. Colony size and build-up can vary and can contribute to inaccuracy.

A.6 Spectrophotometry

Spectrophotometers measure intensity of light transmitted through a sample. Turbid cell suspensions absorb or scatter light and reduce the amount of transmitted light. The higher the cell concentration is, the higher the turbidity. Optical density (OD) is proportional to the biomass in the cell suspension. The culture is placed in a transparent cuvette, the cuvette is placed in the spectrophotometer, and the optical density can be measured immediately. This is an indirect counting method requiring a standard curve generated using the same cell type being measured.

A.7 Impedance-based counter

An impedance-based cell counter, also known as a Coulter principle-based counter, is an instrument for counting and sizing cells in suspension. It is based on the fact that cells function as discrete insulators when suspended in a conductive liquid; in a Coulter counter the cells, suspended in a solution that conducts electricity, are drawn one by one through a small aperture. Flanking the aperture are two electrodes that conduct electricity. When no cell is in the aperture, electricity flows unabated, but when a cell is drawn through the aperture, the current is impeded for a short duration. The path through the aperture, in which the cell is detected, is known as the "electrical sensing zone". The impedance-based cell counter enumerates the number of such events and measures the current (and impedance), which directly correlates to the volume of the passing cell. The cell count is gated by specifying the size range of the cell population of interest. Common sources of error include coincidence (when, occasionally, more than a single cell traverses the aperture simultaneously) and gating choice. A consensus standard that addresses this direct counting method is ASTM 2149-01 (2007).

A.8 Flow cytometry

Single cells in suspension flow in a narrow stream in front of a laser or LED beam. The beam hits them one by one, and a peak light detector picks up the light that is reflected or emitted from the cells. Flow cytometers have many other abilities, such as analysing the shape of cells and their internal and external structures, as well as measuring the amount of specific proteins and other biochemicals in the cells. There are many potential sources of error, including reagent quality and specificity, instrumentation settings (e.g. gating parameters), and data analysis methods. Accuracy may be improved with the availability of cell-based reference materials (e.g. CD4+ cells).

A.9 Automated image analysis

Recent approaches involve the use of high-quality microscopy images and a statistical classification algorithm to perform automated cell detection and counting. A range of image classification techniques can be employed for this purpose. Common sources of error include the use of improper imaging operation, such as focusing, and improper counting of cell aggregates, which can be difficult for image analysis algorithms to appropriately segment. Another major source of error is the establishment of the image analysis counting settings to avoid counting debris and correctly capturing all cells.

A.10 Cell number by total DNA quantification

The total DNA quantity, typically determined by the intensity of a non-selective DNA fluorescent label, is sometimes used as a surrogate measure to determine the total cell number. The accuracy of DNA quantification can be affected by the selectivity of label and efficiency of labelling, and the proportion of lysate DNA extraction efficiency present in the preparation.

A.11 Metabolic activity assays and other assays

A range of bioassays aimed to assess the biological activity is used as a surrogate for measuring the cell number. The assay can include glucose/lactose production, lactate dehydrogenase release, and ATP

levels. It is important to note that only metabolically active cells will be included in measurements such as these.

Other less common assays for cell counting include enzyme-linked immunosorbent assay (ELISA) and a related enzyme-linked immunospot (ELISpot) assay. These methods would be useful for enumerating cells that produce cytokines or cell-specific markers on their cell surface and are amenable to the indirect counting method. Multiplex bead-based systems are also commonly used to identify cytokines and cell-specific markers, and can enumerate multiple markers within single microplate wells.

Annex B
(informative)

Common cell counting methods for various measurement purposes

Table B.1 — Categories of common cell counting methods[a,b,c]

	Direct cell counting	Indirect cell counting
Total cell counting	— Automated microscopy — Impedance-based counter — Flow cytometry — Manual counting	— Automated microscopy — Dry cell mass/weight — NIR (Near InfraRed) — Packed cell height — Spectrophotometry — Total DNA quantification — Turbidity meter
Differential cell counting	— Automated microscopy — Colony Forming Unit (CFU) — ELIspot — Flow cytometry — Manual counter	— Cell-based ELISA immunoassay — Impedance-based biomass monitor — Metabolic activity assays — NIR (Near InfraRed)

[a] The list provided in Table B.1 is by no means an exhaustive inventory of all counting methods.

[b] Some of the listed methods may be placed in other categories based on the experimental design. In some cases, only the primary use is presented.

[c] Methods that may be used for an in processing cell-counting measurement, i.e. in situ measurement that uses a sensor inside of the bioreactor and/or ex situ measurement that uses of a sensor outside of the bioreactor to measure a circulating sample from the bioreactor.

Bibliography

[1] ISO Guide 33, *Reference materials — Good practice in using reference materials*

[2] ISO Guide 98-3, *Uncertainty of measurement, Guide to the expression of uncertainty measurement (GUM:1995)*

[3] ISO/IEC Guide 99:2007, *International vocabulary of metrology — Basic and general concepts and associated terms (VIM)*

[4] ISO 5725-1:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[5] ISO 5725-2:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[6] ISO 5725-3:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 3: Intermediate measures of the precision of a standard measurement method*

[7] ISO 5725-6:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 6: Use in practice of accuracy values*

[8] ISO 8196-3:2009[1)], *Milk — Definition and evaluation of the overall accuracy of alternative methods of milk analysis — Part 3: Protocol for the evaluation and validation of alternative quantitative methods of milk analysis*

[9] ISO 10718:2002, *Cork stoppers — Enumeration of colony-forming units of yeasts, moulds and bacteria capable of growth in an alcoholic medium*

[10] ISO 11843-1:1997, *Capability of detection — Part 1: Terms and definitions*

[11] ISO 13366-1:2008, *Milk — Enumeration of somatic cells — Part 1: Microscopic method (Reference method)*

[12] ISO 13528:2015, *Statistical methods for use in proficiency testing by interlaboratory comparison*

[13] ISO 17511:2003, *In vitro diagnostic medical devices — Measurement of quantities in biological samples — Metrological traceability of values assigned to calibrators and control materials*

[14] ISO 20391-2[2)], *Biotechnology — Cell counting — Part 2: Experimental design and statistical analysis to quantify counting method performance*

[15] ASTM F 2149-01 *(Reapproved 2007), Standard Test Method for Automated Analyses of Cells — the Electrical Sensing Zone Method of Enumerating and Sizing Single Cell Suspensions*

[16] ASTM F2944-12, *Standard Test Method for Automated Colony Forming Unit (CFU) Assays — Image Acquisition and Analysis Method for Enumerating and Characterizing Cells and Colonies in Culture*

[17] ASTM D4455-85 (2014), *Standard Test Method for Enumeration of Aquatic Bacteria by Epifluorescence Microscopy Counting Procedure*

[18] ASTM F 2739-08, *Standard Guide for Quantitating Cell Viability within Biomaterial Scaffolds*

[19] H20-A2. Reference Leukocyte (WBC) Differential Count (Proportional) and Evaluation of Instrumental Methods; Approved Standard – Second Edition

1) Equivalent to IDF 128-3:2009.

2) Under preparation. Stage at time of publication ISO/CD 20391-2:2018.

[20] DIN 58932-1, *Haematology — Determination of the concentration of blood corpuscles in blood — Part 1: Blood collection, sample preparation, biological influence factors, interference factors*

[21] DIN 58932-2, *Haematology — Determination of the concentration of blood corpuscles in blood — Part 2: Characteristic quantities for erythrocytes (erythrocyte indices)*

[22] DIN 58932-3, *Haematology — Determination of the concentration of blood corpuscles in blood — Part 3: Reference method for the determination of the concentration of erythrozytes; Text in German and English*

[23] DIN 58932-4, *Haematology — Determination of the concentration of blood corpuscles in blood — Part 4: Reference procedure for the determination of the concentration of leucocytes*

[24] DIN 58932-5, *Haematology — Determination of the concentration of blood corpuscles in blood — Part 5: Reference method for the determination of the concentration of thrombocytes; Text in German and English*

[25] Reference method for the enumeration of erythrocytes and leucocytes, International Council for Standardization in Haematology; Prepared by the Expert Panel on Cytometry. *Clin. Lab. Haematol*. 1994, **16** pp. 131–138

[26] Platelet Counting by the RBC/ Platelet Ratio Method - A Reference Method, International Council for Standardization in Haematology Expert Panel on Cytometry and International Society of Laboratory Hematology Task Force on Platelet Counting - American Journal Clinical Pathologists 115:460-464 (2001)

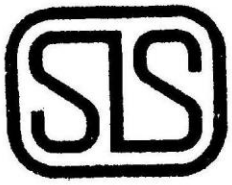
[27] CLSI H44-A2, Methods for Reticulocyte Counting (Automated Blood Cell Counters, Flow Cytometry, and Supravital Dyes); Approved Guidance – Second Edition

ICS 07.080
Price based on 16 pages

SLS CERTIFICATION MARK

The Sri Lanka Standards Institution is the owner of the registered certification mark shown below. Beneath the mark, the number of the Sri Lanka Standard relevant to the product is indicated. This mark may be used only by those who have obtained permits under the SLS certification marks scheme. The presence of this mark on or in relation to a product conveys the assurance that they have been produced to comply with the requirements of the relevant Sri Lanka Standard under a well designed system of quality control inspection and testing operated by the manufacturer and supervised by the SLSI *which includes surveillance inspection of the factory, testing of both factory and market samples.*

Further particulars of the terms and conditions of the permit may be obtained from the Sri *Lanka Standards Institution, 17, Victoria Place, Elvitigala Mawatha, Colombo 08.*



Printed at SLSI (Printing Unit)

SRI LANKA STANDARDS INSTITUTION

The Sri Lanka Standards Institution (SLSI) is the National Standards Organization of Sri Lanka established under the Sri Lanka Standards Institution Act No. 6 of 1984 which repealed and replaced the Bureau of Ceylon Standards Act No. 38 of 1964. The Institution functions under the Ministry of Technology.

The principal objects of the Institution as set out in the Act are to prepare standards and promote their adoption, to provide facilities for examination and testing of products, to operate a Certification Marks Scheme, to certify the quality of products meant for local consumption or exports and to promote standardization and quality control by educational, consultancy and research activity.

The Institution is financed by Government grants, and by the income from the sale of its publications and other services offered for Industry and Business Sector. Financial and administrative control is vested in a Council appointed in accordance with the provisions of the Act.

The development and formulation of National Standards is carried out by Technical Experts and representatives of other interest groups, assisted by the permanent officers of the Institution. These Technical Committees are appointed under the purview of the Sectoral Committees which in turn are appointed by the Council. The Sectoral Committees give the final Technical approval for the Draft National Standards prior to the approval by the Council of the SLSI.

All members of the Technical and Sectoral Committees render their services in an honorary capacity. In this process the Institution endeavours to ensure adequate representation of all view points.

In the International field the Institution represents Sri Lanka in the International Organization for Standardization (ISO), and participates in such fields of standardization as are of special interest to Sri Lanka.

Printed at the Sri Lanka Standards Institution, 17, Victoria Place, Elvitigala Mawatha, Colombo 08.