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IDF 169-1

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Milk and milk products — Quality control in microbiological laboratories —

Part 1: Analyst performance assessment for colony counts

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Lait et produits laitiers — Contrôle de qualité en laboratoires microbiologiques —

Partie 1: Évaluation de la performance des analystes effectuant les comptages de colonies

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Foreword

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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 14461-1|IDF 169-1 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

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ISO 14461|IDF 169 consists of the following parts, under the general title *Milk and milk products — Quality control in microbiological laboratories*:

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— *Part 1: Analyst performance assessment for colony counts*

— *Part 2: Determination of the reliability of colony counts of parallel plates and subsequent dilution steps*

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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 and AOAC International 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 National Committees casting a vote.

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All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Statistics of analytical data*, of the Standing Committee on *Quality assurance, statistics of analytical data and sampling*, under the aegis of its project leaders, Dr. H. Glaeser (EU) and Prof. Dr. H. Weiss (DE).

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This edition of ISO 14461-1|IDF 169-1, together with ISO 14461-2|IDF 169-2, cancels and replaces IDF 169:1994, which has been technically revised.

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ISO 14461|IDF 169 consists of the following parts, under the general title *Milk and milk products — Quality control in microbiological laboratories*:

- *Part 1: Analyst performance assessment for colony counts*
- *Part 2: Determination of the reliability of colony counts of parallel plates and subsequent dilution steps*

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Introduction

Every microbiological method consists of several steps that are followed in a specific sequence (sub-sampling, diluting, plating and counting). The final result has a margin of uncertainty that is determined by the variability of all the steps involved.

In order to obtain results with a margin of uncertainty not much larger than what can be expected from the correct application of the method, it is necessary to follow the rules of Good Laboratory Practice (GLP).

The three most important factors in obtaining a correct plate count are

— the homogeneity of the sample material,

— the exactness with which the dilutions are performed, and

— the technique of inoculation and/or counting of the plates.

By homogenizing a sample material very well, making multiple dilution series, and inoculating several plates from the same dilution, it is possible to assess how well a laboratory can perform the colony-count technique, taking into account the expected variability of the method.

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A too large variability indicates that at least one of the steps in the performance of the method is out of control. The identification of those steps is done by comparison of the replicate inoculations, the different dilution levels and the dilution series. When the steps with excessive variability have been identified, the necessary measures should be taken to bring these steps under control.

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Milk and milk products — Quality control in microbiological laboratories —

Part 1: Analyst performance assessment for colony counts

1 Scope

This part of ISO 14461|IDF 169 describes a procedure for testing the performance of the colony-count technique within a laboratory by establishing the within-laboratory variability of its technique and identifying those steps that are associated with excessive variability.

The procedure is also suitable for checking the proper observance of Good Laboratory Practice (GLP), which may be a prerequisite for participation in interlaboratory tests of colony-count methods.

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EXAMPLE Appropriate test samples are raw milk, pasteurized milk and dried milk.

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2 Normative references

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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 648:1977, *Laboratory glassware — One-mark pipettes*

ISO 835-4, *Laboratory glassware — Graduated pipettes — Part 4: Blow-out pipettes*

ISO 4788, *Laboratory glassware — Graduated measuring cylinders*

ISO 7218, *Microbiology of food and animal feedings stuffs — General rules for microbiological examinations*

ISO 8261|IDF 122, *Milk and milk products — Preparation of samples and dilutions for microbiological examination*

3 Terms and definitions

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

3.1
colony-count technique
counting of the number of microorganisms as determined by the procedure specified in this part of ISO 14461|IDF 169

NOTE The number of microorganisms is expressed per gram or per millilitre of test sample.

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4 **Principle** (see Figure 1)

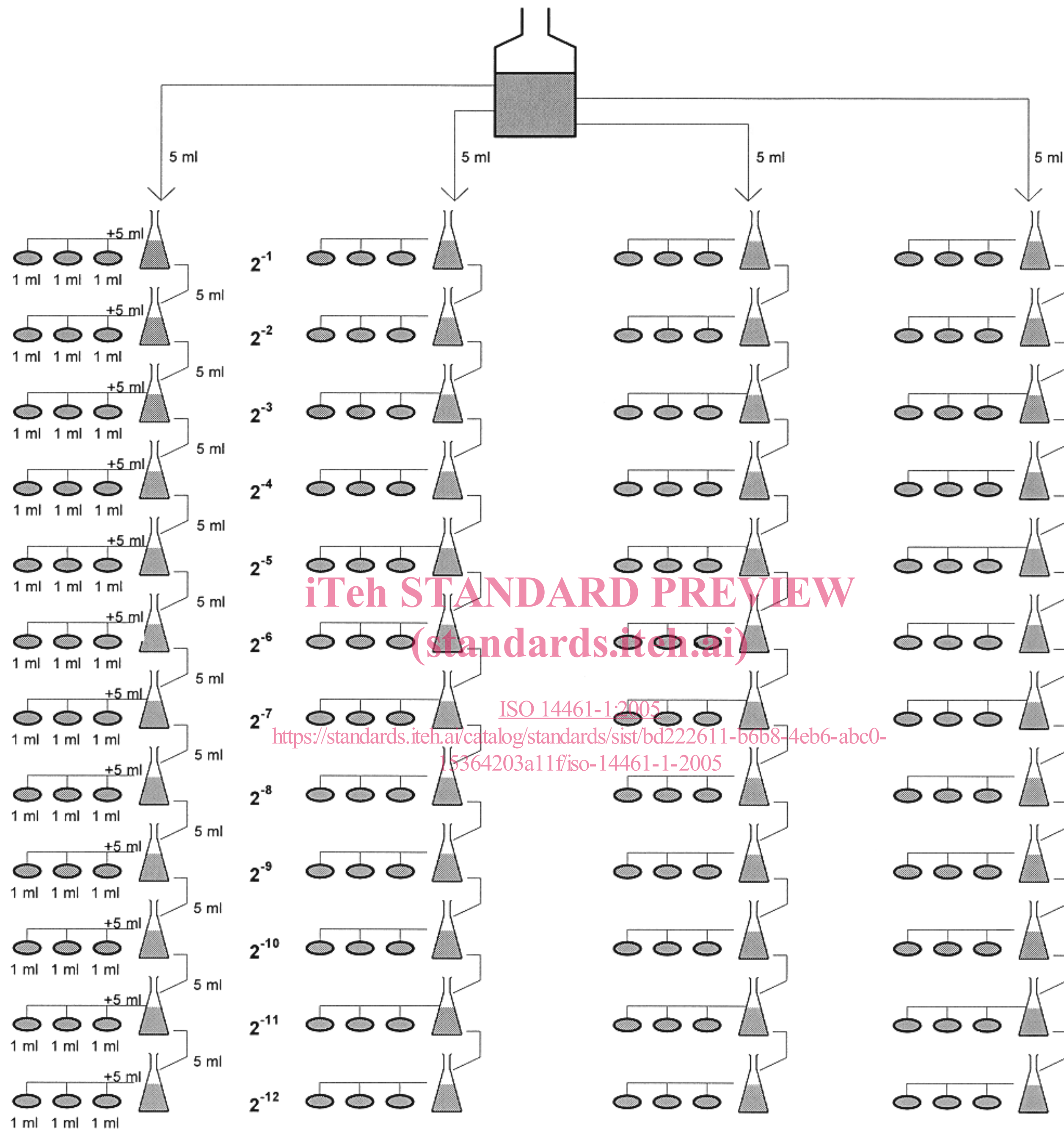

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Figure 1 — Quality assurance in the microbiological laboratory: Design of pilot studies for plate count

4.1 A test sample is homogenized then diluted to a suitable working density. (e.g. 500 CFU to 10 000 CFU per millilitre). A suspension is prepared.

4.2 From this first dilution, four dilution series are prepared, each consisting of 12 binary dilution steps.

NOTE Binary (two-fold) dilution steps are used, not decimal (10-fold) dilutions as is the usual practice. With binary dilutions it is possible to count colonies on plates originating from five to six dilutions, and this large number of counts improves considerably the testing of the dilution steps.

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4.3 Three parallel plates are poured from each dilution of each series.

4.4 The plates are incubated.

4.5 The sequence of the plates is randomized and the colonies on each plate are counted.

4.6 The counts are tabulated and the “statistical homogeneity” of the counts in two steps is calculated.

4.7 If the values obtained are statistically homogeneous, then the quality of the application of the method is satisfactory and no further evaluation is needed.

4.8 If the results are not statistically homogeneous, an analysis of variance (ANOVA) is performed in order to identify the variation of the results with one or more of the factors that were varied (i.e. dilution series, dilution levels, plating). Further investigations are carried out and the factor(s) identified are adjusted.

NOTE Users will designate the particularly important sources of error in the performance of the method.

5 Diluent, culture media and reagents

The operations described in detail in this clause and in Clause 9 shall either be carried out by one person alone or be divided over a group with clearly defined tasks for each participant.

Use only reagents of recognized analytical grade and distilled water or water of at least equivalent purity, unless otherwise specified. The reagents and the water shall be free from substances that may adversely influence the growth of microorganisms under the test conditions. The culture medium shall be of recognized bacteriological quality. Any dehydrated medium shall be prepared according to the manufacturer's instructions.

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5.1 Sodium hydroxide solution or **hydrochloric acid** (approx. 0,1 mol/l), to adjust the pH of the diluent and the culture medium.

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5.2 Culture medium: Tryptone-glucose-yeast extract agar, with addition of skimmed milk powder.

5.2.1 Composition

Yeast extract	2,5 g
Tryptic digest of casein (tryptone)	5,0 g
Glucose monohydrate ($C_6H_{12}O_6 \cdot H_2O$)	1,0 g
Skimmed milk powder	1,0 g
Agar	10 g to 15 g [a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

In all cases it is necessary to add the skimmed milk powder, even if the dehydrated complete medium is purchased and even if the suppliers consider such an addition unnecessary.

5.2.2 Preparation

For the experiment 2 litres of medium of the same lot will be needed. If a commercial dehydrated complete medium is used, follow the manufacturer's instructions but add the skimmed milk powder. Adjust the pH so that after sterilization it is 7,0 ± 0,2 at about 45 °C.

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If the medium is prepared from dehydrated basic components, then dissolve and disperse in preheated water, in the following order: yeast extract, tryptone, glucose and, finally, the skimmed milk powder. Heating the water will assist in the dissolving and dispersion procedure. Add the agar and heat to boiling, while stirring frequently, until the agar is completely dissolved. Alternatively, steam the mixture for about 30 min. Filter the medium through filter paper, if necessary. Adjust the pH so that after sterilization it is 7,0 ± 0,2 at about 45 °C.

Dispense the culture medium in amounts of 250 ml into bottles (6.10). Sterilize all the bottles at one time in the autoclave (6.1) set at 121 °C for 15 min.

Store the prepared medium in the dark at a temperature between 0 °C and 5 °C for no longer than 1 month.

5.3 Diluents: Peptone/salt solution or **quarter-strength Ringer's solution**, from a single lot.

5.3.1 Peptone/salt solution

This is the diluent selected for general use.

5.3.1.1 Composition

Peptone	1,0 g
Sodium chloride (NaCl)	8,5 g
Water up to	1 000 ml

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5.3.1.2 Preparation

Dissolve the components in the water, by heating if necessary. Adjust the pH so that after sterilization it is 7,0 ± 0,2 at 25 °C.

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5.3.2 Quarter-strength Ringer's solution

5.3.2.1 Composition

Sodium chloride (NaCl)	2,25 g
Potassium chloride (KCl)	0,105 g
Calcium chloride, anhydrous ($CaCl_2$)	0,06 g
Sodium hydrogen carbonate ($NaHCO_3$)	0,05 g
Water up to	1 000 ml

5.3.2.2 Preparation

Dissolve the salts in the water. Adjust the pH so that after sterilization it is 6,9 ± 0,2 at 25 °C.

5.3.3 Preparation of the diluent

Sterilize the diluent by autoclaving, in quantities not greater than 500 ml. Then dispense portions of 90 ml at room temperature into sterile dilution bottles (6.8) using sterile graduated measuring cylinders or other dispensing devices (6.11), and portions of 5 ml into sterile test tubes (6.9) using 5 ml one-mark or graduated pipettes or other dispensing devices (6.12). When emptying a pipette touch the tip against an inclined wall of the container in order to ensure correct delivery.

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NOTE Dispensing the portions before autoclaving can lead to unequal evaporation during sterilization, resulting in differences in the final strength of the portions.

Cool and store both the bulk and the dispensed portions of the diluent at a temperature between 0 °C and 5 °C. Use both the bulk and the dispensed portions the next day at the latest.

6 Apparatus and glassware

Sterilize all apparatus that will come into contact with the test sample, the diluent, the dilutions or the culture medium in accordance with ISO 7218 and ISO 8261|IDF 122.

Usual microbiological laboratory equipment and, in particular, the following.

6.1 Autoclave, capable of operating at 121 °C ± 3 °C.

6.2 Hot-air oven, capable of operating at above 180 °C.

6.3 Incubator, capable of operating at 30 °C ± 1 °C at all points within it.

6.4 pH-meter, with temperature compensation, accurate to ± 0,1 pH units.

6.5 Water baths, capable of operating at 20 °C ± 1 °C, 45 °C ± 1 °C and between 44 °C and 47 °C.

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6.6 Lenses, of magnification 2× to 4× and of at least 8×.

6.7 Glass beads, of diameter about 6 mm.

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6.8 Dilution bottles, of nominal volume 150 ml to 250 ml, with watertight stoppers, containing 5 to 10 glass beads (6.7). Add the beads before sterilizing the bottles.

6.9 Test tubes, of height about 150 mm and diameter about 15 mm, with stoppers.

6.10 Bottles, of nominal volume 500 ml, with stoppers, for storing 250 ml portions of culture medium.

6.11 Graduated measuring cylinders, with main-point graduations, complying with ISO 4788, or other dispensing devices with a proven equivalent level of accuracy.

6.12 One-mark or **graduated pipettes**, calibrated, capable of delivering 1 ml, 5 ml and 10 ml, complying with ISO 648:1977, class A, or ISO 835-4, or other dispensing devices with a proven equivalent level of accuracy.

6.13 Petri dishes, made of clear uncoloured glass or plastic material, the bottom having an internal diameter of about 90 mm and no irregularities interfering with colony counting.

6.14 Mechanical stirrer, capable of mixing the contents of the test tubes, working on the principle of eccentric rotation (e.g. a vortex mixer).

6.15 Top-loading balance, of sufficient weighing capacity, capable of weighing to the nearest 0,05 g.

7 Sampling

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

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Sampling is not part of the method specified in this part of ISO 14461|IDF 169. A recommended sampling method is given in ISO 707.

8 Preparation of test sample

8.1 Milk

Agitate the test sample thoroughly by rapidly inverting the sample container 25 times, so that the microorganisms are distributed as evenly as possible. Avoid foaming or allow any foam to disperse. The interval between mixing and removing the test portion shall not exceed 3 min.

8.2 Dried milk

Thoroughly mix the contents of the closed container by repeatedly shaking and inverting it. If the test sample is in the original unopened container and this is too full to permit thorough mixing, transfer it to a larger container, then mix.

9 Procedure

9.1 General

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In colony-count methods plates are often partially or completely uncountable due to various reasons (spreading, mould growth, etc.). For the present method, only a limited number of missing values may be tolerated (see 10.1). Too many missing values indicate either that the material used is not suitable for the test, or that the technique is faulty. In such a case, repeat the procedure with another, more suitable sample material or with stricter adherence to the instructions.

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9.2 Number of decimal dilution steps

The expected microbiological density of the sample determines the number of decimal dilution steps, as follows:

a) when the expected count is less than 100 000 per millilitre or per gram, make a decimal dilution to 0,1 (one decimal step);

b) when the expected count is between 100 000 and 1 000 000 per millilitre or per gram, make a serial dilution to 10^{-2} (two decimal steps);

c) when the expected count is higher than 1 000 000 per millilitre or per gram, make a serial dilution to 10^{-3} (three decimal steps).

9.3 Preparation of first decimal dilution

9.3.1 Milk

Remove 1 ml of test sample (8.1) with a sterile pipette (6.12) and add to 9 ml of diluent (5.3) (or 10 ml of test sample to 90 ml of diluent, or 11 ml of test sample to 99 ml of diluent). Shake this primary dilution [e.g. 25 times with a movement of about 300 mm for 7 s manually or, using a mechanical stirrer (6.14), for 5 s to 10 s] to obtain a 10^{-1} dilution.

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9.3.2 Dried milk

9.3.2.1 Open the container (8.2), remove the amount of test portion required with a spatula and proceed as indicated in 9.3.2.2. Immediately close the container again.

9.3.2.2 Weigh 10 g of test sample into a suitable glass vessel (e.g. a beaker) and then add the powder to the dilution bottle containing a suitable diluent (5.3). Alternatively, weigh 10 g of test sample directly into the bottle with the diluent. To dissolve the test sample, swirl slowly to wet the powder then shake the bottle (e.g. 25 times with a movement of about 300 mm, for about 7 s). A peristaltic-type blender may be used as an alternative to shaking. Allow to stand for 5 min, shaking occasionally.

9.4 Preparation of further decimal dilutions

Prepare further dilutions in accordance with ISO 8261|IDF 122.

9.5 Melting the medium

Before starting the operations described in 9.6, melt the culture medium (5.2) and cool it in the water bath (6.5) set at between 44 °C and 47°C. Check the temperature of the medium by placing a thermometer into a 250 ml portion of agar (e.g. water agar) in a separate container, which is identical to that used for the medium. Pour the molten agar within 2 h after melting.

9.6 Preparation of binary dilutions and inoculation of the medium

9.6.1 First dilution series (S_1)

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Take 12 dilution test tubes (6.9) with 5 ml of diluent from cold storage (5.3.3).

Make serial binary dilutions (D_1, D_2, …) by transferring with a fresh pipette 5 ml of the suspension from the previous dilution (9.4) into a tube with 5 ml of diluent. Mix the suspension 5 times during 5 s with the stirrer (6.14) before each transfer. The first inoculum is taken from the last decimal dilution bottle (9.4), which is immediately placed back in the refrigerator.

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Before starting the next binary dilution series, inoculate three Petri dishes (P_1, P_2 and P_3) from each of the twelve dilutions using 1 ml one-mark or graduated pipettes (6.12). Use a fresh sterile pipette for each dilution level.

After inoculating all plates of the series (S_1), pour 12 ml to 15 ml of molten and tempered (44 °C to 47°C) culture medium (9.5) into each Petri dish in the same working order as the inoculation. Mix the medium carefully with the inoculum by rotating the Petri dishes sufficiently to obtain evenly dispersed colonies after incubation. Allow the mixture to solidify by leaving the Petri dishes to stand on a cool horizontal surface.

9.6.2 Subsequent dilutions (S_2, S_3 and S_4)

After completion of the first series of dilutions and plating, prepare the second, third and fourth dilution series (S_2, S_3 and S_4) similarly, starting each time with the mixing of the contents of the last decimal dilution bottle (9.4) stored in the refrigerator in the meantime. Use two or three 250 ml portions of molten culture medium for plating each dilution series and discard the rest.

9.7 Incubation

Invert the prepared dishes and place them in the incubator (6.3) set at 30 °C for 72 h ± 2 h. Do not stack the dishes more than three high. Mark the position of each dish in a stack (low – middle – high).

NOTE This information may be useful if it turns out that the variability between the plates is too large and a possible stacking effect is to be investigated.

Separate stacks of dishes from one another and from the walls and top of the incubator. Do not leave trays in the incubator.

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