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

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National foreword

This British Standard is the UK implementation of ISO 19344:2015.

The UK participation in its preparation was entrusted to Technical Committee AW/5, Chemical analysis of milk and milk products.

A list of organizations represented on this committee can be obtained on request to its secretary.

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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

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Forewords

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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 on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

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.

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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.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

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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.

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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.

Neither ISO nor IDF take position concerning the evidence, validity and scope of these patent rights.

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INTERNATIONAL STANDARD

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

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.

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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;

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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.

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