
**Milk — Enumeration of somatic cells —
Part 2:
Guidance on the operation
of fluoro-opto-electronic counters**

Lait — Dénombrement des cellules somatiques —

*Partie 2: Lignes directrices pour la mise en œuvre des compteurs
fluoro-opto-électroniques*

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Contents

Page

Foreword.....	iv
Foreword.....	v
1 Scope	1
2 Normative references	1
3 Terms and definitions.....	1
4 Principle	2
5 Factors affecting the results of measurements.....	2
5.1 Sample bottles	2
5.2 Sampling.....	2
5.3 Preservation	3
5.4 Sample storage and transport.....	3
5.5 Interfering substances	3
5.6 Sample quality at analysis	4
5.7 Applied chemicals	4
5.8 Instrument condition	4
5.9 Working factor.....	5
5.10 Testing volumes.....	5
6 Calibration	5
6.1 Reference materials.....	5
6.2 Calibration procedure.....	6
7 Sampling.....	8
8 Determination.....	8
9 Checks on performance in routine operation.....	8
9.1 Blank checks	8
9.2 Carry-over effect	8
9.3 Ratio of reagent volume and test sample volume.....	9
9.4 Pilot checks	9
9.5 Additional instrument surveillance.....	9
9.6 Repeatability.....	9
9.7 Intralaboratory reproducibility	10
9.8 External comparisons	10
10 Specific remarks for the use with milk from different species	10
10.1 General.....	10
10.2 Cow milk	10
10.3 Goat milk.....	10
10.4 Sheep milk.....	11
10.5 Buffalo milk	11
11 Precision.....	11
11.1 Repeatability.....	11
11.2 Intralaboratory reproducibility	11
11.3 Reproducibility.....	12
12 Test report	12
Bibliography	13

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 13366-2|IDF 148-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

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This edition of ISO 13366-2|IDF 148-2 (cancels and replaces ISO 13366-2:1997 and ISO 13366-3:1997, which have been technically revised.

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

- *Part 1: Microscopic method (Reference method)*
- *Part 2: Guidance on the operation of fluoro-opto-electronic counters*

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the IDF National Committees casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. IDF shall not be held responsible for identifying any or all such patent rights.

ISO 13366-2|IDF 148-2 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO-IDF Action Team on *Automated methods*, of the Standing Committee on *Quality assurance, statistics of analytical data and sampling*, under the aegis of its project leaders, Mrs S. Orlandini (IT) and Mr H.J.C.M. van den Bijgaart (NL).

This edition of ISO 13366-2|IDF 148-2 cancels and replaces IDF 148A:1995, methods B and C of which have been technically revised.

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

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Milk — Enumeration of somatic cells —

Part 2:

Guidance on the operation of fluoro-opto-electronic counters

1 Scope

This part of ISO 13366|IDF 148 gives guidance on the operating conditions for counting somatic cells, in both raw and chemically preserved milk, using fluoro-opto-electronic somatic cell counters in which either a rotating disc technique or flow cytometry is applied in the counting section.

The guidance is applicable to the counting of somatic cells in raw cow milk. The guidance is also applicable to raw milk of other species, such as goat, sheep and buffalo, if the specified prerequisites are met.

2 Normative references

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

ISO 8196-1|IDF 128-1, *Milk — Definition and evaluation of the overall accuracy of indirect methods of milk analysis — Part 1: Analytical attributes of indirect methods* — 2006

ISO 8196-2|IDF 128-2, *Milk — Definition and evaluation of the overall accuracy of indirect methods of milk analysis — Part 2: Calibration and quality control in the dairy laboratory*

ISO 13366-1|IDF 148-1, *Milk — Enumeration of somatic cells — Part 1: Microscopic method (Reference method)*

ISO Guide 34, *General requirements for the competence of reference material producers*

ISO Guide 43-1, *Proficiency testing by interlaboratory comparisons — Part 1: Development and operation of proficiency testing schemes*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 8196-1|IDF 128-1, ISO 8196-2|IDF 128-2 and the following apply.

3.1

reference method

method described in ISO 13366-1|IDF 148-1 for the counting of somatic cells

3.2

somatic cells

those cells that show more than a threshold intensity of fluorescence due to the staining of DNA in their nuclei

NOTE The number of somatic cells is expressed in cells per millilitre.

4 Principle

Fluoro-opto-electronic counters contain functions for the uptake of reagents and test sample, a mixing section and a counting section. In the mixing section, the test sample is mixed with a buffer and a stain solution. Part of the resulting mixture is transferred to the counting section and put onto an object plane. Each stained particle observed with a fluorescence microscope produces an electrical pulse that is filtered, amplified and recorded. The resulting pulse height distribution is electronically processed, whereby discrimination is made between noise signals and pulses that are attributed to stained somatic cells. The discriminator level can either be fixed or dynamic.

In the mixing section, closely controlled volumes of test sample and buffer/stain solutions are dosed and mixed. Mixing can take place in a cup, a mixing chamber, a centrifuge, a sample loop or in the tubing leading to a flow cell.

In the counting section, either disc cytometry or flow cytometry can be applied. In the case of disc cytometry, a thin film of the mixture is brought through a nozzle to the top of a vertical rotating disc. This rotating surface acts as a moving object plane for a fluorescence microscope. When using flow cytometry, part of the mixture is placed in the high-speed flow of a surrounding sheath liquid in a capillary flow cell. Through acceleration, the mixture forms a thin string in which the somatic cells are dynamically focused and aligned. This string then passes the objective of a fluorescence microscope.

Some instruments contain two channels in the counting section. In terms of analytical quality assurance, such a situation should be considered equivalent to working with two separate units, so the performance should be evaluated separately for each channel.

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5 Factors affecting the results of measurements

5.1 Sample bottles

Sample bottles should be fit for use, i.e. to transfer test samples from the point of sampling to the laboratory without loss or damage.

Care is to be exercised that sample bottles are leak-proof and that a proper empty volume is left. Too large an empty volume can facilitate churning; too small an empty volume can cause problems with mixing.

5.2 Sampling

5.2.1 General

Sampling materials (i.e. sample bottles, beakers and sampling devices) should be clean and dry. Where automatic samplers are used, these should have been properly validated.

Test samples should preferably be cooled immediately after sampling to between 0 °C and 6 °C and be kept at that temperature until counting (see 5.4) rather than being preserved. Freezing should be avoided. If preservation is necessary, suitable means for chemical preservation of test samples are described in 5.3.

5.2.2 Bulk milk samples

Thorough mixing of the raw bulk milk to be sampled is essential. Somatic cells will concentrate in the upper and lower layers in the case of insufficient stirring.

5.2.3 Milk samples from individual animals

The release of somatic cells in the milk during milking is uneven. When the aim is to produce a representative counting result for a whole milking, it is essential that a representative sample of the whole milking be obtained. For diagnostic purposes, a sample of a partial milking may suffice.

5.3 Preservation

If chemical preservation is considered necessary, the test sample (5.2.1) should be preserved as soon as possible, but in any case within 24 h after sampling. In all cases, the test sample should be kept cool (0 °C to 6 °C) until the addition of the preservative.

Suitable preservatives are the following.

- a) Boric acid: its final concentration in the test sample should not exceed 0,6 g/100 ml. Such preserved samples may be stored at between 6 °C and 12 °C for up to a further 24 h.
- b) Sodium azide: its final concentration in the test sample should not exceed 0,024 g/100 ml. Such preserved samples may be stored at between 2 °C and 10 °C for up to a further 72 h.
- c) Bronopol (2-bromo-2-nitro-1,3-propanediol): its final concentration in the test sample should not exceed 0,05 g/100 ml. Such preserved samples may be stored at between 2 °C and 12 °C for up to a further 6 d.
- d) Potassium dichromate: its final concentration in the test sample should not exceed 0,2 g/100 ml. Such preserved samples may be stored at between 2 °C and 12 °C for up to a further 6 d.

Accompanying colour tracers found to be suitable are

- Patent Blue V with a final concentration in the test sample of up to 0,15 mg/100 ml,
- Yellow Orange S (E110) with a final concentration in the test sample of up to 1 mg/100 ml, and
- a mixture of Patent Blue V and Eosin B with a final concentration in the test sample of up to 0,03 mg and 0,45 mg/100 ml, respectively.

Other preservatives and colour tracers may be used provided that their effectiveness and conditions for use have been soundly validated.

In all cases, properly validate the absence of interference for the counting equipment concerned before application.

In cases where fluoro-opto-electronic cell counters are combined with milk analysers for the measurement of other test sample components, care should be taken that the applied preservatives and colour tracers do not affect the counting result.

5.4 Sample storage and transport

Unpreserved test samples should be stored at between 0 °C and 6 °C and should be counted within 96 h after the completion of sampling. Avoid freezing the test samples. Storage at higher temperatures and/or over longer time scales may result in non-representative counts. The measurement of samples after freezing and thawing may result in lower counts (by 10 % to 20 %). The age of the samples at freezing and the type of thawing process can influence the counting result.

5.5 Interfering substances

The use of substances that interfere in the counting should be avoided. Substances known to influence the instrument read-out are

- a) preservatives and colour tracers at higher concentrations than specified in 5.3, and
- b) Methylene blue at higher concentrations, i.e. > 0,06 mg/100 ml.

5.6 Sample quality at analysis

Breakdown of somatic cells (lysis) will result in an increase of smaller cell fragments. The lower intensity of fluorescence after staining of these particles causes a shift in the pulse height distribution to the left. This will hamper proper differentiation from noise pulses and therefore result in a lower count.

NOTE In several types of instruments, features are available for evaluating the position and the shape of the pulse height distribution. See the relevant instructions and guidance from the instrument manufacturer.

After the processing of problematic samples, the flow path should be checked and possible cleaned. The proper functioning of the instrument should be tested before further use. Possible problematic test samples are

- a) milk samples from severely infected udders, i.e. with clots,
- b) milk samples with impurities,
- c) milk samples with high numbers of erythrocytes,
- d) colostrum,
- e) late lactation milk, and
- f) sour milk.

Where possible, analysis of problematic test samples should be avoided.

5.7 Chemicals used

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All reagents used should be of recognized analytical and bacteriological quality. Water used should be demineralized (remaining conductivity $< 10 \mu\text{S}/\text{cm}$) or water of at least equivalent purity. Follow the manufacturer's instructions for the preparation of the working solutions, the maximum storage time and storage requirements.

Local conditions regarding the use and discharge of applied chemicals and effluents should be observed.

5.8 Instrument condition

5.8.1 General points of attention are

- a) the functioning of the mixing device and the stirrer,
- b) possible disturbances at sample intake and in the flow system due to blocking by impurities, clots or fouling in the mixing and incubation units, and
- c) the condition and the functioning of the light source and the photo multiplier, the gain setting and the signal quality.

5.8.2 Specific points of attention with disc cytometric counters are

- a) the positioning of the film on the rotating disc,
- b) the cleanliness of the object plane and the functioning of the cleaning sponge; timely replacement of the sponge is essential, and
- c) proper emptying of the collection vessel for the rinsing liquid.

5.8.3 A specific point of attention with flow cytometric counters is the variation in the behaviour of the sample string in the flow cell and the sheath liquid flow. Some instrument manufacturers offer special programme features for checking this, thereby indicating possible solutions in the case of deviations.

5.9 Working factor

The working factor is the number by which the actual number of somatic cells counted by an instrument is multiplied in order to arrive at the somatic cell count of the test sample. In theory, precision characteristics and accuracy should benefit from a lower working factor.

5.10 Testing volumes

A proper ratio between the volume of buffer/stain solution and that of the test sample is essential for correct counting.

6 Calibration

6.1 Reference materials

6.1.1 General

Reference materials should be produced under closely controlled conditions; i.e. working with a quality assurance system and fulfilling the requirements as listed in ISO Guide 34.

Reference materials may be

- a) certified reference materials (CRMs) as produced by a recognized official organization,
- b) secondary reference materials (SRMs) as prepared by an external supplier, or
- c) in-house reference materials (IRMs) as prepared by the laboratory itself, whereby traceability is kept with CRMs, SRMs or via interlaboratory proficiency studies.

NOTE CRMs for somatic cell counting are not available. Examples of suitable procedures for the preparation of IRMs are listed below. IRMs with a composition as close as possible to natural milk are preferable.

6.1.2 Preparation of calibration samples

6.1.2.1 Preparation by addition of a bovine leucocyte suspension

- a) Mix 1 000 ml of sterilized or UHT milk with low somatic cell count with 1 ml of polypropylene 2000 and 0,4 g of bronopol.
- b) Add the required amount of a suitable leucocyte suspension to different portions of the mixture in order to obtain a suitable range of cell counts.

6.1.2.2 Preparation by microfiltration

- a) Collect fresh bulk milk and add bronopol to a final mass fraction of 0,02 %.
- b) Skim the milk in a cream separator to a mass fraction of fat of below 0,1 %.
- c) Concentrate the skimmed milk 20-fold by applying tangential microfiltration over a membrane with a pore size of 0,8 μm , resulting in a portion with a high cell content (HCM) and a portion with a low cell content (LCM).
- d) Mix cream, HCM and LCM in the required quantities so as to obtain 5 to 8 milk portions with a mass fraction of fat of 3,5 % and different levels of cell counts covering the range of interest.