TECHNICAL SPECIFICATION

ISO/TS 26844

IDF/RM 215

First edition
2006-11-15

Milk and milk products — Determination of antimicrobial residues — Tube diffusion test

Lait et produits laitiers — Détermination de résidus antimicrobiens — Test de dissémination en tube

Reference numbers
ISO/TS 26844:2006(E)
IDF/RM 215:2006(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

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.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of normative document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

— an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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/TS 26844 | IDFRM 215 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 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.

The main task of technical committees is to prepare International Standards. 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.

In other circumstances, particularly when there is an urgent market requirement for such documents, a Standing Committee may decide to publish an other type of normative document which is called by IDF: *Reviewed method*. Such a method represents an agreement between the members of a Standing Committee and is accepted for publication if it is approved by at least 50 % of the committee members casting a vote. A *Reviewed method* is equal to an ISO/PAS or ISO/TS and will, therefore, also be published jointly under ISO conditions.

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/TS 26844 | IDF/RM 215 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 *Veterinary residues*, of the Standing Committee on *Analytical methods for additives and contaminants*, under the aegis of its project leaders, Mr H. Stegeman (NL) and Mr J. Kerkhof (NL).

Milk and milk products — Determination of antimicrobial residues — Tube diffusion test

1 Scope

This Technical Specification (Reviewed Method) specifies a microbiological inhibitor test for the detection of a broad variety of antimicrobials in milk and milk products.

The method is applicable to raw milk, heat-treated milk and reconstituted dried milk.

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 4833, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of micro-organisms — Colony-count technique at 30 °C*

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

ISO 13969 | IDF 183, *Milk and milk products — Guidelines for a standardized description of microbial inhibitor tests*

ISO 18330 | IDF 188, *Milk and milk products — Guidelines for a standardized description of immunoassays or receptor assays for the detection of antimicrobial residues*

3 Terms and definitions

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

3.1
antimicrobial substances
substances that show an inhibition in the procedure specified in this document

3.2
limits of detection
concentration level at which a defined percentage of positive samples is detected

EXAMPLE 95 %.

4 Principle

A milk sample is added to two test tubes with agar media containing *Geobacillus stearothermophilus* ATCC 10149 (identical to NIZO strain C953). The test tubes differ from each other in pH, in added supplements and synergistic antibiotics. Incubation resulting in normal growth of the organism causes the pH

indicator in the agar to change colour from purple to yellow. When substances that are inhibitory to the growth of microorganisms are present in the milk, the colour of the pH indicator will remain purple.

Test tube A (pH 7,0; chloramphenicol) shows an improved sensitivity for tetracycline residues, and test tube B (pH 8,0; trimethoprim) for beta-lactams, macrolides, aminoglycosides, sulfonamides and trimethoprim residues.

5 Test organism, culture media, standard solutions and control samples

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

5.1 Test organism

Use a suspension of *Geobacillus stearothermophilus* ATCC 10149 (identical to NIZO strain C953) [1)] adjusted to a viable count of approximately 5 000 000 colony-forming units/ml (see Annex B for preparation). Check the quality of each new batch of the test-organism suspension by determining the sensitivity for the standard solutions mentioned in Table 1.

Table 1 — Standard solutions for testing the sensitivity of the test-organism suspension

Standard solution	Content µg/kg milk
Benzylpenicillin (Penicillin-G)	2
Sulfadiazine	150
Neomycin	30
Erythromycin	10
Oxytetracycline	100

Perform the check with standard solutions and control milk in 5-fold, according to the procedure described in Clause 9. Determine the sensitivity of the test-organism suspension for benzylpenicillin and oxytetracycline with tube A (5.2.5) and the sensitivity for sulfadiazine, neomycin and erythromycin with tube B (5.2.6). A positive result should be obtained in all test tubes.

5.2 Culture media

In order to improve the reproducibility of the method, it is recommended to use dehydrated basic components or dehydrated complete media for the preparation of culture media. Follow the manufacturers' instructions.

5.2.1 Basic medium

5.2.1.1 Components

Casein trypton	5,0 g
Yeast extract	2,5 g
Glucose, anhydrous	1,0 g
Agar	10 g to 15 g
Water	1 000 ml

NOTE The basic dehydrated medium is commercially available as Plate Count Agar.

1) Suspension of *Geobacillus stearothermophilus* ATCC 10149 or NIZO strain C953 is an example of a product available commercially. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by ISO or IDF of this product.

5.2.1.2 Preparation

Dissolve the components in the water by heating. Adjust the pH so that after sterilization it is 7,0 ± 0,2.

Autoclave the medium at 121 °C ± 1 °C for 15 min.

The thus-prepared basic medium may be kept for a maximum of 3 months if stored in the dark at 0 °C to +5 °C.

5.2.2 Bromocresolpurple solution

5.2.2.1 Components

Bromocresolpurple	250 mg
Ethanol, 96%	5 ml
Water	100 ml

5.2.2.2 Preparation

Dissolve the bromocresolpurple in the ethanol. Dilute with water to 100 ml.

The bromocresolpurple solution may be kept for a maximum of 6 months if stored in the dark at 0 °C to +5 °C.

5.2.3 Chloramphenicol (CAP) solution

5.2.3.1 Components

Chloramphenicol	20,0 mg
Methanol	5 ml
Water	100 ml

5.2.3.2 Preparation

Dissolve the chloramphenicol in the methanol. Dilute with water to 100 ml.

The chloramphenicol solution may be kept for a maximum of 1 month if stored in the dark at 0 °C to +5 °C.

5.2.4 Trimethoprim (TMP) solution

5.2.4.1 Components

Trimethoprim	25,0 mg
Ethanol, 96 %	5 ml
Water	1 000 ml

5.2.4.2 Preparation

Dissolve the trimethoprim in the ethanol. Dilute with water to 1 000 ml.

The TMP solution may be kept for a maximum of 1 month if stored in the dark at 0 °C to +5 °C.

5.2.5 Test tubes A (pH 7)

Melt the basic medium (5.2.1). Cool the medium in a water bath (6.3) to 63 °C ± 1 °C. Add 1,5 ml of CAP solution (5.2.3) and 2 ml of bromocresolpurple solution (5.2.2) to 100 ml of the preheated basic medium, while keeping the medium in the water bath set at 63 °C. Mix the medium well.

Keeping the medium in the water bath set at 63 °C, adjust the pH (see 6.5) to 7,0 ± 0,1 at that temperature by using 1 mol/l NaOH or 1 mol/l HCl.

Subsequently, add per 100 ml of medium such an amount (approx. 2 ml) of test-organism suspension (5.1) that in the absence of antimicrobial substances the colour change does appear after incubation in the water bath at 63 °C for 4 h 15 min ± 30 min.

Mix and dispense the test medium in portions of 1 ml in tubes (6.4) and leave the medium to solidify.

The test tubes A may be stored at 0 °C to +5 °C for a maximum of 3 days, provided that the tubes are covered to avoid evaporation (e.g. with parafilm).

5.2.6 Test tubes B (pH 8)

Melt the basic medium (5.2.1). Cool the medium in a water bath (6.3) to 63 °C ± 1 °C. Add 0,6 ml of TMP solution (5.2.4) and 2 ml of bromocresolpurple solution (5.2.2) to 100 ml of the preheated basic medium, while keeping the medium in the water bath set at 63 °C. Mix the medium well.

Keeping the medium in the water bath set at 63 °C, adjust the pH (see 6.5) to 8,00 ± 0,02 at that temperature by using 1 mol/l NaOH or 1 mol/l HCl. Take care that while adjusting, the pH of the medium does not exceed a value of 8,05.

Subsequently, add an amount (approx. 2 ml) of test-organism suspension (5.1) per 100 ml of medium so that in the absence of antimicrobial substances the colour change appears after the incubation in the water bath at 63 °C for 4 h 15 min ± 30 min.

Mix and dispense the test medium in portions of 1 ml in tubes (6.4) and leave the medium to solidify.

The test tubes B may be stored at 0 °C to +5 °C for a maximum of 3 days, provided that the tubes are covered to avoid evaporation (e.g. with parafilm).

5.3 Standard solutions and control samples

Correct all weighing for purity and salt contents in accordance with ISO 13969 | IDF 183.

For the preparation of standard solutions and control samples, it may be assumed that 1 ml of solution is equal to 1 g of solution.

5.3.1 Benzylpenicillin standard solutions and control samples

Taking into account the limited stability of benzylpenicillin, it is advisable to prepare all the benzylpenicillin standard solutions freshly and to freeze the control milk samples on the same day at below −18 °C.

5.3.1.1 Benzylpenicillin standard stock solution

Dissolve 20,0 mg ± 0,1 mg of benzylpenicillin in 1 000 ml of water and mix. The thus-prepared benzylpenicillin standard stock solution contains 20 mg/l of benzylpenicillin.

The benzylpenicillin standard stock solution may be kept for a maximum of 2 days if stored at 0 °C to +5 °C.

5.3.1.2 Benzylpenicillin standard working solution

Dilute 10 ml of benzylpenicillin standard stock solution (5.3.1.1) with water to 1 000 ml and mix. The thus-prepared benzylpenicillin standard working solution contains 200 µg/l of benzylpenicillin.

5.3.1.3 Benzylpenicillin control milk sample

Dilute 1 ml of benzylpenicillin standard working solution (5.3.1.2) with negative milk (5.4) to 100 ml and mix. The thus-prepared control milk sample contains 2 µg/l of benzylpenicillin.

The benzylpenicllin control milk sample may be kept for a maximum of 2 months if stored in test tubes (6.4) at below −18 °C.

NOTE 1 mg of pure penicillin-G potassium salt is equivalent to 1 595 International Units of penicillin G. 1 mg of pure penicillin-G sodium salt is equivalent to 1 670 International Units of penicillin G.

5.3.2 Oxytetracycline standard solutions and control samples

5.3.2.1 Oxytetracycline standard stock solution

Dissolve 5,0 mg ± 0,1 mg of oxytetracycline in 10 ml of 0,1 mol/l HCl solution. Dilute to 100 ml with water and mix. The thus-prepared oxytetracycline standard stock solution contains 50 mg/l of oxytetracycline.

The oxytetracycline standard stock solution may be kept for a maximum of 1 week if stored in the dark at 0 °C to +5 °C.

5.3.2.2 Oxytetracycline standard working solution

Dilute 10 ml of oxytetracycline standard stock solution (5.3.2.1) with water to 100 ml and mix. The thus-prepared oxytetracycline standard working solution contains 5 000 µg/l of oxytetracycline.

5.3.2.3 Oxytetracycline control milk sample

Dilute 2 ml of oxytetracycline standard working solution (5.3.2.2) with negative milk (5.4) to 100 ml and mix. The thus-prepared control milk sample contains 100 µg/l of oxytetracycline.

The oxytetracycline control milk sample may be kept for a maximum of 3 months if stored in test tubes (6.4) at below −18 °C.

5.3.3 Sulfadiazine standard solutions and control samples

5.3.3.1 Sulfadiazine standard stock solution

Dissolve 15,0 mg ± 0,1 mg of sulfadiazine in 100 ml water and mix. The thus-prepared sulfadiazine standard stock solution contains 150 mg/l of sulfadiazine.

The sulfadiazine standard stock solution may be kept for a maximum of 2 weeks if stored at 0 °C to +5 °C.

5.3.3.2 Sulfadiazine standard working solution

Dilute 10 ml of sulfadiazine standard stock solution (5.3.3.1) with water to 100 ml and mix. The thus-prepared sulfadiazine standard working solution contains 15 000 µg/l of sulfadiazine.

5.3.3.3 Sulfadiazine control milk sample

Dilute 1 ml of sulfadiazine standard working solution (5.3.3.2) with negative milk (5.4) to 100 ml and mix. The thus-prepared control milk sample contains 150 µg/l of sulfadiazine.

The sulfadiazine control sample may be kept for a maximum of 2 months if stored in test tubes (6.4) at below −18 °C.

5.3.4 Neomycin standard solutions and control samples

5.3.4.1 Neomycin standard stock solution

Dissolve 30,0 mg ± 0,1 mg of neomycin in 5 ml of 0,1 mol/l phosphate buffer (pH 8,0 ± 0,1) and mix. Dilute to 1 000 ml with water and mix again. The thus-prepared neomycin standard stock solution contains 30 mg/l of neomycin.

The neomycin standard stock solution may be kept for a maximum of 2 weeks if stored at 0 °C to +5 °C.

5.3.4.2 Neomycin standard working solution

Dilute 10 ml of neomycin standard stock solution (5.3.4.1) with water to 100 ml and mix. The thus-prepared neomycin standard working solution contains 3 000 µg/l of neomycin.

5.3.4.3 Neomycin control milk sample

Dilute 1 ml of neomycin standard working solution (5.3.4.2) with negative milk (5.4) to 100 ml and mix. The thus-prepared control milk sample contains 30 µg/l of neomycin.

The neomycin control milk sample may be kept for a maximum of 2 months if stored in test tubes (6.4) at below −18 °C.

5.3.5 Erythromycin standard solutions and control samples

5.3.5.1 Erythromycin standard stock solution

Dissolve 20,0 mg ± 0,1 mg of erythromycin in 5 ml of methanol. Dilute to 1 000 ml with water and mix. The thus-prepared erythromycin standard stock solution contains 20 mg/l of erythromycin.

The erythromycin standard stock solution may be kept for a maximum of 2 weeks if stored at 0 °C to +5 °C.

5.3.5.2 Erythromycin standard working solution

Dilute 5 ml of erythromycin standard stock solution (5.3.5.1) with water to 100 ml and mix. The thus-obtained erythromycin standard working solution contains 1 000 µg/l of erythromycin.

5.3.5.3 Erythromycin control milk sample

Dilute 1 ml of erythromycin standard working solution (5.3.5.2) with negative milk (5.4) to 100 ml and mix. The thus-prepared erythromycin control milk sample contains 10 µg/l of erythromycin.

The erythromycin control milk sample may be kept for a maximum of 2 months if stored in test tubes (6.4) at below −18 °C.

5.4 Negative milk (milk free from antimicrobials)

See ISO 13969|IDF 183 for a method of obtaining negative milk, or use UHT sterilized whole milk free of substances inhibitory to microorganisms.

6 Apparatus and glassware

Disposable utensils are an acceptable alternative to reusable glassware if they have suitable specifications.

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

6.1 Autoclave, for wet sterilization.

See ISO 7218.

6.2 Micropipettes, of capacity 1 000 µl.

6.3 Water baths, lidded or covered, thermostatically controlled, capable of circulating water and of maintaining a constant temperature of 63 °C ± 1 °C, 70 °C ± 1°C and 80 °C ± 1°C, respectively.

6.4 Test tubes, of diameter approximately 16 mm and length approximately 80 mm.

6.5 pH meter, accurate to within ± 0,01 pH units, equipped with automatic temperature correction and electrode(s) suitable for measurements in liquids at 63 °C (i.e. Ag/AgCl electrode).

6.6 Analytical balance, capable of weighing to the nearest 0,1 mg, with a readability up to 0,01 mg.

6.7 Tube rack, suitable of holding the test tubes (6.4).

7 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 Technical Specification. A recommended sampling method is given in ISO 707 | IDF 50.

8 Preparation of test sample

It is important that liquid milk samples be tested without delay. Reconstitute dried milk samples in distilled water prior to testing.

9 Procedure

9.1 Control samples

Include in each tube rack (6.7) the following test tubes with 0,3 ml of positive control milk samples or negative milk samples added per tube:

a) test tube A (5.2.5) with benzylpenicillin control milk sample (5.3.1.3);

b) test tube A (5.2.5) with oxytetracycline control milk sample (5.3.2.3);

c) test tube A (5.2.5) with negative milk (5.4);

d) test tube B (5.2.6) with benzylpenicillin control milk sample (5.3.1.3);

e) test tube B (5.2.6) with sulfadiazine control milk sample (5.3.3.3);

f) test tube B (5.2.6) with negative milk (5.4).

When testing a large number of samples while expecting that a large majority of the test samples do not contain antimicrobial substances, these samples may serve as negative milk.

9.2 Test tube preparation

9.2.1 Pipette 10 ml of test sample into a glass tube. Heat in the water bath (6.3) set at 80 °C for 10 min to inactivate heat-labile natural inhibitory substances. Cool promptly to room temperature.

9.2.2 Pipette 0,3 ml of test portion (9.2.1) into the corresponding labelled test tube A (5.2.5) and into the corresponding labelled test tube B (5.2.6).

9.2.3 Keep the test tubes A and B in the rack (6.7) at room temperature for 1 h to allow diffusion of the milk.

9.2.4 Pour off the milk above the agar layer. Cover the test tubes with aluminium foil or cap to prevent evaporation.

Optionally, after diffusion, place the tubes in a water bath (6.3) set at 70 °C for 10 min to activate the spores through a heat shock.

9.3 Incubation

Place the test tubes (9.2.4) in a water bath (6.3) set at 63 °C. Incubate at this temperature for 4 h 15 min ± 30 min until the colour of the test tubes concerned (A or B) with negative milk has just turned from purple to completely yellow.

The colour of the test tubes concerned (A or B) with the control samples should at least be still light purple at the moment of removal. Subsequently, remove all test tubes (A or B) from the water bath and allow them to stand at room temperature for 10 min.

9.4 Interpretation

Observe and register the colour of the test tubes containing test samples and control samples as follows.

a) A completely or partly purple colour of the solid test medium in any of the test sample or control sample tubes indicates the presence of substances inhibitory to the test organism and indicates a positive test.

b) A full yellow colour of the solid test medium in any of the test sample or control sample tubes indicates the absence of substances inhibitory to the test organism and indicates a negative test.

The colour of the test tubes containing negative milk should be yellow, while those containing control milk samples should remain purple. If not, repeat testing. If a repeatedly deviating colour development occurs with control milk samples and/or negative milk, identify the cause.

10 Confirmation (optional)

10.1 General

The procedure for confirmation testing of the presence of beta-lactam and sulfonamide residues has been described in Reference [4]. Further guidance is given in 10.2 and 10.3.

NOTE The presence of combinations of antibiotics and/or other inhibitors in the test sample can cause difficulties in this presumptive confirmation.

10.2 Presumptive confirmation of beta-lactams

Positive test samples with test tube A (5.2.5) and test tube B (5.2.6) may be tested for the presence of penicillin and cephalosporin residues using beta-lactamase. Beta-lactam residues may be considered present if the inhibitory activity in the beta-lactamase-treated sample is counteracted.

Two types of beta-lactamase enzymes may be distinguished:

— penicillinase (beta I activity), which is more active in degrading penicillins, and

— cephalosporinase (beta II activity), which is more active in degrading cephalosporins.

EXAMPLE This is a test procedure with penicillinase. Add 2 ml of penase concentrate containing 10 000 000 International Units of penase/ml (available from Becton, Dickinson and Company [2)]) to 100 ml of the test medium of tube A (5.2.5) before the pH adjustment. Mix and distribute the test medium in 1 ml portions in tubes and leave the medium to solidify. Then follow the test procedure described in Clause 9.

Some beta-lactams (i.e. cephalexin) are less sensitive to beta-lactamase. In such cases, an additional pre-treatment of the milk sample (1 ml of test sample with 0,3 ml of penase concentrate at 37 °C for 2 h) is recommended.

10.3 Presumptive confirmation of sulfonamides

Positive test samples with test tube B (5.2.6) may be tested for the presence of sulfonamide residues using a *p*-amino benzoic acid (PABA) solution. Sulfonamide residues may be considered present if the inhibitory activity in the PABA-treated sample is counteracted.

EXAMPLE Add 2 ml of *p*-amino benzoic acid solution (5 g/kg of water) to 100 ml of test medium of tube B (5.2.6) after the addition of the test organism and before the pH adjustment. Mix and distribute the test medium in 1 ml portions in tubes and leave the medium to solidify. Then follow the test procedure described in Clause 9.

10.4 Confirmation of other inhibitors

If the inhibiting substance is neither identified as a beta-lactam nor a sulfonamide, further identification with other tests, such as a microbiological multiplate test system, immunoassays or chemical methods (HPLC, LC-MS) is required (see ISO 18330 | IDF 188).

11 Expression of results

Express the results by indicating the presence or absence of antimicrobial substances.

12 Precision

See Annex A for limits of detections for some antibiotics with this tube diffusion method in milk.

13 Test report

The test report shall specify:

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

b) the sampling method used;

c) the test method used, with reference to this Technical Specification;

d) all operational details not specified in this Technical Specification, or regarded as optional, together with details of any incidents which may have influenced to test result(s);

e) the test result(s) obtained.

2) Penase concentrate, available from Becton, Dickinson and Company, is an example of a product available commercially. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by ISO or IDF of this product.

Annex A
(informative)

Data from collaborative studies

Limits of detection, in micrograms per kilogram of milk, for the two-tubes method, tube A (pH 7) and tube B (pH 8), were obtained from several collaborative studies [3] with participation of three experienced Dutch laboratories. The results are given in Tables A.1 to A.5.

Table A.1 — Beta-lactams

Beta-lactams	Tube A µg/kg	Tube B µg/kg
Benzylpenicillin	2	3
Amoxicillin		3
Cloxacillin		20
Ceftiofur		50
Cephalexin		80

Table A.2 — Macrolides

Macrolides	Tube A µg/kg	Tube B µg/kg
Erythromycin		10
Spiramycin		200
Tylosin		20

Table A.3 — Aminoglycosides

Aminoglycosides	Tube A µg/kg	Tube B µg/kg
Dihydrostreptomycin		70
Neomycin		30
Kanamycin		500
Gentamicin		20

Table A.4 — Tetracyclines

Tetracyclines	Tube A µg/kg	Tube B µg/kg
Oxytetracycline	100	400
Tetracycline	100	300
Doxycycline	100	
Chlortetracycline	200	>800

Table A.5 — Sulfonamides

Sulfonamides	Tube A µg/kg	Tube B µg/kg
Dapsone		2
Sulphamethazine		400
Sulfadiazine		150

Annex B
(informative)

Preparation of test-organism suspension

B.1 Test organism

Obtain a culture of *Geobacillus stearothermophilus* ATCC 10149 (identical to NIZO strain C953), for example from ATCC (http://www.atcc.org/) [3] or another source.

B.2 Stock culture

B.2.1 Slant agar

B.2.1.1 Components

Yeast extract	2 g
Peptone	5 g
Meat extract	1 g
Sodium chloride (NaCl)	5 g
Manganese sulfate ($MnSO_4 \cdot H_2O$)	35 mg
Agar	15 g
Distilled water	1 000 ml

NOTE The dehydrated basic component mix without manganese sulfate is commercially available as Nutrient Agar.

B.2.1.2 Preparation

Dissolve the components in the water by heating. Adjust the pH so that after sterilization it is 7,4 ± 0,1. Autoclave the medium at 121 °C ± 1 °C for 15 min.

Before solidifying, place in sterile and stopper-capped glass reagent tubes (approx 10 ml) and allow the agar to solidify in a slanted position.

B.2.2 Maintenance of the test culture

Inoculate a tube of the slant agar (B.2.1) in streaks, using a loop of the ATCC test culture, and place for 48 h in an incubator at 63 °C ± 1 °C. After incubation, flame the stopper of the tube, then force a little way into the tube, and close with a sterile rubber stopper. The stock culture tube thus obtained may be kept for several months in a refrigerator at 5 °C or kept as test-organism suspension in a freezer at below −20 °C.

3) This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by ISO or IDF of this product (see also footnote 1).

B.3 Propagation of the test-organism culture

B.3.1 Transfer 20 ml of molten slant agar (B.2.1) aseptically into a sterile 140 mm Petri dish and cool to room temperature.

B.3.2 Using a sterile pipette, add 5 ml of distilled water to a stock culture tube (B.2.2). Prepare a test-organism suspension by gently scraping culture from the slant agar with a loop and rinsing it in the stock culture tube.

B.3.3 Using a sterile pipette, add 0,5 ml of test-organism suspension (B.3.2) to the Petri dishes with solidified agar (B.3.1). Inoculate the whole surface evenly with a glass spatula.

B.3.4 Incubate in an incubator at 63 °C ± 1°C for 72 h.

B.3.5 Using a sterile pipette, add sterile physiological saline/peptone solution (8,5 g of NaCl and 1,0 g of peptone/kg water) to the cultured Petri dish (B.3.4). Spread it over the surface with a glass spatula. Collect the test-organism suspension in a sterile bottle. Close the bottle and shake thoroughly.

B.3.6 Wash the suspension by centrifuging at 2 000 g for 5 min. Decant the supernatant and resuspend the cells into a sterile physiological saline/peptone solution. Execute this step twice.

B.3.7 Suspend cells in physiological saline/peptone solution. Heat for 10 min to promote the building of spores at 80 °C.

B.4 Concentration adjustment of the test-organism suspension

Adjust the concentration of the test-organism suspension so as to arrive at approximately 5 000 000 colony-forming units per millilitre, as determined on plate count agar with incubation at 63° C for 18 h to 24 h (see ISO 4833).

B.5 Storage of test-organism suspension

Pour out into small quantities and store at below −20 °C for a maximum of 1 year.

Bibliography

[1] ISO 707 | IDF 50, *Milk and milk products — Guidance on sampling*

[2] NOUWS, J.F.M., LOEFFEN, G., SCHOUTEN, J., VAN EGMOND, H., KEUKENS, H. and STEGEMAN, H. Improvement of the tube diffusion test with respect to detection of antibiotic residues and sulphonamides in raw milk. *Archiv für Lebensmittelhygiene*, **46**, 1995, pp. 140-141

[3] *Instruction manual for the testing of herd bulk milk samples*. Netherlands Milk Control Station (MCS Nederland), Zutphen, 2003 (in Dutch)

[4] Detection and confirmation of inhibitors in milk and milk products. *IDF Bulletin*, **258**, 1991

[5] SUHREN, G. and BEUKERS, R. Delvotest S.P. for the detection of cloxacillin and sulfamethoxazole in milk. IDF interlaboratory study. *J. AOAC Int*. **81**, 1998, pp. 978-990

ICS 67.100.01

Price based on 13 pages