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BSI Standards Publication

Chemical disinfectants and antiseptics — Preservation of test organisms used for the determination of bactericidal (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) activity

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National foreword

This British Standard is the UK implementation of EN 12353:2013. It supersedes BS EN 12353:2006 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee CH/216, Chemical disinfectants and antiseptics.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Chemical disinfectants and antiseptics - Preservation of test organisms used for the determination of bactericidal (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) activity

Antiseptiques et désinfectants chimiques - Conservation des organismes test utilisés pour la détermination de l'activité bactéricide (Legionella inclus), mycobactéricide, sporicide, fongicide et virucide (bacteriophages inclus)

Chemische Desinfektionsmittel und Antiseptika - Aufbewahrung von Testorganismen für die Prüfung der bakteriziden (einschließlich Legionella), mykobakteriziden, sporiziden, fungiziden und viruziden (einschließlich Bakteriophagen) Wirkung

This European Standard was approved by CEN on 14 December 2012.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

Management Centre: Avenue Marnix 17, B-1000 Brussels

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Foreword

This document (EN 12353:2013) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by August 2013, and conflicting national standards shall be withdrawn at the latest by August 2013.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 12353:2006.

The document was revised to adapt it to the latest state of science, to correct errors and ambiguities. The following are the significant technical changes since the last edition:

— The methods of preservation of Legionella, mycobacteria, bacteriophages and viruses are new and were added. Data obtained by using the former version of EN 12353 are still valid.

According to the CEN/CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Introduction

Standardized tests for the determination of bactericidal (incl. *Legionella pneumophila*), mycobactericidal, sporicidal, fungicidal and virucidal (incl. bacteriophages) activity of chemical disinfectants and antiseptics necessitate the use of test organisms whose purity and identity have been verified and whose biological behaviour remains stable. Therefore it is essential to specify the storage requirements.

This European Standard aims at describing methods for preservation of test organisms used for such purposes.

1 Scope

This European Standard specifies methods for keeping test organisms used and defined in European Standards for the determination of bactericidal (incl. *Legionella pneumophila*), mycobactericidal, sporicidal, fungicidal and virucidal (incl. bacteriophages) activity of chemical disinfectants and antiseptics drawn up by CEN/TC 216. These methods for keeping test organisms can only be carried out in connection with at least one of those standards where a reference to this European Standard is established.

NOTE 1 Annex A (informative) contains a non-exhaustive list of test organisms for which this standard can be applied.

NOTE 2 European Standards (EN and prEN) where this European Standard is referenced are listed in the Bibliography.

NOTE 3 A specific part on the preservation of bacterial spores may be added once the results of the ongoing ring trials are available.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 14885, *Chemical disinfectants and antiseptics — Application of European Standards for chemical disinfectants and antiseptics*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN 14885 apply.

4 Requirements

Each test organism specified in a CEN/TC 216 European Standard and referred to in this European Standard shall be handled as described in this European Standard.

The purity and identity of the preserved test organism shall be verified during the preparation and regularly during the storage, except for viruses where only the identity is checked before the stock virus suspension is stored.

The preserved test organism – except viruses - should be checked at regular intervals (at least in the case of longer storage than 14 months) to ensure that its susceptibility to products has not changed. As long as CEN/TC 216 has not developed specific tests for this purpose any suitable method can be used e.g. EN 1040 for bacteria, EN 1275 for fungi, EN 14348 for mycobacteria, EN 13623 for *Legionella pneumophila*, EN 14476 for viruses or EN 13610 for dairy bacteriophages.

5 Methods

5.1 Principle

A sample of the test organism – in general in freeze dried form - is obtained from a culture collection. This sample is cultured, prepared for storage, filled into storage vessels and placed in the deep freeze.

From this sample a stock culture is prepared and subsequently used to prepare working cultures for the test procedure. In some cases the working cultures are directly prepared from the deep freeze samples.

5.2 Materials and reagents

5.2.1 Test organisms

See Annex A for examples of test organisms.

The origin (culture collection), taxonomic name and reference number, date of receipt and batch number of the freeze dried test organisms shall be recorded (5.11.2).

5.2.2 Culture media and reagents

5.2.2.1 General

The formulas of all media and reagents are given in case commercial ready-to-use material is not used. It is to be checked that each commercial supplier has established an appropriate quality control system.

All weights of chemical substances given in this European Standard refer to the anhydrous salts unless otherwise stated. Hydrated forms may be used as an alternative, but the weights required shall be adjusted to allow for consequent molecular weight differences.

The reagents shall be of analytical grade and/or appropriate for microbiological purposes. They shall be free from substances that are toxic or inhibitory to the test organisms.

To improve reproducibility, it is recommended that whenever possible, commercially available dehydrated material is used for the preparation of culture media. The manufacturer's instructions relating to the preparation of these products should be rigorously followed.

All specified pH values are measured at (20 ± 1) °C.

For each culture medium, cell culture and reagent a limitation for use should be fixed.

5.2.2.2 Water

The water shall be freshly glass distilled water and not demineralized water. If distilled water of adequate quality is not available, water for injections (see bibliographic reference [1]) can be used.

Sterilize in the autoclave (5.3.2.1a)). Sterilization is not necessary if the water is used for e.g. preparation of culture media and subsequently sterilized.

5.2.2.3 Tryptone Soya Broth (TSB) for bacteria, except *Legionella*

Tryptone soya broth, consisting of:

Tryptone, pancreatic digest of casein	17,0 g
Soya peptone, papaic digest of soybean meal	3,0 g
Sodium chloride (NaCl)	5,0 g
Water (5.2.2.2)	800,0 ml
Dipotassium phosphate (K_2HPO_4)	2,5 g
Glucose	2,5 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave (5.3.2.1a)). After sterilization the pH of the medium shall be equivalent to 7,2 ± 0,2.

5.2.2.4 Malt Extract Broth (MEB) for fungi

Malt extract broth, consisting of:

Malt extract (food grade, e.g. Christomalt powder from Difal or equivalent that is not highly purified and not only based on maltose, e.g. malt extract from OXOID)[1]	20,0 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave (5.3.2.1a)). After sterilization the pH of the medium shall be equivalent to 5,6 ± 0,2.

5.2.2.5 Cryoprotectant solution for bacteria, spore-forming bacteria, fungi

Cryoprotectant solution, consisting of:

Beef extract	3,0 g
Tryptone, pancreatic digest of casein	5,0 g
Glycerol ($C_3H_8O_3$) [2]	150,0 g
Water (5.2.2.2)	to 1 000,0 ml

Dissolve the constituents in boiling water. Sterilize in the autoclave (5.3.2.1a)). After sterilization the pH of the solution shall be equivalent to 6,9 ± 0,2.

Any commercially available cryoprotectant containing glycerol for preservation of test organisms equivalent to the solution described above may be used.

If justified, any other equivalent cryoprotectant solution may be used, e.g. for *Legionella* (5.5.2).

5.2.2.6 Middlebrook 7 H 9 broth with 10 % ADC enrichment and glycerol as reconstituent and cryoprotectant solution for mycobacteria (MADC)

Middlebrook 7 H 9 broth, consisting of:

Middlebrook 7 H 9 broth powder	4,7 g
Glycerol ($C_3H_8O_3$) [2]	100,0 ml
Water (5.2.2.2)	800,0 ml

Treat in the autoclave (5.3.2.1a)) for a holding time of only 10 min and cool to 45 °C. Add under aseptic conditions 100 ml Middlebrook ADC enrichment to obtain approximately 1 000,0 ml. The pH of the medium shall be equivalent to 6,6 ± 0,2.

5.2.2.7 Polysorbate 80 solution

Polysorbate 80 solution, consisting of:

Polysorbate 80	0,5 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave (5.3.2.1a)).

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[1] This information is given for the information of users of this standard and does not constitute an endorsement of the products named. Corresponding products supplied by other manufacturers may be used if they can be shown to lead to the same results.

5.2.2.8 DMSO as cryoprotectant for cell culture freezing

Dimethyl sulphoxide (DMSO) is used to help protect the cells from rupture by the formation of ice crystals.

Since DMSO is toxic it should be handled with care. It can be absorbed through the skin and may cause irritation and/or burns. It is teratogenic and an allergen. Latex gloves should be worn when handling it.

5.2.2.9 Glutamine solution, 3 %

Dissolve 12 g Glutamine in 400 ml of water (5.2.2.2) and sterilise by membrane filtration. The solution is stored at (-20 ± 1) °C.

5.2.2.10 TV (Trypsin-Versene)

Dissolve 0,05 g Trypsin in 100 ml of 0,53 mM EDTA (Ethylene diamine tetra acetic acid) and sterilise by membrane filtration. Store at (4 ± 1) °C.

5.2.2.11 Antibiotic suspension

Chemicals

50 million units Penicillin-G	(eg Sigma PEN-K[2])
50 g Streptomycin sulphate (approx. equal to 750 i.u./mg)	(eg Sigma Cat : 56501[2])
25 × 500,000 units Mycostatin	(eg Nystatin : E R Squibb 59150[2])
Water (5.2.2.2) to 2,5 l.	

Preparation

Dissolve vial contents of antibiotics in water (5.2.2.2) and fill up to 2,5 l.
Dispense aseptically into 50 ml and 5 ml aliquots.
Store at – 20 °C. Shake the bottle after thawing.
Use 5 ml per litre of medium to give a final concentration of:

Penicillin	100 units/ml
Streptomycin	100 µg/ml
Mycostatin	25 units/ml

5.2.2.12 Phosphate-buffered saline solution (PBS)

Sodium chloride (NaCl)	8,00 g
Potassium chloride (KCl)	0,20 g
Disodium hydrogen phosphate, 12-hydrate (Na_2HPO_4 x $12H_2O$)	2,89 g
Potassium phosphate, monobasic (KH_2PO_4)	0,20 g
Water (5.2.2.2)	to 1 000,0 ml

5.2.2.13 Foetal calf serum (FCS)

FCS has to be certified free of viruses and mycoplasma.

Extraneous viruses and mycoplasma may interfere with cell and virus growth resulting in false results.

5.2.2.14 Earle's BSS

Sodium chloride (NaCl)	68,0 g
Potassium chloride (KCl)	4,0 g

[2] This information is given for the information of users of this standard and does not constitute an endorsement of the products named. Corresponding products supplied by other manufacturers may be used if they can be shown to lead to the same results.

Calcium chloride ($CaCl_2$)	2,0 g
Magnesium sulphate, 7-hydrate ($MgSO_4 \times 7H_2O$)	2,0 g
Sodium hydrogenphosphate, 2-hydrate ($NaH_2PO_2 \times 2H_2O$)	1,4 g
Glucose	10,0 g
Phenol red, 1 % (5.2.2.15)	20,0 ml
Water (5.2.2.2)	to 1 000,0 ml

$CaCl_2$ should be dissolved separately in 100 ml of water (5.2.2.2) and added to the other dissolved reagents just before the solution is brought to its final volume. The solution is 10-fold concentrated. It is sterilized by membrane filtration through a 0,22 µm Millipore or Seitz-type filter[3] and can be stored at (4 ± 1) °C for 4 weeks.

For use the solution is diluted 10-fold with water (5.2.2.2) and buffered by the addition of 2,5 % of an 8,8 % Sodium hydrogen carbonate ($NaHCO_3$) solution.

5.2.2.15 Phenol red, 1 % solution

a) A 1,0 N Sodium hydroxide (NaOH) solution is prepared.

b) 10 g of alcohol soluble Phenol red, European Pharmacopeia [2] are placed in a 100 ml flask (5.3.2.12); 20 ml of the NaOH solution are added, mixed and allowed to stand for a few minutes.

c) The dissolved dye is transferred in a 1 000 ml volumetric flask (5.3.2.12).

d) Additional 10 ml amounts of the NaOH solution are added to the flask and the dissolved material is added to the volumetric flask. No more than a total of 70 ml of the NaOH solution should be used.

e) The solution is brought to a final volume of 1 000 ml with water (5.2.2.2) and stored at room temperature.

5.2.2.16 Sodium bicarbonate (8,8 % w/v solution)

Dissolve 8,8 g sodium bicarbonate in water (5.2.2.2) to 100 ml and sterilize by autoclaving (5.3.2.1a)). Store at (4 ±1) °C.

5.2.2.17 Eagle's minimum essential medium (MEM) for cell cultures

MEM is used for growth and maintenance of cell cultures. First prepare a stock solution. For use, the stock solution is diluted 10-fold with water (5.2.2.2). 1 % of the 3 % Glutamine solution (= 0,03 %) (5.2.2.9), Antibiotic suspension (5.2.2.11), and 2,5 % of a 8,8 % Sodium bicarbonate solution (5.2.2.16) are added. An appropriate concentration of foetal calf serum (FCS, (5.2.2.13); 10 % for growth, 2 % for maintenance) is added before use.

The following solutions are prepared:

Solution A

	per litre stock solution
L-Arginine HCl	1,05 g
L-Histidine HCl	0,31 g
Lysine HCl	0,58 g
Tryptophane	0,10 g
L-Phenylalanine	0,32 g
L-Threonine	0,48 g
L-Leucine	0,52 g

[3] Millipore® and Seitz® are examples of suitable products available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.

L-Valine	0,46 g
L-Isoleucine	0,52 g
L-Methionine	0,15 g

These amino acids are dissolved with gentle heating (80 °C) in 200 ml of 0,075 N HCl. 0,075 N HCl is prepared by adding 1,5 ml of commercial C.P. HCl (11.9 N) to 236,6 ml water. Take 200 ml from the prepared 238,1 ml.

Solution B

	per litre stock solution
L-Tyrosine	0,36 g
L-Cysteine	0,24 g

These two amino acids are dissolved in 200 ml of 0,075 N hydrochloric acid (HCl) by heating up to 80 °C for 2 h and subsequently cooling to 20 °C.

Solution C

	per litre stock solution
Nìcotinamide	0,20 g
Pyridoxal	0,20 g
Thiamine	0,20 g
Pantothenic acid	0,20 g
Choline	0,20 g
Inositol	0,40 g
Riboflavin	0,02 g

These reagents are dissolved in approximately 175 ml of water (5.2.2.2) then brought to a final volume of 200 ml with water (5.2.2.2). The solution is dispensed in 10 ml volumes.

NOTE The solutions A, B and C are 10-fold concentrated preparations and can be stored in the refrigerator (5.3.2.8).

Solution D

Dissolve 200 mg of Biotin in 150 ml of water (5.2.2.2). To increase stability upon storage, 1 ml of 1 N hydrochloric acid (HCl) is added.

The total volume is brought to 200 ml with water (5.2.2.2) and the solution is dispensed in 10 ml aliquots and stored at (-20 $\pm$ 1) °C.

Solution E

Dissolve 200 mg Folic acid (crystalline) in 200 ml of 10 fold diluted Earle's BSS (5.2.2.14), pH = 7,4. The solution is dispensed in 10 ml amounts and stored at (-20 $\pm$ 1) °C.

Preparation of the final mixture of Eagle's MEM

a) The following reagents are dissolved in 200 ml Solution B :

Sodium chloride (NaCl)	68,0 g
Potassium chloride (KCl)	4,0 g
Magnesium sulphate heptahydrate ($MgSO_4 x\, 7H_2O$)	2,0 g

b) 1,4 g of Sodium dihydrogen orthophosphate monohydrate (NaH_2PO_4 x H_20) are dissolved in 55 ml of water (5.2.2.2) and added to the solution a).

c) 10 g of Glucose dissolved in 50 ml of water (5.2.2.2) and 20 ml of a 1 % Phenol red solution (5.2.2.15) are added to the solution b).

d) The volume of solution c) is brought to 600 ml with water (5.2.2.2) and the following solutions are added:

	per litre of stock solutions
Solution C	10 ml
Solution D	10 ml
Solution E	10 ml

e) In a separate flask containing 160 ml of water (5.2.2.2), 2 g calcium chloride $CaCl_2$ are dissolved and then added to solution d) with vigorous shaking until complete dissolution.

f) 200 ml of solution A is added to solution e); this mixture can be kept overnight in the refrigerator (5.3.2.8).

g) The total volume of f) is brought to exactly 1 000 ml with water (5.2.2.2) and the solution is sterilised by membrane filtration (5.3.2.16). This 10-fold concentrated medium is stored at 4 °C.

h) For use, the medium is diluted 10-fold with water (5.2.2.2). Add 1 % of the 3 % glutamine solution (5.2.2.9) and 2,5 % of the 8,8 % sodium bicarbonate solution (5.2.2.16). The resulting solution cannot be stored longer than 2 h.

5.2.2.18 M17-broth

For maintenance of bacterial host strain (*Lactococcus lactis*) and propagation of dairy bacteriophages

Phytone peptone (from soya meal)	5,00 g
Polypeptone peptone (from casein & animal tissue)	5,00 g
Beef extract powder	5,00 g
Yeast extract	2,50 g
D(+)-lactose	5,00 g
Ascorbic acid	0,50 g
Sodium-ß-glycerophosphate	19,00 g
Magnesium sulphate heptahydrate , 7 H_2O	0,25 g
Water (see 5.2.2.2)	1 000 ml

Sterilize in the autoclave (5.3.2.1a)). After sterilization the pH of the medium shall be equivalent to 7,0 ± 0,2.

5.2.2.19 M17-agar (bottom agar)

Bottom agar for quantitative counting of lysis zones (plaques) derived from single infective bacteriophage particles in the bacterial lawn of the host bacteria.

Add 15 g of agar to 1 000 ml of M17-broth (5.2.2.18). Dissolve the agar by boiling with constant stirring.

Sterilize in the autoclave (5.3.2.1a)). After sterilization the pH of the medium shall be equivalent to 7,0 ± 0,2 when measured at 20 °C. When the agar is cooled down to (47 ± 1) °C, add 10 ml of a sterile 1 mol/l $CaCl_2$-stock solution (5.2.2.21). Mix gently and pour 15 ml to 18 ml of agar into Petri dishes (5.3.2.10).

5.2.2.20 Overlay agar (top agar, soft agar)

For counting bacteriophages: Dissolve 6,5 g agar in 1 000 ml M17-broth (5.2.2.18) and heat until boiling with constant stirring. Dispense the molten agar in test tubes (2,5 to 3 ml each).

Sterilize in the autoclave (5.3.2.1a)).

For achieving clear phage-derived lysis zones (plaques) in the lawn of host bacterial cells only well-defined agar should be used which is specified by the supplier for phage enumeration by the overlay technique.

5.2.2.21 $CaCl_2$-stock solutions (1 mol/l and 0,05 mol/l)

Dissolve either 110,99 or 5,55 g anhydrous $CaCl_2$ in water (5.2.2.2) and dilute to 1 000 ml to obtain the 1 mol/l or the 0,05 mol/l stock solution, respectively. Sterilize in the autoclave (5.3.2.1a)).

5.2.2.22 Skim milk

Prepare reconstituted skim milk (1,5 % fat content) for the test conditions as follows:

— reconstitute skim milk powder, guaranteed free of antibiotics or additives, at a rate of 100 g/l of water (5.2.2.2);

— treat by steaming at 100 °C on 3 successive days (30 min each) and leave between steam treatments at room temperature.

Do not leave between subsequent steamings in the refrigerator!

NOTE Undiluted skim milk is used for maintenance of the bacterial host strain (5.4.1).

Alternatively, treat at a reduced temperature of (115 ± 3) °C for 15 min in an autoclave (5.3.2.1a)).

To obtain a volume fraction of 10 % working solution, dilute 1 part of skim milk with 9 parts of sterile water (5.2.2.2) which is required as an optional interfering substance for the phage suspension test (5.7.2).

Store the volume fraction of 10 % skim milk at 4 °C to 8 °C.

The final concentration of the skim milk in the test procedure (5.7.1) shall be a volume fraction of 1 %.

5.2.2.23 Buffered Charcoal Yeast Extract (BCYE) Broth, for *Legionella*

BCYE agar, consisting of

—	yeast extract (bacteriological grade)	10,0 g;
—	Activated charcoal	2,0 g;
—	α-ketoglutarate, monopotassium salt	1,0 g;
—	ACES buffer (N-2-acetamido-2-aminoethanesulfonic acid)	10,0 g;
—	Potassium hydroxide (KOH) (pellets)	2,8 g;
—	L-cysteine hydrochloride monohydrate	0,4 g;
—	Iron(III) pyrophosphate [$Fe_4 (P_2O_7)_3$]	0,25 g;
—	distilled water	to 1 000,0 ml.

Preparation of BCYE

a) Cysteine and iron solutions

Prepare fresh solutions of L-cysteine hydrochloride and iron (III) pyrophosphate by adding 0,4 g and 0,25 g respectively to 10-ml-volumes of water (5.2.2.2). Sterilize each solution by membrane filtration (5.3.2.16). Store in clean sterile containers at (20 ± 3) °C for not more than three months.

b) ACES buffer

Add the ACES granules to 500 ml of water (5.2.2.2) and dissolve by standing in a water bath at 45 °C to 50 °C. To a separate 480 ml of water (5.2.2.2), add all the potassium hydroxide pellets and dissolve with gentle shaking. To prepare the ACES buffer, mix the two solutions.

NOTE ACES buffer can cause denaturation of the yeast extract if the following sequence is not followed.

c) Final medium

Add sequentially to the 980 ml of ACES buffer, the charcoal, yeast extract and α-ketoglutarate. Prepare a 0,1 mol/l solution of potassium hydroxide (KOH) by dissolving 5,6 g in 1 l of water (5.2.2.2). Prepare a 0,1 mol/l solution of sulphuric acid (H_2SO_4) by carefully adding 5,3 ml of H_2SO_4 to 1 l of water (5.2.2.2). Use the solutions of 0,1 mol/l potassium hydroxide or 0,1 mol/l sulphuric acid as appropriate to adjust the pH to 6,9 ± 0,2. Add the agar, mix and autoclave (5.3.2.1a)). After autoclaving, allow to cool to (47 ± 2) °C in a water bath (5.3.2.2).

Add the L-cysteine and the iron(III) pyrophosphate solutions aseptically, mixing well between additions.

The pH of the final medium is 6,9 ± 0,2 at 25 °C.

Prolonged heating during sterilisation or heating at too high a temperature shall be avoided, as it can affect the nutritional qualities of BCYE medium. Batch-to-batch variation of the ingredients of the medium (particularly α-ketoglurarate) can also affect its performance. Therefore it is essential to check the quality of each newly prepared batch of media for its ability to support the growth of the test organism within three days of incubation. This is assessed quantitatively using either known quantities of the obligatory *Legionella* strain or by direct comparison to previous batches.

5.2.2.24 Page's saline

Saline solution, consisting of:

— Sodium chloride (NaCl) 0,120 g;

— Magnesium sulphate ($MgSO_4 \times 7H_20$) 0,004 g;

— Calcium chloride ($CaCl_2 \times 2H_20$) 0,004 g;

— Disodium hydrogen phosphate (Na_2HPO_4) 0,142 g;

— Potassium dihydrogenphosphate (KH_2PO_4) 0,136 g;

— Water (5.2.2.2) to 1 000,0 ml.

Sterilize in the autoclave (5.3.2.1a)).

To aid accurate preparation, it is recommended that a 10 l volume of Page's Saline is prepared and dispensed in smaller volumes as required for autoclaving. Alternatively the salt solutions may be made up individually in concentrated form for dilution when the product is required.

5.2.3 Cell cultures

5.2.3.1 Storage of cell cultures

a) Actively growing cell cultures (log phase) shall be used for storage. Grow cell culture in MEM (5.2.2.17) supplemented with 10 % FCS (5.2.2.13). Aspirate the growth medium from the flask and rinse the cells with PBS - for a 75 cm^2 flask, use 5 ml to 10 ml. Aspirate PBS (5.2.2.12) and pipette 5 ml of ATV (5.2.2.10) (at 37 °C) onto the monolayer in the flask. Incubate at 37 °C for 2-5 min. Bump the side of the flask against the palm of the hand to help detach the cells. Wait until the cells are detached. Add 5 ml of MEM (5.2.2.17) + 10 % FCS (5.2.2.13) with a 10 ml pipette and vigorously wash to detach any remaining

cell from the bottom of the flask and to separate any cell clumps. Then transfer the cell suspension into a centrifuge tube. Save a sample for counting and then centrifuge at 100 g_N to 150 g_N (5.3.2.14) for 5 min. Remove the supernatant and re-suspend the cells in enough FCS (5.2.2.13) + 6 % to 10 % DMSO (5.2.2.8) to obtain a final cell concentration of 1 to 2 x10^6 cells/ml. The suspension is dispensed into cryovials (approximately 1,8 ml/each).

b) Place tubes briefly on ice: start the freezing procedure within 5 min. The cells should be frozen slowly (at -1 °C/min). This is most easily done using a programmable cooler. If such a cooler is not available, transfer the tubes from ice to an insulated pre-cooled box (or wrapped in cotton wool) and place for 2 hours in a freezer at -20 °C to -25 °C (5.3.2.13); then place in a -70 °C to -90 °C freezer for one or two days. Finally transfer the vials into a deep freezer for storage at -196° C (liquid nitrogen) (5.3.2.13) for long-term storage.

5.2.3.2 Preparation of new cell cultures

a) Remove a vial (5.2.3.1b)) from the liquid nitrogen and rapidly thaw it in a 37 °C water bath. Avoid total immersion.

b) Transfer the suspension into a 15 ml centrifuge tube, add 5 ml of MEM (5.2.2.17) + 10 % FCS (5.2.2.13) and centrifuge at 100 g_N for 5 min (5.3.2.14). Remove the supernatant fluid, resuspend the cells in 5 ml of MEM + 10 % FCS and inoculate in a new flask (5.3.2.15) containing 10 ml to 15 ml of MEM + 10 % FCS – for a 75 cm² flask use 15 ml to 22 ml of culture medium. Incubate at 37 °C (5.3.2.3).

c) When the cell monolayer is confluent, the cell culture can be either infected (5.8) or used to prepare new subcultures.
Cells can be subcultured at 1:3 or higher ratio for up to several passages (sometimes up to 40), before discarding them and taking a fresh aliquot from the liquid nitrogen stocks.
To subculture, aspirate the growth medium from the flask and rinse the cells with PBS (5.2.2.12) - for a 75 cm^2 flask use 5 ml to 10 ml. Aspirate PBS and pipette 5 ml of ATV (5.2.2.10) (at 37 °C) onto the monolayer in the flask. Incubate at 37 °C for 2 min to 5 min. Bump the side of the flask against the palm of the hand to help detach the cells. Wait until the cells are detached. Add 5 ml of MEM (5.2.2.17) + 10 % FCS (5.2.2.13) with a 10 ml pipette and vigorously wash to detach any remaining cell from the bottom of the flask and to separate any cell clumps. Take an aliquot of the cells, appropriate to the desired ratio, and add them into fresh MEM (5.2.2.17) + 10 % FCS (5.2.2.13) in a new flask (15 ml to 22 ml for a 75 cm^2 flask). Cell cultures should be regularly checked for the absence of mycoplasma and contaminating viruses.

5.2.4 Host strains for dairy bacteriophages (*Lactococcus lactis*)

5.2.4.1 Stock culture of host bacteria

Inoculate (1 % volume fraction) *Lactococcus lactis* subsp*. lactis* F 7/2 (DSM 4366) in M17-broth (5.2.2.18) and incubate for 24 h at 30 °C (5.3.2.3).

Inoculate skim milk (5.2.2.22) with a volume fraction of 1 % liquid culture, incubate for 2 h at (30 ± 1) °C (5.3.2.3) and maintain this stock culture of the host strain in skim milk in a refrigerator (5.3.2.8). In 2-weeks-intervals, let these stock cultures grow overnight at (30 ± 1) °C and repeat the method to obtain a fresh stock culture.

If prolonged storage is necessary, freeze the skim milk cultures at -18 °C to -20 °C or lower.

Alternatively use lyophilized cultures and reactivate according to the supplier's recommendation or inoculate *Lactococcus lactis subsp. lactis* F7/2 (DSM 4366) on an M17 agar (5.2.2.19) plate or slope and maintain this stock culture in a refrigerator (5.3.2.8)

5.2.4.2 Working culture of host bacteria

In order to prepare the working culture of host bacteria, subculture from the stock culture (5.2.4.1) in M17-broth (5.2.2.18) and incubate at (30 ± 1) °C (5.3.2.3). Use a volume fraction of 1 % inoculum from liquid culture or a loop of bacteria from a M17-slope or agar plate.

After 16 h to 24 h prepare a second subculture from the first subculture in M17 broth (a volume fraction of 1 % inoculum) and incubate for 16 h to 24 h at (30 ± 1) °C.

From the second subculture, a third subculture may be produced in the same way. The second and/or third subculture are the working culture(s).

If it is not possible to prepare the second subculture on a particular day, a 48 h subculture may be used for subsequent subculturing, provided that the subculture has been kept after a 2-h-incubation at (30 ± 1) °C in the refrigerator at 2 °C to 8 °C. In these circumstances, prepare a further 16 h to 24 h subculture before proceeding. Do not use a fourth subculture.

5.3 Apparatus and glassware

5.3.1 General

Sterilize all glassware and parts of the apparatus that will come into contact with the culture media and reagents or the sample, except those which are supplied sterile, by one of the following methods:

a) by moist heat, in the autoclave (5.3.2.1 a)];

b) by dry heat, in the hot air oven (5.3.2.1 b)].

5.3.2 Usual microbiological laboratory equipment[4]

and, in particular, the following:

5.3.2.1 Apparatus for sterilization (moist and dry heat)

a) for moist heat sterilization, an autoclave capable of being maintained at (121^{+3}_{0}) °C for a minimum holding time of 15 min;

b) for dry heat sterilization, a hot air oven capable of being maintained at (180^{+5}_{0}) °C for a minimum holding time of 30 min, at (170 $^{+5}_{0}$) °C for a minimum holding time of 1 h or at (160^{+5}_{0}) °C for a minimum holding time of 2 h.

5.3.2.2 Water baths, capable of being controlled at (20 ± 1) °C, (37± 1) °C and at (45 ± 1) °C if pour plate technique is used.

5.3.2.3 Incubator, capable of being controlled at either (36 ± 1) °C or at (37± 1) °C (for bacteria (incl. *Legionella*), mycobacteria and viruses) or at (30 ± 1) °C (for fungi and *Lactococcus lactis* host strains for dairy bacteriophages)**.** For mycobacteria and eukaryotic cell cultures a CO_2 – incubator (95 % air, 5 % CO_2) and a temperature of (36 ± 1) ºC is recommended. For mycobacteria, if a CO_2 – incubator is not used, the inoculated plates should be protected from drying by sealing with insulating tape or packing them into polyethylene bags.

5.3.2.4 pH-meter, having an inaccuracy of calibration of no more than ± 0,1 pH units at (20 ± 1) °C. A puncture electrode or a flat membrane electrode should be used for measuring the pH of the agar media (5.4.2).

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4 Disposable sterile equipment is an acceptable alternative to reusable glassware.

5.3.2.5 Fritted filter: porosity of 40 µm to 100 µm (ISO 4793).

5.3.2.6 Electromechanical agitator e.g. Vortex® mixer[5].

5.3.2.7 Forceps or wire.

5.3.2.8 Refrigerator, capable of being controlled at 2 °C to 8 °C.

5.3.2.9 Graduated pipettes, of nominal capacities 10 ml and 1 ml and 0,1 ml. Calibrated automatic pipettes may be used.

5.3.2.10 Petri dishes (plates), of size 90 mm to 100 mm.

5.3.2.11 Glass or ceramic beads, (3 mm to 4 mm in diameter).

5.3.2.12 Volumetric flasks.

5.3.2.13 Equipment for deep freezing test organisms at a temperature of -20 °C, –70 °C and -196 °C – liquid nitrogen, including cryovials of nominal capacity of 0,5 ml (min) to 2,0 ml (max).

5.3.2.14 Centrifuge (100 g_N to 4,000 g_N).

5.3.2.15 Flasks for cell culture use (e.g. 25 cm², 75 cm²)

5.3.2.16 Membrane filtration apparatus, constructed of a material compatible with the substances to be filtered, with a filter holder of at least 50 ml volume, and suitable for use of filters of diameter 47 mm to 50 mm and 0,22 µm pore size for sterilization of liquid materials and reagents. For bacteriophages disposable filter membrane units (pore size 0,45) µm are used

The vacuum source used shall give an even filtration flow rate.

5.3.2.17 Biological safety cabinet, class II or higher, for growing cell cultures and viruses.

5.4 Procedure for preservation of test organisms – General

5.4.1 Handling of freeze dried / frozen test organisms from culture collections

Follow for reconstitution of the freeze dried samples of the test organisms (viruses are shipped mostly in a frozen state) the procedures described in 5.5 to 5.9. If in special cases it is not possible or appropriate, follow the supplier's recommendations.

5.4.2 Choice of incubation procedure, agar medium, cell culture/cell line

Apply always the same incubation procedure (temperature, incubation time and type of incubator (5.3.2.3)] and use the same agar, cell culture/cell line and/or host strain as prescribed for the preparation of the working cultures / test suspension (e.g. "*N*") of test organisms in the corresponding European Standard.

NOTE In most of the CEN/TC 216 Standards this information is given in "5.4.1**"**.

5 Vortex® is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.

5.5 Procedure for preservation of bacteria (incl. *Legionella*, spore-forming bacteria, excl. mycobacteria and bacterial spores) and yeasts[6]

5.5.1 Reconstitution of the freeze dried test organisms

Rehydrate the pellet of the freeze dried sample of the test organism using about 1,0 ml TSB (5.2.2.3) for bacteria, BCYE broth (5.2.2.23) for *Legionella* or MEB (5.2.2.4) for yeasts as suspension medium and allow to swell 30 min. Dilute this suspension in about 5,0 ml of the suspension medium (TSB for bacteria, BCYE broth for *Legionella* or MEB for yeasts) and mix (5.3.2.6) till homogeneity for at least 30 s.

Inoculate two agar plates (5.4.2) with samples of this suspension – one plate with max 0,1 ml, the other with a loopful to achieve single colonies. Incubate (5.4.2). The rest of the suspension can be discarded.

Take a sample from the agar plate with single colonies for verification of purity and identity of the test organism as described in 5.10. Simultaneously continue with 5.5.2.

5.5.2 Preparation for storage

a) Add cryoprotectant solution (5.2.2.5) to the surface of one or both of the agar plates (10 ml per plate) and resuspend the cells in this solution using a glass spreader. Aspirate the cell suspension from the surface of the agar and dilute with cryoprotectant solution (5.2.2.5) to 10 ml or more – depending on the amount needed per year. Mix (5.3.2.6) for 30 s.
For *Legionella,* either water (5.2.2.2) or Page's saline (5.2.2.24) may be used instead of cryoprotectant solution. In case of no growth on the agar BCYE broth (5.2.2.23) may be inoculated and a subculture from this broth may be incubated (5.3.2.3) and prepared as described above.

   Instead of using 10 ml, 5 ml per plate for resuspending with subsequent aspiration may be used. It is possible then to add again 5 ml, resuspend and aspirate again.

b) Immediately after mixing, pipette 0,5 ml quantities of the diluted suspension (a) into cryovials.

   An alternative way of storage is by coating of beads:

   Pipette 1 ml of suspension into a cryovial containing two beads (5.3.2.11). Shake the vial to distribute the suspension onto the beads. Allow to stand for 30 min at 20 °C. Remove the excess cryoprotectant solution with a pipette.

   It is recommended to prepare a sufficient number of cryovials.

c) Place and store the cryovials at a temperature of –70 °C or less (5.3.2.13) for maximum 14 months. Longer periods are possible as long as sufficient viability can be established. The duration is also dependent on the results of susceptibility tests to be performed.

   Discard all cryovials if the purity or identity could not be verified in accordance with 5.5.1 and 5.11.

5.5.3 Preparation of stock culture / working cultures

Defrost the cryovial (5.5.2c)) or a single bead of a cryovial (removed by using a wire or forceps (5.3.2.7)) at room temperature. Inoculate agar plate(s) or slope(s) (5.4.2) with this suspension / this coated bead and incubate (5.4.2). Use the culture(s) as stock culture(s) and store for no more than nine weeks in the refrigerator (5.3.2.8) (*Pseudomonas aeruginosa* for no more than six weeks). From the stock culture(s) working cultures can be prepared according to the corresponding standard.

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6 For a graphical representation of this procedure see Figure B.1 for bacteria and Figure B.3 for yeasts.

5.6 Procedure for preservation of mycobacteria[7]

5.6.1 Reconstitution of the freeze dried test organisms

Rehydrate the pellet of the freeze dried sample of the test organism using about 1,0 ml MADC (5.2.2.6) as suspension medium. Dilute this suspension in about 5,0 ml of the suspension medium MADC (5.2.2.6), and mix (5.3.2.6) till homogeneity for at least 30 s.

Inoculate two agar plates (5.4.2) with samples of this suspension – one plate with max 0,1 ml, the other with a loopful to achieve single colonies. Incubate (5.4.2). The rest of the suspension can be discarded.

Take a sample from the agar plate with single colonies for verification of purity and identity of the test organism as described in 5.10. Simultaneously continue with 5.6.2.

5.6.2 Preparation for storage

a) Add MADC (5.2.2.6) to the surface of one or both of the agar plates (10 ml per plate) and resuspend the cells in this solution using a glass spreader. Aspirate the cell suspension from the surface of the agar and dilute with MADC (5.2.2.6) to 10 ml or more – depending on the amount needed per year. Mix (5.3.2.6) for 30 s.

   Instead of using 10 ml, 5 ml per plate for resuspending with subsequent aspiration may be used. It is possible then to add again 5 ml, resuspend and aspirate again.

b) Immediately after mixing, pipette 0,5 ml quantities of the diluted suspension (a) into cryovials. The amount can be increased up to 2,0 ml per cryovial if more than 2 working culture plates are planned to be prepared (5.6.3).

   An alternative way of storage is by coating of beads:

   Pipette 1 ml of suspension into a cryovial containing two beads (5.3.2.11). Shake the vial to distribute the suspension onto the beads. Allow to stand for 30 min at 20 °C. Remove the excess cryoprotectant solution with a pipette.

   It is recommended to prepare a sufficient number of cryovials.

c) Place and store the cryovials at a temperature of –70 °C or less (5.3.2.13) for maximum 14 months. Longer periods are possible as long as sufficient viability can be established. The duration is also dependent on the results of susceptibility tests to be performed.

   Discard all cryovials if the purity or identity could not be verified in accordance with 5.6.1 and 5.11.

5.6.3 Preparation of working cultures

Defrost the cryovial (5.6.2c)) or a single bead of a cryovial (removed by using a wire or forceps (5.3.2.7)) at room temperature. Inoculate agar plate(s) or slope(s) (5.4.2) with this suspension / this coated bead and incubate (5.4.2). Use the culture(s) as working culture and/or to prepare further working cultures of the test organism as specified in the corresponding standard. Stock cultures are not prepared.

7 For a graphical representation of this procedure see Figure B.2.

5.7 Procedure for preservation of moulds (e.g. *Aspergillus brasiliensis*)[8]

5.7.1 Reconstitution of the freeze dried test organism

Rehydrate the pellet of the freeze dried sample of the test organism using about 1,0 ml MEB (5.2.2.4) as suspension medium. Dilute this suspension in about 5,0 ml of the suspension medium MEB (5.2.2.4) and mix (5.3.2.6) till homogeneity for at least 30 s.

Inoculate two agar plates (5.4.2) with samples of this suspension – one plate with max 0,1 ml, the other with a loopful to achieve single colonies. Incubate (5.4.2). The rest of the suspension can be discarded.

Take a sample from the agar plate with single colonies for verification of purity and identity of the test organism as described in 5.10. Simultaneously continue with 5.8.2.

5.7.2 Preparation for storage

a) Add polysorbate 80 solution (5.2.2.7) to the surface of one or both of the agar plates (10 ml per plate) and detach conidia, the asexual fungal spores (produced exogenously from a hyphal tip) into the solution with the help of a glass spatula or glass beads (5.3.2.11). Transfer the suspension into a flask and shake gently for 1 min together with 5 g of beads (5.3.2.11).

   Filter the suspension through a fritted filter (5.3.2.5) directly or indirectly (via containers) into centrifuge tubes. Centrifuge the filtered suspension at 2 000 g_N (5.3.2.14) for 20 min. Discard the supernatant. Resuspend the conidia in 10 ml or more – depending on the amount needed per year – cryoprotectant solution (5.2.2.5) and mix (5.3.2.6) for 30 s.

   Instead of 10 ml you may use 5 ml per plate for resuspending with subsequent aspiration. You may add then again 5 ml, resuspend and aspirate again.

b) Immediately after mixing, pipette 0,5 ml quantities of the diluted suspension (a) into cryovials.

   An alternative way of storage is by coating of beads:

   Pipette 1 ml of suspension into a cryovial containing two beads (5.3.2.11). Shake the vial to distribute the suspension onto the beads. Allow to stand for 30 min at 20 °C. Remove the excess cryoprotectant solution with a pipette.

   It is recommended to prepare a sufficient number of cryovials.

c) Place and store the cryovials at a temperature of –70 °C or less (5.3.2.13) for maximum 14 months. Longer periods are possible as long as sufficient viability can be established. The duration is also dependent on the results of susceptibility tests to be performed.

   Discard all cryovials if the purity or identity could not be verified in accordance with 5.8.1 and 5.11.

5.7.3 Preparation of stock culture / working cultures

Defrost the cryovial (5.7.2c)) or a single bead of a cryovial (removed by using a wire or forceps (5.3.2.7)) at room temperature. Inoculate agar plate(s) or slope(s) (5.4.2) with this suspension/ coated bead and incubate (5.4.2). Use the culture(s) as stock culture(s) and store for no more than nine weeks in the refrigerator (5.3.2.8). From the stock culture(s) working cultures can be prepared according to the corresponding standard.

8 For a graphical representation of this procedure see Figure B.4.

5.8 Procedure for preservation of viruses (except dairy bacteriophages)[9]

5.8.1 Reconstitution of frozen virus

Virus suspensions are prepared from frozen reference virus obtained from culture collections. The viruses are multiplied in an appropriate cell line which produces high titres of infectious viruses (5.2.3). All procedures handling viruses and/or cell cultures have to be performed in appropriate biological safety cabinets (5.3.2.17).

For virus multiplying, confluent cell cultures have to be used. The growth medium of the cell culture is discarded; the cell monolayer is washed once with PBS (5.2.2.12). The viral suspension is thawed at room temperature and immediately distributed over the monolayer. If necessary, the virus suspension can be kept in an ice bath for not more than 30 min. The culture is incubated at 37 °C for approximately 1 h to allow viral adsorption. At the end of the incubation time, MEM (5.2.2.17) + 2 % FCS (5.2.2.13) is added to the monolayer, and the culture is kept in an incubator (5.3.2.3) for the required time. After virus multiplication, the cultures are frozen/thawed 3 times to break cells, using alternately an ethanol [2]/dry ice bath and a water bath set at 25 °C or a -80 °C freezer and a water bath at 25 °C. The cell debris is separated by centrifugation at 400 g_N (5.3.2.14) for 15 min and discarded.

5.8.2 Preparation for storage of stock virus suspension

The virus suspension obtained by multiplication of the reference virus suspension is divided into approximately 1,0 ml aliquots and kept in a deep freezer (5.3.2.13) at temperatures below -70 °C. For long term storage (more than 1 year), a temperature of -196 °C under liquid nitrogen is required (5.3.2.13).

An aliquot of the suspension is checked for identity, see 5.10.

5.8.3 Preparation of test virus suspension

The deep-freezed stock virus suspension is thawed at room temperature and immediately inoculated on an appropriate cell culture. If necessary, the virus suspension can be kept in an ice bath for not more than 30 min.

For the procedure see 5.8.1.
An alternative could be direct subcultures of 5.8.1.
Aliquots of the virus suspension can be used to inoculate other cell cultures in order to produce additional test virus suspensions for use in a test or for storage (5.8.1). In this latter case, identity has to be checked before freezing.

NOTE Due to safety reasons, only 10 passages from the original seed virus are allowed in case of the polio virus vaccine strain

5.9 Procedure for preservation of bacteriophages

5.9.1 Reconstitution of frozen bacteriophages

Bacterial virus suspensions are prepared from frozen bacteriophage stock samples obtained from culture collections. The viruses are propagated on an appropriate host strain (5.2.4) that produces high titres of infectious bacteriophage particles (5.2.4).

In a test tube, add 0,1 ml of phage lysate to 0,1 ml of the working culture of the host bacteria [16 h to 24 h culture in M17 broth (5.2.4.2)]. Supplement the sample with 10 mmol/l $CaCl_2$ by adding 0,05 ml from a sterile 50 mmol/l $CaCl_2$ stock solution (5.2.2.21). Vortex briefly and incubate for 10 min at room temperature (i.e., 20 °C to 21 °C) to allow the phage to adsorb to the bacterial cells.

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9 For a graphical representation of this procedure see Figure B.5.

Add 10 ml M17 broth (pre-warmed to (30 ± 1) °C) to the test tube, mix briefly and incubate at (30 ± 1) °C (5.3.2.3) until cell lysis occurs. Growth of host bacteria (i.e. turbidity) and cell lysis shall be followed with the naked eye or preferably by measuring the optical density of the culture in a suitable spectrophotometer at a defined wave length (e.g. 620 nm).

Prepare a control test tube in the same way, but replace the phage lysate by 0,1 ml M17-broth (5.2.2.18). Compare the turbidity in the test tube and in the control tube.

After lysis of host bacteria in the phage-containing test tube, pass the phage-containing supernatant through a 0,45 µm membrane filter (5.3.2.16).

Maintain the phage stocks at 4 °C to 8 °C until use.

5.9.2 Preparation for storage

For storage up to 30 months, it is sufficient to store the bacteriophage lysates at 4 °C after sterilization by membrane filtration without cryoprotectant. For longer storage duration glycerol [2] is used as cryoprotectant.

Sterilize by autoclaving (5.3.2.1a)) a 85 % to 88 % glycerol solution and add a 0,5 ml aliquot to 4,5 ml of a bacteriophage lysate, filtered through a membrane filter with 0,45 µm pore size (5.3.2.16). The virus suspension is divided into 1,0 ml aliquots in cryovials and kept in a deep freezer (5.3.2.13) at temperatures below -20° C.

5.9.3 Preparation of bacteriophages working suspensions

Cryovials with the frozen virus suspension are rapidly thawed, e.g. between closed hands, and used for inoculation on an appropriate bacterial host strain.

For the procedure see 5.9.1. An alternative could be direct subcultures of 5.9.1.

Remaining aliquots of the virus suspension can be used to propagate on further bacterial host strains or be kept for storage (5.9.1).

5.10 Verification of the purity and identity of test organisms

5.10.1 General

Verifications shall be carried out during preparation and at intervals during storage of the frozen test organisms. For viruses, identity only shall be verified, see 5.8.2.

5.10.2 Purity

Purity shall be verified by isolation on one or more agar plates (5.4.2) and by microscopic observation after Gram or other staining. Because of fundamental procedural problems the purity of viral cultures is not checked. In case of any doubt about the purity of a given culture it should be discarded rather than tested.

5.10.3 Identity

The identity of the test organism shall be verified in accordance with currently accepted identification methods, e.g. ELISA methods with specific antisera or PCR for viruses.

5.11 Documentation

For each test organism the following information shall be recorded:

5.11.1 Freeze dried test organism / frozen viruses

a) name of the culture collection from which the freeze dried test organism/frozen virus was obtained;

b) taxonomic name and the reference number;

c) date of receipt;

d) batch number given by the supplier.

5.11.2 Cryovials of frozen test organism

a) date on which the frozen sample was reconstituted;

b) number of cryovials originating from this batch (5.5.2b); 5.6.2b); 5.7.2b)) and their laboratory code (e.g. date);

c) the cryoprotectant solution used if different from the one in this European Standard and justification of its equivalence (5.2.2.5).

5.11.3 Stock culture

a) date on which the stock culture was prepared;

b) labelling of the stock culture.

5.11.4 Verification of purity and identity

a) details of methods used to check the purity and identity of the test organism;

b) dates at which the checks were carried out and the results obtained.

Annex A
(informative)

Test organisms – Culture collection references and relation to CEN/TC 216 standards and prENs

A.1 Bacteria (except mycobacteria and spore-forming bacteria)

A.1.1 *Enterobacter cloacae*

Culture collections: CIP 104674, DSM 6234

CEN/TC 216 Standards: EN 1276, EN 13697

A.1.2 *Enterococcus hirae*

Culture collections: ATCC 10541, CIP 58.55, DSM 3320, NCIMB 8192

CEN/TC 216 Standards: EN 1276, EN 1656, EN 13727, EN 13697, EN 14349, EN 14561

A.1.3 *Enterococcus faecium*

Culture collections: ATCC 6057, DSM 2146

CEN/TC 216 Standards: EN 13727

A.1.4 *Escherichia coli* (1)

Culture collections: ATCC 10536, CIP 54.127, DSM 682, NCTC 10418, NCIMB 8879

CEN/TC 216 Standards: EN 1276, EN 13697

A.1.5 *Escherichia coli* (2) K12

Culture collections: NCTC 10538, DSM 11250, CIP 54.117, NCIMB 10083, NCDO 1984 ; IFO 3301 ; NBRC 3301

CEN/TC 216 Standards: EN 1499, EN 1500, EN 13727

A.1.6 *Lactobacillus brevis*

Culture collections: DSM 6235, CIP 103474

CEN/TC 216 Standards: EN 1276, EN 13697

A.1.7 *Legionella pneumophila,* serogroup 1, Philadelphia

Culture collections**:** NCTC 11192, ATCC 33152, CIP 103854 T

CEN/TC 216 Standards : EN 13623

A.1.8 *Proteus hauseri*

Culture collections: ATCC 13315, CIP 58.60, NCIMB 4175, NCTC 4175, DSM 30118, IFO 3851, NBRC 3851

CEN/TC 216 Standards: EN 1656, EN 14349

Proteus vulgaris **see** ***"Proteus hauseri"***

A.1.9 *Pseudomonas aeruginosa*

Culture collections: ATCC 15442, CIP 103467, DSM 939, NCIMB 10421

CEN/TC 216 Standards: EN 1040, EN 1276, EN 1656, EN 13727, EN 13697, EN 14349, EN 14561

A.1.10 *Salmonella enterica* subsp. *enterica*, Serotype *typhimurium*

Culture collections: ATCC 13311, CIP 58.58, NCTC 74, DSM 5569

CEN/TC 216 Standards: EN 1276, EN 13697

Salmonella typhimurium **see** ***"Salmonella enterica"***

A.1.11 *Staphylococcus aureus* subsp. *aureus*

Culture collections: ATCC 6538, CIP 4.83, DSM 799, NCTC 10788, NCIMB 9518

CEN/TC 216 Standards: EN 1040, EN 1276, EN 1656, EN 13727, EN 13697, EN 14349, EN 14561

Steptococcus faecalis **see** ***"Enterococcus hirae"***

A.2 Mycobacteria

A.2.1 *Mycobacterium avium* subsp. *avium*

Culture collections: ATCC 15769, DSM 44157, CIP 105415

CEN/TC 216 Standards: EN 14204, EN 14348, EN 14563

A.2.2 *Mycobacterium terrae*

Culture collections: ATCC 15755, DSM 43227, CIP 104321

CEN/TC 216 Standards: EN 14348, EN 14563

A.3 Spore-forming bacteria

A.3.1 *Bacillus cereus*

Culture collections: ATCC 12826, CIP 105151

CEN/TC 216 Standards: EN 13704, EN 14347

A.3.2 *Bacillus subtilis* subsp. *spizizenii*

Culture collections: ATCC 6633, DSM 347, NCTC 10400, CIP 52.62, CCM 19999, IAM 1069, NCIB 8054

CEN/TC 216 Standards: EN 13704, EN 14347

A.3.3 *Clostridium sporogenes*

Culture collections: CIP 79.39

CEN/TC 216 Standards: EN 13704

A.4 Fungi (moulds and yeasts)

A.4.1 *Aspergillus brasiliensis* (former "*A. niger")* (mould)

Culture collections: ATCC 16404, IP 1431.83, DSM 1988, CBS 733.88, NCTC 2275, CMI 149007, BCCM/MUCL 30113, BCCM/IHEM 3961

CEN/TC 216 Standards: EN 1275, EN 1650, EN 1657, EN 13624, EN 13697, EN 14562

A.4.2 *Candida albicans* (yeast)

Culture collections: ATCC 10231, IP 4872, DSM 1386, CBS 6431, NCTC 3179, BCCM/IHEM 3731

CEN/TC 216 Standards: EN 1275, EN 1650, EN 1657, EN 13624, EN 13697, EN 14562

A.4.3 *Saccharomyces cerevisiae* (1) (yeast)

Culture collections: ATCC 9763, DSM 1333, IP 143283, CBS 5900

CEN/TC 216 Standards: EN 1650, EN 13697

A.4.4 *Saccharomyces cerevisiae* (2) *var. diastaticus* (yeast)

Culture collections: DSM 70487, BCCM/MUCL 27817

CEN/TC 216 Standards: EN 1650, EN 13697

A.5 Viruses

A.5.1 *Adenovirus* type 5, strain Adenoid 75

Culture collections: ATCC VR-5

CEN/TC 216 Standards: EN 14476

A.5.2 *Bovine Enterovirus* Type 1 (ECBO)

Culture collections: ATCC VR-248

CEN/TC 216 Standards: EN 14675

A.5.3 *Bovine Parvovirus*, strain Haden

Culture collections: ATCC VR-767

CEN/TC 216 Standards: EN 14476

A.5.4 *Poliovirus* type 1, Sabin strain LSc-2ab

Culture collections: NIBSC 01/528

CEN/TC 216 Standards: EN 14476

A.6 Bacteriophages

A.6.1 *Lactococcus lactis* subsp. *lactis* bacteriophage P008

Culture collection : DSM 10567

CEN/TC 216 Standards : EN 13610

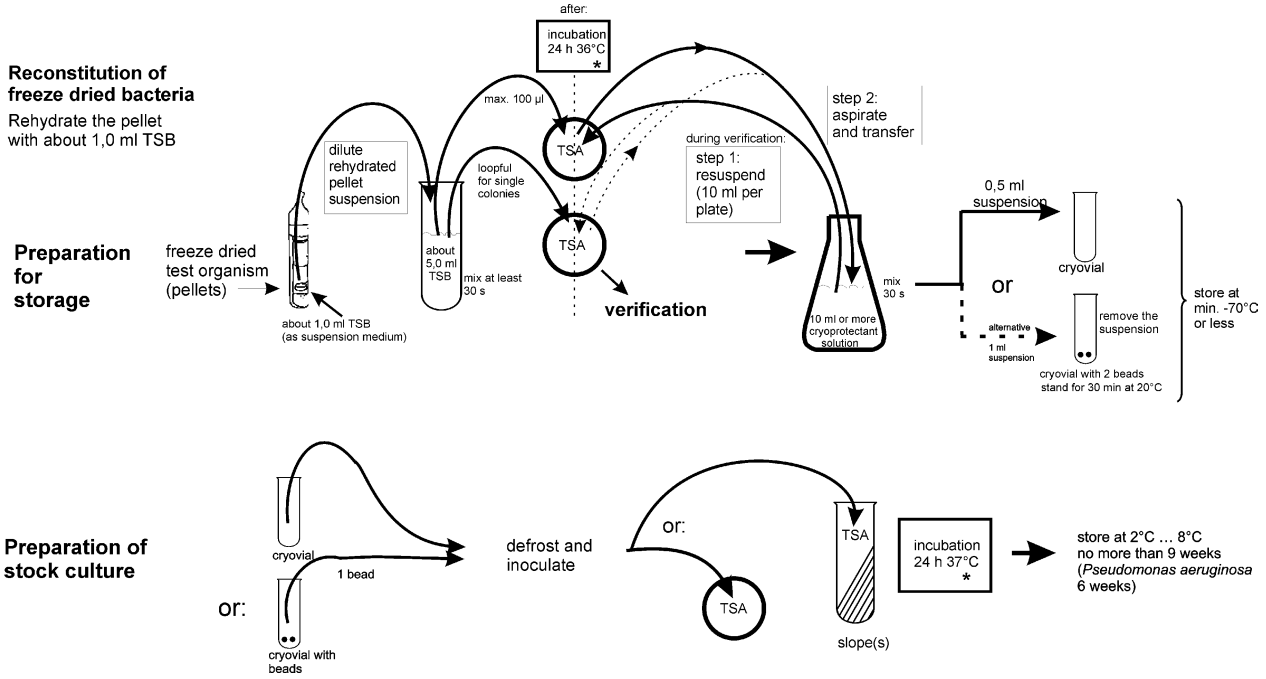
A.6.2 *Lactococcus lactis* subsp. *lactis* bacteriophage P001

Culture collection : DSM 4262

CEN/TC 216 Standards : EN 13610

Annex B
(informative)

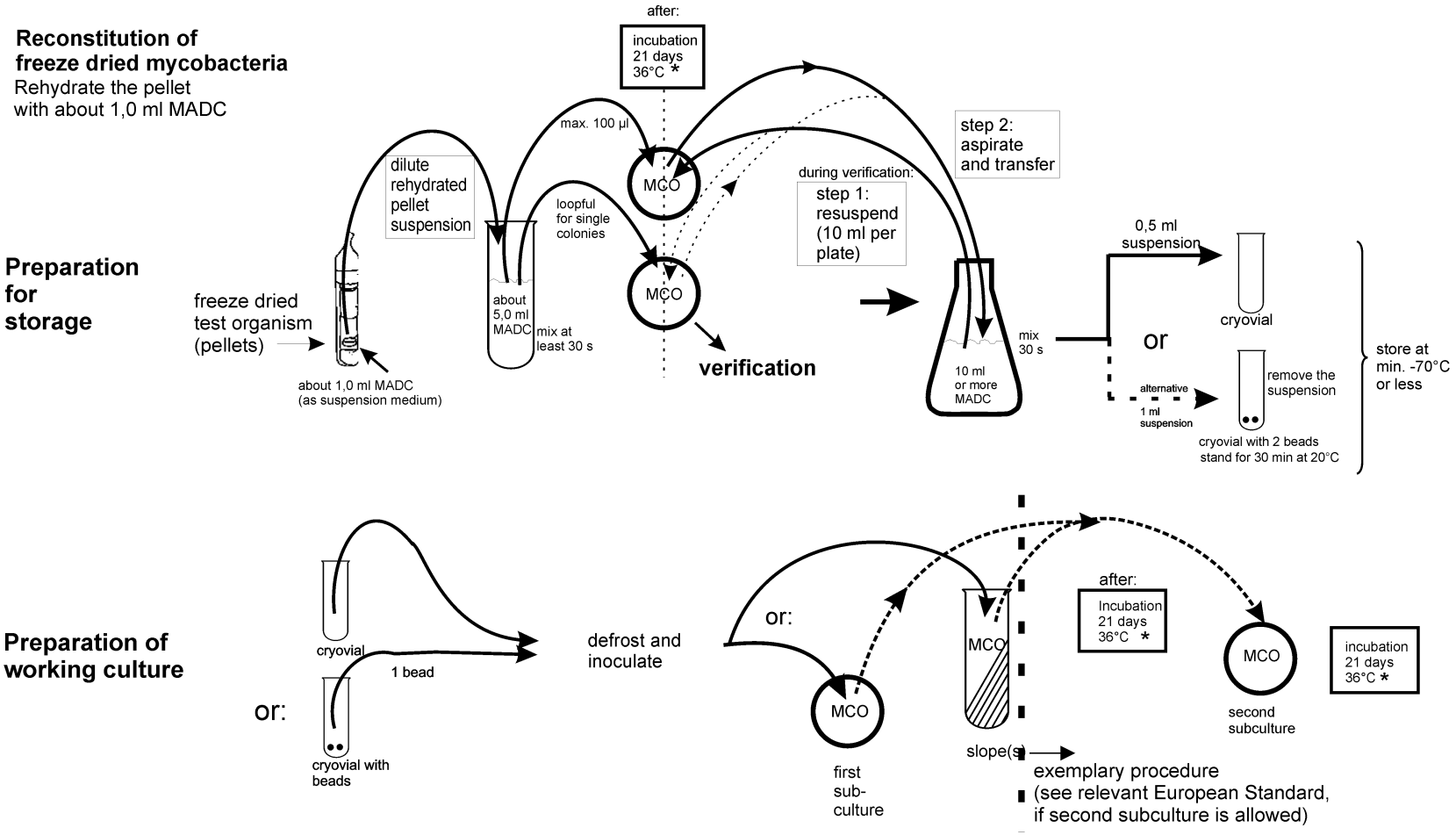
Graphical representations



Key

TSB = Tryptone Soya Broth TSA = Tryptone Soya Agar (example) * example, see the relevant standard

Figure B.1 – Preservation of bacteria (except mycobacteria) – See 5.5



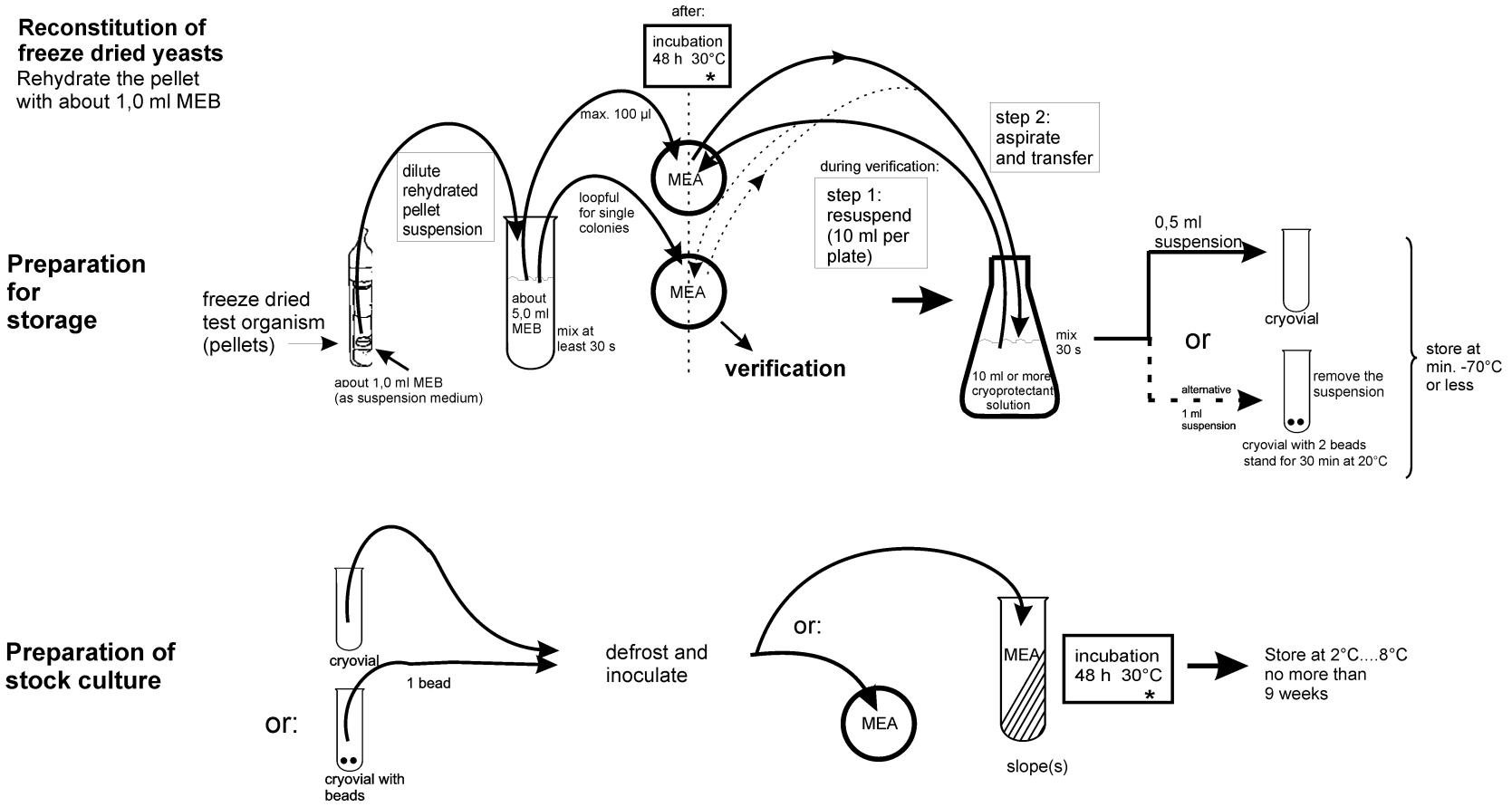
Key

MADC = Middlebrook 7H9 broth with 10 % ADC enrichment and glycerol

MCO = Middlebrook and Cohn 7H10 Agar with 10 % OADC enrichment (example)

* example, see the relevant standard

Figure B.2 – Preservation of mycobacteria – See 5.6



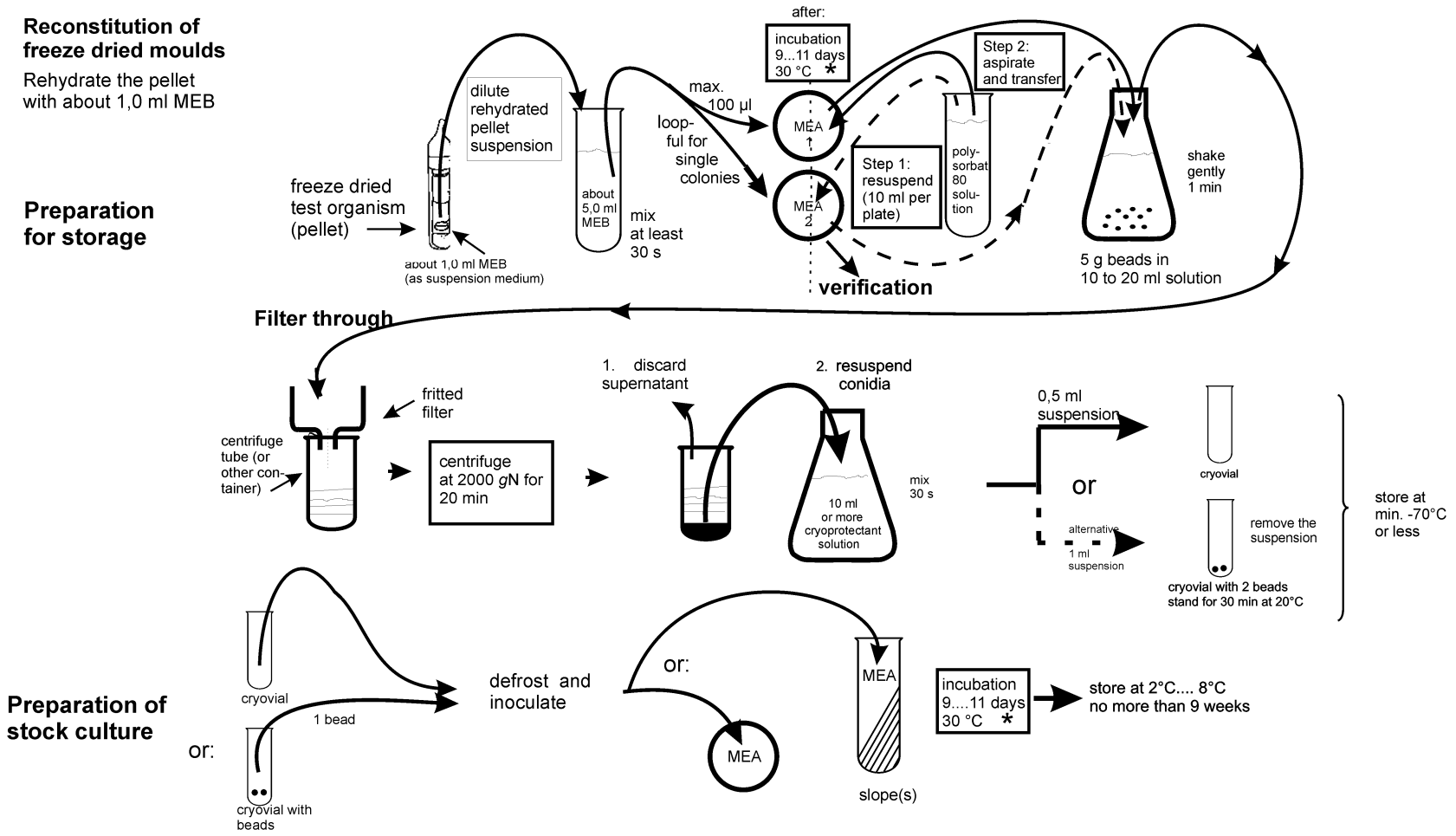
Key

MEB = Malt Extract Broth

MEA = Malt Extract Agar (example)

* example, see the relevant standard

Figure B.3 – Preservation of yeasts – See 5.5



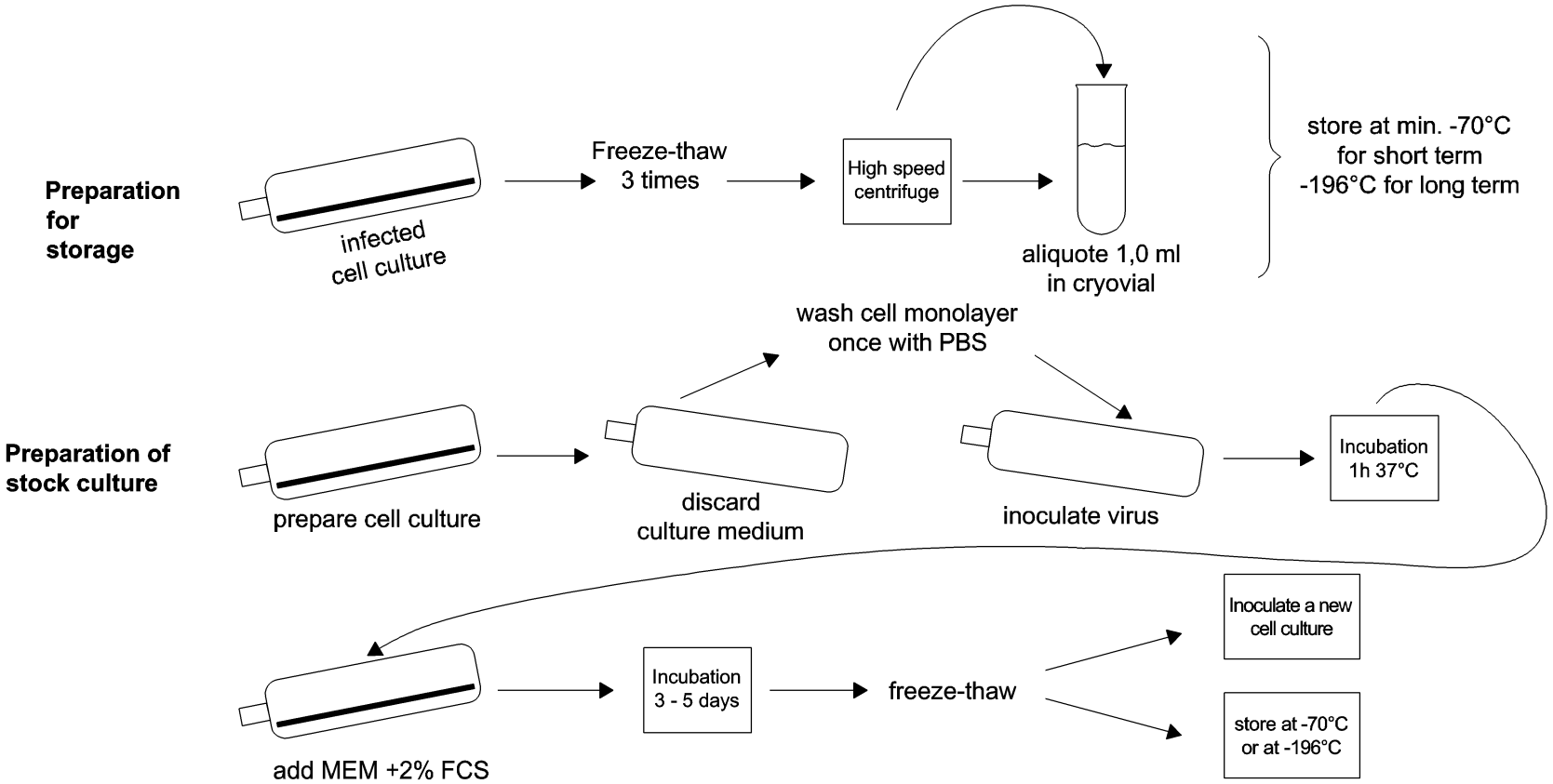
Key

MEB = Malt Extract Broth

MEA = Malt Extract Agar (example)

* example, see the relevant standard

Figure B.4 – Preservation of moulds – See 5.7



PBS = phosphate buffered saline **FCS = fetal calf serum** **MEM = minimal essential medium of Eagle**

Figure B.5 -Preservation of viruses – See 5.8

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[7] EN 1500 *, Chemical disinfectants and antiseptics — Hygienic handrub — Test method and requirements (phase 2, step 2)*

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[14] EN 13697, *Chemical disinfectants and antiseptics — Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas — Test method and requirements without mechanical action (phase 2/step2)*

[15] EN 13704, *Chemical disinfectants — Quantitative suspension test for the evaluation of sporicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas — Test method and requirements (phase 2, step 1)*

[16] EN 13727, *Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of bactericidal activity in the medical area — Test method and requirements (phase 2, step 1)*

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[24] EN 14563, *Chemical disinfectants and antiseptics — Quantitative carrier test for the evaluation of mycobactericidal or tuberculocidal activity of chemical disinfectants used for instruments in the medical area —Test method and requirements (phase 2, step 2)*

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