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Nanotechnologies — Performance evaluation requirements for quantifying biomolecules using fluorescent nanoparticles in immunohistochemistry

Nanotechnologies — Exigences d'évaluation des performances pour la quantification de biomolécules en immunohistochimie à l'aide de nanoparticules fluorescentes

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Foreword

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This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

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

Fluorescent nanoparticles are expanding their market into bio-technological and research fields as a labelling material to be used for immunohistochemical staining.

Conventionally, various fluorescent dyes, including FITC (fluorescent isothiocyanate), rhodamine-isothiocyanate and sulforhodamine 101 acid chloride, have been used for immunohistochemical staining. They are still powerful tools for identifying localization of target biomolecules, for example, proteins and sugar chains, mainly for qualitative analyses. They are also applied to quantitative analysis in combination with various algorithms for calculating signal intensity related to the quantity of the target biomolecules. The quantification system generally consists of sample preparation, staining, microscopic observation and photography, and image processing for obtaining quantification results as shown in Figure 1. For reliable measurement results of quantification, fluorescent dyes that are brighter and more photostable by exposure to the excitation light are more appropriate.

Large number of fluorescent nanoparticles are available in the market. Generally, they show higher brightness and are more resistant to photobleaching, compared to the conventional fluorescent dyes. The characteristics of fluorescent nanoparticles can be an advantage for the quantification of target biomolecules by immunohistochemical methods also combining with the same algorithm employed for the quantification with conventional fluorescent dyes.[1][2][3][4]

In this context, various staining kits with fluorescent nanoparticles and various quantification systems have been developed and are available in the market.[5] Thus, the needs to realise the compatibility of various systems are expanding in the research and industrial fields.

In this document the minimum requirements for performance evaluation of products and application using fluorescent nanoparticles is addressed. This document provides information ensuring the comparability of the results of relative quantification by using fluorescent nanoparticles.

This document does not provide industry segment specific performance criteria for the workflow of measuring biomolecules. When applicable, users can also additionally consult existing industry specific standards.

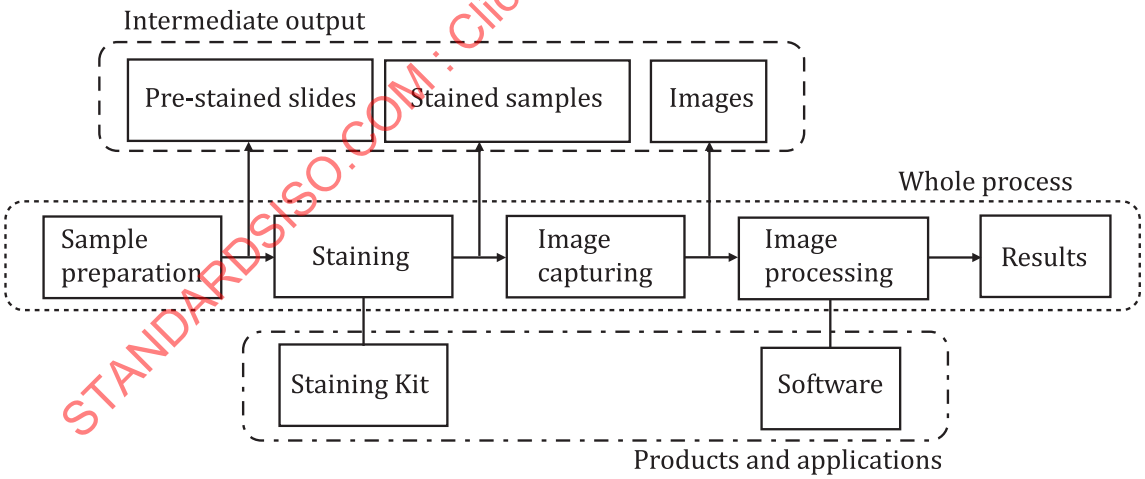

Figure 1 — Target quantification process by using of fluorescent nanoparticles

Nanotechnologies — Performance evaluation requirements for quantifying biomolecules using fluorescent nanoparticles in immunohistochemistry

1 Scope

This document describes minimum requirements for performance evaluation of applying fluorescent nanoparticles in quantitative immuno-histochemistry.

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
fluorescent nanoparticle
nanoparticle emitting fluorescence excited by light of specific wavelength

3.2
cell block array
paraffin block re-embedded with plural cylinders gouged out from paraffin embedded cell suspension for histopathology

3.3
cell block array section
thin slice of cell block array obtained by cutting cell block array using microtome, and mounted onto a glass slide

3.4
agglomerate
collection of weakly or medium strongly bound particles where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2022, 3.1.2]

3.5
agglomeration
process through which agglomerates form

3.6
aggregate
particle comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces, for example, covalent or ionic bonds, or those resulting from sintering or complex physical entanglement.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2022, 3.1.3, modified — Note 1 to entry has been adapted.]

3.7
aggregation
process through which aggregates form

3.8
quantum yield
number of quanta emitted per quantum absorbed

[SOURCE: ISO 22493:2014, 6.6.9]

3.9
molar extinction coefficient
optical density of 1 M fluorescent substance per 1 cm of optical path of absorption cell

3.10
photobleaching
destruction of fluorescing properties of molecules by light, resulting in reduced fluorescence of the sample

[SOURCE: ISO 10934:2020, 3.2.31]

4 Principle

Immunofluorescence methods are based on staining of thin tissue sections with specific antibodies recognizing intended molecules, e.g. proteins and sugars. The specific antibodies are prepared by immunizing animals, e.g. goat, rabbit or mouse, with target molecules. While monoclonal and polyclonal antibodies are used for immunofluorescence methods, antibodies need to be labelled with fluorescent substances, e.g. dyes or nanoparticles. For investigating localization and quantity of the target molecules, tissue sections are stained with fluorescence labelled antibodies. There are two basic methods for staining with labelled antibodies. One is the direct immunofluorescence method, the other is an indirect immunofluorescence method. The direct immunofluorescence method is performed by using a single fluorescence-labelled antibody recognizing the target molecules. The indirect immunofluorescence method is performed with a non-labelled antibody recognizing the target molecules (first antibody) and antibody recognizing the first antibody (second antibody). When the first antibody (e.g. IgG) is produced in mouse, anti-mouse IgG antibody should be selected for the second antibody. In addition, other molecular systems enhancing fluorescence signal are also available in market. For example, the avidin-biotin system is well known for this application. In the avidin-biotin system, the second antibody can be labelled with biotin and reacted with avidin conjugated with fluorescent materials including nanoparticles, e.g. quantum dots (QD), i.e. semiconductor particles with a few nanometres in size. The first antibody-second antibody-avidin-QD complex emits a strong fluorescence signal with less photo bleaching. It is preferred for the quantification of the target molecules by using nanoparticles including QD.

Fluorescence signal from the stained specimen is measured by fluorescence microscopy, which is an optical imaging technique that detects simultaneously the emitted fluorescence from the field of view using a camera. Fluorescence intensity, namely the emitted fluorescence, can be measured as an intensity value in fluorescence microscopy, which is computed by summing together the intensity

values from a group of individual pixels in a digital image acquired using a digital camera. In addition to the measurement of integrated fluorescence intensity, counting bright spots is used to quantify the target biomolecules.

Quantitative comparison of the intensity data or the numbers of the bright spots requires sound experimental design and appropriate operation of the whole quantitative photometric system including a digital camera e.g. a charge coupled device (CCD) or a scientific complementary metal oxide semiconductor (sCMOS). Issues of the quantitative comparison of intensity data involving the digital camera and controller software settings, including collection of dark count images to estimate the offset, flat-field correction, background correction, benchmarking of the excitation lamp and the fluorescent collection optics are described in Reference [15].

For quantitative analysis by immunofluorescence microscopy, fluorescence intensity or the number of bright spots can be compared to the measured value by enzyme-linked immuno-sorbent assay (ELISA) or fluorescence activated cell sorter (FACS) and a calibration curve drawn (see A.3). Fluorescence microscopy measures the fluorescence intensity or the number of bright spots from immunostained thin slice of tissues and cells, however, it does not measure the biomolecule number per cell. It measures the fluorescence intensity or the number of bright spots from a slice of a population of the cells although it is corelated to the number of biomolecules in cells when fluorescence intensity or the number of the bright spots is measured from a sufficient number of the cells in a field of view. In this sense, microscopic measurement of fluorescence intensity or the number of bright spots is a relative measurement.

Fluorescence intensity does not in itself have an associated SI unit, because it is a relative measurement. The number of the bright spots has “unit one” but it is also a relative measurement in principle. A relative intensity measurement (RIM) is determined as the ratio of one intensity measurement or bright spot count to another. The fluorescence intensity measurement is an accurate estimate of the ratio of the irradiance from part or all of a specimen, to the irradiance from part or all of the same or another specimen. For the counting of bright spots, the results can be interpreted in a similar way with the integrated fluorescence intensity measurements.

Indices for performance evaluation of quantitative values and application of CBA (cell block array) for realizing comparability among values that form various quantification systems are described in the following clauses.

5 Selection of fluorescent nanoparticles

5.1 General

Nanoparticles shall be selected to fit the purpose of the quantification system. Required performance of nanoparticles varies with respect to quantity of target biomolecules, performance of imaging systems including sensitivity, available excitation wavelengths, colours for staining including multicolour staining. For the selection of nanoparticle-labelled antibodies, including commercially available labelled antibodies, the performance of nanoparticles and the titre of the antibodies should be evaluated (see 5.2).

5.2 Characteristics of nanoparticle

When nanoparticle labelling is performed in the laboratory, the nanoparticle shall be selected based on the characteristics of nanoparticle. The characteristics of nanoparticles to be evaluated shall include but not limited to the following.

a) Brightness.

   Brightness is important for the quantification of biomolecules by immunohistochemistry. Initial brightness can be used as a characteristic for the selection of nanoparticles. It is a relative brightness that is proportional to molar extinction coefficient and quantum yield. For the selection of nanoparticles, these parameters, i.e. molar extinction coefficient and quantum yield, should be evaluated.

Molar extinction coefficient is defined as the optical density of 1 M fluorescent substance per 1 cm of optical path of absorption cell, measured by absorption photometry. Quantum yield is defined as the number of quanta emitted per quantum absorbed and can be measured with a fluorospectrophotometer.

b) Photobleaching time.

When fluorescent dyes or nanoparticles are continuously irradiated by excitation light, their emission output decreases and is eventually bleached. It can be characterized by the half-time of bleaching when the number of emitted photons per hour is halved.

c) Particle size and size distribution.

Average particle size should be analysed with coefficient of variation (CV). Dynamic light scattering (DLS) and scanning electron microscopy (SEM) can be used for this analysis. For SEM analysis some standards[16] are helpful.

d) Aggregation and agglomeration.

Agglomeration is the process or degree to form agglomerates that are a collection of weakly or medium strongly bound particles. In agglomeration, the resulting external surface area is similar to the sum of the surface areas of the individual particles. Aggregation is the process or degree to form aggregates that are particles comprising strongly bonded or fused particles. In aggregation, the resulting external surface area is significantly smaller than the sum of the surface areas of the individual particles (see Clause 3).

An example of an evaluation method of aggregation is shown in Annex B.

NOTE That includes QD aggregate and agglomerate in biological media[6] as well as the other types of nanoparticles.[7][8][9][10] The excess aggregated QDs can be separated and removed.[11]

e) Non-specific absorption of nanoparticles.

Nonspecific absorption of fluorescent nanoparticles can be a part of the background noise, diminish the quality of fluoromicroscopic images, and hinder the relative-quantification analysis. Nonspecific absorption of nanoparticles should be evaluated (see 7.1).

f) Uniformity of nanoparticle(s).

When nanoparticles are used as fluorescent substances, variation in particle size can reduce the level of correlation between quantity of biomolecules and fluorescence intensity. Particle size shall be evaluated along with CV and reported.

6 Quantification system

6.1 Overall design

When designing a quantification system for selected fluorescent nanoparticles, the intended use shall be defined and documented. The quantification system described in this document uses technology of immunohistochemistry that is an application staining thin sections of tissues with an antibody specifically recognizing a target biomolecule. The thin section can be prepared from formalin fixed paraffin embedded and frozen tissues. They are subsequently stained with antibodies linking to nanoparticles. In some cases, another molecular system including biotin-avidin conjugation can be used to detect the antibody binding to the target molecules (see 6.2.1). For the quantification, stained slides are observed with fluorescence microscope and images of tissues are captured with a digital camera, followed by quantification of the fluorescence signals from the tissue image with the image analysis software. The design of the quantification system should cover all the steps of the process, from the prepared tissue sections and the experimentally measured values, to the image processing and final quantification of the data with the processing software.

The design description shall contain but not be limited to the following specifications: the fluorescent nanoparticle to be used and its performance, antibodies including nanoparticle labelled and non-labelled antibodies when used, staining conditions, microscope system with imaging equipment, spectral characteristics of the light source used, and image analysis software. The design shall be reviewed to ensure conformity to the requirements of the system.

For selecting image processing software, requirements of the software shall be described. Minimum exposure time for imaging is critical for the quality of image processing by the software as well as other instrument configurations stated in 6.4. Staining procedure, imaging and image processing software are not independent of each other. When quantification analysis method is newly developed or changed, the design description shall be reviewed.

6.2 Antibody

6.2.1 General

Various antibodies are used in the immunohistochemistry. For example, a single labelled antibody for direct immunofluorescence methods and a set of two antibodies comprising primary antibody recognizing a target biomolecule and labelled second antibody recognizing the first antibody molecule. The labelled antibody is conjugated with a nanoparticle directly or molecules attached to the other molecules, for example biotin attached to avidin. By any method, the antibody recognizing the target biomolecule and nanoparticle are linked after staining to mark the target biomolecules.

NOTE Various labelling designs can be employed for immunohistochemistry. Typical labelling schemes are as follows.

a) Primary antibody - Biotinylated secondary antibody -avidinated nanoparticles.

b) Biotinylated antibody -avidinated nanoparticles.

c) Nanoparticles with primary antibody bound to the surface.

Antibodies shall be selected based on various characteristics including antibody type, target antigen, applicability to method, animal species, immunoglobulin type, titre and affinity. The specific issues described below should be considered.

6.2.2 Antibody type

Antibody type, i.e. monoclonal vs polyclonal should be considered for the choice of the quantification method.

Monoclonal antibodies generally bind to a single recognition site (single epitope), leading to 1:1 ratio with respect to biomarker molecules, and allowing direct quantification (e.g. by fluorescence intensity).

On the contrary, polyclonal antibodies can bind to multiple epitopes within the same biomolecule, providing some advantages in terms of tagging efficiency but precluding any intensity-based quantification. Depending on spatial resolution, polyclonal antibodies can still be used for quantification of biomolecules by using the method of counting the number of fluorescent points in the image (see 6.4.2).

6.2.3 Target antigen

The target antigen is selected according to the role of the antibody used in the staining process.

For antibody to be used for recognizing biomolecules, e.g. antibodies for the direct immunofluorescence method and primary antibodies for the indirect immunofluorescence method, the antigen shall be confirmed as the target biomolecule.

A monoclonal antibody recognizes the epitope that is a specific part of the antigen. The epitope can be determined by various epitope mapping technologies,[13][14] when appropriate. Information on the epitope can be helpful for quantification of biomolecules.

On the other hand, the antigen of the second antibody are the primary antibodies recognizing the target biomolecules. The antigenicity of the primary antibodies is determined mainly by the animal species (see 6.2.5) and immunoglobulin type (see 6.2.6).

6.2.4 Applicability of antibody to method

The antibody to be used for microscopic quantification shall be confirmed by its applicability to immunohistochemistry. In many cases, commercially available antibodies have been validated for specific application including western blotting, flow cytometry, chromatin immunoprecipitation assay (ChIP) and immunohistochemistry. Even if the antibody was validated for immunohistochemistry, the applicability of the antibody shall be confirmed for the fitness of purpose before use.

6.2.5 Animal species

An antibody to be used for the immunohistochemistry is normally produced based on the immune reaction of the animals against the immunogen. A polyclonal antibody is prepared from serum of the immunized animal. A monoclonal antibody is produced with the cells obtained from the immunized animal, by various technologies including hybridoma and phage display technologies. In both cases, the origin of immunoglobulin of the antibody is the immunized animal. In the indirect immunofluorescence method, second antibody, for immunostaining is selected based on the antigen recognized. Thus, the antigen of the second antibody is the immunoglobulin of the primary antibody, e.g. mouse IgG and rabbit IgG.

6.2.6 Immunoglobulin class

Antibodies are classified mainly into five isotypes (or classes) of immunoglobulins including IgG, IgM, IgA, IgD, and IgE, divided by the types of heavy chain in the antibody molecule structured by molecular class switching. Due to the differences in heavy chain polypeptides, these immunoglobulins can play different roles in the specific processes of the immune response. There are differences in the number of Y-shaped monomers, consisting of a combination of two light chains and two heavy chains in a complete protein. For example, IgM molecule consists of five Y-shape monomers, whereas IgG molecule consists of a single monomer.

The immunoglobulin class of antibody is important information, when a monoclonal antibody is used for the indirect immunofluorescence method. When the polyclonal antibody is used for the primary antibody, major isotype, IgG, can be presumed. Monoclonal antibody is, however, produced from a clone of the antibody producing cell. Thus, the clone produces a single isotype of immunoglobulin. When a monoclonal antibody is used, immunoglobulin isotyping of this producing antibody shall be performed for the selection of the second antibody. The second antibodies shall be selected to fit to the isotype of the primary antibody, for example, anti-mouse IgG antibody can be selected for the primary monoclonal antibody produced from mouse hybridoma.

6.2.7 Titre

Regarding the antibody used, concentration, titre, or both should be evaluated to be fit for purpose of staining before measurement.

The antibody concentration is the total amount of antibody (protein) per unit volume regardless of the degree of the antibody function. Concentration of pure antibody can be estimated by measuring OD 280 nm. OD of 1 % (10 mg/ml) antibody solution is expected to be 13 to 14. Commercially available protein assay kits can also be used for the estimation of the concentration. Titre can be defined as a functional concentration estimated by the immunoglobulin specific measurement method including ELISA.

Antibodies are supplied, however, without concentration and titre but only with recommended dilutions for various application. In these cases, the dilution factor of stock solution of the antibody can be used as an index of antibody function instead of titre.

Information on concentration, titre and dilution factors are helpful for obtaining stable results from quantification by fluorescence immunohistochemistry including staining processes.

6.2.8 Specificity

The specificity of an antibody can be evaluated by various methods. Examples are shown as follows:

— western blotting;

— analysis with cells known to express the target biomolecules;

— competitive analysis with synthesized peptide;

— immunoprecipitation-mass spectrometry;

— negativity check with knock out or reduction check with knock down animals;

— peptide array;

— comparison with the other antibodies that were used in the past;

— analysis with phosphatase treated tissues or cells for phosphorylation modification specific antibody.

Re-evaluation of purchased antibodies are not realistic. Practically, specificity evaluation by antibody manufacturer can be used as a premise to select the antibody. Originally prepared antibodies should be evaluated by the appropriate method, e.g. western blotting, other than the quantification method to be used, when applicable.

6.3 Staining procedure

6.3.1 Staining conditions

The following points should be considered for designing staining procedure:

— pre-treatment conditions including deparaffinization;

— antigen retrieval;

— blocking reagent, blocking time, and washing conditions after blocking;

NOTE Serum of an animal species matching that of secondary antibody is frequently used for blocking in routine methods.

— concentration of antibodies, antibody staining time and washing conditions;

— mounting procedure including cover-glass application.

For designing the staining procedure, the conditions are optimized by maximizing signals for positive samples and linear relationship between the quantity of target and signal intensity, and by minimizing non-specific binding for negative samples. There are, however, trade-offs in some cases.

6.3.2 Robustness of staining procedure

For designing the staining procedure, its robustness shall be considered. Especially, the influence of laboratory temperature should be considered. Room temperature is uneven with respect to climatic region and the presence of air-conditioning. The temperature range is important for specifying robustness of the staining process. An automatic stainer or robotics should be used in the staining

procedure, especially for improving the accuracy of quantification. If staining is performed by personnel, their personnel skills should be included in the estimation of robustness.

6.4 Image processing

6.4.1 Image quality and relevant factors

Intrinsic image parameters including but not limited to, signal to noise ratio, intensity saturation and pixel resolution can be the indicators of the image quality, enabling the imaging processing.

The images to be used for the quantitative comparison of a relative intensity measurement (RIM) can be influenced by the following factors[15]:

— the non-uniformity of signal intensities across the field of view of the microscope;

— the presence of an offset in the pixel values in the recorded digital image;

— the intensity signals in the image exceeding the linear dynamic range of the camera;

— the inaccurate recording of the pixel values in image data files due to factors such as a lossy compression operation or unexpected modification of the pixel bit depth when saving each file;

— low signal-to-noise ratio of the measured light signal intensities;

— instability in the optical power of the illumination source.

For signal intensity determination, the image analysis protocol should address corrections for signal offset inherent to the detector, non-uniform signal detection across the field, and possible saturation of the detector. These issues and possible normalization strategies are described in Reference [15].

Instrument configuration, such as excitation intensity, digital gain, dynamic range and pinhole size shall be set appropriately and recorded.

When larger and brighter nanoparticles are available, the number of bright spots can be used as the measured value instead of the fluorescence intensity. In this case, the resolution of a single bright spot in the detection of a bright spot can be representative of the quality of the image.

6.4.2 Selection of image processing software

The quantification methods shall be selected to be fit for the documented purpose of the system (see 5.1), although various quantification methods and algorithms are available for the bio-molecular detection. [1] There are two categories of quantification methods, more specifically measurements based on the detected fluorescence signal intensity[3] and counting of the number of fluorescent points. [4][5]

For example, RESA/PLACE (rapid exponential subtraction algorithm/pixel-based locale assignment for compartmentalization of expression) algorithms are used for quantification.[12] These algorithms carry out definition of the target fluorescence signal as well as the definition of localization by the identification of tumour locale with in the histospot (“tumour mask”) and identification of subcellular compartments (e.g. nuclei, membranes, cytoplasm).

In addition to the quantification of signal intensity, target biomolecule can be measured by counting the number of nanoparticles. For example, the number of nanoparticles in the bright spot in a fluorescence image can be estimated by three steps, comprising discrimination of a bright spot arising from nanoparticle fluorescence and not from noise, definition of the area of a bright spot derived from nanoparticles and estimation of the number of nanoparticles in the bright spot.[3]

7 Comparability of results

7.1 Light source adjustment

For comparison of the results of relative quantification, the light source should be adjusted to illuminate the view of the microscope appropriately.

Light source can be adjusted by monitoring the intensity of the light from the light source directly or the fluorescence intensity of the stable fluorescence material illuminated with the light source. The intensity of the light source can be measured with a power meter calibrated to be SI-traceable. A commercially available standard florescent slide can be used as the stable fluorescence material.

The measured light intensity or fluorescence intensity can be an indicator of the illumination intensity used for the relative quantification microscopy. Light source operation at constant light intensity is important to obtain stable fluorescence images. It is also helpful for considering the comparability of quantification results. The measurement results of light intensity or fluorescence intensity should be recorded.

7.2 Reference material

Quantification with fluorescence microscopy is a relative quantification. The measurand has no unit and different values to the same fluorescence intensity when different measurement systems are used. In order to compare the quantification results from different measurement systems or different laboratories, the conversion factor should be calculated based on the results from the same sample, i.e. reference material, with different quantification system.

Reference material shall be established for ensuring comparability of the results from different quantification systems.

NOTE CBA can be used for reference material (see Annex A).

When appropriate reference material can be identified, the quantity of target biomolecules, as a specified property of reference material, should be measured by the method other than the designed measurement method including FACS, ELISA, and western blotting (see Annex A). Because quantification with immunofluorescence microscopy is a relative quantification, measured values cannot be directly compared with the above-mentioned other quantification methods. It is just a rough indication of the amount of the biomolecules, but can be helpful for the discussion of the abundance of biomolecules in the target tissues or cells in some cases.

Fluorescence intensity can be affected by the FFPE slice thickness and permeability of cells. The slice thickness should be set to that normally used for the quantification (e.g. 4 µm). Various tissue permeability cannot mimic culture cells, even if the cells are chosen carefully. In this case thinner slices can be considered to mitigate the effect of permeability.

Lot-to-lot differences of the reference material shall be considered. The comparative data between the current lot and the new lot of reference materials is useful for adjusting the differences between the lots.

8 Performance characteristics

8.1 Background

8.1.1 General

Background fluorescence intensity for each tissue to be measured shall be determined. The image obtained from staining with nanoparticle conjugated molecules without primary antibody should be used for the determination of background. Three indicators should be considered. For example, when biotinylated antibody -avidinated nanoparticle labelling is used, the background can be determined

from the image of tissues stained only with avidinated nanoparticles without first and second antibodies.

The following three indicators influencing the background should be evaluated:

a) autofluorescence;

b) nonspecific absorption of nanoparticles;

c) nonspecific absorptions of antibodies.

Every indicator needs to be evaluated for each tissue to be measured. Because statistical analysis with multiple views is not practical, outliers are confirmed by measuring in at least three views.

8.1.2 Autofluorescence

Autofluorescence can be evaluated by staining with the first antibody and nanoparticle conjugated molecules. For example, maximum fluorescence intensity from three views of tissues stained with the first antibody, biotin labelled second antibody without avidinated nanoparticles can be used as the autofluorescence value of the tissue.

8.1.3 Nonspecific absorption of nanoparticles

Nonspecific absorption of nanoparticles can be evaluated by staining only with the nanoparticle conjugated molecules. For example, maximum fluorescence intensity from three views of tissues stained only with avidinated nanoparticles without first and biotinylated second antibodies, can be used as the value of nonspecific absorption of nanoparticles.

8.1.4 Nonspecific absorption of antibodies

Nonspecific absorption of antibodies can be evaluated by staining the same combination of antibodies and nanoparticles, and precluding or substituting an antibody by a non-target specific isotype antibody.

The nonspecific absorption of the first antibody can be, for example, evaluated by measuring samples stained with a non-target specific isotype antibody instead of a target specific antibody and appropriate secondary antibody with nanoparticle conjugated molecules. These combinations can be used as a control for the qualitative analysis. Maximum fluorescence intensity from three views of staining can be used as the value of nonspecific absorption of the first antibody.

The nonspecific absorption of the second antibody can be, for example, evaluated by measuring signals from samples stained only with the secondary antibody and nanoparticle conjugated molecules, without a target specific antibody. Maximum fluorescence intensity from three views of staining can be used as the value of nonspecific absorption of the second antibody.

8.1.5 Loss of intensity and interferences

When the field of view is continuously illuminated, fluorescence intensity can be gradually reduced. The half-time of the bleaching of nanoparticles (see 5.2) used for the measurement can be an index of the loss of intensity.

For mitigating the loss of intensity, use of an alternative fluorescent nanoparticle, lower illumination, short exposure time, normalization according to the fluorescence photobleaching curve and anti-photobleaching material can be considered.

The loss of signal intensity due to quenching or absorption of the material shall also be taken into account.

For the bright spot counting measurements, loss of intensity and interferences are critical to the count of bright spots. For this purpose, the decrease of the fluorescence intensity of nanoparticles

can be ignored as long as sufficient brightness of the nanoparticles is maintained during the counting measurement.

8.2 Reference material dependent indices

8.2.1 General

Quantification in fluorescence microscopy is a relative quantification. The measured value is unitless. Therefore, the indices described here are valid if the same reference materials are used.

8.2.2 Limit of detection (LOD)

Estimation of LOD shall be performed. Practically, LOD is analysed based on the standard deviation (SD) of determined background signals in a field of view. For example, LOD can be set to a sum of the mean of the signal intensities of background and three times of SD. When counting method for quantification, LOD can be set to the mean of the number of nanoparticles per view of background plus three times of SD. Reference material, e.g. CBA, should be used for the measurement of the background signals to avoid the differences in the slides.

The determined LOD is also helpful for the interpretation of the results of bright spot counting including the detection of the bright spot by image analysis.

When another definition of LOD is used, it shall be documented and reported.

8.2.3 Limit of quantification (LOQ)

LOQ should be determined to the minimum fluorescence from a slice of a cylinder emitting the weakest fluorescence in the CBA slices. The cells in one of the cylinders of CBA should be those expressing a small enough amount of the target biomolecules for quantification.

For the bright spot counting, LOQ depends on the limit of bright spot detection. When the brightness of the nanoparticle is enough for the detection of the bright spot, LOQ is considered to be a single spot.

8.2.4 Linearity and dynamic range

By measuring of fluorescence intensity from the stained CBA prepared with cells in which the expression levels of proteins were measured by another method, e.g. including FACS or ELISA, in advance (i.e. given value), it is possible to graph the relationship between the quantitative measurement results and the pre-measured values.

A regression line can be drawn when the measured value and the given value are graphed. The sum of the distances of each measurement point from the regression line can be an indicator of linearity. At the measurement points of low fluorescence intensity and high fluorescence intensity concentration, the distance from the regression line tends to be large. In that case, a threshold value can be determined and a measurement point with a large distance can be removed from the regression line. Finally, a regression line drawn by a group of points that are sufficiently short in distance from the regression line is obtained, and the dynamic range can be set from the lowest fluorescence intensity to the highest intensity of the regression line. Therefore, in order to verify a sufficient dynamic range, it is necessary to prepare a cell line with a sufficiently low or high expression level of the target biomolecules. In any case, the measured maximum or minimum fluorescence intensity from a slice of cylinder in CBA should be the maximum or minimum fluorescence intensity of the dynamic range.

Linearity and dynamic range of the bright spot counting can be determined in the same way by replacing the fluorescence intensity with the number of bright spots.

8.3 Robustness

The effect of small deviations in the relevant method parameter on the method performance and the measurement results should be examined to determine method robustness. Relevant method parameters that influence the results are the concentration of antibody (both first and second antibodies for indirect immune fluorescence quantification), the degree of washing process, and the excitation light (laser) strength should be varied during robustness testing.

In addition, the photochemical process in the deactivation process of the excited fluorescent molecules and the quenching due to the collision of the fluorescent molecule with the quencher are affected by the temperature, and rise with temperature. This reduces the fluorescence intensity. Therefore, robustness evaluation includes not only temperature effects on the staining step, but also on the actual fluorescence imaging and measurement step, depending on instrument type, because not all instruments will have a temperature-controlled incubator or stage.

For counting the number of bright spots, the effect of temperature should be evaluated to ensure that the temperature does not affect the detection of bright spots in the range that the measurements are performed.

9 Validation and verification

9.1 General

The quantification system using fluorescent nanoparticles shall be validated before the measurement. In the beginning of the validation process, purpose of the measurement and requirements for the purpose shall be defined. The reference material, e.g. CBA should be designed appropriately for collecting data to prove the quantification system fulfils the requirements and be adequate for the measurement purpose. During the validation processes, the performance characteristics described in Clause 8 should be evaluated.

When the purpose of the quantification contains additional decision processes, including interpretation of results, acceptance criteria for validation should be designed based on the final decision process.

Validated methods used without modification shall be subjected to independent verification by the users of the methods before being used to measure the targets. The validation process often clarifies the objectives that should be provided during validation to ensure that the requirements are fulfilled. The same reference materials used in the validation process can be used for the verification to collect comparable data regarding the performance characteristics.

9.2 Single lab precision

Precision is a measure of the variability in independent measurement results obtained for the same sample under stipulated conditions.

Repeatability of the quantification method using fluorescent nanoparticles should be determined. For example, reference material, e.g. CBA, can be used for the determination of repeatability of tissue sample analyses. Measurement should be repeated for statistically meaningful times, for example, an independent measurement of three samples.

Intermediate precision is also helpful for the performance evaluation of the relative-quantification method. The estimation of intermediate precision can be designed based on the repeatability by changing operators or measurement days, for example, three different days. The CBA are also used for the determination of intermediate precision depending on the purpose of measurement with relative-quantification system.

9.3 Reproducibility

Quantitative fluorescence microscopy with immunohistochemistry is a relative quantification method, and the results of the measurements, e.g. fluorescence signal intensity or the number of bright spots, cannot be compared directly to those from the other quantification systems, especially in the other laboratories.

When the quantification results are compared with those from the other quantification systems, especially in another laboratory, the same reference materials, e.g. CBA, shall be used, and the conversion factor for the comparison should be calculated.

The staining process has a large degree of variability. Therefore, reproducibility should be evaluated by taking the dyeing process into account.

10 Reporting

The data report shall contain sufficient detail to allow independent assessment of the target quantification results.

Reporting elements shall include, but are not limited to:

— sample - ID, source description;

— reagents – name, source, lot number;

— antibodies – first and second antibodies for indirect immunofluorescence;

— reference material used;

— background and related indicators;

— LOD, LOQ, dynamic range (see 8.2);

— result of validation, verification, or both;

— description of the whole measurement system;

— unexpected observations.

Annex A
(informative)

Example of the reference material

A.1 Principle

Cell block array can be an example of the reference material to evaluate the quantification methods of biomolecules using fluorescent nanoparticles.

A.2 Cultured cells

A.2.1 General

Cells are selected based on the target expression level so that it can be a representative signal when the calibration curve is formed.

A.2.2 Cells

Human mammary gland derived epithelial cells, MCF7 (ATCC HTB-22), T47D (ATCC HTB-113), CAMA-1 (ATCC HTB21), ZR-75 (ATCC CRL-1500), UACC-812 (ATCC CRL-1897), and inflammatory breast cancer derived cells, KPL4, were used.

A.2.3 Cell block array

The selected cells are individually embedded in paraffin. A solid cylinder of paraffin containing cells is die-cut out from a cell-condensed portion. The cylinder is termed a “cell block”. Combination of cell blocks are designed so that each representative target concentration is proper for building the calibration curve. Cell blocks are arranged to be arrayed and embedded again in paraffin. A cell block array section is prepared by thin slicing the resultant block.

A.3 Calibration curve

For quantitative analysis by immunofluorescence microscopy, fluorescence intensity or the number of bright spots can be compared to the measured value by ELISA (enzyme-linked immunosorbent assay) or FACS (fluorescence activated cell sorter) to produce a calibration curve. Figure A.1 shows an example of calibration curve comparing the number of bright spots with the measured signal from FACS.