INTERNATIONAL STANDARD

**ISO 26323**

**IDF 213**

First edition
2009-07-01

# Milk products — Determination of the acidification activity of dairy cultures by continuous pH measurement (CpH)

*Produits laitiers — Détermination de l'activité acidifiante des cultures laitières par mesurage continu de pH (CpH)*



Reference numbers
ISO 26323:2009(E)
IDF 213:2009(E)



ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

International Dairy Federation
Diamant Building • Boulevard Auguste Reyers 80 • B-1030 Brussels
Tel. + 32 2 733 98 88
Fax + 32 2 733 04 13
E-mail info@fil-idf.org
Web www.fil-idf.org

Published in Switzerland

 

# Contents

Page

Foreword ..... iv
Foreword ..... v
1 Scope ..... 1
2 Normative references ..... 1
3 Terms and definitions ..... 1
4 Principle ..... 2
5 Diluents, culture media and reagents ..... 2
6 Apparatus ..... 4
7 Sampling ..... 5
8 Preparation ..... 5
8.1 Milk preparation ..... 5
8.2 Cleaning and calibration of pH electrodes ..... 5
8.3 Protein and fat cleaning of the electrode ..... 6
8.4 Stabilization and storage of the pH electrode ..... 6
8.5 Calibration of the pH electrode ..... 6
8.6 Disinfection of the pH electrode with ethanol ..... 6
8.7 Decontamination of the pH electrode by heat treatment ..... 6
9 Procedure ..... 6
9.1 Frozen cultures ..... 6
9.2 Freeze-dried products ..... 8
9.3 Termination of the analysis ..... 9
10 Precision ..... 10
10.1 Interlaboratory test ..... 10
10.2 Repeatability ..... 10
10.3 Reproducibility ..... 10
11 Test report ..... 11
Annex A (informative) Interlaboratory test — A CpH ring trial ..... 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 26323 | IDF 213 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.

# 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 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 26323 | IDF 213 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 ISO-IDF Joint Action Team on *Lactic acid bacteria and starters* of the Standing Committee on *Microbiological methods of analysis* under the aegis of its project leader, Mr. L. V. Jørgensen (DK).

# Milk products — Determination of the acidification activity of dairy cultures by continuous pH measurement (CpH)

## 1 Scope

This International Standard specifies a method for the measurement of the acidification activity of lactic acid bacteria by continuous measurement of pH.

NOTE The method is based on Reference [9].

The method is applicable to dairy starter cultures where these characteristic microorganisms are present.

Two types of standardized milk are specified in the procedure: boiled milk with 9,5 % mass fraction dry matter (B-milk 9,5); and autoclaved milk with 9,5 % mass fraction dry matter (A-milk 9,5). It is possible that heat treatment of B-milk 9,5 does not inactivate all enzymes that are present, which can affect the activity of some cultures. In that case, cultures are tested with A-milk 9,5 in which all enzymes have been inactivated.

## 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 6887-5, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products* [1)]

## 3 Terms and definitions

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

**3.1**
**acidification activity**
ability of a dairy starter culture to acidify standardized milk determined according to the procedure described in this International Standard

NOTE The acidification activity can be quantified by the following parameters.

a) $t_a$ — The time it takes to start acidifying the standardized milk, i.e. time for pH to drop 0,08 pH-units from initial pH (after 15 min). Time $t_a$ is measured in minutes from the inoculation time, $t = 0$. If the software collects data every 4 min, $t_a$ is determined by interpolation.

b) $pH_{t\,h}$ — The pH after $t$ h (e.g. 4 h, 6 h, 12 h or 16 h) of acidification in the standardized milk at 30 °C, 37 °C, 40 °C or 43 °C. The actual time for the parameter depends on the characteristics of the starter culture.

c) $t_{pH\,x}$ — The time it takes to acidify the standardized milk to a certain pH, e.g. pH 4,50. The actual time for the parameter depends on the characteristics of the starter culture and the application for which it is used.

1) Supersedes ISO 8261 | IDF 122.

## 4 Principle

A specified quantity of starter culture is diluted and inoculated in a specified amount of standardized milk. The inoculated culture is incubated at a specified constant temperature of 30 °C, 37 °C, 40 °C or 43 °C for a given time depending on the characteristics of the starter culture. During incubation, the acidification activity is measured by continuous pH measurements using a pH electrode and a data logger. When the fermentation curve is obtained, a number of curve parameters can be calculated or extracted.

## 5 Diluents, culture media and reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and only distilled or demineralized water or water of equivalent purity. The water shall be free of substances likely to inhibit or influence the growth of microorganisms in the reconstituted milk. If chlorinated water is used, neutralize the chlorine prior to use.

Observe the following rules for the water quality: the cell count shall be below 50 cell/ml and conductivity below 5 µS/cm. Tests to determine the suitability of water for microbiological applications appear in ISO/TS 11133-1 [5].

### 5.1 Basic materials

**SAFETY PRECAUTIONS — Take the required safety precautions when using the electrode cleaner (5.1.2), potassium chloride solution (5.1.3) or diaphragm cleaner (5.1.4) as these can irritate skin and eyes.**

#### 5.1.1 Buffer solutions

**5.1.1.1 Buffer solution of pH 4,00**, capable of buffering at 20 °C; e.g. Merck/VWR CertiPUR (Order No. 1.09475) [2] or equivalent.

**5.1.1.2 Buffer solution of pH 7,00**, capable of buffering at 20 °C; e.g. Merck/VWR CertiPUR (Order No. 1.09477) [2] or equivalent.

**5.1.2 Electrode cleaner**, capable of cleaning the electrode; e.g. pepsin/HCl solution from Mettler Toledo (Order no. 9891) [2] or equivalent.

**5.1.3 Potassium chloride solution**, of concentration $c$(KCl) = 3 mol/l; e.g. Mettler Toledo (Order no. 9823) [2] or equivalent.

**5.1.4 Diaphragm cleaner**, capable of cleaning the diaphragm; e.g. thiourea/HCl solution from Mettler Toledo (Order no. 9892) [2] or equivalent.

**5.1.5 Ethanol solution**, $\varphi$ ($C_2H_5OH$) = 70 % volume fraction in water, used for disinfection.

**5.1.6 Medium-heat, low fat, spray-dried milk powder**. Use medium-heat, low fat, spray-dried milk powder produced from milk of good quality and with no detectable antibiotics residues. The spray-dried milk powder shall be of good microbiological quality (see Reference [8]) having a natural, pleasant taste and flavour of fresh skimmed milk and shall consist of the components specified in Table 1.

2) Example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by either ISO or IDF of this product. Other products may be used if they can be shown to give similar results.

**Table 1 — Medium-heat, low fat, spray-dried milk powder compound[a], e.g. from Chr. Hansen[3)]**

| Component | Mass fraction<br>% |
|---|---|
| Milk protein | 34 to 38 |
| Lactose | 48 to 56 |
| Milk fat | < 1,25 |
| Ash | 7 to 9 |
| Moisture | <4 |
| Titratable acidity, lactic acid | < 0,15 |
| [a] The pH of a 10 % mass fraction solution should be in the range 6,5 to 6,8. | |

## 5.2 Substrates

### 5.2.1 QC-Milk 9,5

**5.2.1.1 Composition**. Use only standardized milk with a specified dry matter content prepared from spray-dried skimmed milk powder according to the composition in Table 2.

The prepared amount may be smaller than that specified in Table 2. However, do not prepare less than ~1,1 kg of milk. If using a small amount, use the analytical balance (6.1) to weigh the milk powder to the nearest 1 mg.

The aim is to prepare milk with a dry matter content of 9,5 % ± 0,2 % mass fraction, similar to that of bottled and sterilized milk.

**Table 2 — Composition of milk made up from spray-dried milk powder**

| Component | Mass<br>kg |
|---|---|
| Medium-heat milk powder [a] | 10,9 to 11,2 |
| Water | 100,0 |
| [a] The quantity varies due to water content of milk powder and water loss during the preparation and heat treatment. | |

**5.2.1.2 Preparation**. Dissolve the medium-heat milk powder in water for not more than 30 min. If needed, warm the water up to 40 °C to completely dissolve the powder.

Dispense the milk obtained into cylindrical bottles to reach a volume of 200 ml after heat treatment. Check the volume by weighing the bottle until it reaches a net mass of 207 g ± 2 g (total mass minus that of the bottle). The density of QC-milk 9,5 is 1,033 g/ml.

Alternatively, dispense the milk obtained into cylindrical bottles to reach a mass of 200 g ± 2 g (net mass). Compensate for the mass difference in the inoculation procedure.

3) Example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by either ISO or IDF of this product. Other products may be used if they can be shown to give similar results.

**5.2.2 Heat treatment of B-milk 9,5**. Heat treat the prepared milk (5.2.1.2) according to the temperature programme in Table 3.

**Table 3 — Temperature programme for heat treating B-milk 9,5**

| Procedure | Temperature °C | Time min |
|---|---|---|
| Heating | > 99 ± 1 | < 20 |
| Holding | 99 ± 1 | 30 ± 1 |
| Cooling | 99 ± 1 to 40 ± 5 | < 40 |

Then, store the B-milk 9,5 obtained at below 7 °C for at least 16 h.

Record the evaporation during heat treatment and the tolerance of mass after the heat treatment.

**5.2.3 Shelf life of B-milk 9,5**. Before use, store the B-milk 9,5 (5.2.2) for a minimum of 16 h and a maximum of 12 d.

Enzymes such as proteases may not have been inactivated and can affect acidification activity of some cultures.

**5.2.4 Heat treatment of A-milk 9,5**. Use A-milk 9,5 for cultures where B-milk 9,5 does not give the required repeatability, e.g. due to residual protease activity.

Sterilize the prepared milk (5.2.1.2) according to the temperature programme in Table 4.

**Table 4 — Temperature programme for heat treating A-milk 9,5**

| Procedure | Temperature °C | Time min |
|---|---|---|
| Heating | 115 ± 1 | < 20 |
| Holding | 115 ± 1 | 15 ± 1 |
| Cooling | 115 ± 1 to 40 ± 5 | < 40 |

Then, store the A-milk 9,5 obtained at below 7 °C for at least 16 h.

Record the evaporation during heat treatment and the tolerance of mass after that treatment.

**5.2.5 Shelf life of A-milk 9,5**. Before use, store the A-milk 9,5 (5.2.4) for a minimum of 16 h and a maximum of 12 d.

## 6 Apparatus

Usual microbiological laboratory equipment and, in particular, the equipment required for the preparation of test samples and dilutions specified in ISO 6887-5, as well as the following.

**6.1 Analytical balances**, capable of weighing to the nearest 0,01 g and 1 mg (see 5.2.1.1), respectively.

**6.2 Autoclave**, capable of operating at 99 °C ± 1 °C and 115 °C ± 1 °C.

**6.3 Water baths**, capable of being maintained at 21 °C ± 1 °C, 30,0 °C ± 0,2 °C, 37,0 °C ± 0,2 °C, 40,0 °C ± 0,2 °C and 43,0 °C ± 0,2 °C, under thermostat control.

**6.4 pH electrodes**, suitable for measuring the required pH; e.g. Mettler Toledo [4)] 405-DPAS-SC-K8S/150 or equivalent electrodes.

**6.5 Cylindrical bottles**, capacity 250 ml, of height 16,5 cm and internal diameter 5,5 cm.

**6.6 Temperature probes**, calibration of accuracy ± 0,1 °C.

**6.7 Data logger**, equipped with pH and temperature channels; connected to a computer capable of logging data from pH and temperature probes.

## 7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 | IDF 50[1].

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

## 8 Preparation

### 8.1 Milk preparation

At least 16 h before starting the analysis, loosen the lids of the milk bottles (second weighing) to be used in the activity analysis. Allow oxygen and carbon dioxide concentrations to reach equilibrium between milk and surroundings.

Allow the water baths to pre-warm to the temperature of inoculation.

Cool the milk bottles used for dilution (first weighing) to between 4 °C and 12 °C to minimize the microbial activity.

Pre-warm the milk bottles for activity analysis (second or third weighing, see 9.1.2) to the temperature of inoculation in the water bath at the required temperature for 20 min to 40 min. The total time depends on the incubation temperature and may not exceed 60 min prior to inoculation.

Set the temperature for continuous measurement by a calibrated temperature probe in a control bottle. Check the temperature before inoculation. Alternatively, calibrated thermometers can be used.

### 8.2 Cleaning and calibration of pH electrodes

Warm the buffer solution of pH 4,00 (5.1.1.1), the buffer solution of pH 7,00 (5.1.1.2), and the pH electrodes (6.4) to the incubation temperature of the water bath (6.3) maintained at 30,0 °C ± 0,2 °C, 37,0 °C ± 0,2 °C, 40,0 °C ± 0,2 °C or 43,0 °C ± 0,2 °C for at least 10 min before calibrating the pH probes.

Use the pH-value of the buffers at a temperature of 30 °C, 37 °C, 40 °C or 43 °C as supplied by the manufacturer when calibrating the pH probes.

NOTE The pH values of the buffers are temperature dependent and, on request, can be obtained from the manufacturer or supplier.

4) Example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by either ISO or IDF of this product. Other products may be used if they can be shown to give similar results.

### 8.3 Protein and fat cleaning of the electrode

Between each of the fermentations and after each run, rinse the electrode with water so that no visible milk residues remain. Remove any remaining fat by cleaning the electrode with the ethanol solution (5.1.5).

Disinfect the electrode. Immerse the electrode in new electrode cleaner (5.1.2) for at least 15 min. Rinse the electrode with water. Take care to use new electrode cleaner each time.

### 8.4 Stabilization and storage of the pH electrode

Immerse the pH electrode (6.4) in the potassium chloride solution (5.1.3) for 30 min to allow stabilization.

Store the electrode in the potassium chloride solution (5.1.3), if needed, and renew the solution at least every week.

### 8.5 Calibration of the pH electrode

Rinse the pH electrode (6.4) with water. Gently wipe it dry with lens paper (or similar soft paper). Immerse the electrode in buffer solution of pH 7,00 (5.1.1.2) to initiate calibration.

Rinse the electrode with water. Gently wipe dry with lens paper (or similar soft paper). Immerse the electrode in buffer solution of pH 4,00 (5.1.1.1) to finalize calibration. The slope obtained should be 93 % or more and the intercept between –30 mV and 30 mV.

Renew the calibration buffers every day.

### 8.6 Disinfection of the pH electrode with ethanol

Disinfect the pH electrode (6.4) with the ethanol solution (5.1.5). Then gently dry the electrode with lens paper (or similar soft paper). The electrode is now ready for use.

### 8.7 Decontamination of the pH electrode by heat treatment

Decontamination may be required for pH electrodes (6.4), e.g. when electrodes have been contaminated with bacteriophages. If so, decontaminate the contaminated pH electrode by using a heat treatment at 99 °C for 30 min.

## 9 Procedure

### 9.1 Frozen cultures

#### 9.1.1 Thawing of frozen samples before inoculation

Take a representative test sample. Use a test sample of 10 g ± 4 g for single component products. For blended products, use about half the container content, i.e. 250 g ± 10 g for 500 g cartons and 500 g ± 50 g for 1 000 g bags.

If the product is inhomogeneous, thaw the whole carton/bag, e.g. for layered products or products with low percentage of one component.

Transfer the test sample to a sterile stomacher bag. Place the bag in a second robust plastic bag. If possible, draw light vacuum on the bag to improve heat transfer.

Thaw the bag and its content in a water bath (6.3) at 21 °C until the whole sample is just thawed (in less than 20 min). When thawing, observe the sample and massage the bag to ensure that it can be removed from the water bath when the whole sample is just thawed.

Dry the outside of the bag with a paper towel. Homogenize the sample by massaging the bag.

Take care that the temperature of the thawed culture does not exceed 5 °C. After the culture is thawed, inspect the bags for leakage, indicated by the presence of culture solution or water between the two bags.

### 9.1.2 Weighing and inoculation

Divide the inoculation into two operations; a first weighing, $m_{I1}$, and a second weighing, $m_{I2}$. Based on these two weighing procedures the inoculation amount is calculated.

Calculate the inoculation amount, $w_I$, expressed as a percentage, using Equation (1):

$$w_I = \frac{m_{I1}}{m_1} \times \frac{m_{I2}}{m_2} \times 100 \quad (1)$$

where

$m_1$ is the mass, in grams, of the milk and culture;

$m_2$ is the mass, in grams, of the milk and culture in the second dilution.

Limits for individual weighing are given in Table 5.

**Table 5 — Limits for the individual weighing**

| Inoculation<br>% | $m_{I1}$<br>g | $m_{I2}$<br>g |
|---|---|---|
| [0,005 to 0,01] ± 0,002 | 1,5 to 2,5 | 0,8 to 1,5 |
| 0,01 ± 0,002 | 1,5 to 2,5 | 1,7 to 2,9 |
| > 0,01 ± 0,002[a] | > 3 | 1,7 to 2,9 |

[a] Higher inoculation percentages can be obtained by variation of the value of the first weighing $m_{I1}$.

### 9.1.3 Inoculation of the dilution bottle (1st weighing)

Place the milk bottle for dilution of the first weighing on the analytical balance (6.1) set to zero. Weigh the thawed sample directly into the milk bottle. Record the actual mass, in grams, of the inoculum (first weighing) to two decimal places.

Gently mix the milk and culture it by inverting 10 times. Let the bottle stand for at least 30 s but not more than 10 min before the inoculation of the activity milk bottle (2nd weighing).

### 9.1.4 Inoculation of the preheated milk bottle (2nd weighing)

Take the preheated milk bottle for the second weighing from the water bath (6.3) maintained at 30,0 °C ± 0,2 °C, 37,0 °C ± 0,2 °C, 40,0 °C ± 0,2 °C or 43,0 °C ± 0,2 °C. Dry the outside of the bottle by using a paper towel. Place it on the analytical balance (6.1) set to zero.

Mix the dilution bottle by inverting it 10 times before the second weighing is carried out. Directly weigh the inoculum from the dilution bottle into the activity bottle. Record its mass, in grams (second weighing), to two decimal places.

Mix the activity bottle carefully by inverting it 10 times. Then place the bottle in the water bath (6.3) maintained at 30,0 °C ± 0,2 °C, 37,0 °C ± 0,2 °C, 40,0 °C ± 0,2 °C or 43,0 °C ± 0,2 °C.

Disinfect the calibrated pH electrode with soft paper (e.g. lens tissue) soaked in ethanol solution (5.1.5). Place the electrode in the warm activity milk bottle. Set the software for continuous measurement of pH to start monitoring, according to the instructions of the manufacturer.

Repeat the inoculation procedure for samples and the control culture until all samples are incubated.

Place a calibrated temperature probe (6.6) in an uninoculated milk or water bottle placed in the water bath (6.3) maintained at 30,0 °C ± 0,2 °C, 37,0 °C ± 0,2 °C, 40,0 °C ± 0,2 °C or 43,0 °C ± 0,2 °C.

Always run a control culture with a known activity in parallel with the sample using the same time and temperature combination, and water bath.

## 9.2 Freeze-dried products

### 9.2.1 Thawing of freeze-dried final samples

Take a representative test sample. Acclimatize the freeze-dried cultures to room temperature for ~15 min before opening the pouch. Use a sample size of ~10 g.

If the content of the pouches weighs less than 10 g, use the whole pouch content.

### 9.2.2 Weighing and inoculation

Calculate the target inoculation, $w_t$, expressed as a percentage, by using Equation (2):

$$w_t = \frac{m_{c0}}{m_m} \times 100 \quad (2)$$

where

$m_{c0}$ is the mass, in grams, of culture blend;

$m_m$ is the specified amount, in grams, of milk to be inoculated with this culture.

The inoculation is divided into three weighing procedures: $m_{I1}$, $m_{I2}$ and $m_{I3}$.

Based on the three weighings, calculate the inoculation, $w_{fd}$, expressed as a percentage, by using Equation (3):

$$w_{fd} = \frac{m_{I1}}{m_1} \times \frac{m_{I2}}{m_2} \times \frac{m_{I3}}{m_3} \times 100 \quad (3)$$

where

$m_1$ is the mass, in grams, of the milk and culture;

$m_2$ is the mass, in grams, of the milk and culture after the second dilution;

$m_3$ is the total mass, in grams, of milk and culture after the third dilution.

Limits for individual weighing are given in Table 6.

**Table 6 — Limits for the individual weighing**

| Pouch size<br>g | $m_{I1}$<br>g | $m_{I3}$[a]<br>g |
|---|---|---|
| < 10 | Whole pouch | 0,5 to 10 |
| > 10 | 10 to 12 | 0,5 to 10 |

[a] If the limits for $m_{I3}$ are exceeded, no second dilution is needed. This is equivalent to an inoculation of ⩾ 0,01 % (see 9.1.2).

EXAMPLE

A starter culture for 250 kg (250 000 g) of milk contains, for instance, 12 g of culture. Therefore the target inoculation, $w_t$, expressed as a percentage, is given by

$$w_t = 100 \times \frac{12}{250\,000} = 0{,}004\,8$$

With

$m_{I1}$ = 11,9 g $m_1$ = 218,8 g $m_{I2}$ = 10 g $m_2$ = 216,9 g $m_{I3}$ = 4,06 g $m_3$ = 211,0 g

the inoculation, $w_{fd}$, expressed as a percentage, is

$$w_{fd} = \frac{11{,}9}{218{,}8} \times \frac{10{,}0}{216{,}9} \times \frac{4{,}06}{211{,}0} \times 100 = 0{,}004\,8$$

### 9.2.3 First weighing

Weigh directly ~10 g of culture into a stomacher bag. Add one bottle (e.g. 200 ml) of A- or B-milk 9,5 to the stomacher bag. Record the actual mass, in grams, of the inoculum, $m_{I1}$, to two decimal places.

Use a stomacher bag to mix at 200 r/min for 2 min. Check the suspension for homogeneity. If needed, mix it again using the stomacher bag for an additional 2 min. Allow the suspension to settle for at least 30 s, but not more than 2 min, before the second weighing. Continue this procedure until the suspension is homogenous.

### 9.2.4 Second weighing

Transfer ~10 g of the content from the first dilution bottle, $m_{I1}$, into a milk bottle (e.g. 200 ml). Note the actual mass, in grams, of the inoculum, $m_{I2}$, to two decimal places.

Gently mix the milk and culture by inverting 10 times. Let the bottle stand for at least 30 s but no more than 10 min before the inoculation of the activity milk bottle (third weighing).

### 9.2.5 Inoculation of warm activity milk bottle (third weighing)

Based on the first and second weighing, calculate, $m_{I3}$, in grams. Transfer, using a pipette, the calculated amount into the warm activity milk bottle. Record the actual mass of inoculum, $m_{I3}$, to two decimal places.

Mix the activity bottle carefully by inverting 10 times. Then place the bottle in the water bath (6.3) maintained at 30,0 °C ± 0,2 °C, 37,0 °C ± 0,2 °C, 40,0 °C ± 0,2 °C or 43,0 °C ± 0,2 °C.

Disinfect the calibrated pH electrode with soft paper (e.g. lens tissue) soaked in 70 % ethanol. Place the electrode in the warm activity milk bottle. Set the software to start monitoring according to the instructions of the manufacturer.

Repeat the inoculation procedure for samples and control culture until all are incubated.

Place a calibrated temperature probe (6.6) in an uninoculated milk or water bottle that is placed in the water bath (6.3) maintained at 30,0 °C ± 0,2 °C, 37,0 °C ± 0,2 °C, 40,0 °C ± 0,2 °C or 43,0 °C ± 0,2 °C.

Always run a control culture with a known activity in parallel with the sample using the same time and temperature combination, and water bath.

## 9.3 Termination of the analysis

Check the temperature profile of the water bath which is measured by using a temperature probe (6.6) in an uninoculated bottle. Alternatively, use calibrated thermometers. The run is approved when the temperature variation in the uninoculated bottle always remains within ± 0,5 °C of the intended temperature.

# 10 Precision

## 10.1 Interlaboratory test

Details of the interlaboratory test on the precision of the method are summarized in Annex A. The repeatability and the reproducibility limits were determined by using three dairy cultures of lactic acid bacteria available commercially in Europe, North America, and South America to acidify milk to produce fermented dairy products.

The values derived from the interlaboratory test may not be applicable to concentration ranges, cultures and matrices other than those given, especially non-acidifying and/or very texturing cultures of lactic acid bacteria.

The concentration ranges tested and the characteristics of the dairy cultures of lactic acid bacteria among the products selected are representative of the worldwide market and are in accordance with ISO 27205 | IDF 149 [7].

## 10.2 Repeatability

The absolute difference between two individual single test results, obtained with 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, shall in not more than 5 % of cases be greater than the values given in Table 7.

NOTE As indicator of the repeatability limit, $r$, Table 7 shows the estimate of the repeatability of different starter cultures of lactic acid bacteria in Reference [10]. Further details are given in Annex A.

**Table 7 — Repeatability limit, $r$ [$t_a$, $pH_{4\,h}$, $pH_{6\,h}$ and $pH_{16\,h}$]**

| Product | Description | Parameter [a] | $r$ [b] |
|---|---|---|---|
| Samples 1 | A mesophilic O-starter culture of *Lactococcus lactis* strains. | $t_a$ | 7,3 |
| | | $pH_{6\,h}$ | 0,039 |
| | | $pH_{16\,h}$ | 0,032 |
| Samples 2 | A mesophilic LD-starter culture of *Lactococcus lactis, Leuconostoc mesenteriodes* and *Lactococcus lactis* subsp. *lactis* biovar. diacetylactis strains. | $t_a$ | 7,8 |
| | | $pH_{6\,h}$ | 0,062 |
| | | $pH_{16\,h}$ | 0,028 |
| Samples 3 | A thermophilic starter culture of *Streptococcus thermophilus* strains. | $t_a$ | 4,0 |
| | | $pH_{4\,h}$ | 0,048 |
| | | $pH_{16\,h}$ | 0,039 |

[a] A pH measured after 4 h is indicated by $pH_{4\,h}$; after 6 h, by $pH_{6\,h}$; and after 16 h, by $pH_{16\,h}$, respectively.

[b] The results are obtained from Reference [10].

## 10.3 Reproducibility

The absolute difference between two individual single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, shall in not more than 5 % of cases be greater than the values given in Table 8.

NOTE As indication of the reproducibility limit, $R$, Table 8 shows the estimation of the reproducibility of different starter cultures of lactic acid bacteria in Reference [10]. Further details are given in Annex A.