INTERNATIONAL STANDARD

ISO 19344

IDF 232

First edition
2015-12-15

Milk and milk products — Starter cultures, probiotics and fermented products — Quantification of lactic acid bacteria by flow cytometry

Lait et produits laitiers — Cultures, probiotiques et produits fermentés — Quantification de bactéries lactiques par cytométrie en flux

Reference numbers
ISO 19344:2015(E)
IDF 232:2015(E)

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Forewords

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The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products* and the International Dairy Federation (IDF). This document is being published jointly by ISO and IDF.

IDF (the International Dairy Federation) is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/food control authorities.

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ISO 19344 | IDF 232 was prepared by the IDF Standing Committee on *Analytical Methods for Dairy Microorganisms* and the ISO Technical Committee ISO/TC 34 on *Food products*, Subcommittee SC 5 on *Milk and milk products*.

The work was carried out by the IDF/ISO Project Group on *Quantification of Lactic Acid Bacteria by Flow Cytometry* of the Standing Committee on *Analytical Methods for Dairy Microorganisms* under the aegis of its project leader, Sandra Casani (DK), Ph.D.

Introduction

Quantification of lactic acid bacteria is an important factor in assessing the quality of starter cultures, probiotics and fermented milk products. Examination of lactic acid bacteria in these products can be done following different method principles, with plate count techniques being the most traditional and widely used. Newer techniques include flow cytometry, which is able to determine cells as active and/or total units. Advantages of the use of flow cytometry include low variation, differentiation between active and total cells, and possibility of high analysis throughout. Furthermore, the quantification and use of the fraction of active cells per total cells is a key feature and an important flow cytometry tool to evaluate the fitness of a given cell population. This is of special relevance for certain applications such as optimization of production process and stability assessment during shelf-life.

The International Organization for Standardization (ISO) and the International Dairy Federation (IDF) draw attention to the fact that compliance with this document may involve the use of patents concerning the staining of protocol C as described in this document.

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Milk and milk products — Starter cultures, probiotics and fermented products — Quantification of lactic acid bacteria by flow cytometry

WARNING — The use of this International Standard may involve hazardous materials and operations. This International Standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this International Standard to establish safety and health practices and to determine the applicability of regulatory limitations prior to use.

1 Scope

This International Standard specifies a standardized method for the quantification of active and/or total lactic acid bacteria and probiotic strains in starter cultures used in dairy products by means of flow cytometry. The method is also applicable to probiotics used in dairy products and to fermented milk products such as yogurts containing primarily lactic acid bacteria.

This International Standard does not apply to taxonomical differentiation of bacteria. Due to its non-specificity, the method may quantify other bacteria than those within the scope of this International Standard, when present in the sample. This may lead to overestimation of the counts.

The minimum bacterial cell concentration in the sample before applying this standardized method depends on the dilution rates used in the individual protocols. Typically 10^6 cells per gram or ml are considered within the minimum range.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6887-1:1999, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

ISO 6887-5:2010, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 7889 | IDF 117, *Yogurt — Enumeration of characteristic microorganisms — Colony-count technique at 37 °C*

ISO 15214, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of mesophilic lactic acid bacteria — Colony-count technique at 30 °C*

3 Terms and definitions

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

3.1
lactic acid bacteria
gram-positive, non-motile, non-spore forming, catalase-negative, nitrate-reductase-negative and cytochrome oxidase-negative bacterium that does not liquefy gelatine or produce indole

Note 1 to entry: Lactic acid bacteria have a fermentative metabolism which is mainly saccharolytic. Lactic acid is the major end product from carbohydrate utilization.

EXAMPLE Lactic acid bacteria of importance for the dairy industry are: *Streptococcus thermophilus*, *Lactococcus lactis*, *Pediococcus*, *Enterococcus*, *Leuconostoc* and *Lactobacillus*.

3.2
probiotic strains
probiotic strains are live microorganisms which, when administered in adequate amounts, are intended to confer a health benefit to the host

EXAMPLE Probiotic strains of importance are: *Bifidobacterium animalis*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus plantarum* and *Propionibacterium freudenreichii*.

Note 1 to entry: See Reference [1].

3.3
active fluorescent units
AFU
events counted in a gate specific for scatter/fluorescence characteristics of presumed live cells, i.e. cells stained for the specific activity indicator used in the protocol

3.4
non-active fluorescent units
n-AFU
events counted in a gate specific for scatter/fluorescence characteristics of presumed dead cells, i.e. cells damaged to an extend that they do not stain for the specific activity indicator used in the protocol

3.5
total fluorescent units
TFU
sum of AFU and n-AFU

3.6
% active fluorescent units
% AFU
percentage ratio of AFU to TFU

4 Principle

4.1 A test portion or sample is prepared, and diluted if necessary.

4.2 Initial suspensions, and/or dilutions if needed, are stained according to one of the following three protocols, differing on the target of fluorescent cell staining, in order to discriminate active and total fluorescent units:

a) dual staining targeting nucleic acid with the non-permeant red-fluorescent dye propidium iodide (PI) and intracelullar enzyme activity based on cleavage of 5(6)-carboxyfluorescein diacetate (cFDA) mixed isomers to green-fluorescent carboxyfluorescein by intracellular esterases;

b) dual nucleic acid staining with PI and a cell-permeant green fluorescent dye, i.e. SYTO®[1)] 24 green fluorescent cell-permeant nucleic acid stain;

c) single staining with the membrane-potential-sensitive cyanine dye 3,3'-diethyloxacarbocyanine iodide ($DiOC_2$). Wavelength of emitted light changes with metabolic activation of cells.

The choice of the staining protocol depends on the user's preferences or possibilities.

4.3 The stained samples are analysed by means of a flow cytometer using a combination of light scattering (LS) and detection of emitted fluorescent light. As cells pass into the flow cytometer, each cell is counted and the fluorescence is recorded.

4.4 Gating is conducted to separate cells from noise and to differentiate AFUs and n-AFUs.

4.5 Calculation of the concentration in the original sample is a multiplication of AFUs (or TFUs) per volume of analysed sample and the dilution factors employed in the sample preparation.

5 Diluents and reagents

5.1 General

Unless otherwise specified, use only reagents of recognized analytical grade, and distilled or deionized water or water of equivalent purity, according to ISO 7218.

Prepare the initial suspension (common for all protocols) with the diluent as specified in 5.2.

The composition and the preparation of all the reagents used in each of the three staining protocols (A, B and C) are specified in 5.3. An overview of the diluents and reagents per protocol is given in Table 1.

5.2 Peptone-salt solution

The composition and preparation of the peptone-salt solution is according to ISO 6887-5:2010, 5.2.1.

NOTE For the preparation of the initial suspension, and dilutions if needed, other diluents for general use mentioned in ISO 6887-5:2010 can be used if they can be shown to lead to the same results.

5.3 Diluents and reagents for staining protocols

WARNING — Propidium iodide is a potential mutagen. Proper actions for deactivation should be taken in case of spilling. Preparation and application of the dye solution shall be carried out in a fume cupboard, using protective equipment and following good laboratory practices.

Table 1 — Reagents used per protocol

Reagent	Protocol A	Protocol B	Protocol C
Diluent	PBS (5.3.1.1)	PBS (5.3.2.1)	MRS or M17 broth (5.3.3.1 or 5.3.3.2)
Dye solution	cFDA (5.3.1.2) PI (5.3.1.3)	PI (5.3.2.2) SYTO® 24 (5.3.2.3)	Glucose solution (5.3.3.3.1) $DiOC_2$ (5.3.3.3.2) Buffer solution (5.3.3.3.3)

1) SYTO® 24 green fluorescent cell-permeant nucleic acid stain is supplied by Life Technologies. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.3.1 Protocol A

5.3.1.1 Phosphate-buffered saline (PBS)

5.3.1.1.1 Composition

— 9 g sodium chloride (NaCl)

— 795 mg sodium hydrogenphosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$)

— 144 mg potassium dihydrogen phosphate (KH_2PO_4)

5.3.1.1.2 Preparation

Dissolve the components (see 5.3.1.1.1) in water. Add water to a final volume of 1 000 ml. Adjust the pH with HCl to 7,4 ± 0,05, if necessary. Distribute the solution into aliquots and sterilize in an autoclave set at 121 °C ± 1 °C (liquid cycle) for 15 min. The diluent can be stored at cooling temperature (3 °C ± 2 °C) for up to 6 months.

5.3.1.2 5(6)-Carboxyfluorescein diacetate (cFDA) mixed isomers solution

5.3.1.2.1 Composition

— 230 mg 5(6)-cFDA mixed isomers

— 100 ml dimethyl sulfoxide (DMSO)

5.3.1.2.2 Preparation

A 5 mmol/l solution is prepared by dissolving cFDA in DMSO at the amounts specified in 5.3.1.2.1. The solution can be stored at −18 °C ± 2 °C, protected from light, for up to 6 months.

5.3.1.3 Propidium iodide (PI)

5.3.1.3.1 Composition

— 100 mg PI

— 100 ml ultrapure water

5.3.1.3.2 Preparation

Dissolve the PI in ultrapure water to a final concentration of 1,0 mg/ml, corresponding to approximately 1,5 mmol/l. This can be stored at 3 °C ± 2 °C, protected from light, for up to 6 months.

NOTE The concentration of the PI solution used is 0,1 % and the final concentration is 0,002 %. This is below the potential toxicity level.

5.3.2 Protocol B

5.3.2.1 Phosphate-buffered saline (PBS)

See 5.3.1.1.

5.3.2.2 Propidium iodide (PI)

See 5.3.1.3 for the preparation of the PI solution. The PI solution shall be further diluted to 0,2 mmol/l with water prior to use.

NOTE The concentration of the PI solution used is 0,01 % and the final concentration is 0,000 1 %. This is below the potential toxicity level.

5.3.2.3 SYTO® 24 green fluorescent cell-permeant nucleic acid stain

The stain is a 5 mmol/l solution in DMSO. Store at −20 °C, protected from light, for up to 12 months. The solution shall be diluted to 0,1 mmol/l with water before use.

5.3.3 Protocol C

5.3.3.1 MRS broth

The composition and the preparation are specified in ISO 15214 except for no addition of agar.

5.3.3.2 M17 broth

The composition and the preparation are specified in ISO 7889 except for no addition of agar.

5.3.3.3 Stain mixture

The stain mixture consists of 210 µl 50 % glucose solution (5.3.3.3.1), 210 µl 1,5 mmol/l $DiOC_2$ (5.3.3.3.2) and 50 ml buffer solution (5.3.3.3.3). The stain mixture is prepared the same day as it is used.

5.3.3.3.1 Glucose solution

5.3.3.3.1.1 Composition

— 50 g D(+)-glucose monohydrate

— 50 g water

5.3.3.3.1.2 Preparation

A 50 % glucose solution is prepared by dissolving the glucose in the water. This is aided by warming the solution to below the boiling point. Avoid evaporation. The solution is autoclaved at 121 °C ± 1 °C for 15 min and can be stored unopened at 3 °C ± 2 °C for up to 3 months.

5.3.3.3.2 3,3'-diethyloxacarbocyanine iodide ($DiOC_2$)

5.3.3.3.2.1 Composition

— 69 mg 3,3'-$DiOC_2$, ≥ 98 %

— 100 ml dimethyl sulfoxide (DMSO)

5.3.3.3.2.2 Preparation

The $DiOC_2$ staining is prepared as a 1,5 mmol/l solution by weighing $DiOC_2$ into DMSO at the amounts specified in 5.3.3.3.2.1. Dispense in, e.g., 1 ml tubes. Keep dark, as $DiOC_2$ is unstable in light, at 5 °C ± 3 °C for up to 12 months.

5.3.3.3.3 Buffer solution

5.3.3.3.3.1 Composition

— 7,6 g sodium chloride (NaCl; 130 mmol/l)

— 0,5 g sodium dihydrogenphosphate dihydrate ($NaH_2PO_4 \cdot H_2O$; 3 mmol/l)

— 1,24 g sodium hydrogenphosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$; 7 mmol/l)

— 1 000 ml water

5.3.3.3.3.2 Preparation

Weigh and dissolve the three salts in the water at the amounts specified in 5.3.3.3.3.1. Stirring is applied until the salts are dissolved. Adjust pH to 6,5 ± 0,05 with 2,5 mol/l HCl. The solution shall then be filtered through a 0,22 µm filter. The mixture can be kept at 3 °C ± 2 °C for up to one week. For longer periods, up to 6 months, storage at −20 °C is recommended.

6 Apparatus

Usual laboratory equipment and, in particular, the equipment required for the preparation of test samples and dilutions specified in ISO 6887-5, as well as the following, shall be used.

6.1 Water bath, capable of operating at 21 °C ± 1 °C.

6.2 Analytical balance, capable of weighing to the nearest 1 mg, with readability to 0,1 mg.

6.3 pH-meter, with temperature compensation, accurate to ± 0,1 pH unit.

6.4 Incubator, heating block or **equivalent**, capable of operating at the temperatures specified in Table 2.

Table 2 — Incubation temperatures required per protocol

Protocol	Incubation temperatures
A	30 °C ± 1 °C and 37 °C ± 1 °C
B	37 °C ± 1 °C
C	30 °C ± 1 °C and 37 °C ± 1 °C

6.5 Flasks, bottles and test tubes, of sufficient capacity to contain the required volumes and leave adequate head-space for mixing. The capacity depends on the staining protocol and on the flow cytometry equipment.

6.6 Pipettes, sterile, calibrated for bacteriological use, accurate to within 2 % of the volume being pipetted.

6.7 Vortex mixer.

6.8 Filter, sterile, with membrane filters of a pore size 0,22 µm and 25 µm.

6.9 Flow cytometer, instrument capable of detecting and counting particles or cells when passing individually in a directed flow through a beam of excitation light. The instrument must be equipped with a blue laser emitting at 488 nm and with light detectors (fluorescence emission and light scattering). Further details on instrument properties and settings are given in 9.3.2.

6.10 Automated sample preparation unit, automated sample processor capable of handling liquids for sample dilution, mixing, incubation and/or injection into the flow cytometer. This equipment is optional.

7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 | IDF 50[2].

A representative sample is collected for analysis. Unless frozen, test samples shall be cooled after sampling to between 3 °C ± 2 °C and kept at that temperature until testing or freezing. The age of the samples at testing and freezing and the storage conditions may influence the counting result.

The sample shall not be damaged or changed during transportation or storage. The sample shall be homogenous and representative of the batch of product to be tested.

Commercial starter cultures and commercial dairy products shall be stored prior to testing as recommended by the manufacturer.

8 Preparation of test sample

8.1 General

General requirements are in accordance with ISO 6887-1:1999 and ISO 6887-5:2010.

The sample shall be handled for testing in accordance with good laboratory practices.

Initial suspensions and, if needed, further dilutions of the samples to be tested, i.e. freeze-dried cultures, frozen cultures and fermented milk products containing cultures, are prepared as specified in 8.2 to 8.4. The appropriate dilution required to be analysed by flow cytometry depends on the initial concentration of cells in the sample, the staining protocol and the flow cytometry equipment.

8.2 Freeze-dried cultures

Prior to preparation of the initial suspension, allow the freeze-dried sample to acclimatize to room temperature before opening the pouch or container. Alternatively, mix the content of the original sample thoroughly, remove the needed test portion with a sterile spatula, and transfer it to a sterile container or pour the needed test portion into a sterile container. Allow the sample to reach ambient temperature of 20 °C to 25 °C.

Thoroughly mix the contents of the closed container/bag by repeatedly shaking and inverting it.

Prepare an initial suspension (between 10 and 100 fold) by weighing the test sample into a suitable sterile vessel and then adding the required amount of diluent. Alternatively, weigh the test sample directly into the bottle with the diluent. The diluent shall be peptone-salt solution (5.2). The temperature of the diluent shall be the same as that of the test sample in order to avoid damaging the microorganisms by sudden changes in temperature.

For rehydration, the initial suspension is left at ambient temperature of 20 °C to 25 °C for approximately 10 min, and no longer than 45 min, shaking occasionally or continuously (e.g. using a peristaltic homogeniser), before further dilution.

Make sure that the sample is dissolved before proceeding immediately with further testing steps. If needed, further dilutions are prepared by thoroughly mixing a specific amount of the initial suspension with the appropriate volume of diluent (5.2).

As an alternative to manual sample preparation, an automated sample preparation module dedicated to sample preparation, dilution and/or injection can be used.

8.3 Frozen cultures

Prior to initiating preparation of the initial suspension, the test sample shall be thawed. This can be done by leaving the sample at an ambient temperature of 20 °C to 25 °C until the sample has just thawed. Alternatively, place the sample in a water bath at 21 °C ± 1 °C and keep it in the water bath until the test portion has just thawed, see ISO 26323 | IDF 213[3]. Samples shall be tested as soon as they have been thawed. Mix the test sample carefully after thawing.

Prepare the initial suspension, and appropriate dilution(s) if needed, as stated in 8.2. The test sample is measured by volume or weight. The unit of the reported final result shall reflect the choice of unit for the preparation of the test sample.

8.4 Fermented milk products

Samples from fermented milk products, e.g. yogurts, are prepared as follows.

a) Add 90 ml ± 0,1 ml of peptone-salt solution (5.2) at an ambient temperature of 20 °C to 25 °C to a sterile bottle.

b) Mix the sample (e.g. yogurt) either by inverting and shaking or by mixing it with a sterile spatula.

c) Weigh 10 g ± 0,1 g of the sample and add it into the diluent in order to prepare dilution 10^{-1}. The temperature of the diluent shall be the same as that of the test sample in order to avoid damaging the microorganisms by sudden changes in temperature.

d) Shake the bottle slowly 10 times by inverting and shaking.

e) If needed, immediately prepare the serial dilution to obtain the adequate dilution for testing.

NOTE For a final dilution of 10^{-4} to 10^{-5}, there will be so little background interference in the flow cytometer from yogurt matrices that no specific pre-treatment is needed. For yogurt samples containing particles, e.g. fruit pieces or vanilla, the debris might be removed by filtering the final dilution through a 25 µm filter before staining.

9 Procedure

9.1 General

Following preparation of the test sample, testing includes the following steps:

— staining (9.2);

— flow cytometry analysis (9.3);

— gating (9.4);

— calculation of concentrations (Clause 10).

9.2 Staining

Initial suspensions, and/or dilutions from the test sample if needed, are processed and stained depending on the chosen protocol as specified in 9.2.1, 9.2.2 and 9.2.3 prior to quantification by flow cytometry. Diagrams for the three staining protocols are found in Annex A.

The individual staining protocols include dilution steps to achieve an appropriate cell concentration. For further details on appropriate dilution see Clause 11 and for a calculation example see Annex B.

9.2.1 Protocol A

The staining principle is based on the enzymatic activity of cells. In active cells, the non-fluorescent dye cFDA is cleaved by cellular esterase releasing the green fluorescent carboxyfluorescein (maximum fluorescence emission at 520 nm). For a better separation of active and non-active or damaged cells, a counterstaining with PI is performed with a fluorescence emission maximum at 620 nm (red fluorescence).

The test sample (8.2, 8.3 or 8.4) is diluted appropriately in PBS (5.3.1.1). In the last dilution step, 870 µl PBS and 100 µl sample are added to 10 µl cFDA (5.3.1.2; 5 mmol/l in DMSO).

The solution is mixed thoroughly using, e.g., a mechanical stirrer for 5 s and incubated 15 min in the dark at 30 °C for mesophilic strains and 37 °C for thermophilic strains.

20 µl PI (5.3.1.3; 1,5 mmol/l in water) are added to the mixture above and the solution is mixed thoroughly and incubated for 15 min in the dark at room temperature.

The mixture is then ready for flow cytometry analysis (9.3). Analyse the sample within 45 min after adding the cFDA (see A.1).

9.2.2 Protocol B

The staining principle is based on a dual nucleic acid staining with cell permeant dye SYTO® 24 (fluorescence emission maximum at 515 nm) and cell impermeant dye PI (fluorescence emission maximum at 620 nm). SYTO® 24 permeates the membrane of total cells and stains the nucleic acids with green fluorescence. PI penetrates only bacteria with damaged membranes, causing a reduction in SYTO® 24 green fluorescence when both dyes are present. Thus, live bacteria with intact cell membranes fluoresce bright green (defined as active fluorescent cells), bacteria with slightly damaged membranes exhibit both green and red fluorescence (defined as damaged cells) and bacteria with broken membranes fluoresce red (defined as non-active fluorescent cells).

The test sample (8.2, 8.3 or 8.4) is diluted appropriately in PBS (5.3.2.1). In the last dilution step, 100 µl of the sample is added to 880 µl of PBS (5.3.2.1) and mixed thoroughly.

10 µl PI (5.3.2.2; 0,2 mmol/l in water) and 10 µl SYTO® 24 (5.3.2.3; 0,1 mmol/l in water) are added to the mixture. This is mixed thoroughly using, e.g., a mechanical stirrer for 5 s and incubated in the dark for 15 min at 37 °C.

After the dual staining procedure, the sample shall be analysed by flow cytometry (9.3) immediately (see A.2).

9.2.3 Protocol C

The staining principle is based on the membrane-potential-sensitive $DiOC_2$, which changes emission wavelength when active cells build up membrane potential. In all cells, the $DiOC_2$ binds to the membrane with a green fluorescence emission maximum at 500 nm. When cells are activated, the maximum fluorescence emission wavelength is red-shifted. The degree of the red-shift is strain dependent.

The test sample (8.2, 8.3 or 8.4) is diluted appropriately in MRS broth (5.3.3.1) for activation of the metabolism. The exception to this general rule is *Streptococcus thermophilus* which activates better in M17 broth (5.3.3.2).

The dilution for this protocol is divided into two steps. Firstly, the sample is diluted in MRS (or M17) to activate the cells. To ensure optimal activation, the test sample shall be diluted at least 10 times and it shall be incubated for 30 min at 30 °C for mesophilic and 37 °C for thermophilic strains. Secondly, the sample is diluted 25 times into the stain mixture (5.3.3.3) to reach the appropriate dilution. This is incubated at room temperature for 30 min.

The cells are then ready to be analysed by means of flow cytometry (9.3). Flow cytometry testing shall be completed within 30 min after the end of staining (see A.3).

9.3 Flow cytometry analysis

9.3.1 General

The stained samples are analysed by flow cytometry.

Flow cytometry is a technique for rapid quantification of cells combining light source, optics, flow chamber, liquid sample delivery system, light detectors, electronics and software. The flow cytometer provides a constant flow of sheath fluid through a cuvette, singularizing and preparing the cells for analysis. The cuvette is traversed by a beam of light to illuminate flowing cells. Around the cuvette, detectors collect scattered light in two angles [forward scatter channel (FSC) and side scatter channel (SSC)] and fluorescence in different colours (e.g. green, yellow, red). When a cell reaches the flow cuvette, a proportion of the light beam is scattered and cellular fluorescence markers are excited to emit fluorescence. The detection of the scattered light and the concomitant emission of fluorescence are referred to as an event, i.e. each cell passing through gives rise to one event, each of which shows the state of the cell.

Whereas the detected fluorescence is linked to the state of the cell, i.e. enzyme activity, membrane integrity and/or membrane potential, the FSC provides information on cell size and optical density. The SSC provides information on cell morphology, reflectivity and granularity.

The recorded events per microlitre sample indicate how many cells are counted and at the same time differentiate, based on fluorescence parameters, the cells into two categories: active fluorescent and non-active fluorescent.

9.3.2 Instruments and settings

Flow cytometer configuration settings for the optimal functioning of the three protocols are given in Table 3. Some of the parameters, e.g. excitation source and filters for the detectors, are important for the proper performance of the individual protocols, whereas some are less important, e.g. event rate is equipment dependent and varies significantly from instrument to instrument.

The flow cytometer shall be properly calibrated in accordance with manufacturer instructions. Most flow cytometers are calibrated for accurate volumetric determination of the analysed sample for easy calculation of the cell concentration. For instruments without calibrated volumetric determination, the number of AFUs or TFUs in the sample is calibrated against standardized fluorescent beads, added to the sample as an internal standard, with a known concentration.

Even for instruments with calibrated volumetric determination, the use of standard fluorescent beads is mandatory as it greatly increases the ability to trace inaccuracies in small volume determinations and enables verification of proper performance of the detectors. These beads with known concentration and fluorescence intensities shall be used to demonstrate appropriate detection of relevant wavelengths and intensities of the emitted light as well as to document calibration status. The use of standard beads also provides the ability to make accurate comparisons of data from sample to sample, from day to day or from laboratory to laboratory.

The flow cytometer instrument shall be operated by a trained technician and as described in the instruction manual provided by the manufacturer. The flow cytometer should also be cleaned and serviced regularly in accordance with the manufacturer's instructions.

Table 3 — Recommendations for flow cytometer: configuration and settings

Optical configuration	Excitation source	Laser 488 nm, minimum 20 mW
	Detectors	Emission: minimum 2 fluorescence channels and scatter channels
		FL1: Green channel Protocol A: 500–570 nm Protocol B: 500–540 nm Protocol C: 515–545 nm
		FL2 or FL3: Orange or red channel Protocol A: Orange/red > 570 nm Protocol B: Red > 630 nm Protocol C: Red > 650 nm
		Light scattering: Side scatter channel (SSC) and forward scatter channel (FSC)
Fluidic configuration	Sample flow rate	15 to 120 µl per min[a]
	Sample volume analysed	20 µl to 250 µl
	Event rate	Max: 20 000 events/sec[a]
Overall analysis parameters	Triggering parameters used	SSC or both FSC and SSC
	Amplifier and signal conditioning (linearity or logarithmic scale)	Log scale

[a] Settings for these parameters depend on the instrument. Manufacturer guidelines should be followed.

9.4 Gating

9.4.1 General

Gating refers to the evaluation of FSC, SSC and fluorescence results for each of the recorded events. Typically, these results are presented by the instrument software in histograms and/or dot plots with optional axis. The data shall be evaluated in a systematic way to firstly separate cells from noise and secondly differentiate AFUs and n-AFUs.

Thus, gating may be conducted in accordance with the following general recommendations.

a) Separate the cells from noise and media debris in a FSC vs. SSC plot.

b) Gate all cells and only do subsequent data plots on these.

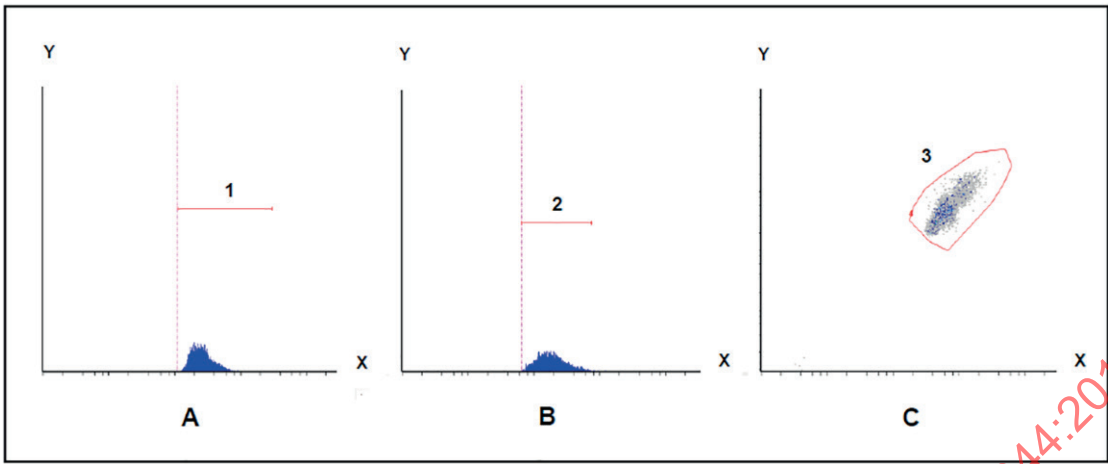
c) Plot the intensity of the red vs. the green fluorescence to differentiate AFUs from n-AFUs.

d) If it is difficult to discriminate the two populations, it is suggested to plot a histogram of counts vs. the ratio of red over green intensity to help the eye discriminate between cell populations. This improves greatly the ability to discriminate cell populations.

e) If still in doubt whether a population is active or non-active, inactivate the active cells either chemically or by heating and thereby document that the presumed live population is shifted to the presumed dead ones. Damaged cells shall not be included in the count of active cells.

9.4.2 Protocol A

To discriminate background noise from cells, the FSC and SSC thresholds are set to exclude background signals from cells (see Figure 1). Gating is conducted as described in 9.4.1.



Key

A forward scatter channel (LS1)
X forward scatter channel
Y counts

B side scatter channel (LS2)
X side scatter channel
Y counts

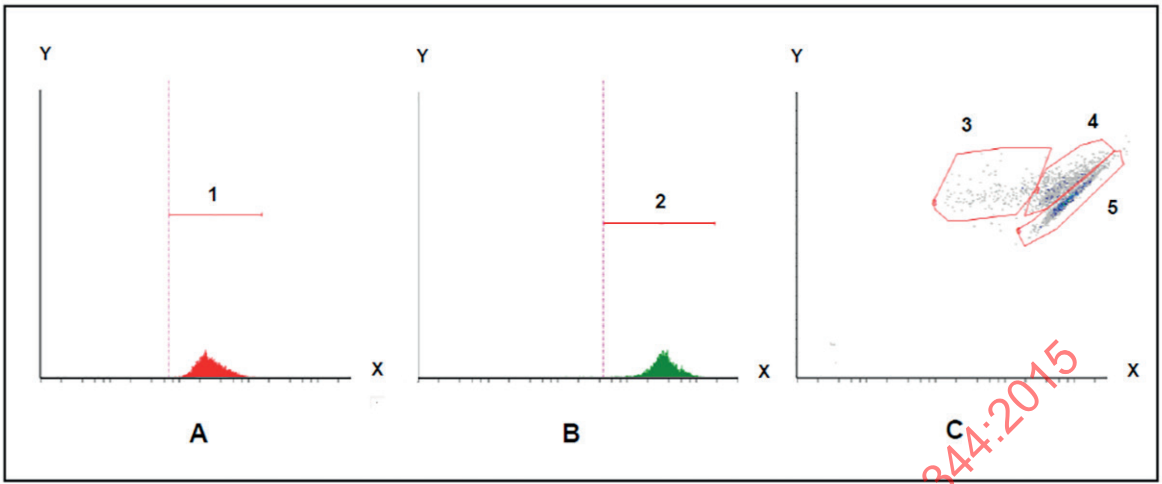
C forward scatter channel vs. side scatter channel
X forward scatter channel
Y side scatter channel

1 LS1 plot (forward light scatter)
2 LS2 plot (sideward light scatter)
3 LS1 and LS2 dot plot showing cell population

NOTE The figure demonstrates the threshold to discriminate cells from noise. The cells are gated in the FSC vs. SSC plot.

Figure 1 — Protocol A, Example (*Streptococcus thermophilus*)

By gating red vs. green fluorescence, two to three populations (active/damaged/non-active) can be differentiated in the dot plot (see Figure 2).



Key

A fluorescent light plot (FL) 3 (red)
X FL3
Y counts

B fluorescent light plot (FL) 1 (green)
X FL1
Y counts

C FL1 vs. FL3
X FL1
Y FL3

1 FL 3 (detector for red fluorescence)
2 FL 1 (detector for green fluorescence)
3 non-active cells
4 damaged cells
5 active cells

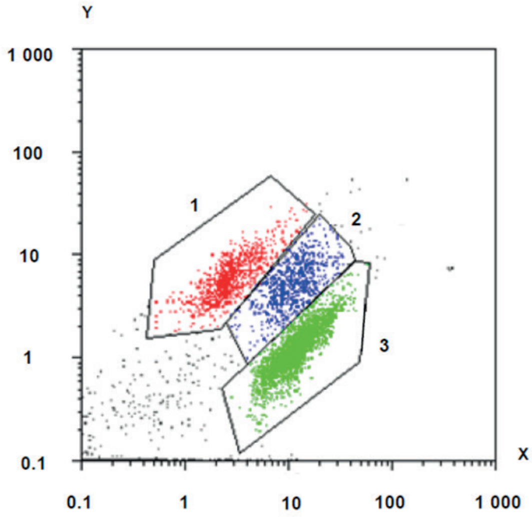
NOTE The figure demonstrates the threshold settings to discriminate fluorescent cells from noise. The active cells, non-active cells and an intermediate cell population are gated in the FL red vs. FL green plot.

Figure 2 — Protocol A, Example (*Streptococcus thermophilus*, freeze dried)

9.4.3 Protocol B

As described in the general recommendations for gating, the parameters FSC for the size, SSC for the structure, fluorescent light plot (FL) 1 for green fluorescence and FL 3 for red fluorescence are evaluated.

In the resulting dot plot of FL1 vs. FL3, according to the principle of the dual labelling with Syto® 24 and PI, three different populations are identified and defined in the gates of active fluorescent cells, non-active fluorescent cells and an intermediate population, here termed “damaged” (see Figure 3). Only the population in the gate active will be calculated as the quantification result of AFUs for the sample.



Key

X FL1

Y FL3

1 non-active

2 damaged

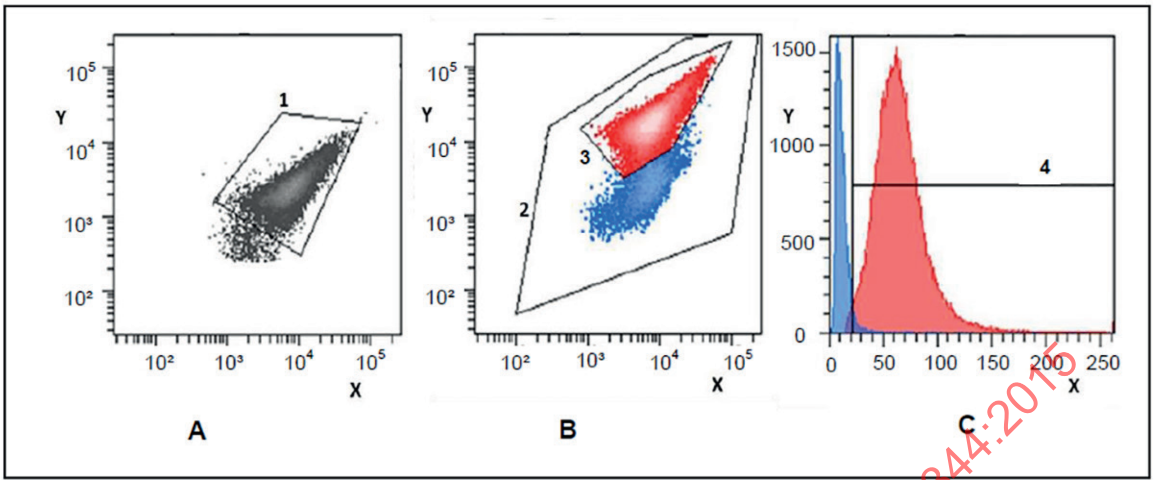
3 active

NOTE The figure demonstrates flow cytometry multiparameter dot plots of green fluorescence (FL1) vs. red fluorescence (FL3)

Figure 3 — Protocol B, Example (*Lactobacillus casei paracasei*, freeze-dried)

9.4.4 Protocol C

Cells (active and non-active) are discriminated from background noise in the SSC vs. green fluorescence plot. This total population of cells is then gated in the far red (> 650 nm) vs. green plot (515 nm – 545 nm) as shown in Figure 4 (left and centre).



Key

A	**cells gated from background**	1	cells gated from background
X	intensity green fluorescence (515 nm – 545 nm)	2	total fluorescent cells
Y	intensity side scatter light	3	red-shifted active fluorescent cells
		4	red-shifted active fluorescent cells
B	**active cells gated from non-active cells**		
X	intensity green fluorescence (515 nm – 545 nm)		
Y	intensity far red fluorescence (> 650 nm)		
C	**ratio far red over green fluorescence**		
X	ratio far red over green fluorescence		
Y	counts		

Figure 4 — Protocol C, Examples of gating cells (*Bifidobacterium*, freeze dried)

The active cells (high intensity far red) are clearly separated from the non-active (lower intensity far red). In more difficult situations where the borderline between active and non-active cells is not so clear, a plot of counts vs. ratio of far red over green (Figure 4, C) gives a clear indication of the border between active and non-active cells.

As the protocol discriminates between active and non-active based on the intensity of red fluorescence, it is important to tune the instrument to achieve the same log intensity of red and green fluorescence for the active cells.

10 Calculation and expression of results

Four categories of results can be obtained by following this standardized method:

a) active fluorescent units per millilitre or gram (AFU/ml or AFU/g): number of active fluorescent unit events divided by the volume or weight of tested sample multiplied by the dilution factor;

b) non-active fluorescent units per millilitre or gram (n-AFU/ml or n-AFU/g): number of non-active fluorescent unit events divided by the volume or weight of tested sample multiplied by the dilution factor;

c) total fluorescent units (TFU/ml or TFU/g): sum of active and non-active units per millilitre or gram;

d) % active fluorescent units (% AFU): percentage ratio of active fluorescent units (a) to total fluorescent units (c).

For a), b) and c), round the calculated result to two significant figures. For a three-figure number, round the third figure to the nearest zero. If the third figure is 5, round to the figure below if the second figure is even and to the figure above if the second figure is odd. The result shall be expressed as a number from 1,0 to 9,9 multiplied by the appropriate power of 10.

For the calculation examples below, the following parameters have been used:

— injection volume (V) on flow cytometer: 20 µl;

— recorded AFU events (n): 2 000 events;

— recorded n-AFU events (m): 4 000 events;

— initial dilution (a): 100 fold;

— additional dilutions (b): 1 × 10 fold;

— protocol staining dilution (c): 10.

EXAMPLE 1 Calculating AFU/ml using protocol A:

$$\frac{AFU}{ml}=\frac{1000\ \mu l}{V}\times(a\times b\times c)\times n$$

$$\frac{AFU}{ml}=\frac{1\ 000\ \mu l}{20 \mu l}\times(100\times 10\times 10)\times 2\ 000=1\times 10^{9}$$

EXAMPLE 2 Calculating n-AFU/ml using protocol A:

$$\frac{nAFU}{ml}=\frac{1000\ \mu l}{V}\times(a\times b\times c)\times m$$

$$\frac{nAFU}{ml}=\frac{1\ 000\ \mu l}{20 \mu l}\times(100\times 10\times 10)\times 4\ 000=2\times 10^{9}$$

EXAMPLE 3 Calculating TFU/ml:

$$\frac{TFU}{ml}=\frac{AFU}{ml}+\frac{nAFU}{ml}=\frac{1\times 10^{9}}{ml}+\frac{2\times 10^{9}}{ml}=\frac{3\times 10^{9}}{ml}$$

EXAMPLE 4 Calculating % AFU:

$$\%AFU=\frac{AFU}{TFU}\times 100\ \%=\frac{1\times 10^{9}}{3\times 10^{9}}\times 100\ \%=33,3\ \%$$

11 Critical factors affecting results

In order to obtain valid and reliable results, the following factors should be considered carefully.

a) Homogeneity and representativeness of the sample. Inhomogeneous samples shall be replicated or, alternatively, the sample size shall be sufficiently large to include the expected variations.

b) Appropriate dilution of the samples. Samples shall be diluted to a degree that enables detection of cells:

   1) around the optimal rate for the instrument in order to avoid shadowing of events, i.e. two events counted as one, and to optimize the cell to noise response;

   2) with a minimum of 1 000 cell events per sample. This number reflects the need to be absolutely sure that the counted events are not noise stemming from particulate matter in the media and that the number of counted events shall be sufficient to give robust counting statistics.

   This means that the appropriate dilution depends on the flow rate through the instrument; the volume available for the analysis; the time available for each counting; and finally the maximum counting speed of the flow cytometer.

   The appropriate dilution ensures that the maximum counting speed is not exceeded; the minimum number of counted events is obtained within an acceptable time; and the available volume of diluted sample is sufficient.

   See Annex B for a calculation example of the appropriate dilution.

c) The flow cytometer shall be properly maintained, calibrated and operated. Special care shall be taken so that the laser is aligned correctly as described by the manufacturer. As described in 9.3.2, the performance of the instrument shall be optimized and verified on a routine basis by standard fluorescent beads.

d) The excitation laser and the filter and mirror settings on the detectors shall be suitable for the chosen protocol. The different fluorophores emit light at different wavelengths. The correct and unambiguous detection and separation of AFUs, n-AFUs and noise depends on this.

e) The detected intensities of light will to some extent be sensitive to cell aggregation. This is most easily seen in a plot of SSC vs. FSC, where the larger aggregations of cells will result in an elongated distribution of cells. Thus, the trained operator will be able to conclude whether cells are not completely separated into single cells.

f) Air bubbles shall be avoided as they distort the light in the cuvette and thus may obscure the detected events. Typically, instruments hold an option to purge the liquid path to remove bubbles.

g) The sample matrix may contain excessive amounts of particulate matter that can be detrimental to the proper analysis of the sample. As an example, for yogurt samples, an appropriate maximum dilution is recommended to reduce the adverse effect of the yogurt matrix. See Annex B for a calculation example of the appropriate maximum dilution.

12 Precision

12.1 Interlaboratory test

Details of the interlaboratory test on the precision of the method are summarized in Table C.1 and Table C.2 (see also Reference [4]). The values for the repeatability and reproducibility were determined by using nine commercially available starter cultures of lactic acid bacteria or probiotic strains (frozen or freeze-dried) used in fermented milk products worldwide. Furthermore, one commercial yogurt product containing two strains of lactic acid bacteria was also included in the interlaboratory test. For each of the ten sample types, two samples (batches at different concentrations) were tested to obtain the reported precision data.

The interlaboratory test was carried out in accordance with ISO 5725-1[5], ISO 5725-2[6] and Reference [7]. The values derived from this interlaboratory test may not be applicable to concentration ranges, cultures and matrices other than those given. The concentration ranges tested, the homogeneity of the samples and the characteristics of the lactic acid bacteria or probiotic strains among the products selected were representative of the worldwide market and were in accordance with ISO 27205 | IDF 149[8].

The precision data reported has been calculated from active and total fluorescent results (log_{10} transformed) obtained by testing the samples with the three staining protocols in parallel.

12.2 Repeatability

The absolute difference between two individual single test results (AFU per gram or TFU per gram, log_{10} transformed), obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will not be greater, in more than 5 % of cases, than the values given in Table 4.

12.3 Reproducibility

The absolute difference between two individual single test results (AFU per gram or TFU per gram, log_{10} transformed), obtained using the same method on identical test material in different laboratories with different operators using different equipment, will not be greater, in more than 5 % of cases, than the values given in Table 4.

Table 4 — Repeatability limits r and reproducibility limits R collectively defined for the product types, log_{10} AFU/g and log_{10} TFU/g

Parameters[a]	Product type[b]	Mean	s_r	s_R	*r*	*R*
AFU/g	Frozen and freeze-dried lactic acid bacteria or probiotic strains	11,345	0,023	0,160	0,06	0,45
	Yogurt	8,868	0,030	0,279	0,08	0,76
TFU/g	Frozen and freeze-dried lactic acid bacteria or probiotic strains	11,484	0,026	0,134	0,07	0,38
	Yogurt	9,185	0,040	0,421	0,10	1,17

a All precision data are expressed as decadic logarithms of AFU or TFU per gram sample.

b See Annex C for further details on strains.

13 Test report

The test report shall contain at least the following information:

a) all information necessary for complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this International Standard, i.e. ISO 19344 | IDF 232, and the relevant flow cytometry protocol;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result(s) obtained, or, if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Diagram of staining protocols

A.1 Protocol A