
**Milk, milk products and mesophilic
starter cultures — Enumeration of citrate-
fermenting lactic acid bacteria — Colony-
count technique at 25 °C**

*Lait, produits laitiers et ferments mésophiles — Dénombrement des
bactéries lactiques fermentant le citrate — Technique de comptage des
colonies à 25 °C*

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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 17792|IDF 180 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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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 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 17792|IDF 180 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 *Lactic acid bacteria starters*, of the Standing Committee on *Microbiological methods of analysis*, under the aegis of its project leader, Prof. B. Bianchi-Salvadori (IT).

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This edition of ISO 17792|IDF 180 ~~(cancels and replaces IDF 180:1997)~~, which has been technically revised.

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Milk, milk products and mesophilic starter cultures — Enumeration of citrate-fermenting lactic acid bacteria — Colony-count technique at 25 °C

1 Scope

This International Standard specifies methods for the enumeration of citrate-fermenting lactic acid bacteria using a colony-count technique at 25 °C.

The methods are applicable to dairy starter cultures and dairy products where these characteristic microorganisms are present.

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 565, *Test sieves — Metal wire cloth, perforated, metal, plate and electroformed sheet — Nominal sizes of openings*

ISO 17792:2006

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

ISO 8261|IDF 122, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

3 Terms and definitions

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

3.1

citrate-fermenting bacteria

homofermentative and heterofermentative lactic acid bacteria that form lenticular colonies with a diameter between 0,5 mm and 1,2 mm in selective media containing citrate and special indicators as specified in this International Standard

NOTE The most important citrate-fermenting mesophilic lactic acid bacteria belong to the following species.

a) For lactococci:

— *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*;

microscopic appearance: lactococci are normally spherical or ovoid cells (0,4 µm to 1,0 µm), occurring in pairs or chains; they are non-spore-forming, Gram-positive, non-motile and catalase-negative.

b) For leuconostocs:

- *Leuconostoc mesenteroides* subsp. *cremoris*;
- *Leuconostoc mesenteroides* subsp. *mesenteroides*;
- *Leuconostoc mesenteroides* subsp. *dextranicum*;
- *Leuconostoc lactis*;

microscopic appearance: leuconostocs are generally spherical or lenticular cells (0,4 µm to 0,5 µm), occurring in pairs or chains; they are non-spore-forming, Gram-positive, non-motile and catalase-negative.

4 Principle

4.1 Decimal dilutions of the sample are inoculated into:

- a) Nickels and Leesment medium [4], modified [3], followed by aerobic incubation at 25 °C for 72 h, for the count of citrate (zones) and non-citrate (no zones) fermenting bacteria. Then X-gal is added followed by aerobic incubation at room temperature for 24 h to differentiate between *L. lactis* subsp. *lactis* biovar *diacetylactis* (white colonies with zones) and leuconostoc species (blue colonies with or without zones).
- b) Nickels and Leesment medium [4] plus vancomycin, followed by aerobic incubation at 25 °C for 3 days to 5 days, for the enumeration of leuconostocs.

4.2 Colonies are counted and confirmed by means of appropriate tests.

4.3 The number of characteristic microorganisms per gram of sample is calculated from the number of colonies obtained on plates at dilution levels so as to give a significant result.

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5 Diluents, culture media and reagents

5.1 General

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

Tests to determine the suitability of water for microbiological applications have been published [2].

CAUTION — Some reagents are toxic and/or dangerous and can cause allergy through inhalation and skin contact.

5.2 Basic materials

See ISO 8261 | IDF 122 and ISO 7218.

5.3 Diluent

See ISO 8261 | IDF 122.

5.4 Culture media

5.4.1 Nickels and Leesment medium + X-gal^[3]

NOTE The main problem with the use of modified Nickels and Leesment medium is the poor growth of non-fermenting bacteria. These bacteria grow abundantly but are so small that they can be mistaken for the particles of insoluble calcium citrate.

5.4.1.1 Basic medium

5.4.1.1.1 Composition

Tryptic digest of casein	20,0 g
Yeast extract	5,0 g
Gelatine	2,5 g
Glucose (C ₆ H ₁₂ O ₆)	5,0 g
Lactose (C ₁₂ H ₂₂ O ₁₁)	5,0 g
Sodium chloride (NaCl)	4,0 g
Trisodium citrate dihydrate (C ₆ H ₅ Na ₃ O ₇ ·2H ₂ O)	2,0 g
Agar	(12,0 to 15,0) g ^a
Water up to	1 000 ml

^a Depending on the gel strength of the agar.

5.4.1.1.2 Preparation

Suspend separately each of the above-mentioned components in the water. Heat the suspension to boiling under frequent agitation to dissolve all components completely. Mix the thus-dissolved components well. Add water to a final volume of 1 000 ml.

Distribute the medium into bottles and sterilize in an autoclave set at 121 °C ± 1 °C for 15 min. If necessary, adjust the pH by using the required pH-adjusting reagent (5.5) and pH meter (6.7), so that after sterilization the pH is between 6,6 and 6,7.

5.4.1.2 Calcium lactate solution

5.4.1.2.1 Composition

Calcium lactate pentahydrate (C ₆ H ₁₀ CaO ₆ ·5H ₂ O)	8,0 g
Water	100 ml

5.4.1.2.2 Preparation

Dissolve the calcium lactate pentahydrate in the water by heating. Sterilize in an autoclave set at 121 °C ± 1 °C for 15 min.

5.4.1.3 Calcium citrate suspension

5.4.1.3.1 Composition

Tricalcium dicitrate tetrahydrate ($C_{12}H_{10}Ca_3O_{14}\cdot 4H_2O$)	13,3 g
Carboxymethylcellulose (CMC)	0,8 g
Water up to	100 ml

5.4.1.3.2 Preparation

Grind the calcium citrate tetrahydrate, which has previously been sifted through a sieve of 0,8 mm nominal aperture size (see ISO 565), and the CMC together in a mortar.

Slowly add prewarmed water at approximately 45 °C to a final volume of 100 ml. Blend the obtained mixture for 10 min and vacuum filter through cotton cloth. Sterilize the filtered suspension in an autoclave set at 121 °C ± 1 °C for 15 min.

NOTE Approximately 30 % of the calcium citrate is lost during filtration.

5.4.1.4 Starter culture serum

Prepare the starter culture medium by growing a L-, D- or LD-starter culture in autoclaved skimmed or autoclaved recombined skimmed milk at 25 °C ± 1 °C for 24 h.

Filter through filter paper and sterilize the filtrate at 115 °C ± 1 °C for 15 min. Remove any sediment by decantation and sterilize 200 ml of filtrate once again.

5.4.1.5 X-gal solution <https://standards.iteh.ai/catalog/standards/sist/57cccf5-3d28-4d4d-ac45-5aee845ba2fc/iso-17792-2006>

CAUTION — X-gal and NMP are toxic and must be handled in a fume cupboard.

5.4.1.5.1 Composition

5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal)	400 mg
N-Methyl-2-pyrrolidone (NMP)	100 ml

5.4.1.5.2 Preparation

Dissolve the X-gal in NMP and sterilize by filtration (see 6.13) through a 0,45 μ m Durapore filter (e.g. from Millipore¹). Store the solution at 20 °C.

1) Millipore is the name of the producer of a product available commercially. This information is given for the convenience of the users of this International Standard and does not constitute an endorsement by either ISO or IDF of this product.

5.4.1.6 Complete medium

5.4.1.6.1 Composition

Basic medium (5.4.1.1)	900 ml
Calcium lactate solution (5.4.1.2)	100 ml
Calcium citrate suspension (5.4.1.3)	50 ml
Starter culture serum (5.4.1.4)	100 ml

5.4.1.6.2 Preparation

Just before use, melt the basic medium (5.4.1.1) in a boiling water bath. When melted, cool the basic medium to between 48 °C and 50 °C.

Prewarm the calcium lactate solution (5.4.1.2), the calcium citrate suspension (5.4.1.3) and the starter culture serum (5.4.1.4) in a water bath (6.6) set at between 48 °C and 50 °C. Aseptically, add each of them to the melted basic medium and mix.

5.4.2 Nickels and Leesment medium ^[4] plus vancomycin

5.4.2.1 Basic medium

See 5.4.1.1.

5.4.2.2 Calcium lactate solution

See 5.4.1.2.

5.4.2.3 Calcium citrate suspension

See 5.4.1.3.

5.4.2.4 Starter culture serum

See 5.4.1.4.

5.4.2.5 Vancomycin solution (of volume fraction 2 %)

CAUTION — Vancomycin can cause allergy through inhalation and skin contact. Pregnant and breastfeeding women must not work with this agent.

5.4.2.5.1 Composition

Vancomycin	360 mg to 440 mg
Water	20 ml

5.4.2.5.2 Preparation

Dissolve the vancomycin in the distilled water and sterilize by filtration.

The vancomycin solution may be stored at between 4 °C and 7 °C for 1 week or at –20 °C for 4 weeks.