INTERNATIONAL STANDARD

ISO 13366-3

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Milk — Enumeration of somatic cells —

Part 3:
Fluoro-opto-electronic method

Lait — Dénombrement des cellules somatiques —

Partie 3: Méthode fluoro-opto-électronique

Reference number
ISO 13366-3:1997(E)

Foreword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 13366 was prepared by Technical Committee ISO/TC 34, *Agricultural food products,* Subcommittee SC 5, *Milk and milk products,* in collaboration with the International Dairy Federation (IDF) and AOAC INTERNATIONAL, and will also be published by these organizations.

ISO 13366 consists of the following parts, under the general title *Milk — Enumeration of somatic cells*:

— *Part 1: Microscopic method*

— *Part 2: Electronic particle counter method*

— *Part 3: Fluoro-opto-electronic method*

Annexes A to D of this part of ISO 13366 are for information only.

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Milk — Enumeration of somatic cells —

Part 3:
Fluoro-opto-electronic method

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

1 Scope

This part of ISO 13366 specifies a method for counting somatic cells in both raw and chemically preserved milk, using a fluoro-opto-electronic counting instrument[1].

NOTE — Counting of cells in unpreserved samples within the first 24 h after milking could give unreliable results with older instruments (e.g. Fossomatic 90 and 215).

2 Definition

For the purposes of this part of ISO 13366, the following definition applies.

2.1 somatic cells: Those cells that have a minimum intensity of fluorescence due to the staining of DNA in their nuclei.

3 Principle

Mixing of the milk to be examined with a buffer and stain solution. Transference of the mixture in the form of a thin film to a rotating disc, serving as an object plane for a microscope. Each stained cell observed by the microscope produces an electrical pulse that is amplified and recorded. Direct reading of the number of somatic cells in thousands per millilitre.

4 Reagents

WARNING — Ethidium bromide is toxic. The preparation and application of the basic and working solutions shall be carried out in a fume cupboard. Use gloves for protection.

1) The Fossomatic counting instrument (250, 300 or 360) supplied by Foss Electric, Hillerod, Denmark is an example of suitable equipment available commercially. This information is given for the convenience of users of this part of ISO 13366 and does not constitute an endorsement by ISO of the equipment named.

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or deionized water or water of equivalent purity.

4.1 Basic solutions

4.1.1 Dye-buffer solution

4.1.1.1 Composition

Ethidium bromide	2,5 g
Tripotassium citrate	400 g
Citric acid	14,5 g
Deionized water	5 litres
Poly(ethylene glycol) mono-*p*-(1,1,3,3-tetramethylbutyl) phenyl ether[1)]	50 ml

1) For example, Triton X-100 concentrate.

4.1.1.2 Preparation

Dissolve the ethidium bromide in 1 litre of water in a 5 litre container. Stir gently until the ethidium bromide is completely dissolved. The process can be speeded up by heating to between 40 °C and 60 °C. Add the tripotassium citrate and citric acid to the ethidium bromide solution. Add 4 litres of water. Stir gently until the solids are completely dissolved. Add the poly(ethylene glycol) ether concentrate while stirring. Even when stored under light-proof, airtight and cool conditions, the solution shall be kept for no longer than 90 days.

4.1.2 Poly(ethylene glycol) mono-*p*-(1,1,3,3-tetramethylbutyl) phenyl ether solution

4.1.2.1 Composition

Poly(ethylene glycol) mono-*p*-(1,1,3,3-tetramethylbutyl) phenyl ether[1)]	10 ml
Water	1 litre

1) For example, Triton X-100 concentrate.

4.1.2.2 Preparation

Dissolve the poly(ethylene glycol) ether in 1 litre of pre-heated water at approx. 60 °C. Even when stored under airtight and cool conditions, this solution shall be kept for no longer than 25 days.

4.2 Working solution

4.2.1 Dye-buffer working solution

Mix 1 part of the dye-buffer basic solution (4.1.1) with 9 parts of water. (This should be enough for approx. 2 700 samples.) Do not use working solutions older than 7 days.

4.2.2 Rinsing liquid

4.2.2.1 Composition

Poly(ethylene glycol) mono-*p*-(1,1,3,3-tetramethylbutyl) phenyl ether[1)]	10 ml
Ammonia solution, 25 % (*V*/*V*)	25 ml
Water	10 litres
1) For example, Triton X-100 concentrate.	

4.2.2.2 Preparation

Add the poly(ethylene glycol) ether and the ammonia solution to the water.

The composition of the reagents might vary depending on the counting system used. Therefore follow the manufacturer's instructions exactly.

4.3 Preservatives

Boric acid, potassium dichromate, sodium azide or bronopol may be used.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Counting instrument, operating according to the fluorescence optical principle (e.g. Fossomatic). Calibrate in accordance with the manufacturer's instructions. For calibration it is necessary to use milk samples whose cell count has been made by the microscopic method (details are given in ISO 13366-1).

NOTE — Cell count standards are available from the manufacturer.

5.2 Water bath, with circulation, capable of being maintained at a temperature of 40 °C ± 1 °C.

5.3 Sample tubes, with leak-proof seal.

6 Sampling

6.1 It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 [1].

6.2 If automatic samplers are used, they shall be tested properly.

6.3 Prior to testing or preservation, samples should be stored at a temperature of between 2 °C and 6 °C.

6.4 Preservation, if necessary, shall be carried out as soon as possible after sampling, but in any case within 24 h, by addition of one of the following preservatives.

a) **Boric acid** (H_3BO_3): Add the boric acid to the test sample. The final concentration of this preservative in the sample shall not exceed 0,6 g per 100 ml. Such a preserved test sample may be stored at a temperature of between 6 °C and 12 °C for up to a further 24 h.

b) **Potassium dichromate** ($K_2Cr_2O_7$): Add the potassium dichromate to the test sample. The final concentration of this preservative in the test sample shall not exceed 0,2 g per 100 ml. Such a preserved test sample may be stored at a temperature of between 6 °C and 12 °C for up to further 72 h. Local conditions regarding the discharge of effluents shall be observed for samples preserved with potassium dichromate.

c) **Sodium azide:** Immediately after sampling, add the sodium azide to the test sample. The final concentration of this preservative in the sample shall not exceed 0,024 g per 100 ml. Such a preserved test sample may be stored at temperature of between 2 °C and 6 °C. Examination should be carried out within 48 h of sampling.

d) **Bronopol** (2-bromo-2-nitropropan-1,3-diol): Immediately after sampling, add the bronopol to the test sample. The final concentration of this preservative in the sample shall not exceed 0,05 g per 100 ml (preferably 0,02 g per 100 ml). Such a preserved test sample may be stored at a temperature of between 2 °C and 6 °C. Examination should be carried out within 72 h of sampling.

NOTES

1 A test sample already preserved with boric acid may be further preserved for up to 48 h using potassium dichromate.

2 The time of storage of test samples with added bronopol can increase up to 5 days under good conditions and with verification of the quality of the cells using a modern metering device. However this involves the immediate addition of the preservative and keeping the sample in a cold place until testing.

7 Preparation of test sample

7.1 Store the unpreserved test sample for at least 24 h after milking, at a temperature of between 2 °C and 6 °C. If examination of the unpreserved sample has nonetheless to be performed within 24 h after milking, the test sample shall be pretreated by the addition of potassium dichromate (6.4) and left to stand for at least 3 h.

7.2 Heat both the unpreserved and the preserved samples in the water bath (5.2) set at 40 °C and keep them at room temperature for no longer than 30 min.

8 Procedure

8.1 Test portion

Further dilution of the test sample and preparation of the test portion take place automatically in the instrument (5.1).

8.2 Determination

Ensure that cell counting is carried out within 30 min of the end of heating (7.2) and before the temperature is below 30 °C. Ensure that the instrument stirrer is operating correctly so as to obtain as homogeneous a distribution of the cells as possible. If no instrument stirrer is available, thoroughly mix the test portion immediately before counting.

9 Expression of results

Express the number of somatic cells in thousands per millilitre of milk.

NOTE — For a discussion of the use of cell-count standard samples, see annex C.

10 Precision

Details of an interlaboratory test on the precision of the method are summarized in annex A. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

11 Test report

The test report shall specify:

— the method in accordance with which sampling was carried out, if known;

— the method used;

— the test result(s) obtained; and

— if the repeatability has been checked, the final quoted result obtained.

It shall also mention all operating details not specified in this part of ISO 13366, or regarded as optional, together with details of any incidents which may have influenced the test result(s).

The test report shall include all information necessary for the complete identification of the sample.

Annex A
(informative)

Results of interlaboratory test

An interlaboratory test (37 participating laboratories) gave the results shown in table A.1 for r (repeatability limit) and R (reproducibility limit) in thousands of cells per millilitre.

Table A.1

Milk sample	Mean number of cells per millilitre	s_r	r	s_R	R
2	210	13,7	38,9	36,7	103,7
4	438	21,2	59,9	51,3	145,0
6	609	32,6	92,3	89,4	253,0

It should be noted that under practical conditions the geometric mean of several (e.g. three) determinations is used.

NOTE — For the targets of precision, see annex B.

Annex B
(informative)

Quality control in the laboratory

B.1 Purpose

The purpose of quality control procedures is to ensure close agreement between cell counts determined in the routine way and the "true" cell count of the samples. Poor agreement may be due to random errors in individual determinations (such as may arise from inadequate mixing or inaccurate pipetting) or it may be due to systematic errors or bias (such as that introduced by incorrect calibration of instruments). The magnitude of both kinds of error may vary with the true cell count of the sample. Figure B.1 illustrates the effect of both random and systematic errors on the relationship between true and observed cell counts.

Repeatability is a measure of the variation between replicate determinations in one laboratory using the same sample. Reproducibility is a measure of the variation between determinations carried out in different laboratories using the same sample. Neither repeatability nor reproducibility, as defined by ISO 5725-1[2], attempts to measure the bias in measurements relative to "true" values. The procedures recommended in this annex aim to do both, with a combination of routine checks within laboratories and periodic collaborative trials to assess the relative performance of different laboratories.

B.2 Routine monitoring within laboratories

B.2.1 Repeatability

For routine monitoring of the repeatability of counts, any sample with about 500 000 cells per millilitre should be counted at regular intervals (e.g. after every 20th or 50th sample) throughout the working day. At the end of the day, the coefficient of variation of the counts should be calculated. If it is greater than 5 %, the laboratory procedure should be checked, in particular to see that sufficient care is being taken over mixing and pipetting.

B.2.2 Bias

In order to assess the counting bias within a laboratory, standard samples with known "true" counts must be available. Milk samples whose cell count has been estimated by microscopic counting could be used, but normal milk samples will keep for only a few days and it would be expensive to get accurate counts for fresh samples so frequently. Alternatively, standard leucocyte suspensions or milk samples suitably preserved to guarantee a shelf-life of at least 1 month should be used.

Two standards with about 300 000 cells per millilitre and 600 000 cells per millilitre should be prepared and the "true" count of each sample should be determined microscopically or by electronic analysis in at least three different laboratories. The standards should be counted five times by each laboratory at the beginning of each series of analyses and, if the mean count for either standard differs from its "true" count by more than 5 % to 10 %, the calibration of the instrument or any other possible cause of systematic errors should be checked.

B.2.3 Additional requirements

In addition to B.2.1 and B.2.2, the following procedures should be carried out:

— calibration of instrument with relation to the slope;

— visual inspection of the instruments;

— check on zero-setting; and

— determination of the carry-over factor.

B.3 Collaborative trials

B.3.1 Objective

The purpose of collaborative trials is to obtain estimates of the repeatability and the reproducibility of counts for the same samples of milk in different laboratories and to measure the bias in each laboratory's counts relative to the best available estimate of the "true" count of each sample. In addition to providing absolute measures of the reliability of individual counts, the results of these trials demonstrate to inexperienced laboratories the levels of repeatability and bias attained in experienced laboratories.

B.3.2 Design

Ten batches of milk with cell counts spread evenly over the range 200 000 cells per millilitre to 800 000 cells per millilitre should be prepared by the organizing laboratory.

Four 15 ml samples of each milk should be distributed to each participating laboratory, coded in such a way that only the trial coordinators know the identity of the 40 samples.

Each laboratory should count each sample four times and report the individual counts to the trial coordinators.

B.3.3 Statistical analyses

B.3.3.1 In an optional description of collaborative trials, the linear values of cell counts are used. Statistical analyses can also be performed using their logarithms or their square-root values. Bias defines the difference between the observed mean and the reference mean.

B.3.3.2 The laboratory means and the overall mean for each of the 10 milks should be calculated.

B.3.3.3 For each of the 10 milks in each laboratory, the following analysis of variance should be carried out:

Source of variation	v	M
Samples of the same milk	3	$s^2 + 4s_s^2$
Replicate counts	12	s^2

where

v is the number of degrees of freedom;

M is the mean square value;

s is the standard deviation of replicate counts;

s_S is the standard deviation of samples of the same milk.

From the observed mean square values, the repeatability limit, r, is calculated as

$$r = 2{,}8\left(s^2 + s_S^2\right)^{1/2}$$

The laboratories should be ranked according to the maximum r value for any sample. The laboratories, not exceeding 15 % of the total number, giving the largest maximum r value should be identified. The arbitrary exclusion rate of 15 % ensures that reference means in small trials are based on at least five laboratories after exclusion of repeatability and bias.

B.3.3.4 For each laboratory, calculate the regression of its sample means on the sample overall mean. From the regression line, calculate the maximum bias for each laboratory within the range of observed data.

Rank the laboratories according to the maximum bias. Identify the laboratories, not exceeding 15 % of the total number, with the largest maximum biases.

B.3.3.5 Calculate reference means for each milk excluding those laboratories identified in steps B.3.3.3 and B.3.3.4 as having the poorest repeatabilities and the largest biases.

B.3.3.6 For each laboratory, calculate a new regression of sample means on reference means.

B.3.4 Presentation

B.3.4.1 The laboratory means for each milk should be tabulated, and the overall mean and reference mean for each milk sample should appear at the foot of the table.

B.3.4.2 A single standard deviation for repeatability, pooled over all samples, should be given for each laboratory, and the laboratories should be ranked on this parameter.

B.3.4.3 The intercept and slope of the regression of each laboratory's means on the reference mean should be given. The maximum bias within the range of observed means should also be given, and the laboratories should be ranked on this parameter.

B.3.4.4 Each laboratory should receive a graph on which its own individual counts are plotted against the reference mean, and the 45° line and the laboratory's regression line should be shown.

B.3.4.5 The calculation of the reproducibility limit (R) or of s, is desirable.

B.3.5 Comparison between trials

The distributions of repeatability and bias should be monitored from one trial to the next. From pooled repeatabilities and bias in each trial, a histogram should be constructed and the position of the laboratory in the distribution should be identified. Those laboratories with national responsibilities should be identified so that their absolute performance over successive trials and their performance relative to other laboratories within a trial can be seen.

B.3.6 Targets for precision

Analysis of international and national intercomparison trials suggests that the following figures are reasonable targets.

a) Cell count level between 400 000 per millilitre and 500 000 per millilitre:

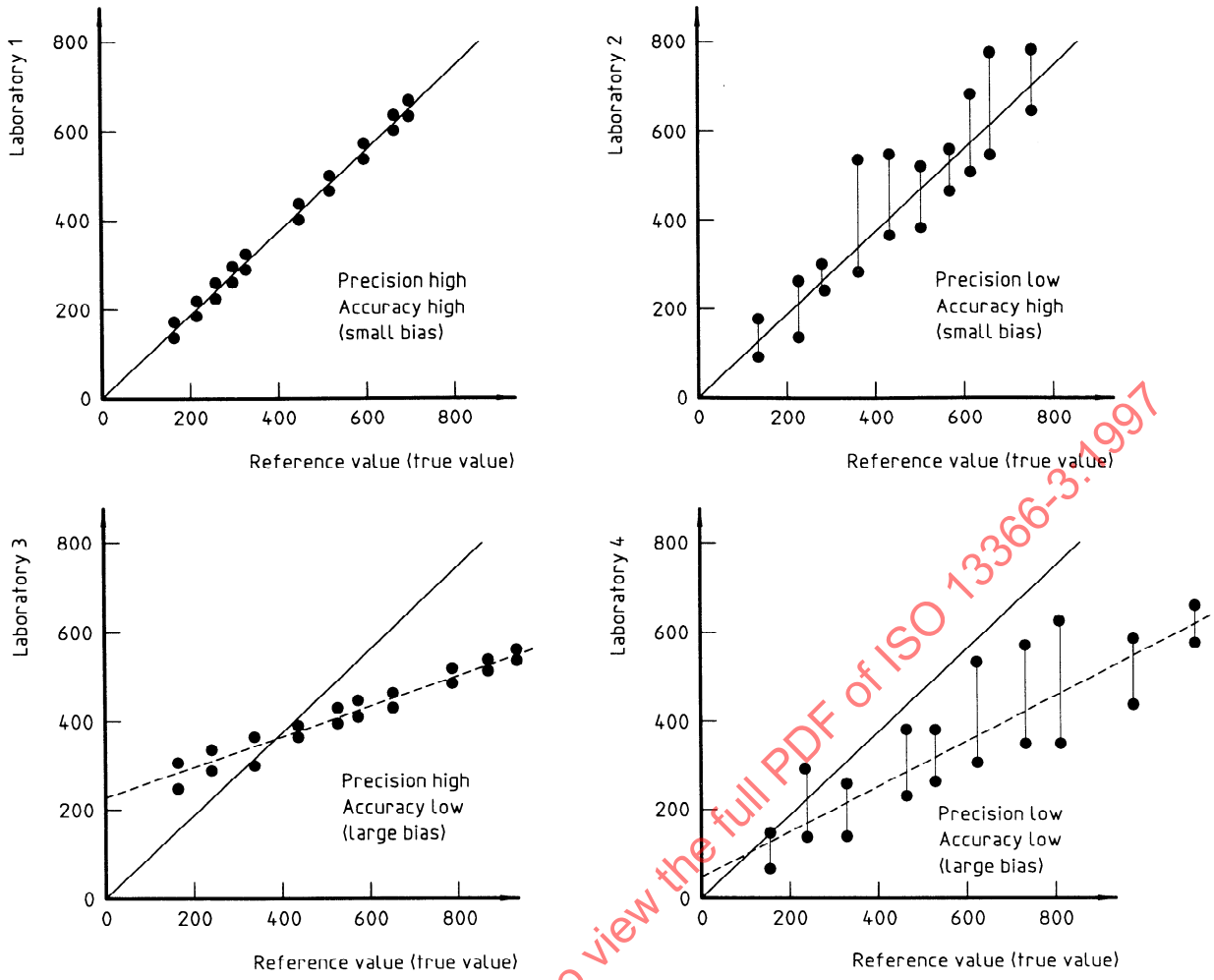
repeatability limit (r) = 2,8 × 20 000 = 56 000 per millilitre (this is equivalent to a coefficient of variation of 4 % to 5 %);

reproducibility limit (R) = 2,8 × 50 000 = 140 000 per millilitre (this is equivalent to a coefficient of variation of 10 % to 12 %).

b) Cell count level between 100 000 per millilitre and 200 000 per millilitre:

repeatability limit (r) = 2,8 × 10 000 = 28 000 per millilitre (this is equivalent to coefficient of variation of 5 % to 10 %);

reproducibility limit (R) = 2,8 × 20 000 = 56 000 per millilitre (this is equivalent to coefficient of variation of 10 % to 20 %).



NOTE — The graphs represent the terms "precision" and "accuracy of the mean". The counts obtained in a single laboratory with reference values and the mean of several laboratories are compared. Ten counts each of two subsamples were made.

Figure B.1 — Precision and accuracy: Types of error