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Milk — Enumeration of somatic cells —

Part 1: Microscopic method (Reference method)

Lait — Dénombrement des cellules somatiques —

Partie 1: Méthode au microscope (Méthode de référence)

Reference numbers
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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 13366-1|IDF 148-1 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.

This second edition of ISO 13366-1|IDF 148-1 cancels and replaces the first edition (ISO 13366-1:1997), of which it constitutes a technical revision.

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*

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Foreword

IDF (the International Dairy Federation) is a non-profit organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have the right to be represented at 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 13366-1|IDF 148-1 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 *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 13366-1|IDF 148-1 cancels and replaces IDF 148A:1995.

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Milk — Enumeration of somatic cells —

Part 1:
Méthode au microscope (Méthode de référence)

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1 Scope

This part of ISO 13366|IDF 148 specifies a microscopic method (reference method) for the counting of somatic cells in both raw and chemically preserved milk.

This part of ISO 13366|IDF 148 is applicable for the counting of somatic cells in cows' milk, provided that the eventually mentioned prerequisites are met.

This method is suitable for preparing standard test samples and determining reference method values that are required for calibrating mechanized and automated cell-counting methods.

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 determine the applicability of regulatory limitations prior to use.

2 Terms and definitions

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

2.1
somatic cells
those cells with nuclei, that is all leucocytes and epithelial cells, determined according to the procedure described in this part of ISO 13366|IDF 148

3 Principle

A test portion of milk to be examined is spread over a slide to form a smear. The smear is dried. During this process, the cells are stained. Subsequently, the stained cells are counted using a microscope. The number of cells counted in a defined area are multiplied by a working factor, to give the number of cells per millilitre.

4 Reagents

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

4.1 Dye solutions

WARNING — Tetrachloroethane is poisonous. Ethidium bromide is mutagenic. 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.

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4.1.1 Modified Newman-Lampert stain solution (Levowitz-Weber modification)

4.1.1.1 Components

Ethanol, 95 % (volume fraction)	54,0 ml
Tetrachloroethane [a]	40,0 ml
Methylene blue	0,6 g
Acetic acid, glacial	6,0 ml

[a] Xylene can be used as an alternative in the same volume amount as mentioned for tetrachloroethane.

4.1.1.2 Preparation

Mix the ethanol and the tetrachloroethane and stopper the bottle. Heat the mixture in a water bath (5.1) set at 65 °C. Add the methylene blue under a fume cupboard and carefully mix. Cool the mixture in a refrigerator to 4 °C.

Then add the glacial acetic acid and carefully mix again. Pass the obtained solution through an appropriate filter (5.2) into an airtight bottle and store it as such.

Filter the Newman-Lampert stain solution again before use.

4.1.2 Ethidium bromide stain solution

4.1.2.1 Stain stock solution

4.1.2.1.1 Composition

Ethidium bromide	0,25 g
Demineralized water	100 ml

4.1.2.1.2 Preparation

Dissolve the ethidium bromide in demineralized water preheated to 40 °C. Cool the solution to room temperature. Adjust to 100 ml with demineralized water.

The ethidium bromide stain stock solution can be kept for two months at a maximum when stored in the dark at 2 °C ± 2 °C.

4.1.2.2 Buffer solution

4.1.2.2.1 Composition

Potassium hydrogenphthalate	0,51 g
Potassium hydroxide	0,162 g
Demineralized water	100 ml

4.1.2.2.2 Preparation

Separately dissolve the potassium hydrogenphthalate and the potassium hydroxide in the demineralized water.

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The buffer solution can be kept for two months at a maximum when stored in the dark at 2 °C ± 2 °C.

4.1.2.3 Ethidium bromide stain working solution

4.1.2.3.1 Components

Ethidium bromide stain stock solution[a] (4.1.2.1)	2 ml
Buffer solution (4.1.2.2)	8 ml
Triton X-100	0,1 ml
Demineralized water	90 ml

[a] A high temperature may reduce the staining capability of ethidium bromide.

4.1.2.3.2 Preparation

Successively add the ethidium bromide stain stock solution, the buffer solution and the Triton X-100 to the demineralized water and carefully mix.

Freshly prepare the ethidium bromide stain working solution directly before use.

4.2 Phosphate Buffer Solution (PBS)

4.2.1 Components

NaCl	8 g
KCl	0,2 g
$Na_2HPO_4 \cdot 7H_2O$	1,15 g
KH_2PO_4	0,2 g
Demineralized water	1 000 ml

4.2.2 Preparation

Dissolve the salts in demineralized water. Adjust to 1 000 ml with the remaining demineralized water.

Adjust the pH to 7,2 ± 0,1.

NOTE It is also possible to use a commercially available phosphate buffer solution with pH = 7,2.

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5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Water baths, capable of maintaining a temperature of 40 °C ± 2 °C, 50 °C ± 2 °C and 65 °C ± 2 °C.

5.2 Filter, resistant to the solvents used, with a pore size of 10 µm to 12 µm.

5.3 Microscope, with a magnification of 500× to 1 000×. Objectives for oil immersion can be used.

When using ethidium bromide, the microscope shall have fluorescence equipment.

5.4 Microsyringe, for dispensing a fixed volume of 0,01 ml of milk, with a maximum tolerance of 2 %.

5.5 Micrometer, to be certified.

5.6 Slides, premarked with an outline shape (rectangular or circular), with an area of 1 cm^2 ± 5 % (95 mm^2 to 105 mm^2), or a standard slide with a template of dimensions 20 mm × 5 mm or having a diameter, d, of 11,28 mm.

5.6.1 Selection of slides

Preferably, work with a fixed premarked area or a template, in order to avoid the recalculation of the working factor with each counting.

5.6.2 Shapes

For a rectangular shape, the upper and lower internal widths, on the one hand, and the left and right internal heights, on the other hand, should not differ by more than 0,2 mm.

For a circular shape, the vertical and horizontal internal diameters should not differ by more than 0,2 mm.

6 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this part of ISO 13366|IDF 148. A recommended sampling method is given in ISO 707|IDF 50.

If using automatic samplers, they shall have been validated properly.

7 Preparation of test sample

7.1 Storage

Prior to testing or preservation, store the test samples at a temperature of 4 °C ± 2 °C.

Analyse the test samples within 6 h after sampling. In the case of longer storage, add chemical preservatives such as boric acid, bronopol or potassium dichromate. The final concentration of boric acid shall not exceed 0,6 g per 100 ml of test sample. The final concentration of bronopol shall not exceed 0,05 g per 100 ml of test sample. The final concentration of potassium dichromate shall not exceed 0,1 g per 100 ml of test sample. Store the thus preserved test samples at a temperature of 4 °C ± 2 °C for no longer than 6 days.

For environmental reasons, it is recommended to restrict the use of potassium dichromate to samples that require a long shelf life only.

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7.2 Preparation

Heat the test sample (see 7.1) in a water bath (5.1) set at 40 °C. Mix the test sample carefully. Cool the sample to the temperature at which the microsyringe (5.4) has been calibrated, for example to 20 °C.

Dilute test samples with an estimated somatic cell count of above 1 000 000 cells/ml with a phosphate buffer solution (4.2) to obtain a somatic cell count of about 500 000 cells/ml for each diluted test sample.

$$d = \frac{V_s}{V_s \times V_b}$$

where

d is the dilution factor to obtain an appropriate somatic cell account in the test sample of about 500 000 cells/ml;

V_s is the volume, in ml, of the test sample;

V_b is the volume, in ml, of the buffer used for diluting the test sample.

Record the required dilution factor, d, the volume of test sample, V_s, and the volume of buffer, V_b, used to obtain the required dilution.

8 Procedure

Prepare, for each test sample, at least two smears and count the best one (e.g. a smear not damaged by the dyeing process). Dip the slides (5.6) in ethanol (of volume fraction 95 %). Flame and cool.

8.1 Preparation of the smear and staining

Follow either 8.1.1 or 8.1.2 for preparation of the smear and staining.

NOTE Staining for goats' milk is described in Annex B.

8.1.1 Preparation of the smear and staining with Newman-Lampert stain solution

Using the microsyringe (5.4), take 0,01 ml of the test sample (eventually diluted) (see 7.2). Rinse the microsyringe with the test sample. If necessary, carefully and gently clean the outside of the microsyringe which has been in contact with the test sample.

Place the mixture on a clean slide with an area of 1 cm^2 (5.6). Using the needle, spread the test sample evenly over the entire area defined, while ensuring that the area close to the perimeter is evenly covered. Dry the smear at room temperature until it is completely dry.

Dip the dried smear on the slide in the modified Newman-Lampert stain solution (4.1.1) for at least 15 min. Dry the smear at ambient temperature.

Then dip the smear gently in tap water until all surplus dye is washed away. Dry again and store with a protection against dust.

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8.1.2 Staining with ethidium bromide stain solution and preparation of the smear

Mix 1 ml of the prepared test sample (see 7.2) with 1 ml of ethidium bromide stain working solution (4.1.2.3) in a reagent tube. Keep the mixture protected from light. Heat the tube in a water bath (5.1) set at 50 °C for 3 min. Cool to room temperature.

Using the microsyringe (5.4), take 0,01 ml of the mixture. Rinse the microsyringe with the mixture. If necessary, carefully and gently clean the outside of the microsyringe which has been in contact with the mixture.

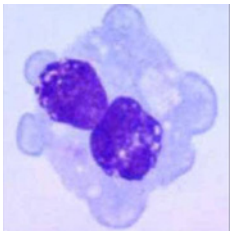
Place the mixture on a clean slide with an area of 1 cm^2 (5.6). Using the needle, spread the mixture evenly over the entire area defined, while ensuring that the area close to the perimeter is evenly covered. Dry the smear at room temperature until it is completely dry.

8.2 Determination

8.2.1 Reading optimization

Using the microscope (5.3), count the cell nuclei in the obtained smear (8.1.1 or 8.1.2) of fields entirely filled with milk smear only. Choose the best magnification (from 500× to 1 000×), in order to have an average maximum number of 20 cells in each field.

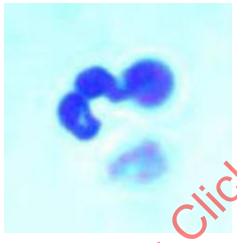
The cells possess a stained nucleus. The cells generally are 8 µm or larger. Do not count cells less than 4 µm (see Figure 1). Count fragments only if more than 50 % of nuclear material is visible. Count cell clusters as one, unless the nuclear unit(s) is (are) clearly separated. See also Figures 2 and 3.



Macrophage

8-30 µm

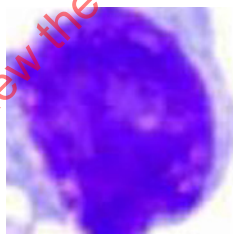
The relation between cytophasma/nucleus is big. Phagocytosis, antigen presentation, secretion chemoattractants



PMN

10-14 µm

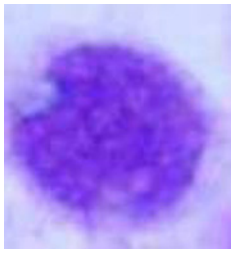
90 % acute mastitis
60 % chronic. The relation between cytoplasma/nucleus is small. Phagocytosis. First line of defense against mastitis



Lymphocyte

5-10 µm

The relation between cytoplasma/nucleus is small. Nucleous intensively stained T helper
T suppressor B cell



Epithelial cell

10-14 µm

Nucleus round. Cytoplasma weakly stained

Figure 1 — Examples of cells

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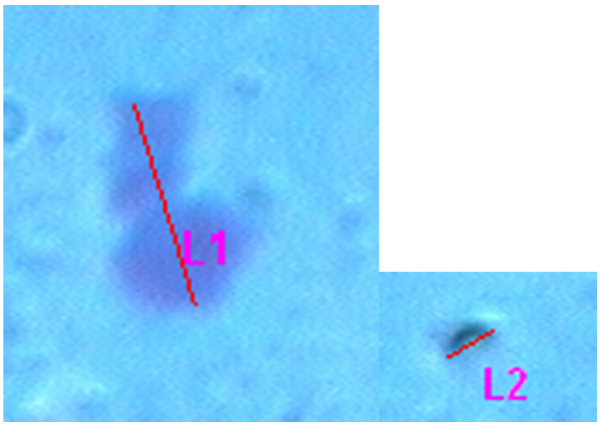
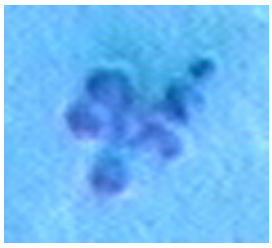
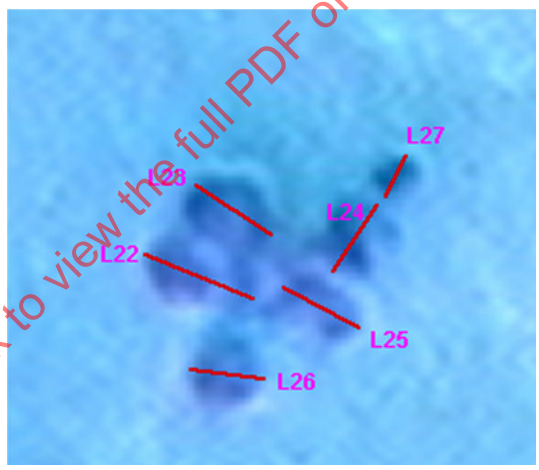


Cell lengths: L1 = 9,79 µm and
L2 = 2,77 µm

Figure 2 — Examples of cells from bulk cows' milk (1 000× magnification)



(500× magnification)



(1 000× magnification)

Cell length: L22 = 9,08 µm; L23 = 8,27 µm; L24 = 4,95 µm; L25 = 7,39 µm; L26 = 6,37 µm and L27 = 3,58 µm.

Figure 3 — Examples from cells from bulk cows' milk

In the example of a cluster as shown in Figure 3, five cells have to be counted. L27 is omitted because its diameter is less than 4 µm.

NOTE The training and skill of the analyst is the main critical success factor for a proper performance of the method. Frequent execution of the method and participation in interlaboratory trials are essential to safeguard a correct level of counting.

Generally, cells in milk are distributed according to a Poisson distribution (see Annex C). The minimum number (N) of cells to be counted in relation to the cell count level, in order to arrive at the listed coefficient of variation, are listed in Table 1.

For a proper execution of the method, it is essential that the listed minimum number of cells be counted. Fields and strips to be counted shall be chosen so as to obtain a representative count for the entire smear.

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Table 1 — Minimum number (N) of cells

Concentration (× 1 000 cells/ml)	CV %	N cells
< 150	10	100
150 to 250	7	200
250 to 400	6	300
⩾ 400	5	400

8.2.2 Counting in successive fields

Count nuclei in successive fields, in vertical strips in regularly spaced fields (see Figure 4 and Table 1).

a) Start at about a distance, d_L, from the left side. For a circular shape, start from a sufficient distance, d_L, from the left side of the horizontal diameter, so as to allow counting of a minimum of 5 fields from the top of the strip. A distance, d_L, of 0,5 mm is suitable for both rectangular and circular shapes.

b) Place the upper or lower edge of the field circumference tangentially onto the internal upper or lower border of the template (the latter should not appear in the field). In the case of an uncovered surface near the border of the template, adjust the field to the border of the smear.

c) After having counted the first field, move the objective with a fixed space distance, d_H, down or up to the next graduation in the direction of the lower or upper edge and count the new field. A distance, d_H, of 1 mm is considered appropriate.

d) From the last field counted, repeat the operation in c), until the opposite side (bottom or top) of the strip is reached. Choose between the following two options.

   — Option 1: The latter field is not to be counted.

   — Option 2: If the lower or upper border appears and it fills less than half the field surface, counting is performed after moving up the objective until the border disappears again completely from the field, which then only covers smear.

e) Then move the objective to the right by a distance, d_W (e.g. d_W = 1,5 mm or d_W = 2 mm depending on the number of fields needed), and start a new strip in the opposite direction (top or bottom).

f) Repeat from b) to e) until the right side of the template is reached.

g) In case an insufficient number of fields is counted (see Table 1), supplementary fields can be counted. To do this, focus the microscope objective on other locations (e.g. by changing the starting place and/or adapting the step-by-step moving distances) so as to obtain appropriate field numbers that are representative of the entire smear.

h) Calculate the result as described in 9.1 for a rectangular shape, or that described in 9.3 for a circular shape.

NOTE With rectangular shapes, 5 fields spaced at 1 mm are possible in vertical strips and 10 strips spaced at 2 mm, making it possible to count 50 fields. Approximately the same field number is obtained with circular shapes, using the same distances. The distances of shifts (spaces) are measured from the same location on the fields with the vernier (adjustment on the upper or lower edge) so that they include the field diameter.

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8.2.3 Rectangular-shape counting by bands

Count nuclei in regularly spaced vertical bands (see Figure 5 and Table 1):

a) Start at about a distance, d_L, from the left side. A distance, d_L, of 0,5 mm is considered appropriate.

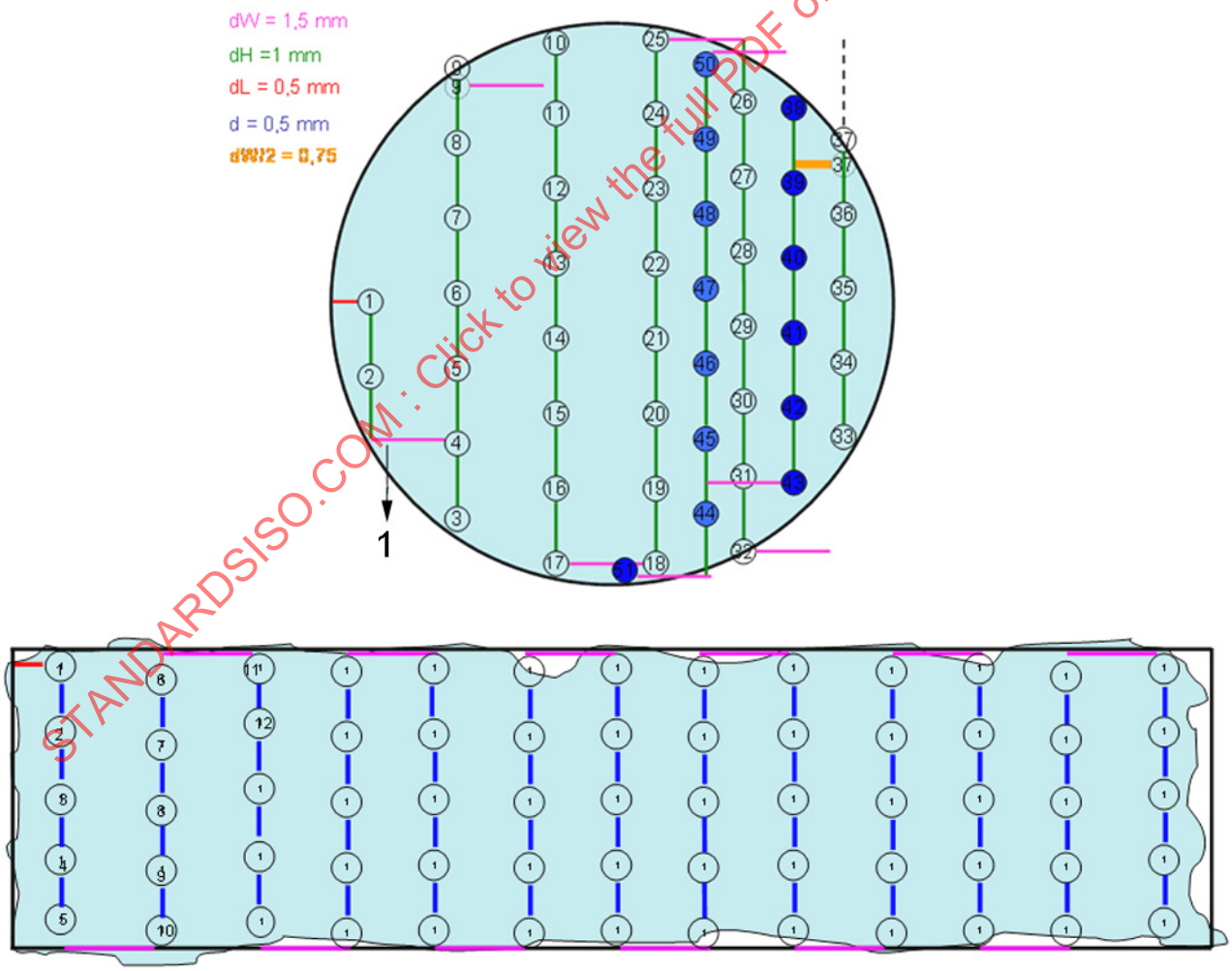
b) Start to count from the upper or lower border of the rectangular area. Place the border of the area in the middle of the microscope field. After having counted all the cells, move the objective in the direction of the opposite border and count all the cells that appear in this band until the opposite border is reached. Record the number of cells counted.

c) Then move the objective to the right with a distance, d_W, and start counting a new band (e.g. d_W = 3 mm to 4 mm, depending on the number of bands needed for a representative counting of the whole smear).

Repeat b) and c) until the right side of the template is reached.

In case an insufficient number of bands is counted (see Table 1), supplementary bands can be counted. In this case, focus the microscope objective on other locations (e.g. by changing the starting place and/or adapting d_W) so as to obtain appropriate bands that are representative of the entire smear.

Calculate the result as described in 9.2.



Key

1 down until lower edge

Figure 4 — Vertical strips in regularly spaced fields for a circular or rectangular shape

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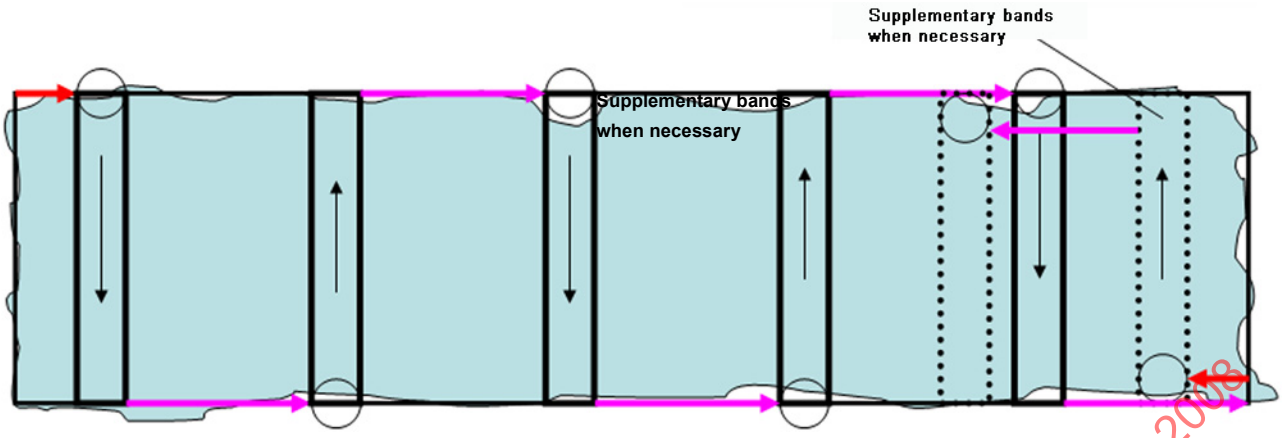

Figure 5 — Regularly spaced vertical bands

9 Calculation and expression of results

9.1 Rectangular shape counting in successive fields

Check the 20,0 and 5,0 mm target values for the length, L_s, and the width, W_s, of the smear, using the graduations and vernier of the microscope.

Calculate the total concentration, c, of cells by using the following equation:

$$c = \frac{W_s \times L_s \times N_t}{\pi \times \left(\frac{D_f}{2}\right)^2 \times N_f \times V_m} \times \frac{1}{d} \quad (1)$$

or

$$c = f_w \times \left[\frac{N_t}{N_f} \times \frac{1}{d}\right]$$

or with the constant working factor, f_w

$$f_w = \frac{W_s \times L_s}{\pi \times \left(\frac{D_f}{2}\right)^2 \times V_m}$$

where

c is the total concentration, expressed in number of cells per millilitre;

W_s is the width, in millimetres, of the smear;

L_s is the length, in millimetres, of the smear;

N_t is the total number of cells counted;

D_f is the diameter, in millimetres, of the microscope field;

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N_f is the number of fields counted completely;

V_m is the volume, in millilitres, of the test sample smeared (see 8.1.1 or 8.1.2) [if the modified Newman-Lampert stain working solution was used for staining (8.1.1), V_m = 0,01 ml. If the ethidium bromide stain solution was used for staining (8.1.2), V_m = 0,005 ml];

d is the dilution factor used in 7.2. (If no dilution is required, d = 1; with a 1:1 dilution, d = 0,5.)

9.2 Rectangular shape counting in bands

Check the 20,0 and 5,0 mm target values for the length and the width of the smear, using the graduations and vernier of the microscope.

Calculate the total concentration, c, of cells by using the following equation:

$$c = \frac{W_s \times N_t}{D_f \times N_b \times V_m} \times \frac{1}{d} \quad (2)$$

or

$$c = f_w \times \left(\frac{N_t}{N_b} \times \frac{1}{d} \right)$$

with the constant working factor, f_w

$$f_w = \frac{W_s}{D_f \times V_m}$$

where N_b is the number of bands counted completely.

The other symbols are defined in 9.1.

9.3 Circular shape counting in successive fields

Check the 11,28 mm diameter of the smear, using the graduations and vernier of the microscope.

Calculate the total concentration, c, of cells by using the following equation:

$$c = \frac{{D_c}^2 \times N_t}{{D_f}^2 \times N_f \times V_m} \times \frac{1}{d} \quad (3)$$

or

$$c = f_w \times \left(\frac{N_t}{N_f} \times \frac{1}{d} \right)$$

with the constant working factor, f_w

$$f_w = \frac{{D_c}^2}{D_f^2 \times V_m}$$

where D_c is the diameter, in millimetres, of the smear.

The other symbols are defined in 9.1.

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9.4 Expression of results

Express the test results in whole figures of thousands (for example: express 401 586 cells/ml as 402 000 cells/ml).

10 Precision

The values for the repeatability and reproducibility were derived from the result of an interlaboratory test carried out in accordance with ISO 5725-1 and ISO 5725-2. Details of this interlaboratory test 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.

10.1 Repeatability

The absolute difference between two independent single test results, 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 in not more than 5 % of cases be greater than the values given in Table 2:

Table 2 — Repeatability values

Concentration (× 1 000 cells/ml)	s_r (× 1 000 cells/ml)	r (× 1 000 cells/ml)
245	38	107
455	43	121
679	69	192
791	110	308

10.2 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than the values given in Table 3:

Table 3 — Reproducibility values

Concentration (× 1 000 cells/ml)	s_R (× 1 000 cells/ml)	R (× 1 000 cells/ml)
245	41	114
455	62	174
679	78	218
791	110	308

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11 Test report

The test report shall specify

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

b) the sampling method used, if known;

c) the test method used, with reference to this part of ISO 13366|IDF 148;

d) all operating details not specified in this part of ISO 13366|IDF 148, 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.

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