
**Milk and milk products — Microbial
coagulants — Determination of total milk-
clotting activity**

*Lait et produits laitiers — Coagulants microbiens — Détermination de
l'activité totale de coagulation du lait*

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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 International Standard may be the subject of patent rights. IDF shall not be held responsible for identifying any or all such patent rights.

ISO 15174|IDF 176 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 second edition of ISO 15174|IDF 176 cancels and replaces the first edition (ISO 15174|IDF 176:2002), which has been technically revised.

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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.

The main task of Standing Committees is to prepare International Standards. Draft International Standards adopted by Standing Committees are circulated to the National Committees for endorsement prior to publication as International Standards. Publication as an International Standard requires approval by at least 50 % of IDF National Committees casting a vote.

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ISO 15174|IDF 176 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 Project Group on *Microbial coagulants* of the Standing Committee on *Analytical methods for processing aids and indicators* under the aegis of its project leader, Mrs. M. Harboe (DK) and Prof. A. Andrén (SE).

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Introduction

Microbial coagulants are derived from various microbial sources, the most common sources being *Rhizomucor miehei* (EC 3.4.23.23), *R. pusillus* (EC 3.4.23.23) and *Cryphonectria parasitica*, formerly named *Endothia parasitica* (EC 3.4.23.22).

Each of these enzymes has its own characteristics as far as milk-clotting activity and cheese-making properties are concerned. These are differences in temperature sensitivity, pH sensitivity, sensitivity to calcium ions, and effect on the rheology of the milk-gel formed.

For practical and economic reasons, therefore, it is very important to have an international method for determination of the total milk-clotting activity of microbial coagulants relative to an international recognized reference standard. Also for practical reasons, it was decided to use the *R. miehei* enzyme as a microbial coagulant reference standard for all types of microbial coagulants.

The method is in accordance with the relative milk-clotting activity test for bovine rennets described in ISO 11815|IDF 157.

A qualitative determination of the microbial coagulants in a sample can be performed according to ISO 15163|IDF 110:—^[7], Annex A. For mixtures of different clotting enzymes, no correct determination of the total milk-clotting activity for the sample can be obtained.

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Milk and milk products — Microbial coagulants — Determination of total milk-clotting activity

1 Scope

This International Standard specifies a method for comparison of the total milk-clotting activity of a microbial coagulant sample with the milk-clotting activity of an international microbial coagulant reference standard on a standard milk substrate prepared with a calcium chloride solution of concentration 0,5 g/l (pH ~6,5).

2 Principle

The time needed for a visible flocculation of a standard milk substrate prepared with a 0,5 g/l calcium chloride solution (pH ~6,5) is determined. The clotting time of a microbial coagulant sample is compared under identical chemical and physical conditions to that of the microbial coagulant reference standard with known milk-clotting activity.

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3 Reagents and materials (standards.iteh.ai)

Unless otherwise specified, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity. <https://standards.iteh.ai/catalog/standards/sist/08b079d9-a315-4791-8e1f-f143f28ee97/iso-15174-2012>

3.1 Buffer solution, pH 5,5. Add, using a pipette (4.1), 10,0 ml of 1 mol/l acetic acid (CH_3COOH) to 10,0 g of sodium acetate trihydrate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$) and mix. Dilute with water to 1 000 ml. If necessary, adjust the pH to 5,5 with 1 mol/l acetic acid or with 1 mol/l sodium acetate solution.

3.2 Calcium chloride stock solution, $\rho(\text{CaCl}_2) = 500$ g/l. Calcium chloride solutions with the required accurate calcium chloride concentration of 500 g/l and the actual density stated are commercially available.¹⁾ Store the solution according to the manufacturer's instructions.

Prior to use, bring the calcium chloride stock solution to room temperature (18 °C to 22 °C). Check the concentration of the calcium chloride solution by titration with EDTA (ethylenediaminetetraacetic acid) every year.

3.3 Calcium chloride working solution, $\rho(\text{CaCl}_2) = 0,5$ g/l. Use the density of the calcium chloride stock solution (3.2) to calculate the mass needed to get a final calcium chloride concentration of 0,5 g/l in the working solution.

The mass of the solution should be equivalent to the addition of 2,00 ml of the stock solution with exact concentration of $\rho(\text{CaCl}_2) = 500$ g/l, in which case the solution mass is ~2,70 g.

Weighing of the calcium chloride stock solution (3.2) is recommended to be able to prepare the calcium chloride working solution, as the viscous solution is difficult to pipette.

1) Chr. Hansen's A/S, Hvidovre, Denmark is an example of a suitable supplier. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this supplier.

Weigh, to the nearest 0,01 g, about 2,70 g of the calcium chloride stock solution (3.2) of exact known concentration at room temperature (18 °C to 22 °C) in a 2 000 ml one-mark volumetric flask. Dilute with water to the 2 000 ml mark and mix. The calcium chloride solution shall be freshly prepared on the day of its use.

NOTE Alternatively, an intermediate calcium chloride solution of 50 g/l can be prepared and further diluted before use.

3.4 Low-heat, low-fat spray-dried milk powders, of good renneting and bacteriological quality.

NOTE Low-heat, low-fat spray-dried milk powders meeting the requirements are commercially available.^{1),2)}

3.5 Microbial coagulant reference standard powder (*Rhizomucor miehei*), in glass ampoules. The exact total milk-clotting activity is labelled on the ampoules.

Store the microbial coagulant reference standard powder in the dark at –18 °C, protected against moisture. For short periods, e.g. during transport, the powder may be kept at ambient temperatures.

The microbial coagulant reference standard powder is a primary reference standard; a secondary liquid standard may be made and used if it is assured that the same result is obtained.

The total milk-clotting activity of the international microbial coagulant reference standard powder (*R. miehei*) is the amount of activity set relative to the first batch of international calf rennet reference standard powder, which was defined to contain 1 000 IMCU/g (see ISO 11815|IDF 157^[6]).

NOTE 1 The total milk-clotting activity is expressed as a percentage relative to the arithmetic mean of test results.

The total milk clotting activity of the microbial coagulant reference standard powder is labelled on the glass ampoules and/or stated on the certificate provided. There is a requirement for future preparations of microbial reference standards to be set relative to the previous batch of the microbial reference standard.

NOTE 2 The total proteolytic (milk-clotting) activity of the microbial coagulant reference standard powder is checked every second year by an alternative method, e.g. on a synthetic hexapeptide substrate of NIZO³⁾.

The international microbial coagulant reference standard powder is commercially available from DSM Food Specialties⁴⁾.

4 Apparatus

Usual laboratory equipment and, in particular, the following.

4.1 Micropipette or any other pipette, capable of delivering 0,5 ml in less than 1 s with a repeatability of 0,2 % or better.

4.2 One-mark pipettes, for delivering appropriate volumes, ISO 648^[1], class A.

Alternatively a **dilutor** (e.g. a Hamilton diluter) with the same precision may be used for diluting the coagulants. For measuring substrate, a **syringe** or a **dispenser** delivering the appropriate amount with repeatability of 0,4 % may also be used.

2) Cecalait, Poligny, France, is an example of a suitable supplier. This information is given for the convenience of users of this document Standard and does not constitute an endorsement by ISO or IDF of this supplier.

3) NIZO Food Research BV, Ede, Netherlands is an example of a suitable supplier. This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO or IDF of this supplier.

4) DSM Food Specialities, Dairy Ingredients Group, Delft, Netherlands is an example of a suitable supplier. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this supplier.

- 4.3 One-mark volumetric flasks**, of required capacities, ISO 1042^[3], class A.
- 4.4 Thermometer**, calibrated, graduated between 20 °C and 45 °C, with a precision $\pm 0,1$ °C.
- 4.5 pH meter**, capable of being read to the nearest 0,01 pH unit.
- 4.6 Analytical balance**, capable of being read to the nearest 1 mg.
- 4.7 Stopwatch**, capable of being read to the nearest second.
- 4.8 Flasks or test-tubes**, for milk-clotting testing, with suitable capacity (see 7.4).
- 4.9 Water bath**, capable of maintaining a temperature of $32\text{ °C} \pm 1\text{ °C}$, but also capable of maintaining the temperature constant to within $\pm 0,2\text{ °C}$ throughout the bath. The water bath should be equipped with the following attachments.
- 4.9.1 Electric motor**, provided with a rotating spindle to which the flask or test tubes (4.8) can be attached, capable of rotating at a suitable angle of about 30° with the water surface of the water bath.

NOTE The rotational frequency is not important for this International Standard. A rotational frequency of 2 r/min to 4 r/min is suitable.

- 4.9.2 Electric lamp**, placed so as to illuminate the flask or test-tube (4.8) effectively.

NOTE A screen with a dark background, placed in the water bath, can be used to improve observation of the milk-clotting in the flask or test-tube.

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5 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given for liquid microbial coagulant (6.1) in ISO 707 | IDF 50:2008^[2], Clause 9 and for powdered microbial coagulant (6.2) in ISO 707 | IDF 50:2008^[2], Clause 13.

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

Store test samples in the dark at a temperature between 0 °C and 5 °C.

6 Preparation of test sample

6.1 Liquid microbial coagulant

Mix the test sample by swirling while avoiding foam formation. Bring the sample to room temperature (18 °C to 22 °C) prior to starting the preparation of the coagulant test solution (7.3).

Liquid coagulant is rather viscous. When pipetting the sample, use the correct technique. Alternatively, accurate and precise dilutions, especially for high strength coagulants, can be made. This is done by weighing the liquid samples on an analytical balance and by calculating its volume, in millilitres, by dividing its mass by the density of the coagulant used.

6.2 Powdered microbial coagulant

Mix the test sample thoroughly to obtain a homogeneous powder. Bring the sample to room temperature (18 °C to 22 °C) before preparing the coagulant test solution (7.3).

NOTE Powdered products can rapidly separate.