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Biotechnology — Minimum requirements for optical signal measurements in photometric methods for biological samples

Biotechnologie — Exigences minimales relatives aux mesures de signaux optiques dans les méthodes photométriques pour les échantillons biologiques

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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*.

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

This document defines terms and provides general guidance for accurate measurement of optical signals used for analysis of biological samples in photometric methods. These photometric methods can use optical signal measurements, including bioluminescence, chemiluminescence, fluorescence or absorption measurement, that can be applied in the fields of biotechnology, life science and medicine. A measured optical signal value is applied for evaluating biological parameters qualitatively or quantitatively, including cellular and metabolic activities, and gene expressions. Photometric methods are used in applications such as toxicity testing, environmental risk assessment, biomanufacturing, drug development, regenerative medicine and biobanking.

There are significant needs for both manufacturers and users for high quality optical signal measurement in photometric methods in industry to increase confidence in the repeatability, intermediate precision and reproducibility for analysis of biological samples. While repeatability of the photometric method is already sufficient for qualitative characterization of biological samples, quantitative characterization requires more accurate intermediate precision and reproducibility of optical signal measurement. It requires proper optical signal measurements, and it also requires assessment of deviations from the ideal proportionality of the optical signal and the output of the photometric method. Requirements for proper optical signal measurement are an important component of the description of specific applications of photometric methods.

This document provides a general framework to support proper measurement of an optical signal in a photometric method. It focuses on the utilization of optical references and relevant technical issues for optical signal measurement in photometric methods, including procedures for verification of instruments, continual performance monitoring of instruments and photometric method validation. Optical references can be used to verify instruments to increase confidence in the repeatability, intermediate precision, and reproducibility of optical signal measurement. For example, an optical signal emitted from biological samples can be compared on a common measurement scale within a laboratory, between manufacturer and manufacturer, manufacturer and user, or user and user.

Biotechnology — Minimum requirements for optical signal measurements in photometric methods for biological samples

1 Scope

This document specifies minimum requirements to support accurate measurement of optical signals in photometric methods used for qualitative or quantitative characterization of biological samples.

This document is applicable to optical signals that are generated, for example, by bioluminescence, chemiluminescence and fluorescence, and optical signals that are detected as changes of light due to absorption.

This document addresses the verification of optical signal measurement instruments used in photometric methods for measurement of biological samples including considerations for the use of optical references.

This document does not provide sector- or application-specific performance criteria for the workflow of measuring biological samples. When applicable, users can also consult existing sector- or application-specific standards, or both.

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 terminology databases for use in standardization at the following addresses:

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

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

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 “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: The term “measurement accuracy” should not be used for measurement trueness and the term “measurement precision” should not be used for “measurement accuracy”, which, however, is related to both these concepts.

Note 3 to entry: “Measurement accuracy” is sometimes understood as closeness of agreement between measured quantity values that are being attributed to the measurand.

Note 4 to entry: ISO 5725-1:1994 uses a different definition for “accuracy”.

[SOURCE: ISO/IEC Guide 99:2007, 2.13, modified — “measurement accuracy” and “accuracy of measurement” deleted as terms. Note 4 to entry added.]

3.2
biological sample
material or object of biological origin

3.3
dynamic range
range of *optical signal* (3.6) values that can be measured quantitatively

[SOURCE: ISO 2041:2018, 3.4.17, modified — "optical signal" and "quantitatively" added to the definition.]

3.4
light source
optical device emitting appropriate wavelength(s) in a specified spectral region

Note 1 to entry: A light source can be a part of an *optical signal* (3.6) measurement instrument.

[SOURCE: ISO 25178-604:2013, 2.3.1 modified — "wavelength(s)" replaced "range of wavelengths". Note 1 to entry added.]

3.5
optical reference
material, *light source* (3.4) or photodetector, sufficiently reproducible and stable with respect to optical properties, that has been established to be fit for its intended use

EXAMPLE Light emitting diode (LED)-based *reference light source* (3.11), laser, slide of fluorescent glass, fluorescent dye in solution or other matrix (e.g. fluorescent bead), slide embedded fluorescent material, reference filter, reference cuvette, reference film, reference solution, *power meter* (3.9) (see Annex B).

Note 1 to entry: The term "optical reference" includes both uncalibrated references and calibrated standards. Optical references can be distributed by an internal organization or prepared by a laboratory (e.g. in-house standard, in-house reference material).

Note 2 to entry: Optical references can be used for *verification* (3.14) of *optical signal* (3.6) measurement instruments (see Annexes D, E, G, H, I and J).

3.6
optical signal
light emitted or changes of light due to absorption caused by transmitting light through samples or chromogenic substances

Note 1 to entry: The optical signal measurement involves, for example, bioluminescence, chemiluminescence, fluorescence and absorption measurements. Annex A gives information about optical signals.

Note 2 to entry: In this document, the term "optical signal" focuses on light before detection.

3.7
optical signal intensity
strength of an *optical signal* (3.6)

Note 1 to entry: Intensity can be used to express the absolute strength or relative strength of an optical signal. An appropriate unit can be used in order to express the intensity of a particular optical signal.

3.8
photometric method
analytical technique using *optical signal* (3.6) measurement(s) to determine components or biological parameters of *biological samples* (3.2)

Note 1 to entry: The photometric method includes preanalytical, optical signal measurement and data analysis procedures.

Note 2 to entry: Biological parameters of biological samples include, for example, cellular and metabolic activities, and gene expressions.

Note 3 to entry: Examples for representative photometric methods are shown in Annex B.

Note 4 to entry: Analysis and assay results of photometric methods can be expressed qualitatively or quantitatively.

Note 5 to entry: The term "radiometric" is widely used instead of "photometric" in the field of optical engineering (e.g. IEC 60050-845).

3.9
power meter
optical power meter
measurement device to determine the radiant power of light used as an *optical reference* (3.5)

Note 1 to entry: The watt (W) is used as a unit to express radiant power.

3.10
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 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 5725-3:1994).

Note 3 to entry: Measurement precision is used to define measurement repeatability, intermediate measurement precision, and measurement reproducibility.

Note 4 to entry: Sometimes "measurement precision" is erroneously used to mean measurement accuracy.

Note 5 to entry: ISO 5725-1:1994 uses a different definition for "precision".

[SOURCE: ISO/IEC Guide 99:2007, 2.15, modified — "measurement precision" deleted as a term. Note 5 to entry added.]

3.11
reference light source
light source (3.4) used as an *optical reference* (3.5)

EXAMPLE Characterized or calibrated LED and laser.

3.12
reference material for calibration curve
material with known value of concentration or amount of a specific substance, for intended purpose

Note 1 to entry: It is identical to or commutable with the measurement object of a *biological sample* (3.2).

Note 2 to entry: Examples for expressing concentration and amount are mol/l and mol, respectively.

3.13
validation
confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled

Note 1 to entry: The objective evidence needed for a validation is the result of a test or other form of determination such as performing alternative calculations or reviewing documents.

Note 2 to entry: The word "validated" is used to designate the corresponding status.

Note 3 to entry: The use conditions for validation can be real or simulated.

Note 4 to entry: ISO/TS 16393:2019 uses the term "validation" in a different meaning in defining "validation experiment". ISO/IEC Guide 99:2007 uses a different definition for "validation".

[SOURCE: ISO 9000:2015, 3.8.13, modified — Note 4 to entry added.]

3.14
verification
confirmation, through the provision of objective evidence, that specified requirements have been fulfilled

Note 1 to entry: The objective evidence needed for a verification can be the result of an inspection or of other forms of determination such as performing alternative calculations or reviewing documents.

Note 2 to entry: The activities carried out for verification are sometimes called a "qualification process".

Note 3 to entry: The word "verified" is used to designate the corresponding status.

Note 4 to entry: ISO/IEC Guide 99:2007 uses a different definition for "verification".

[SOURCE: ISO 9000:2015, 3.8.12, modified — Note 4 to entry added.]

4 Principles

4.1 General

Optical signal measurements, including bioluminescence, chemiluminescence, fluorescence and absorption measurements, are used in photometric methods. Optical signal measurements are often used for biological samples to determine a diverse set of biological parameters qualitatively and quantitatively, including cellular and metabolic activities, and gene expressions (see Annex A for more information). In the photometric methods, the optical signal intensity and spectrum from biological samples are measured using instruments.

NOTE 1 Examples of instruments are luminometers, imaging analysers, fluorescence plate readers, flow cytometers, microarray readers, spectrofluorometers, plate readers, spectrophotometers and DNA sequencers (see Annex B).

Accuracy, precision, repeatability and reproducibility represent some of the important metrological factors used for evaluating the effectiveness of photometric method applied.

Photometric methods can be qualitatively validated using positive and negative control materials.

NOTE 2 The performance characteristics of qualitative photometric methods and their validation can be determined with appropriate statistical models depending on the method, structure of data and statistical experience (e.g. ISO/TS 16393).

Accurate analysis and assay results are obtained by measuring the optical signal with an appropriate selection of experimental materials, including the reagents generating the optical signal from the sample, and the use of suitable instruments for the intended purpose.

Sample preparation is also an important factor governing the performance of a photometric method.

Optical signal measurements produce relative and absolute optical signal values that are functionally related to the quantity of specific characteristics of biological samples or biological parameters. In spectral-resolved measurements, spectral characteristics are indicative for the interaction of particular molecules, structural elements of molecules, or molecular interaction with electromagnetic radiation of different energy.

In some cases, calibration curves constructed using a reference material for calibration curve are required for quantification of the absolute amount of biological sample. A calibration curve can be also

used to determine an effective amount of a test article (e.g. an amount that elicits 50 % response across the calibration curve or ED_{50}).

NOTE 3 Annex H gives an example for the construction of a calibration curve.

For measurement of biological samples, it is sometimes necessary to label or stain biological samples, introduce a reporter gene into cells, tissues and whole organisms, or trigger chemical reactions.

NOTE 4 Reagent quality and its photophysical and chemical properties affects optical signals from the sample. Activity of cells can sometimes affect optical signals.

NOTE 5 Ambient light radiation can cause deterioration of bioluminescent reagents, chemiluminescent reagents, fluorescent materials and fading absorption.

When cells are used in photometric methods, the robustness of analysis and assay results is less reliable if the cellular activity is unstable. In particular, optical signal measurement results are directly affected by the stability of the cellular activity during long-term storage/subculturing and by the stability of responsiveness to the target bioactive substance. The incident measuring light can also affect cellular functions and properties, in particular if the cells are exposed to the light for a long period. Accordingly, the reliability of optical signal measurement results can be increased by maintaining cell stability.

NOTE 6 Examples are assays to evaluate cellular activity, including viability, toxicity and metabolic activity by means of cell-based assays.

NOTE 7 Relevant standards that describe representative methods by means of optical signal measurements are listed in Annex C.

Preanalytical procedures applied before performing optical signal measurements, including cell lysis, antigen-antibody reaction, dye labelling or staining, can affect analysis and assay results.

4.2 Instruments and measurements

Photodetectors, including photomultipliers, photodiodes and image sensors, have specific spectral responsivities. Optical signals, including bioluminescence, chemiluminescence, fluorescence and absorption, can be measured accurately by using spectrally suited photodetectors and colour filters.

NOTE 1 Annex K gives examples for spectral responsivity data of photodetectors.

The optical signal measurement instruments are affected by environmental conditions, including laboratory temperature and exposure to direct sunlight. Adjustment of the spatial resolution of an instrument can be required depending on the application.

Optical signals can be measured quantitatively when the signal intensity is within the dynamic range of the photodetector. Photodetectors have specific linear or nonlinear responsivities within this dynamic range, which can be determined with test measurements.

NOTE 2 The limits of linearity can be determined statistically.

Most instruments perform relative measurements of optical signals. The output values, therefore, depend on the instrument unless a reference material is available to establish a calibration curve. Only when the instruments are absolutely calibrated in radiometric values including the number of photons, can the measured optical signal values be expressed as absolute radiometric quantities.

Background signals can affect optical signal measurement results. Typical sources of background signals are electrical noises (e.g. dark count and read-out noise) and optical noises (e.g. stray light and external light).

Background signals can exist even in the absence of optical signals. Background signals are automatically or manually subtracted after optical signal measurement.

4.3 Optical references

Optical references can be used to confirm the performance of optical signal measurement instruments, including repeatability, intermediate precision, reproducibility, dynamic range and other related instrument performance.

Consistency of optical characteristics between the optical reference and the biological sample increases confidence in instrument performance and the analysis and assay results by measuring the optical signals of biological samples.

NOTE Examples for optical references are an LED-based reference light source (see Annex D for an exemplary luminometer qualification with LED), laser, slide of fluorescent glass, fluorescent dye in solution or other matrix (e.g. fluorescent bead), slide embedded fluorescent material, reference filter, reference cuvette, reference film, reference solution and power meter (see Annex B).

Optical references can be used for installation qualification, operational qualification and performance qualification. Optical references can also be used to compare photodetector responsivity between instruments.

Optical references can be used to calibrate optical signals to amounts or relative amounts, or potencies of target biological samples.

5 Minimum requirements to support optical signal measurement

5.1 Elements of photometric methods

Standardized approaches should be followed to provide accurate analysis and assay results by measuring the optical signal in photometric methods for analysis of biological samples.

Instruments, reagents, biological samples including cells, and other experimental materials used for optical signal measurement in the photometric methods shall be selected for the intended purpose and procedures. Reagents and biological samples shall be properly stored and maintained.

NOTE 1 Stability of reagents and biological samples can change during long-term storage and maintenance.

NOTE 2 In cells expressing reporter gene(s), including bioluminescence, chemiluminescence, fluorescence or colorimetric reporter gene, expression level of the reporter gene(s) can change during storage and subculturing. The copy number of the reporter gene(s) can also change during long-term subculturing.

The manufacturer's instructions should be followed for storage and use of reagents and biological samples.

Optical components of instruments (photodetectors, optics and light sources) used for optical signal measurements shall be selected in accordance with the optical characteristics, including spectral properties, of the photometric methods and biological sample.

Interference or enhancement of the optical signal by the apparatus, reagents, solvent and biological sample used should be taken into account.

NOTE 3 Some apparatus, reagents and solutions have inappropriate characteristics for optical signal measurements, including inhibition or enhancement of reactions creating interfering signals due to absorptive/fluorescent/quenching/phosphorescent/corrosive properties.

NOTE 4 Ancillary materials including phenol red (due to absorption) can alter optical signal measurement results.

NOTE 5 Adhesion to container walls can cause false concentration particularly for low concentration samples. Depending on instrument design, the concentration can also be too high due to carry over from a preceding measurement of high concentration samples.

5.2 Verification of optical signal measurement instruments

5.2.1 Optical references

Optical signal measurement instruments shall be verified using optical references.

NOTE 1 Examples of optical references and instruments are listed in Annex B.

Optical references can be used to verify repeatability, intermediate precision and reproducibility.

Optical signal measurement instruments that have been verified by the manufacturer should be maintained according to the manufacturer's instructions.

Reference light sources, including light emitting diode (LED) or laser, can be used to verify responsivity of luminometers, fluorescence plate readers, microarray readers and imaging analysers. Pulsed LEDs can be used as a reference light source for photodetectors in flow cytometers.

NOTE 2 Annex E gives information and an example for the application of reference light sources for comparison measurements of luminescent biological samples using luminometers.

Reference fluorescent materials, including fluorescent substance, solution or beads, can be used to verify responsivity of imaging analysers, flow cytometers, spectrofluorometers and fluorescence plate readers.

NOTE 3 Further guidance on characterization and assessment of suitable reference materials can be found in ISO Guide 35:2017.

A power meter (an optical power meter) can be used to verify excitation light power (W) of imaging analysers, flow cytometers, spectrofluorometers, microarray readers, fluorescence plate readers, spectrophotometers and plate readers.

A reference cuvette or reference material for absorbance measurement can be used to verify responsivity of plate readers, spectrophotometers and imaging analysers.

A reference light source whose optical signal value is calibrated absolutely against power (W) or number of photons can be applied to calibrate absolute sensitivity of the instruments.

NOTE 4 Annex J gives an example for the calibration of reference light sources and luminometers.

In-house standards (e.g. authentic materials) can be used as optical references when the reproducibility or the stability is confirmed.

5.2.2 Dynamic range

The dynamic range of the photodetector in an instrument to quantitatively measure optical signal(s) from biological sample(s) shall be determined.

NOTE 1 Evaluation of dynamic range is generally conducted as installation qualification and/or operational qualification of optical signal measurement instruments.

NOTE 2 The lower limit of dynamic range can be given by the limit of quantification, whereas the upper limit of dynamic range can be characterized by onset of unacceptable anomalies in sensitivity.

NOTE 3 Examples for the dynamic range determination of luminometers can be found in Annex G. Annex H gives an example for the construction of a calibration curve and the dynamic range determination of fluorescence plate readers. Annex I gives an example of the dynamic range determination for flow cytometers.

The dynamic range can be determined using an LED reference light source, serial dilution of reference material or reporter proteins, including bioluminescence, chemiluminescence, fluorescence and chromogenic proteins, chemiluminescent reagents, and reference cuvettes or reference materials for absorbance measurement.

Reference fluorescent material series with variable and previously determined relative densities or concentrations can be used to determine, adjust and correct the dynamic range of fluorescence measurement instruments.

5.2.3 Background signals

Background signals associated with instruments shall be determined by measurements taken in conditions without the interference of optical signals from analytes. Background signal subtraction should be appropriate for the intended purpose.

NOTE 1 Examples of background signals are dark count and read-out noise.

NOTE 2 Background signals can be evaluated using an untreated sample or without a sample.

Error sources related to optical signal measurements, including stray light from neighbouring samples and spectral overlapping between multiple detection channels, should be evaluated using appropriate samples or optical references.

NOTE 3 Spectral overlapping can be determined and compensated by using single-coloured samples, which are stained or labelled with the same fluorophore or luminophore as the test sample.

Error sources from biological samples and containers, including autofluorescence, photobleaching, quenching and phototoxicity, should be taken into account.

To reduce photobleaching and phototoxicity, the upper limit of the excitation light and the exposure time should be controlled.

When multi-well plates are used as a measurement container, applicable well-to-well crosstalk should be taken into account.

NOTE 4 The colour of multi-well plates influences detection sensitivity and well-to-well crosstalk. Additional information is given in Annex F.

5.3 Optical signal measurement of biological samples

5.3.1 Optical signal measurement

Optical signal measurements shall be performed using instruments that have been verified according to 5.2.

For quantitative evaluation, the optical signal intensity from a biological sample shall be within the dynamic range of the instrument used, as described in 5.2.2. Beyond this range results can only be considered for qualitative evaluation.

NOTE 1 Sometimes the signal intensity detected by the instruments can be brought within the dynamic range by adjusting the sample concentration.

NOTE 2 Incident light on some photodetectors can eventually be controlled within the dynamic range of the instruments by adjusting the power of light source.

5.3.2 Calibration curve

The target biological samples shall be quantified within the dynamic range of a calibration curve.

NOTE 1 The calibration curve can be constructed using a reference material for calibration curve wherever applicable.

NOTE 2 There are some quantification methods that can be performed without using calibration curve methods, but only if a calibrated optical density (OD) measurement method is available and a molar attenuation coefficient (ε) has previously been determined.

NOTE 3 Annex H gives an example for the construction of a calibration curve.

NOTE 4 Calibration curves can be used for verification of instruments, especially for determination of dynamic range.

NOTE 5 There are some quantification methods that do not need the construction of calibration curves (e.g. digital polymerase chain reaction (PCR)).

5.3.3 Photometric methods

Photometric methods originally developed in the same laboratory or modified from the validated methods shall be validated for each specified intended use.

When predefined acceptance criteria are provided for the validation of a photometric method, the method shall be validated for laboratory use based on the same criteria.

For photometric methods intended to evaluate each relative activity of target bioactive substances, optical signal measurement data, including fold-change values, should be validated by a comparison experiment using appropriate positive and negative control materials.

NOTE Accurate analysis and assay results by measuring optical signal can be validated by participating in external quality assessment (EQA) or proficiency testing (PT).

5.3.4 Personnel

Optical signal measurement in photometric methods shall be performed by personnel, including the operator, who are appropriately trained and understand the concepts of the requirements in 5.2, 5.3.1 and 5.3.2.

NOTE Specific guidance on personnel in medical laboratories can be found in ISO 15189.

Devices and instruments specifically designed and developed for untrained non-professional operation should be used according to the manufacturer's instructions.

5.4 Data analysis and reporting

Reporting shall incorporate details to allow independent assessment.

Reports should include the following elements, wherever applicable:

a) optical signal measurement procedure:

   1) the measurement protocol and data analysis method;

   2) calculation formulae (e.g. subtraction of absorbance at certain wavelengths, order of such subtractions);

   3) the reaction time (from the addition of reagent before optical signal measurement);

   4) the environmental condition(s) (e.g. temperature, humidity);

b) optical signal measurement instruments:

   1) the component of instrument (e.g. photodetector, filter, spectroscopy);

   2) instrument specifications and settings used (e.g. gate or exposure time for optical signal detection, wavelength resolution);

   3) the suitability of instrument characteristics to optical properties of sample (e.g. spectral matching);

c) optical references:

   1) the type and name;

2) specifications as applicable (e.g. power (W) or photon number for reference light source, spectrum and intensity for fluorescence material, responsivity for power meter, absorbance for reference cuvette or material);

3) the measurement conditions (e.g. settings of instrument and optical reference);

4) the measurement result of optical references as applicable;

5) the precision when statistical analysis is performed;

NOTE 1 Measurement result can be expressed as relative or absolute values.

d) biological samples:

1) the type and specifications of each sample (e.g. lot number, source, passage number of cells) as applicable;

2) the date and sampling procedure(s) as applicable;

3) the transport and storage conditions as applicable;

e) reagents and substances:

1) the name, source (e.g. lot number) and country of origin;

2) the transport and storage conditions as applicable;

3) the description of a reference material for calibration curve, and appropriate positive and negative control materials used as applicable;

f) results of the optical signal measurement of biological samples:

1) the measurement result;

2) the precision when statistical analysis is performed;

3) the person responsible for the results including the operator;

4) information on the units of optical signal; measurement results given on the basis of relative or arbitrary units should be expressed in the unit of the optical reference;

NOTE 2 Units can be expressed as relative or absolute values.

5) the date and time of optical signal measurement;

g) unexpected observations:

1) any unexpected observations made during analysis;

2) validated modifications from the manufacturer's instructions or applicable standard operation procedures;

3) the observed interference factors.

NOTE 3 The information in the above list can be included in a quantification result, calibration, instrument verification and method validation report.

Annex A
(informative)

Principles of bioluminescence, chemiluminescence, fluorescence and absorption

A.1 General

Components of biological samples and biological parameters, including cellular and metabolic activities, and gene expressions, are measured and evaluated qualitatively or quantitatively by photometric methods. In the photometric methods, the optical signal intensity and spectrum from samples associated with the quantity of biological samples or changes in biological parameters are measured using optical signal measurement instruments. Optical signals to be measured originate from bioluminescence, chemiluminescence, fluorescence, absorption and other related optical signals. In this annex, representative principles of optical signal measurements in photometric methods are described.

NOTE 1 This document describes measurements of bioluminescence, chemiluminescence, fluorescence and absorption because they are widely utilized in photometric methods. There are, however, some other possible optical signals that can be measured including phosphorescence, electroluminescence and Raman scattering. There are also some techniques related to fluorescence, including Foerster energy transfer, fluorescence lifetime, fluorescence polarization and multi-photon excitation. Nephelometry measuring turbidity is also related to optical signal measurement.

NOTE 2 In the photometry and radiometry field, luminous intensity (in units of candela) and radiant intensity (in units of W/sr) are strictly defined for a point source. However, the term “intensity”, including “optical signal intensity”, in this document simply indicates strength of optical signal and is not used only for a point source.

A.2 Bioluminescence and chemiluminescence

Light emission resulting from chemical reactions of certain chemicals including luminol is defined as “chemiluminescence” and the responding chemical reaction is often called “chemiluminescence reaction”. In a chemiluminescence reaction, enzymes including peroxidase and phosphatase are often used as a catalyst that is widely employed in photometric methods such as enzyme-linked immunosorbent assay. In cases where the enzyme is luciferase or photoprotein, the reaction is called “bioluminescence reaction”. As the reaction substrate used for bioluminescence reaction is called “luciferin”, bioluminescence reaction is called “luciferin-luciferase reaction”. The term “bioluminescence” covers the production and emission of light by a living organism or emission of light by a laboratory biochemical system. Therefore, bioluminescence reaction can be considered as a form of chemiluminescence reaction.

Bioluminescence reporter genes are widely used for reporter gene assays to evaluate gene expressions, protein–protein interactions and biological activity of substances.

Bioluminescence enzymes are also used to quantify cellular adenosine triphosphate (ATP) amounts and enzymatic activity.

In cell-based assays by means of bioluminescence measurement methods, cells are generally treated with the target bioactive substance and incubated for a certain period. Afterwards, the cells are lysed, and the bioluminescence signal is measured by adding the bioluminescent reagent into the solution.

NOTE 1 Bioluminescent reagent generally contains luciferin and multiple chemicals for the enhancement and stabilization of the luciferin-luciferase reaction.

Biological activity of a substance can also be evaluated quantitatively or qualitatively without disrupting the cells by means of real-time bioluminescence monitoring or bioluminescence imaging.

Bioluminescence and chemiluminescence reactions emit light in different spectra depending on the reactant.

In bioluminescence and chemiluminescence reactions, optical signal intensity changes with time following the initiation of the reaction by the addition of a bioluminescent reagent or a chemiluminescent substrate, respectively.

The degree of time-dependent alterations of optical signal intensity (i.e. reaction kinetics) largely depends on enzyme and substrate combinations. These alterations of optical signal intensities are further affected by ambient conditions, including temperature.

NOTE 2 When the measured bioluminescence or chemiluminescence value is expressed as an absolute power (in units of W) or as the number of photons, it is an absolute value; in other cases, the value is relative.

NOTE 3 Absolute bioluminescence or chemiluminescence signal value can be measured using an optical signal measurement instrument with photodetector that is calibrated absolute responsivity.

A.3 Fluorescence

Light emission process in which absorption of light of a given wavelength by substance is followed by the emission of light at a longer wavelength is recognized as fluorescence. "Phosphorescence" is also known as the light emission after light absorption, despite the emission delays remarkably after excitation by light absorption. Thus, both fluorescence and phosphorescence are categorized as "photoluminescence" in the broad scientific fields. Scientifically, fluorescence and phosphorescence are described as the emission from the excited singlet or triplet state of the molecule, respectively.

NOTE 1 In ISO 9555-4:1992, 3.1, fluorescence is described as follows: "The emission of electromagnetic waves of characteristic energy when atoms or molecules decay from an excited state to a lower energy state. The excitation may be induced by subjecting the substance to radiation of slightly higher energy (shorter wavelength) than that of the characteristic emission, and it ceases as soon as the external source is removed."

NOTE 2 In IEC 60050-845:2020, fluorescence is described as follows: "emission of optical radiation when a substance is exposed to any type of electromagnetic radiation, where the emitted radiation generally appears within 10 nanoseconds after the excitation".

Fluorescent materials in biological samples, including fluorescent dyes, fluorescent proteins and biological samples, emit fluorescence signals produced by irradiation with excitation light. Fluorescence dyes and reporter genes are used in bioassays by labelling/staining or connecting to the target objects, respectively.

NOTE 3 Absolute power (in units of W) of fluorescence emission can be measured using an optical signal measurement instrument with calibrated absolute responsivity, including quantum efficiency of photodetector.

NOTE 4 Some chromotropic compounds are used, which are, for example, made to change fluorescence spectrum and to be from non-fluorescent to fluorescent, during the assay procedure.

Fluorescent materials bind to specific biological components (proteins, nucleic acids and molecules) and/or specifically localize inside or outside cells in a time-dependent manner.

Fluorescent dyes, fluorescent proteins and biological samples have specific excitation and emission spectral properties.

The fluorescence signal intensity from the samples is usually proportional to the irradiation of excitation light.

The fluorescence signal intensity can be reduced by irradiation of excitation light, which is known as "photobleaching".

A.4 Absorption

Dyes and chromogenic substrates are detected by absorbance measurement. Also, absorption properties of biological samples are utilized to spectrometric measurements. Absorbance measurement methods, including the enzyme-linked immunosorbent assay, spectrophotometric analysis and reporter gene assays, are used to quantify biological samples, or cellular function assessment.

NOTE 1 Absorption is caused by transmitting light through samples or chromogenic substances. Transmittance looks at extinction which also includes contributions from refraction and scattering.

NOTE 2 Some chromotropic compounds change absorption or absorption spectra during the assay procedure.

NOTE 3 Cellular functions include enzymatic activity, cellular uptake and organelle activity.

Chromogenic reagents bind to specific biological samples and localize inside or outside cells in a time-dependent manner.

NOTE 4 Qualitative evaluation of chromogenic assays is sometimes based on visual inspection of colour. Quantitative evaluation uses measurement of absorption at different wavelength.

The optical signal intensity from chromogenic reactions depends on the reaction time and environmental factors, including temperature.

Annex B
(informative)

List of optical references, optical signal measurement instruments and representative photometric methods

Examples of optical references, optical signal measurement instruments, and representative measurement methods for photometric methods are shown in Table B.1.

NOTE A reference plate can be used as an optical reference for quantitative real-time PCR.

Table B.1 — List of optical references, optical signal measurement instruments and representative photometric methods

Optical signal	Optical reference	Optical signal measurement instrument	Representative photometric method
Bioluminescence	LED	Tube or microplate-type luminometer	Reporter gene assay ATP assay Immunoassay
		Imaging analyser	Bioluminescence imaging
Chemiluminescence	LED	Tube or microplate-type luminometer	Immunoassay Reporter gene assay
		Imaging analyser	Western blotting
Fluorescence	Fluorescent glass Fluorescent solution	Microplate reader Spectrofluorometer	Immunoassay Reporter gene assay
	Fluorescent dye Fluorescent bead Pulsed LED Laser	Flow cytometer	Flow cytometry Immunoassay
		DNA sequencer	Sequencing analysis
	Glass slide embedded fluorescent material Laser	Imaging analyser	Fluorescence imaging High content analysis
		Microarray reader	Transcriptome analysis
	Power meter	Excitation light source	
Absorption	Reference filter Reference cuvette Reference film Reference solution	Spectrophotometer	Immunoassay Optical density measurement
	Reference filter Reference film Reference solution	Microplate reader	Immunoassay Optical density measurement Reporter gene assay
	Test target	Imaging analyser	Electrophoresis
	Power meter	Light source	

Annex C
(informative)

List of relevant standards describing representative methods by means of optical signal measurements

This document mainly describes procedures for the verification of optical signal measurement instruments using optical references. Preanalytical procedures including sample preparation are also important components for optical signal measurement accuracy. This document can complement relevant standards describing various analytical and assay methods by means of optical signal measurement. Examples of relevant standards and publications are as follows:

a) cell characterization methods: see ISO 23033;

b) reporter gene assay (bioluminescence measurement): see ISO 19040-1, ISO 19040-3 and References [25], [27], [28] and [31];

c) reporter gene assay (absorbance measurement): see ISO 19040-1 and ISO 19040-2;

d) flow cytometry: see ISO 19344, ISO/TS 19006, ISO 20391-1, ISO 20391-2 and References [26] and [28];

e) cell viability (bioluminescence measurement, ATP assay): see ISO 13629-1;

f) cell viability (absorbance measurement): see ISO 10993-5, ISO 19007 and References [29] and [30];

g) immunoassay (absorbance measurement): see ISO 15089 and Reference [26];

h) spectrophotometry: see Reference [24].

Annex D
(informative)

Example of qualification of luminometer using LED reference light source

D.1 General

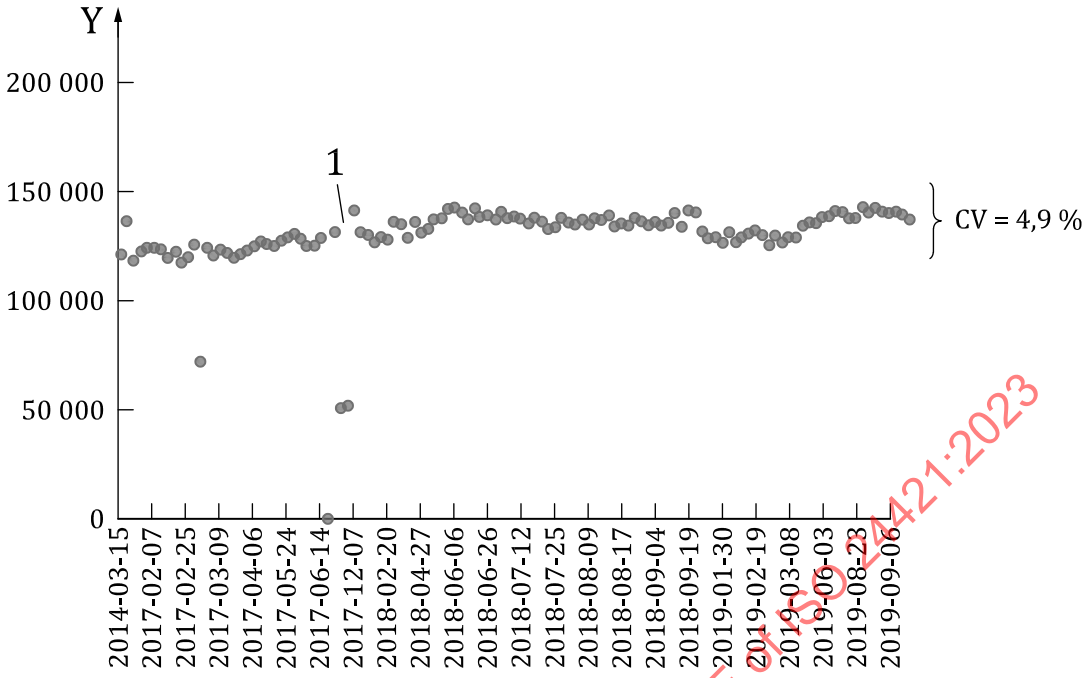
For the qualification of a luminometer, a reference light source, including an LED reference light source, whose stability and reproducibility have been verified, can be used in testing laboratories. By recording data measured with the LED reference light source, day-to-day and experiment-to-experiment reproducibility of the luminometer is evaluated.

D.2 Reproducibility evaluation using LED reference light source

Figure D.1 shows an example of the reproducibility evaluation of a luminometer using an LED reference light source. A plate-type LED reference light source that emits green light (λ_{max} = 527 nm) was set on a microplate luminometer. Output value was measured for 5 s at room temperature. Measurement results were recorded regularly.

Repeated measurements with the LED reference light source were performed for five years. The results show that the responsivity of the luminometer is stable, namely, there is less than 5 % coefficient of variation (CV %), excluding the irregular values recorded before repair. The intermediate precision of the optical signal measurements, made with this luminometer during the experimental period, was ensured (see, for example, Reference [32]).

NOTE In June 2017, signal intensity from the LED reference light source continuously showed irregular values due to luminometer malfunction (see Figure D.1). After repair, the signal intensity showed regular values again.



Key

Y detector count rate (1/s)

1 repair of luminometer

Figure D.1 — Repeated measurements with luminometer using LED reference light source

Annex E
(informative)

Example of application of reference light source for comparison measurements of bioluminescent sample using luminometers

E.1 General

This annex describes an example of comparison of data obtained by measurements with different luminometers, using a reference light source. In the measurement of bioluminescent samples, such as bioluminescent cells, using a luminometer, output value is expressed as relative count value. Therefore, it is impossible to directly compare data when measurements are conducted using different luminometers. Data comparison is also difficult even when measurements are performed with luminometers produced by the same manufacturer due to lot-to-lot variation of the photodetector, indicating difficulty of data comparison between facilities. However, data can be compared on a common measurement scale by evaluating the difference in responsivity between luminometers using a reference light source, including an LED reference light source.

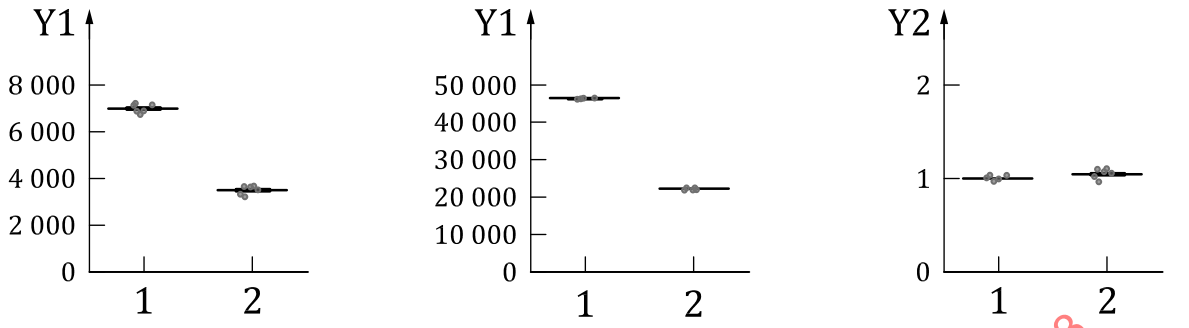
E.2 Data comparison between facilities

Figure E.1 a) shows optical signal intensity of luminescent cells expressing red-emitting beetle luciferase (λ_{max} = 620 nm), provided by laboratory A to laboratory B, measured independently by both laboratories. In the measurements, common commercially available bioluminescent reagent and multi-well plates were used, but luminometers produced by different manufacturers were used. As a result, optical signal intensity expressed as relative count in laboratory B was approximately half of that in laboratory A.

Figure E.1 b) shows optical signal intensity from an LED reference light source (λ_{max} = 624 nm), provided by laboratory A to laboratory B, measured in both laboratories. The optical signal intensity in laboratory B was approximately half of that in laboratory A, similar to Figure E.1 a).

As shown in Figure E.1 c), approximately the same values were obtained when optical signal intensity from bioluminescent cells (see Figure E.1 a)) was normalized by the intensity from the LED reference light source (see Figure E.1 b)), where relative value in laboratory A is set to 1. Thus, it became possible to compare data measured with different luminometers (different facilities) on a common measurement scale by utilizing the reference light source.

This method is helpful when comparing measurement results to ensure the performance of a bioluminescent sample within a laboratory, between manufacturer and manufacturer, manufacturer and user, or user and user.



a) Bioluminescent cells **b) LED reference light source** **c) Normalized intensity**

Key

Y1 detector count rate (1/s)
Y2 normalized intensity
1 laboratory A
2 laboratory B

Figure E.1 — Light intensities comparison

Annex F
(informative)

Example of determination of well-to-well crosstalk in multi-well plates

F.1 General

This annex describes and gives an example of the determination of well-to-well crosstalk when multi-well plates are used as measurement containers mainly in bioluminescence and chemiluminescence measurements.

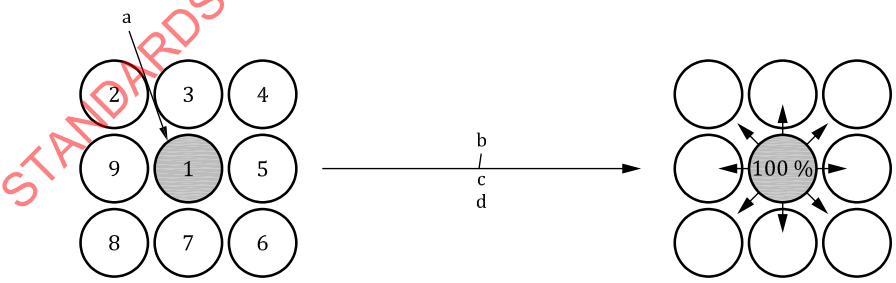
Well-to-well crosstalk occurs when stray light enters adjacent wells, reducing the accuracy of measurement results.

Well-to-well crosstalk is strongly dependent on the instrument and the type of multi-well plate (clear, black, grey or white). In general, the crosstalk value is the highest for clear plates. The value for black multi-well plates is lower than that for white multi-well plates, although higher signal intensity can be obtained with white multi-well plates. Thus, black multi-well plates show the lowest crosstalk value, but due to their low signal efficiency, black multi-well plates can be used only when the signal intensity of the sample is sufficiently high.

F.2 Determination of well-to-well crosstalk

Figure F.1 shows a schematic for the determination of well-to-well crosstalk in multi-well plates. A light-emitting sample, including luciferase solution, a chemiluminescent reagent, or another light source if applicable, was added into a well, and signal intensities of the well and its surrounding wells were measured. In general, crosstalk values for the surrounding wells are expressed as % when the intensity of the well containing the sample is set to 100 %.

NOTE Figure F.1 shows an example of determination of the crosstalk value for a specific well. Crosstalk values sometimes vary by well location. Therefore, multiple wells can be measured to determine crosstalk values for the entire plate.



a Sample (e.g. luciferase-expressing cells or lysates, luciferase enzyme, chemiluminescent reagent or other light source).

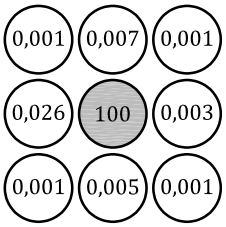
b Measure intensity.

c Sample well (1).

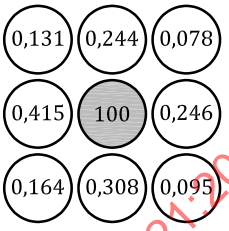
d Surrounding wells (approximately 2 to 9).

Figure F.1 — Schematic of determination of well-to-well crosstalk in multi-well plates

Figure F.2 shows an example of the determination of well-to-well crosstalk using luciferase-expressing cells. The cells were seeded into 96-well black and white clear bottom plates. After incubation for one day, the cells were treated with a commercially available bioluminescent reagent according to the manufacturer's instructions, and signal intensities of the well and its surrounding wells (without cells) were measured. After subtracting the background value (dark counts) from the measurement values, the signal intensity of the cells was set to 100 %, and crosstalk values for the surrounding wells were estimated.



a) Black 96-well clear bottom plates



b) White 96-well clear bottom plates

NOTE Values in white circles indicate well-to-well crosstalk values (%).

Figure F.2 — Determination of well-to-well crosstalk using luciferase-expressing cells

Annex G
(informative)

Examples of dynamic range determination of luminometer

G.1 General

This annex describes an example of the dynamic range determination of luminometer using luciferase enzymes (see Clause G.2) and an LED reference light source (see Clause G.3). Dynamic range evaluation is generally conducted as an installation qualification and/or operational qualification of a luminometer.

G.2 Dynamic range determination using a luciferase enzyme

To determine the dynamic range of a luminometer, a serial dilution of luciferase enzyme solution can be used. Luciferase enzyme is diluted with an appropriate buffer and used to prepare a serial dilution. Then the dilution solution is dispensed into a measurement container including multi-well plates. An appropriate volume of luciferin (bioluminescent reagent) is added to the luciferase enzyme solution and the generated signal is measured using a luminometer.

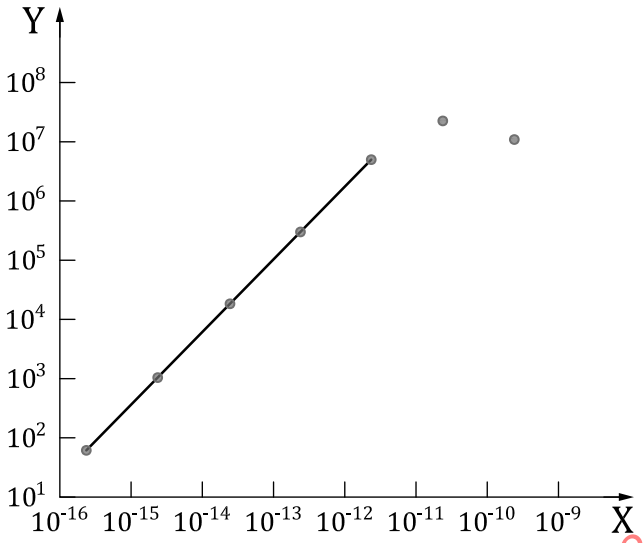
NOTE 1 Purified or crude (e.g. luciferase-expressing cell lysate) luciferase enzyme can be used to determine the dynamic range.

NOTE 2 In the preparation of the dilution solution, the temperature and composition of buffer can affect the activity of luciferase enzyme due to aggregation or deactivation.

NOTE 3 Measurement timing after the addition of bioluminescent reagent is controlled to ensure reproducibility.

Figure G.1 shows an example of the dynamic range determination of a microplate luminometer. Purified green-emitting beetle luciferase enzymes were diluted with 100 mM Tris/HCl (pH 8,0) supplemented with 100 mM NaCl and 8 % (m/v) glycerol, and this was used to prepare a serial dilution using the same buffer. A 10 µl aliquot of the enzyme solution was dispensed into each well of a 96-well black clear bottom plate and mixed with 90 µl of a commercially available bioluminescent reagent. According to the manufacturer's instructions, the signal was measured for 10 s at room temperature.

NOTE 4 In general, bioluminescent reagents contain excess amount of luciferin against luciferase.



Key

X luciferase (mol)

Y detector count rate (1/s)

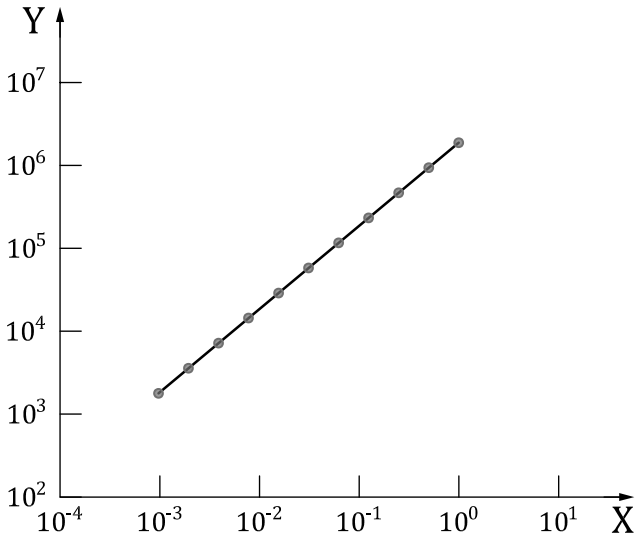
Figure G.1 — Dynamic range determination of a microplate luminometer using purified green-emitting beetle luciferase

G.3 Dynamic range determination using LED reference light source

An LED reference light source whose light output is variable can be used to determine the dynamic range of a luminometer. The appropriate shape of the LED reference light source is selected because various shapes, including button, tube or plate shape, are available.

Figure G.2 shows an example of the dynamic range determination of a microplate luminometer using a plate-type LED reference light source. The LED reference light source, which emits green light (λ_{max} = 527 nm), was set on a microplate luminometer, and signals were measured for 3 s at room temperature. Light output power was changed serially (11 steps).

It is recommended that the temperature of an LED reference light source in a luminometer is kept constant during measurement, because the signal intensity from the LED reference light source is strongly dependent on temperature.



Key

X intensity (LED, arbitrary unit (arb.u.))

Y detector count rate (1/s)

Figure G.2 — Dynamic range determination of a microplate luminometer using an LED reference light source

Annex H
(informative)

Example of construction of calibration curve and dynamic range determination of fluorescence plate reader

H.1 General

This annex describes an example of the dynamic range determination of a fluorescence plate reader, which can be helpful for testing laboratories.

A fluorescence plate reader measures the fluorescence signal emitted by fluorescent substances excited by a light source in the instrument. Excitation and emission filters are employed to separate light having the wavelength intended for use. The fluorescence signal is detected by a photodetector and expressed as arbitrary units. For background correction, a buffer solution not containing fluorescent molecules is measured as a blank and the obtained background value is subtracted from each measured signal value of the fluorescent sample.

H.2 Construction of calibration curve

A calibration curve can be constructed by measuring a serial dilution of reference fluorescent molecules that have the same fluorescence property as the measurement sample (see Figure H.1). Standard materials are suitable for the construction of the calibration curve. The quantity of the measurement sample can be determined by comparing with fluorescence readout and the calibration curve.

NOTE When fluorescence staining of nucleic acids or proteins is performed, sample-to-sample variation due to difference in target molecular species can be observed even if the same fluorescence staining reagent is used.

H.3 Dynamic range determination

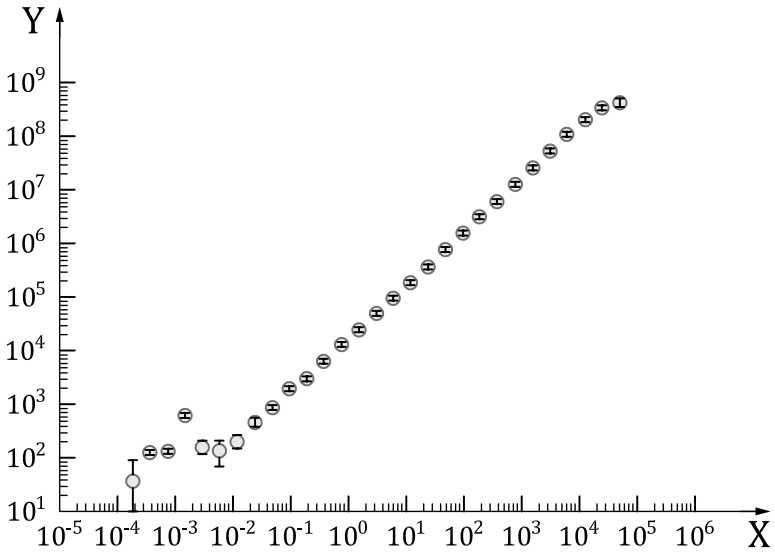
The dynamic range of the fluorescence plate reader can be determined from a calibration curve constructed from a reference material for calibration curve (see Figure H.1). Photodetector settings, including amplification voltage and exposure time, can be adjusted to control the dynamic range.

NOTE 1 In fluorescence measurements, the irradiation power of excitation light and its stability also affect the measurement readout.

NOTE 2 An LED reference light source can be used to determine the dynamic range of a detection unit in a fluorescence plate reader.

NOTE 3 In high concentration fluorescent measurement samples, the linear increase of the fluorescence readout can be inhibited because most of the excitation light is absorbed on the surface and cannot reach the sample solution.

NOTE 4 Properties of fluorescent samples, such as quenching and/or changing of the fluorescence spectrum due to solvent conditions, can affect the measurement readout.



Key

X dye concentration (nM)

Y detector reading (arb.u.)

Figure H.1 — Dynamic range determination of fluorescence plate reader from calibration curve constructed by measuring a serial dilution of fluorescein solution

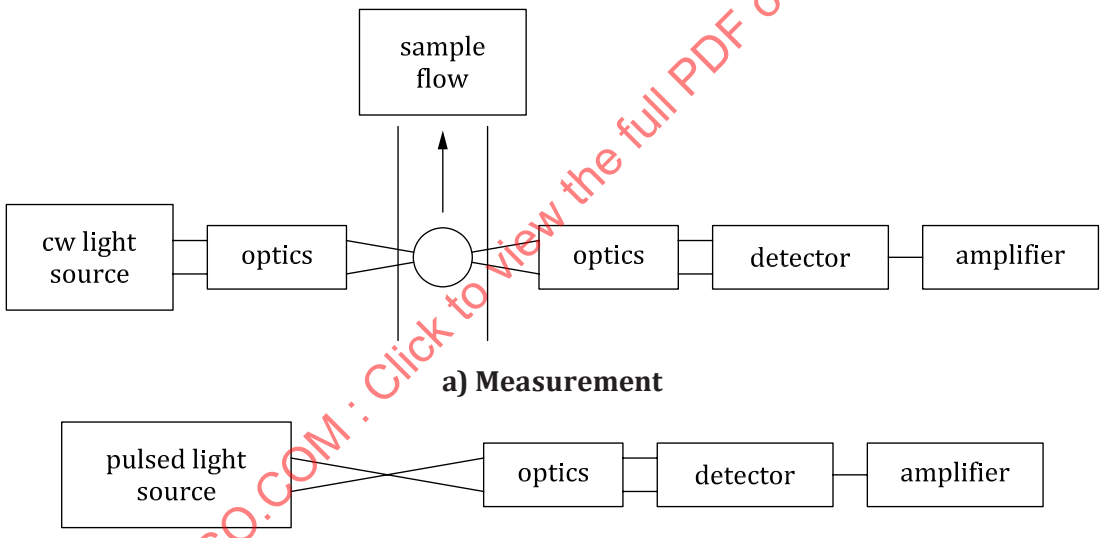
Annex I
(informative)

Example of dynamic range determination of a flow cytometer

I.1 General

Flow cytometers and droplet digital PCR readers use photodetectors to measure fluorescence emitted from particles that flow through an optical detection zone (see Figure I.1 a)). The detection zone is illuminated by a continuous light source. The passing particle causes a fluorescence light pulse that is detected and amplified. The amplifier can be linear or nonlinear depending on the application or the desired dynamic range to be covered. The electric output signal is digitized to determine pulse height or other parameters characterizing the particle analysed.

EXAMPLE The area under the pulse can be exploited to improve sensitivity at low count rate.



a) Measurement

b) Determination of dynamic range

Figure I.1 — Optical configuration

I.2 Dynamic range measurement

The dynamic range of the detector can be determined by replacing the sample stream by a pulsed light source (see Figure I.1 b)). The pulsed light source can be based on one or more LEDs with controlled emission characteristics. The analytical instruments typically have two or more fluorescence detection channels that use different spectral filters in the respective detector optics. It should be considered to remove the spectral filter for determination of the dynamic range with a pulsed light source, since the spectral emission range of the LED can be limited. The pulse duration, pulse height and pulse repletion rate of the optical signal should be adjustable in the range required for testing.

NOTE 1 Typical values of the pulse duration are in the range from 0,5 µs to 50 µs for measurement with flow cytometers. Typical pulse repetition rates are in the range from 0,1 kHz to 20 kHz.

The pulse height of the pulsed light source should be varied in regular steps on a linear or on a logarithmic scale, depending on application. Figure I.2 shows an example of variation on a logarithmic