BRITISH STANDARD

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13610:2002

Chemical disinfectants — Quantitative suspension test for the evaluation of virucidal activity against bacteriophages of chemical disinfectants used in food and industrial areas — Test method and requirements (phase 2, step 1)

The European Standard EN 13610:2002 has the status of a British Standard

ICS 11.080.20; 67.050; 71.100.35

National foreword

This British Standard is the official English language version of EN 13610:2002.

The UK participation in its preparation was entrusted to Technical Committee CH/216, Chemical disinfectants and antiseptics, which has the responsibility to:

— aid enquirers to understand the text;

— present to the responsible international/European committee any enquiries on the interpretation, or proposals for change, and keep the UK interests informed;

— monitor related international and European developments and promulgate them in the UK.

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

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English version

Chemical disinfectants - Quantitative suspension test for the evaluation of virucidal activity against bacteriophages of chemical disinfectants used in food and industrial areas - Test method and requirements (phase 2, step 1)

Désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité virucide contre les bactériophages des désinfectants chimiques utilisés dans le domaine de l'agro-alimentaire et dans l'industrie - Méthode d'essai et exigences (phase 2, étape 1)

Chemische Desinfektionsmittel - Quantitativer Suspensionsversuch zur Bestimmung der viruziden Wirkung gegenüber Bakteriophagen von chemischen Desinfektionsmitteln in den Bereichen Lebensmittel, und Industrie - Prüfverfahren und Anforderung (Phase 2, Stufe 1)

This European Standard was approved by CEN on 23 October 2002.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the Management Centre or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the Management Centre has the same status as the official versions.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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EUROPÄISCHES KOMITEE FÜR NORMUNG

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Contents

Foreword

This document (EN 13610:2002) has been prepared by Technical Committee CEN /TC 216, "Antiseptics and chemical disinfectants" 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 June 2003, and conflicting national standards shall be withdrawn at the latest by June 2003.

In this European Standard the Annex A is normative and the Annexes B, C, D and E are informative.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Malta, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

Introduction

This European Standard describes a suspension test method intended to establish whether a product proposed as a disinfectant in the fields described in clause 1 has or does not have virucidal activity against bacteriophages.

NOTE Virulent bacteriophages (phages) lytic for (i.e. virulent for) starter cultures of *Lactococcus lactis* subsp. *lactis* used for the production of cheese and other fermented milk products are used as model viruses to test the virucidal activity of a product.

The laboratory test closely simulates practical conditions of application. Chosen conditions (contact time, temperature, viruses [i.e. bacteriophages] in suspension, ...) reflect parameters which are found in practical situations including conditions which may influence the action of disinfectants.

Each utilization concentration found from this test corresponds to defined experimental conditions.

The conditions are intended to cover general purposes and to allow reference between laboratories and product types.

However for some applications the recommendations of use may differ and therefore additional test conditions need to be used.

1 Scope

This European Standard specifies a test method (phase 2, step 1) and requirements for the minimum virucidal activity against bacteriophages of chemical disinfectants that form a homogeneous, physically stable preparation in hard water and that are used in food and industrial areas, excluding areas and situations where disinfection is medically indicated and excluding products used on living tissues.

This European Standard applies at least to the following:

a) processing, distribution and retailing of:

   1) food of animal origin:

      ¾ milk and milk products;

      ¾ meat and meat products;

      ¾ fish, seafood, and their products;

      ¾ eggs and egg products;

      ¾ animal feeds;

      ¾ etc.;

   2) food of vegetable origin:

      ¾ beverages;

      ¾ fruits, vegetables and their derivatives (including sugar, distillery ...);

      ¾ flour, milling and baking;

      ¾ animal feeds;

      ¾ ….;

b) other industrial areas:

      ¾ biotechnology (yeast, proteins, enzymes, ...).

Using this European Standard, it is not possible to determine the virucidal activity against bacteriophages of undiluted product as some dilution is always produced by adding the inoculum and interfering substance.

For chemical disinfectants that can be used without dilution it is not possible to determine whether these products, at a concentration above 80 % have a virucidal activity against bacteriophages.

NOTE The method described is intended to determine the activity of commercial formulations or active substances on viruses (bacteriophages) in the conditions in which they are used.

This European Standard is applicable only to disinfectants, complying with the validation test (see annex A).

2 Normative references

Not applicable.

3 Terms and definitions

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

3.1
product (for chemical disinfection and/or antisepsis)
chemical agent or formulation used as a chemical disinfectant or antiseptic

[EN 1040:1997]

3.2
virucide against bacteriophages
product which inactivates the bacteriophages

NOTE The adjective derived from "virucide" is "virucidal".

3.3
virucidal activity against bacteriophages
capability of a product to reduce the infectivity of intact bacteriophages belonging to reference bacteriophage strains P001 and P008 for at least 4 lg under the conditions defined by this standard

3.4
infectivity of bacteriophages
the ability of a bacteriophage to propagate in a suitable host bacterial cell resulting in the release of bacteriophage progeny

3.5
inactivation of bacteriophage
the reduction of infectivity of a bacteriophage by a product specified as a chemical disinfectant

3.6
reference bacteriophage suspension
bacteriophage suspension of a defined virus strain maintained in reference centers and which should not be passaged more than 10 times

3.7
stock bacteriophage suspension
bacteriophage suspension of a defined strain that has been multiplied on a large scale to obtain a bacteriophage suspension revealing identical characteristics as the reference bacteriophage suspension

NOTE This stock bacteriophage suspension is used to prepare a high-titer test bacteriophage suspension for the bacteriophage inactivation test.

3.8
high-titer bacteriophage suspension
the high-titer bacteriophages suspension obtained from agar plates revealing confluent lysis in the bacterial lawn that is used to prepare the bacteriophage test suspension in the virucidal testing of the disinfectant

3.9
bacteriophage test suspension
the bacteriophage suspension of a defined titer that is used in the virucidal testing of the disinfectant

4 Requirements

The product diluted in hard water when tested in accordance with clause 5 shall demonstrate at least a 4 $\log_{10}$ reduction of infectivity of bacteriophages when tested in the presence of a volume fraction of 1 % acidic whey (prepared from acidified low-fat milk) or optionally in the presence of a volume fraction of 1 % skim milk as the interfering substance according to its practical applications and under the required test conditions (20 °C, 15 min, 2 reference bacteriophage strains).

The virucidal activity against bacteriophages shall be evaluated using the two virulent bacteriophages *Lactococcus lactis* subsp. *lactis* bacteriophage P001 and *Lactococcus lactis* subsp. *lactis* bacteriophage P008.

Both phages shall be propagated on the host strain *Lactococcus lactis* subsp. *lactis* F7/2.

The determined virucidal concentration of the tested product is suggested as being suitable for practical situations of use.

Where appropriate, additional specific virucidal activity against bacteriophages shall be determined under other conditions of time, temperature, additional strains and interfering substances in accordance with (see 5.7.1) in order to take into account intended specific use conditions.

NOTE For these additional conditions, the concentration defined as a result can be lower than the one obtained under the initial test conditions of 20 °C, 15 min, 2 selected bacteriophage reference strains.

5 Test methods

5.1 Principle

5.1.1 A test suspension of bacteriophages in a solution of interfering substances is added to a prepared sample of the product under test diluted in hard water.

The mixture is maintained at 20 °C ± 1 °C for 15 min ± 10 s (required obligatory test conditions).

At the end of the contact time, aliquots are taken and the virucidal activity against bacteriophages in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization with a validated neutralizer. If a suitable neutralizer is not available for a specific product, removal of the product by molecular sieving (i.e. gel filtration) shall be used.

The number of surviving bacteriophage particles and the number of bacteriophage particles in the test suspension are determined from appropriate dilution series with a factor of 10 prepared in medium in test tubes.

5.1.2 Additional and optional exposure times, temperatures and interfering substances are specified (see 5.7.1).

NOTE 1 The test described is based on an assessment (under specific conditions) which gives a reduction of at least 99,99 % (4 lg) of the infectivity of the different phages after different contact times.

NOTE 2 For principal reasons, the result of an inactivation applied to a viral population is not necessarily equal to 100 %: i.e., one cannot conclude that there is a 100 % inactivation when on conducting the experiment no infectious phage are found within a limit number of sampling.

5.2 Material and reagents

5.2.1 Test organisms

The virucidal activity against bacteriophages shall be evaluated using the two following bacteriophage strains :

¾ *Lactococcus lactis* subsp. *lactis* bacteriophage P001 DSM 4262 [1)];

¾ *Lactococcus lactis* subsp. *lactis* bacteriophage P008 DSM 10567.

Both phages shall be propagated on the following host strain:

¾ *Lactococcus lactis* subsp. *lactis* F7/2 DSM 4366.

5.2.2 Culture media and reagents

5.2.2.1 General

The reagents shall be of analytical grade and/or appropriate for microbiological purposes. They shall be free from substances that cause toxic or inactivating effects either to the bacteriophages or to the host bacteria used for counting phage-derived plaques.

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

5.2.2.2 Water

The water shall be freshly glass distilled water and not demineralized water.

Sterilize in the autoclave (see 5.3.1).

NOTE 1 If the water is sterilized during the sterilization of the reagents, this is not necessary.

NOTE 2 If distilled water of adequate quality is not available, water for injectable preparation (European Pharmacopoeia) can be used.

5.2.2.3 M17-broth

For maintenance of bacterial host strain, propagation of bacteriophages and for formulation of phage diluent (see 5.2.2.6).

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 sulfate , 7 H_2O	0,25 g
Water (see 5.2.2.2)	1 000 ml

Sterilize in the autoclave (see 5.3.1). After sterilization the pH of the medium shall be equivalent to 7,0 0,2 when measured at 20 °C. When M17-broth is the diluent for neutralizer formulation (see 5.2.2.12 and Annex B), double

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1) DSM 4262, DSM 10567 and DSM 4366 are the collection numbers of bacteriophage and bacterial strains supplied by the DSMZ (Deutsche Sammlung von Mikroorganismen und ZellKulturen). This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of the culture collection named. Corresponding strains supplied by other culture collections may be used if they can be shown to lead to the same results.

concentrated M17-broth shall be used for preparation (i.e., all reagents shall be added in double concentration to 1 000 ml water).

5.2.2.4 M17-agar (bottom agar)

Bottom agar for quantitative counting of lysis zones (plaques) obtained 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 (see 5.2.2.3). Dissolve the agar by boiling with constant stirring.

Sterilize in the autoclave (see 5.3.1). 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 °C ± 1 °C, add 10 ml of a sterile 1 mol/l $CaCl_2$-stock solution (see 5.2.2.8). Mix gently and pour 15 ml to 18 ml of agar into Petri dishes (see 5.3.2.10).

5.2.2.5 Overlay agar (top agar, soft agar)

For counting bacteriophages: Dissolve 6,5 g agar in 1 000 ml M17-broth (see 5.2.2.3) and heat until boiling with constant stirring. Dispense the molten agar in test tubes (2,5 to 3 ml each).

Sterilize in the autoclave (see 5.3.1).

NOTE 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 (see 5.5.2 and 5.5.3).

5.2.2.6 Phage diluent (on basis of ¼ strength Ringer's solution)

For preparing dilution series for titration of phage (counting of phage-derived lysis zones):

¾ **1/4-strength Ringer's solution:**

¾ sodium chloride	2,250 g;
¾ potassium chloride	0,105 g;
¾ calcium chloride, anhydrous	0,06 g;
¾ sodium hydrogen carbonate	0,050 g;
¾ water (see 5.2.2.2)	to 1 000 ml.

Add 10 ml M17-broth (see 5.2.2.3) to 90 ml of 1/4-strength Ringer's solution.

Sterilize in the autoclave (see 5.3.1). Before use, add 1 ml from an 1 mol/l $CaCl_2$-stock solution (see 5.2.2.8) to 100 ml of the dilution broth.

NOTE Ringer's solution can be prepared from ready-to-use tablets according to the supplier's recommendations.

5.2.2.7 SM-buffer

For resuspension and storage of intact phage particles:

¾ Tris-HCl	2,4 g;
¾ NaCl	5,8 g;

¾ $MgSO_4$, 7 H_2O 2,5 g;

¾ water (see 5.2.2.2) to 1 000 ml.

Adjust the pH of the buffer to 7,4 ± 0,1. Sterilize in the autoclave (see 5.3.1).

5.2.2.8 $CaCl_2$-stock solutions (1 mol/l and 0,05 mol/l)

Dissolve either 110,99 or 5,55 g anhydrous $CaCl_2$ in water (see 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 (see 5.3.1).

5.2.2.9 Lactic acid solution (a volume fraction of 10 %)

For acidification of low-fat milk to prepare acidic whey.

Dilute a volume fraction of 90 % stock solution of lactic acid with water (see 5.2.2.2) to obtain a volume fraction of 10 % working solution. For this, 8 parts of water are added to 1 part of stock solution. Sterilize in the autoclave (see 5.3.1).

5.2.2.10 Phosphate-buffered saline

Prepare first a 10 mmol/l sodium phosphate buffer (pH 7,2):

¾ solution A: 1,42 g anhydrous Na_2HP0_4 are dissolved in water (see 5.2.2.2) and diluted to 1 000 ml with water;

¾ solution B: 1,20 g anhydrous NaH_2P0_4 are dissolved in water (see 5.2.2.2) and diluted to 1 000 ml with water.

Mix solutions A and B under constant stirring to obtain a final solution with a pH of 7,2.

Dissolve 8,5 g NaCl in 10 mmol/l sodium phosphate buffer (pH 7,2) and dilute to 1 000 ml with this buffer. Sterilize in the autoclave (see 5.3.1).

5.2.2.11 Sephadex® [2] G-25 gel for molecular sieving (i.e. gel filtration)

Resuspend 22 g of Sephadex®[2] G-25 powder in 100 ml phosphate-buffered saline (see 5.2.2.10). Sterilize in the autoclave (see 5.3.1). After cooling down to room temperature, fill 20 ml of the gel suspension into sterile plastic syringes placed in a sterile centrifuge bottle. Remove excess of buffer by centrifugation in a bench-top centrifuge (see 5.3.2.13) at 1 000 x *g* for 10 min under aseptic conditions. Store these ready-to-use units at 4 °C to 8 °C. They shall be used within a 4 h-period.

NOTE Alternatively, commercially available, disposable ready-to-use columns of suitable capacity can be used.

5.2.2.12 Neutralizer

The neutralizer shall be validated for the product under test in accordance with Annex A. The neutralizer shall be sterile.

NOTE Information on neutralizers that have been found to be suitable for some categories of products is given in Annex B.

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[2] Analytical quality of cross-linked dextran beads for molecular sieving (i.e. gel filtration). Sephadex® G-25 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.

5.2.2.13 Hard water for dilution of products

Hard water shall be prepared as follows:

- solution A: dissolve 19,84 g anhydrous magnesium chloride ($MgCl_2$) or an equivalent of hydrated magnesium chloride and 46,24 g anhydrous calcium chloride ($CaCl_2$) or an equivalent of hydrated calcium chloride in water (see 5.2.2.2) and dilute to 1 000 ml.

  Sterilize in the autoclave (see 5.3.1). Store the solution at 2 °C to 8 °C for no longer than one month;

- solution B: dissolve 35,02 sodium bicarbonate ($NaHCO_3$) in water (see 5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (see 5.3.2.7). Store the solution at 2 °C to 8 °C for no longer than one week.

Hard Water: For the preparation of 1 litre, place at least 600 ml water (see 5.2.2.2) in a 1 000 ml volumetric flask (see 5.3.2.12) and add 6,0 ml of solution A, then 8,0 ml of solution B. Mix and dilute to 1 000 ml with water (see 5.2.2.2). The pH of the hard water shall be 7,0 ± 0,2.

If necessary adjust the pH by using a solution of approximately 40 g/l (about 1 mol/l) of sodium hydroxide (NaOH) or approximately 36,5 g/l (about 1 mol/l) of hydrochloric acid (HCl).

The hard water shall be freshly prepared under aseptic conditions and used within 12 h.

NOTE When preparing the working culture (see 5.4.2) the addition of the product to this hard water produces a different final water hardness in each test tube.

In any case the final hardness is lower than 300 mg/kg of calcium carbonate ($CaCO_3$) in the test tube.

5.2.2.14 Interfering substances

5.2.2.14.1 General

The ionic composition (pH, calcium and/or magnesium hardness) and chemical composition (mineral substances, protein, glycosides, lipids, detergents ...) shall be fully defined.

The interfering substance shall be chosen according to the conditions of use laid down for the product.

The interfering substance shall be sterile and prepared at 10-times of its final concentration in the test.

The method of preparation and sterilization together with the composition shall be noted in the test report (see 5.9).

5.2.2.14.2 Whey solution

Prepare acidic whey solution from pasteurized low fat milk (1,5 % fat content) for the test conditions as follows:

- add 0,3 ml of a volume fraction of 10 % lactic acid solution (see 5.2.2.9) to 10 ml milk, mix (see 5.3.2.6) and keep the sample for 30 min at room temperature. Mix (see 5.3.2.6) occasionally during this 30 min period. Subsequently sediment the precipitated milk proteins in a bench top centrifuge (see 5.3.2.13) at maximum speed (4 000 x *g* at minimum) for 30 min. Sterilize the supernatant (whey) by membrane filtration (0,45 µm pore size) (see 5.3.2.7) and store at 4 °C to 8 °C;

- to obtain a volume fraction of 10 % working solution which is required as the obligatory interfering substance for the phage suspension test (see 5.7.2), dilute 1 part of acidic whey broth with 9 parts of water (see 5.2.2.2). Store the volume fraction of 10 % whey solution at 4 °C to 8 °C.

The whey solutions shall be stored for up to 1 month at 4 °C to 8 °C. For longer storage periods, they shall be kept frozen at - 18 °C to - 20 °C or lower.

The final concentration of the whey solution in the test procedure (see 5.7.1) shall be a volume fraction of 1,0 %.

5.2.2.14.3 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 (see 5.2.2.2);
- sterilize by steaming at 100 °C on 3 successive days (30 min each) and leave between steamings 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 (see 5.4.1).

Alternatively, sterilize at $(115\ ^{+3}_{0})$ °C for 15 min.

To obtain a volume fraction of 10 % working solution, dilute 1 part of skim milk with 9 parts of sterile water (see 5.2.2.2) which is required as an optional interfering substance for the phage suspension test (see 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 (see 5.7.1) shall be a volume fraction of 1 %.

5.3 Apparatus and glassware

5.3.1 General

Sterilize all glassware and parts of 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) in the autoclave (see 5.3.2.1) by maintaining it at $(121\ ^{+3}_{0})$ °C for a minimum holding time of 15 min;

b) in the dry heat sterilizer (see 5.3.2.1) by maintaining it at 180 °C for a minimum holding time of 30 min, at 170 °C for a minimum holding time of 1 h or at 160 °C for a minimum holding time of 2 h.

5.3.2 Usual microbiological laboratory equipment [3)] - in particular the following:

5.3.2.1 Apparatus for sterilization:

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 °C for a minimum holding time of 30 min, at 170 °C for a minimum holding time of 1 h or at 160 °C for a minimum holding time of 2 h.

5.3.2.2 Water baths capable of being controlled at 20 °C ± 1 °C, at additional test temperatures ± 1 °C (see 5.7.1), and at 4 °C ± 1 °C for cooling down melted M17 top agar (see 5.5.2 and 5.5.3).

5.3.2.3 Incubator, capable of being controlled at 30 °C ± 1 °C

3) Disposable equipment is an acceptable alternative to reusable glassware.

5.3.2.4 **pH-meter**, having an accuracy of calibration of ± 0,1 pH units at 25 °C.

5.3.2.5 **Stopwatch.**

5.3.2.6 **Electromechanical agitator**, (i.e. Vortexâ mixer [4)]).

5.3.2.7 **Membrane filtration apparatus** with a filter holder and suitable for use with filters of 0,45 µm pore size.

NOTE Disposable equipment is strongly recommended.

5.3.2.8 **Containers:** Test tubes, culture bottles or flasks of suitable capacity.

5.3.2.9 **Graduated pipettes** of nominal capacities 10 ml and 1 ml and 0,1 ml.

Calibrated automatic pipettes may be used.

NOTE Care should be taken to avoid contamination of the automatic pipettes with virus (i.e. bacteriophage) aerosols.

5.3.2.10 **Petri dishes** of size 90 mm to 100 mm.

5.3.2.11 **Volumetric flasks**.

5.3.2.12 **Mechanical shaker** / **stirrer**.

5.3.2.13 **Benchtop centrifuge**, capable to achieve a minimum centrifugation force of 4 000 x *g*.

5.3.2.14 **Microwave oven**.

Extreme care shall be taken to avoid uncontrolled overheating in the oven which may cause uncontrolled overboiling of liquefied agar. Lids shall be unscrewed loosely covered on the containers to avoid the hazard of explosion.

5.3.2.15 **Visible-light spectrophotometer** (optional) equipped with suitable optical filters. Optionally with suitable sample holders for glass test tubes to allow a direct reading of the optical density (e.g. at 620 nm) of bacterial suspensions.

5.4 Preparation of host bacteria suspensions

5.4.1 Stock culture of host bacteria

Inoculate reconstituted skim milk (see 5.2.2.14.3) with a volume fraction of 1 % liquid culture or with a loop of bacteria from a M17 slope or agar plate, incubate for 2 h at 30 °C ± 1 °C (see 5.3.2.3) and maintain this stock culture of the host strain in reconstituted skim milk in a refrigerator at 4 °C to 8 °C. In 2-weeks-intervals, let these stock cultures grow overnight at 30 °C ± 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.

[4)] Vortex[â] 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.

5.4.2 Working culture of host bacteria

In order to prepare the working culture of host bacteria (see 5.2.1), subculture from the stock culture (see 5.4.1) in M17-broth (see 5.2.2.3) and incubate at 30 °C ± 1 °C (see 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 °C ± 1 °C.

From the second subculture, you may produce a third subculture 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 °C ± 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.

5.5 Preparation of bacteriophage suspension

5.5.1 Stocks of bacteriophages

In a test tube, add 0,1 ml of reference phage lysate (see 5.2.1) to 0,1 ml of the working culture of the host bacteria (16 h to 24 h culture in M17 broth [see 5.4.2]). Supplement the sample with 10 mmol/l $CaCl_2$ by adding 0,05 ml from a sterile 50 mmol/l $CaCl_2$ stock solution (see 5.2.2.8). 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.

Add 10 ml M17 broth (prewarmed to 30 °C ± 1 °C) to the test tube, mix briefly and incubate at 30 °C ± 1 °C (see 5.3.2.3) until cell lysis occurs. Growth of host bacteria (i.e. turbidity) and cell lysis shall be followed:

a) with the naked eye;

b) or preferably by measuring the optical density of the culture in a suitable spectrophotometer (see 5.3.2.15) 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 (see 5.2.2.3). 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 membrane filter (0,45 µm pore size) (see 5.3.2.7).

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

5.5.2 High titer bacteriophage suspensions

Prepare from the stock bacteriophage suspensions (see 5.5.1) a series of decimal dilutions in phage diluent (see 5.2.2.6). For this, add 1,0 ml of the phage stock suspensions to 9,0 ml phage diluent. Mix (see 5.3.2.6) and prepare the following dilution steps in the same way until the 10^{-5} dilution.

Take a sample of 0,1 ml from each dilution step in duplicate and transfer each 0,1 ml sample into a separate test tube. Add 0,3 ml of a 16 h to 24 h bacterial working culture grown in M17-broth (see 5.4.2). Add 0,1 ml from a 50 mmol/l $CaCl_2$ solution (see 5.2.2.8), mix briefly (see 5.3.2.6) and incubate for 10 min at room temperature to allow the phage to adsorb to the host bacterial cells.

Add 2,5 ml to 3 ml of M17-overlay agar (see 5.2.2.5) melted before in a boiling water bath or in a microwave oven (see 5.3.2.14) and subsequently cooled down in a 47 °C ± 1 °C water bath (see 5.3.2.2). Mix briefly and pour the samples evenly onto M17-bottom agar plates (see 5.2.2.4). For homogeneous repartition of the samples, the agar plates shall be tilted slowly by hand before hardening of the overlay-agar.

Extreme care shall be taken to avoid uncontrolled overheating in the microwave oven which may cause uncontrolled overboiling of liquified agar. Lids shall be unscrewed loosely covered on the containers to avoid the hazard of explosion.

Incubate the plates for at least 6 h or overnight for 16 h to 24 h at 30 °C ± 1 °C (see 5.3.2.3).

Examine the plates for the appearance of phage-derived uniform clear lysis zones (plaques) in the lawn of the host bacteria.

NOTE 1 Plaques from phage P008 should reveal sharp edges and a diameter of 1 mm to 2 mm. Plaques from phage P001 should be larger (2 mm to 3 mm in diameter) and should be surrounded by turbid haloes (see annex E).

Select plates from the dilution series which show confluent lysis. On these plates, plaques shall touch each other, and a fine web of remaining unlysed cells shall merely been visible[5)]. Do not choose plates on which the bacterial lawn has been cleared totally or on which single plaques do not touch each other (i.e. on which plaques are totally isolated), since high-titer lysates cannot be obtained from these plates.

Harvest the phages from agar plates revealing confluent lysis by scraping the soft agar with a sterile, bent glass rod and transfer into a (centrifuge) test tube or flask.

Rinse each plate carefully with 5 ml SM-buffer (see 5.2.2.7) and transfer also into the same (centrifuge) test tube.

Shake the tubes gently for at least 15 min at room temperature.

Sediment the soft agar and the cell debries in a bench top centrifuge (i.e. for 15 min at 4 000 x *g*) (see 5.3.2.13).

The supernatant shall be passed through a membrane filter (see 5.3.2.7).

Phages from one strain harvested from different plates shall be pooled prior to determination of the phage titer.

NOTE 2 A phage lysate prepared according to this method is essentially free of protein contamination. In general, the obtained phage titers are in the range of 1×10^{10} to 5×10^{11} PFU/ml [6)].

Maintain the high-titer bacteriophage suspension at 4 °C to 8 °C for up to 3 months.

For counting of the high-titer bacteriophage suspensions prepare serial dilutions using phage diluent (see 5.2.2.6). For this, add 1,0 ml of the high-titer bacteriophage suspension to 9,0 ml phage diluent. Mix (see 5.3.2.6) and prepare the following dilution steps in the same way until the 10^{-9} dilution. Take a sample of 0,1 ml from the 10^{-8} and the 10^{-9} dilution steps in duplicate and transfer each 0,1 ml sample into a separate test tube. Add 0,3 ml of a 16 h to 24 h bacterial working culture grown in M17-broth (see 5.4.2). Add 0,1 ml from a 50 mmol/l $CaCl_2$ solution (see 5.2.2.8), mix briefly (see 5.3.2.6) and incubate for 10 min at room temperature to allow the phage to adsorb to the host bacterial cells.

Add 2,5 ml to 3 ml of M17-overlay agar (see 5.2.2.5) melted before in a boiling water bath or in a microwave oven and subsequently cooled down in a 47 °C ± 1 °C water bath (see 5.3.2.2). Mix briefly and pour the samples evenly onto M17-bottom agar plates (see 5.2.2.4). For homogeneous repartition of the samples, the agar plates shall be tilted slowly by hand before hardening of the overlay-agar.

5.5.3 Preparation of the bacteriophage test suspension

Prior to performing the test procedure for determining the virucidal activity of products, the phage titer of the high-titer bacteriophage suspension (see 5.5.2) is adjusted to 8×10^8 to 3×10^9 PFU/ml [6)] with SM-buffer (see 5.2.2.7) to obtain the final bacteriophage test suspension for the test procedure.

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5) Confluent lysis usually occurs on agar plates derived from the 10^{-3} or 10^{-4} dilution step.

6) PFU/ml = Plaque-forming units per ml.

Before performing the actual test (see 5.7.2.2 and 5.7.2.3) verify the actual phage titer by preparing serial dilutions of the bacteriophage test suspension using phage diluent (see 5.2.2.6). For this, add 1,0 ml of the bacteriophage test suspension to 9,0 ml phage diluent. Mix (see 5.3.2.6) and prepare the following dilution steps in the same way until the 10^{-7} dilution. Take a sample of 0,1 ml from the 10^{-6} and 10^{-7} dilution steps in duplicate and transfer each 0,1 ml sample into a separate test tube. Add 0,3 ml of a 16 h to 24 h bacterial working culture grown in M17-broth (see 5.4.2). Add 0,1 ml from a 50 mmol/l $CaCl_2$ solution (see 5.2.2.8), mix briefly (see 5.3.2.6) and incubate for 10 min at room temperature to allow the phage to adsorb to the host bacterial cells.

Add 2,5 ml to 3 ml of M17-overlay agar (see 5.2.2.5) melted before in a boiling water bath or in a microwave oven and subsequently cooled down in a 47 °C ± 1 °C water bath (see 5.3.2.2). Mix briefly and pour the samples evenly onto M17-bottom agar plates (see 5.2.2.4). For homogeneous repartition of the samples, the agar plates shall be tilted slowly by hand before hardening of the overlay-agar.

Extreme care shall be taken to avoid uncontrolled overheating in the microwave oven which may cause uncontrolled overboiling of liquified agar. Lids shall be unscrewed loosely covered on the containers to avoid the hazard of explosion.

5.5.4 Counting of high titer bacteriophage suspension and bacteriophage test suspension

Incubate the plates at 30 °C ± 1 °C (see 5.3.2.3) for at least 6 h or overnight for 16 h to 24 h. Discard any plates which are not countable (for any reason). Count the phage-derived plaques and determine the plaque-forming units per ml. Calculate the number of PFU/ml in the bacteriophage test suspension (N) using the method given in 5.8.

5.6 Product test solution

Details of samples of the product as received shall be recorded.

Product test solutions shall be prepared in hard water (see 5.2.2.13) at three different concentrations to include the active and the non active range. The concentration of the product test solution shall be 1,25 times the required test concentration.

For solid products, dissolve the product as received by weighting at least 1 g ± 10 mg of the product in a volumetric flask (5.3.2.11) and filling up with hard water (see 5.2.2.13). Subsequent dilutions shall be prepared in volumetric flasks (5.3.2.11) on a volume/volume basis in hard water (see 5.2.2.13).

For liquid products, dilutions of the product shall be prepared in hard water (see 5.2.2.13) on a volume/volume basis in volumetric flasks.

For products supplied in a ready to use state, water (see 5.2.2.2) shall be used to prepare dilutions.

When the product is diluted in hard water, it shall give a physically homogeneous stable preparation.

The product test solutions and dilutions of it shall be prepared freshly and used within 60 min (or less in case of low stability).

The concentration of the product stated in the test report shall be the test concentration. Record the test concentration in terms of weight per volume or volume per volume.

5.7 Procedure

5.7.1 Choice of experimental conditions

Select the contact temperature, contact time and interfering substances from the following, in accordance with practical use for the products (see clause 4):

a) temperature : (in °C):

- the obligatory temperature to be tested is 20 °C ± 1 °C;
- additional temperatures may be chosen from 4 °C ± 1 °C, 10 °C ± 1 °C or 40 °C ± 1 °C;

b) contact time : *t* (in min):

- the obligatory contact time to be tested is 15 min ± 10 s;
- additional contact times may be chosen from 5 min ± 10 s, 30 min ± 10 s or 60 min ± 10 s;

c) bacteriophage strains and bacterial host strain:

- bacteriophages and host strain shall be as given in 5.2.1;

d) interfering substances:

- the obligatory interfering substance to be tested is acidic whey (see 5.2.2.14.2) prepared from acidified low-fat milk in a final concentration of a volume fraction of 1 %;
- in case of additional requirements, a volume fraction of 1 % reconstituted skim milk as given in 5.2.2.14.3 may also be tested.

The product shall not cause the formation of any precipitate in the experimental conditions used.

Each selected experimental condition (, *t*, bacteriophage strain and interfering substance) shall be validated in accordance with Annex A.

5.7.2 Test procedure for assessing virucidal effect against bacteriophages of the product (bacteriophage suspension test)

5.7.2.1 General

The method of choice is the dilution-neutralization method. Carry out the validation of the dilution-neutralization method (see A.4.1) using a suitable neutralizer, chosen according to laboratory experience and published data.

If this neutralizer is not valid, repeat the validation test using an alternative neutralizer containing a combination of polysorbate 80 [7)] (30 g/l), sodium thiosulfate (3 g/l), L-cysteine (3 g/l) and L-histidine (3 g/l) in M17-broth (see 5.2.2.12 and Annex B).

If both neutralizers are found to be ineffective, the molecular sieving method may be used in place of the dilution-neutralization method.

The inactivation of the virucidal activity against bacteriophages of the product shall be validated for each of the tested bacteriophage strains and for each of the chosen experimental conditions (see 5.7.1).

5.7.2.2 Dilution-neutralization method (see Figure A.1)

5.7.2.2.1 General

Prior to testing, equilibrate all reagents (product test solution, bacteriophage test suspension, interfering substances) to the test temperature of ± 1 °C using the water bath (see 5.3.2.2) controlled at ± 1 °C. Check that

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7) Analytical quality, non-hydrolized in accordance with European Pharmacopoeia volume 1. TWEEN 80® 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.

the temperature of the reagents is stabilized at ± 1 °C. The neutralizer shall be equilibrated at a temperature of 20 °C ± 1 °C.

5.7.2.2.2 Test procedure for virucidal activity of products against bacteriophages

Pipette 1,0 ml of interfering substance (see 5.2.2.14) into a test tube. Add 1,0 ml of one of the bacteriophage test suspensions (see 5.5.3) containing 8 x 10^8 PFU/ml to 3 x 10^9 PFU/ml.

Start immediately a stopwatch (see 5.3.2.5), mix (see 5.3.2.6) and place the test tube in the water bath at ± 1 °C for 2 min ± 10 s. At the end of the equilibration time, add 8,0 ml of one of the product test solutions. Start immediately a stopwatch (see 5.3.2.5), mix (see 5.3.2.6) and place the test tube in the water bath controlled at ± 1 °C for the appropriate contact time t ± 10 s.

Just before the end of the contact time, mix (see 5.3.2.6). At the end of the contact time pipette 0,2 ml of the test mixture into a tube containing 9,6 ml neutralizer (see 5.2.2.12) and 0,2 ml water (see 5.2.2.2). Mix (see 5.3.2.6) and place in a water bath controlled at 20 °C ± 1 °C.

At the end of the neutralization time of for 5 min ± 10 s, immediately take a sample of 0,1 ml in duplicate of neutralized mixture (neutralizer, product test solution, interfering substance, bacteriophage test suspension) and of a 10^{-1} serial dilution in phage diluent (see 5.2.2.6) and determine the phage titers (plaque-forming units per ml) (see 5.5.2 and 5.5.3).

Perform this procedure using the other test solutions and the other bacteriophage test suspensions.

5.7.2.2.3 Counting of the test mixture

Incubate the plates for at least 6 h or overnight for 16 h to 24 h at 30 °C ± 1 °C (see 5.3.2.3). Count the phage-derived plaques and determine the number of plaque-forming units for each plate. Any plates which are not countable (for any reason) should be discarded. Calculate the number of PFU/ml in the neutralized mixture (N_n) using the method given in 5.8. For calculation of the number of PFU/ml in the test mixture (N_a), the number of PFU/ml in the neutralized mixture (N_n) shall be multiplied by 5 x 10^1.

5.7.2.3 Molecular sieving (i.e. gel filtration) method (see Figure A.3)

5.7.2.3.1 General

Prior to testing, equilibrate all reagents (product test solution, bacteriophage test suspension, interfering substances) to the test temperature of ± 1 °C using the water bath (see 5.3.2.2) controlled at ± 1 °C. Check that the temperature of the reagents is stabilized at ± 1 °C.

5.7.2.3.2 Test procedure for virucidal activity of products against bacteriophages

Pipette 1,0 ml of interfering substance (see 5.2.2.14) into a test tube. Add 1,0 ml of one of the bacteriophage test suspensions (see 5.5.3) containing 8 x 10^8 PFU/ml [8)] to 3 x 10^9 PFU/ml [8)].

Start immediately a stopwatch (see 5.3.2.5), mix (see 5.3.2.6) and place the test tube in the water bath at ± 1 °C for 2 min ± 10 s. At the end of the equilibration time, add 8,0 ml of one of the product test solutions. Start immediately a stopwatch (see 5.3.2.5), mix (see 5.3.2.6) and place the test tube in the water bath at ± 1 °C for the appropriate contact time t ± 10 s.

8) PFU/ml = Plaque-forming units per ml.

Just before the end of the contact time, mix (see 5.3.2.6). At the end of the contact time, transfer 2,0 ml of the test mixture onto a Sephadex®[9] column (see 5.2.2.11). Centrifuge at 1 000 x *g* for 10 min ± 10 s (see 5.3.2.13).

Immediately take a sample of the detoxified test mixture (interfering substance, bacteriophage test suspension) and prepare serial dilutions in phage diluent (see 5.2.2.6) until the 10^{-2} dilution. From the 10^{-1} and the 10^{-2} dilutions, take a 0,1 ml sample in duplicate and determine the phage titers (plaque-forming units per ml) (see 5.5.2 and 5.5.3).

Perform this procedure using the other test solutions and the other bacteriophage test suspensions.

5.7.2.3.3 Counting of the test mixture

Incubate the plates for at least 6 h or overnight for 16 h to 24 h at 30 °C ± 1 °C (see 5.3.2.3). Count the phage-derived plaques and determine the number of plaque forming units for each plate. Any plates which are not countable (for any reason) should be discarded. Calculate the number of PFU/ml in the detoxified test mixture (N_a) using the method given in 5.8.

5.7.3 Validation of dilution-neutralization method and molecular sieving method

The dilution-neutralization and molecular sieving methods shall have been validated for each of the test bacteriophages and experimental conditions (see 5.7.1) according to Annex A. The validation test (see Annex A) shall also be carried out at the same time as the test procedure (see 5.7.2) using only the highest product concentration and the same conditions (bacteriophage test suspension, product test solution, neutralizer or molecular sieving column, interfering substances, hard water) as used in the test (see 5.7.2.2 or 5.7.2.3).

5.8 Calculation and expression of results

5.8.1 Calculation of phage-derived plaques PFU/ml[10] (i.e., determination of phage titers)

5.8.1.1 General

Phage titers shall be calculated from phage-derived plaques in the lawn of the host bacterial cells as follows:

¾ only plaque counts which are either less than 300 PFU/plate (for phage P008) or less than 200 PFU/plate (for phage P001) shall be used for calculation of plaque counts (see NOTE). For a result to be valid at least one plate has to contain 15 or more plaques. Phage titers shall be calculated using at least one pair of plates, where one or both plates contain more than 15 plaques and both plates contain less than 300 plaques.

NOTE Counting individual plaques on a plate is limited by the beginning of confluent lysis in the lawn of the bacterial host cells. Since the plaques of phage P001 are significantly larger than the plaques from bacteriophage P008 (see 5.5.2), the upper limit for counting plaques derived from bacteriophage P001 is in fact lower than 300 plaques per plate (i.e. 200 plaques per plate);

a) if plates from 2 dilutions fall within this range calculate the number of PFU/ml as the weighted mean count.

For calculation of the weighted mean count use equation (1):

$$\frac{c}{(n_1 + 0{,}1\, n_2) \times d \times V} \quad \text{in PFU/ml [10]} \qquad (1)$$

[9] Analytical quality of cross-linked dextran beads for molecular sieving (i.e. gel filtration). Sephadex® G-25 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.

[10] PFU/ml = Plaque forming units per ml.

where

c is the sum of the plaques counted on all the plates taken into account;

n_1 is the number of plates taken into account at the first dilution;

n_2 is the number of plates taken into account at the second dilution;

d is the dilution factor corresponding to the first dilution taken into account;

V is the sample volume (0,1 ml).

Round off the results calculated to two significant figures. For this:

¾ if the last figure is below 5, the preceding figure is not modified;

¾ if the last figure is equal to 5, round off the preceding figure to the next nearest even figure.

Proceed stepwise until two significant figures are obtained.

As a result, the number of PFU/ml is expressed by a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

EXAMPLE

$$\frac{(192 + 188 + 20 + 18)}{(2 + 0{,}1 \quad 2) \quad 10^{6} \quad 0{,}1} = \frac{418}{2{,}2 \quad 10^{6} \quad 0{,}1} = 1{,}9 \quad 10^{9} \text{ PFU/ml}$$

b) if plates from one dilution fall within the range to calculate the number of PFU/ml, the following method shall be used:

- only plaque counts which are either less than 300 PFU/plate (for phage P008) or less than 200 PFU/plate (for phage P001) shall be used for calculation of plaque counts (see Note above). Phage titers shall be calculated using plaque counts from 2 plates. When at least one plate counts 15 or more plaques, use equation (2) for calculation:

$$\frac{c}{n \times d \times V} \quad \text{in PFU/ml} \qquad (2)$$

where

- c is the sum of the plaques counted on both plates;
- n is the number of plates taken into account;
- d is the dilution factor corresponding to the dilution taken into account;
- V is the sample volume (0,1 ml).

5.8.1.2 Calculation for dilution neutralization test procedure

For the dilution neutralization test procedure (5.7.2.2.2) where the number of PFU on all plates derived from the undiluted test mixture (i.e. the 10^0 dilution step) counted is less than 15, record the phage titer of the neutralized mixture as N_n lower than $1{,}5 \times 10^2$ PFU/ml and the phage titer of the corresponding test mixture as N_a lower than $7{,}5 \times 10^3$ PFU/ml. Where the number of PFU on all plates derived from the 10^{-1} dilution step counted is either higher than 300 (for phage P008) or higher than 200 (for phage P001), record the phage titer of the neutralized mixture either as N_n higher than 3×10^4 PFU/ml (for phage P008) or as N_n higher than 2×10^4 PFU/ml (for phage P001) and the phage titer of the corresponding test mixture either as N_a higher than $1{,}5 \times 10^6$ PFU/ml (for phage P008) or as N_a higher than $1{,}0 \times 10^6$ PFU/ml (for phage P001).

5.8.1.3 Calculation for molecular sieving test procedure

For the molecular sieving test procedure (5.7.2.3.2) where the number of PFU on all plates derived from 10^{-1} dilution steps counted is less than 15, record the phage titer of the detoxified mixture as N_a lower than $1{,}5 \times 10^3$ PFU/ml. Where the number of PFU on all plates derived from the 10^{-2} dilution step counted is either higher than 300 (for phage P008) or higher than 200 (for phage P001), record the phage titer of the detoxified mixture either as N_a higher than 3×10^5 PFU/ml (for phage P008) or as N_a higher than 2×10^5 PFU/ml (for phage P001).

5.8.2 Verification of methodology

For each test organism check that:

a) N is between 8×10^8 PFU/ml and 3×10^9 PFU/ml;

b) N_v is between 2 x 10^4 PFU/ml and 1 x 10^5 PFU/ml;

c) B is equal to or greater than 0,01 · N_v (dilution-neutralization method) or 0,05 · N_v (molecular sieving method);

d) C is equal to or greater than 0,5 · B;

e) A is equal to or greater than 0,05 · N_v;

where

N is the number of PFU/ml of the bacteriophage test suspension (see 5.5.3);

N_v is the number of PFU/ml of the diluted phage suspension (see clause A.2) for validation;

B is the number of PFU/ml of the neutralizer toxicity control test mixture (see A.4.1) or of the phage non retention control test mixture (see A.4.2);

C is the number of PFU/ml of the dilution-neutralization control test mixture (see A.4.1) or of the product elimination control test mixture (see A.4.2);

A is the number of PFU/ml of the experimental conditions control (see A.4.1 or A.4.2).

5.8.3 Expression of results

For each test bacteriophage strain record the number of PFU/ml:

¾ in the bacteriophage test suspension (*N*) (see 5.5.4);

¾ and after the test procedure for virucidal activity of the product against bacteriophage (N_a) (see 5.7.2.2.2 and 5.7.2.3.2).

For validation of the dilution-neutralization method (see A.4.1), record the number of PFU/ml in the diluted phage suspension (N_v) (see clause A.2), in experimental conditions control (A), in the neutralizer toxicity control (B) and in the dilution-neutralization control (C).

For validation of the molecular sieving method (see A.4.2), record the number of PFU/ml in the diluted phage suspension (N_v) (see clause A.2), in experimental conditions control (A), in the phage non retention control (B) and in the product elimination control (C).

For each test bacteriophage and product test concentration calculate and record the reduction in phage activity as follows:

reduction in phage activity $(R) = \frac{N \times 10^{1}}{N_a}$

5.8.4 Conclusion

5.8.4.1 Virucidal activity for general purposes

Virucidal activity against bacteriophages for general purposes is characterized by the concentration of the tested product for which criteria 5.8.1 and 5.8.2 are met and for which a 4 lg or more reduction in viability shall be demonstrated, when the test viruses are:

¾ bacteriophage P001 (DSM 4262);

¾ bacteriophage P008 (DSM 10567);

¾ which are both lytic for *Lactococcus lactis* subsp. *lactis* F7/2 (DSM 4366);

under the required test condition : 15 min ± 10 s, 20 °C ± 1 °C and in the presence of interfering substance (see clause 4).

5.8.4.2 Virucidal activity for specific purpose

Virucidal activity against bacteriophages for specific purpose is characterized by the concentration of the tested product for which criteria 5.8.1 and 5.8.2 are met and for which a 4 lg or more reduction in viability shall be demonstrated, when the test viruses are :

¾ bacteriophage P001 (DSM 4262);

¾ bacteriophage P008 (DSM 10567);

¾ which are both lytic for *Lactococcus lactis* subsp. *lactis* F7/2 (DSM 4366);

under additional conditions (see clause 4): *t* in min, in °C and interfering substances.

It is accepted that for certain applications, this suspension test can provide sufficient information for the particular application.

5.9 Test report

The test report [10)] shall refer to this standard (i.e. EN xxx).

The test report shall state, at least, the following information:

a) identification of the laboratory;

b) identification of the sample:

- ¾ name of the product;
- ¾ batch number;
- ¾ manufacturer;
- ¾ date of delivery;
- ¾ storage conditions;
- ¾ product diluent recommended by the manufacturer for use;
- ¾ active substance(s) and its/their concentration(s) (optional);

c) experimental conditions:

- ¾ period of analysis;
- ¾ product diluent used during the test;

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10) An example of a typical test report is given in Annex C.

- product test concentrations;
- appearance of product dilutions;
- contact time(s);
- test temperature(s);
- interfering substance;
- stability of the mixture (interfering substance and product diluted in hard water);
- temperature of incubation;
- neutralizer or matrix used for molecular sieving;
- identification of the bacteriophages and the host indicator strain(s) used;

d) operating procedure:

- **if the dilution-neutralization method is used** full details of the test for validation of the neutralization medium shall be given;
- **if the molecular sieving method is used** the same full details of the procedure which was carried out in order to justify the use of the molecular sieving method shall be given;

e) test results:

- validation results;
- evaluation of virucidal activity;

f) conclusion;

g) date and signature(s).

NOTE An example is given in Annex C (Tables C.1 and C.2).

Annex A
(normative)

Test for validation of dilution-neutralization and molecular sieving methods

A.1 Principle

A neutralizer is chosen for each product in accordance with A.4.1 (see 5.7). If a suitable neutralizer cannot be found, the molecular sieving method in accordance with A.4.2 is used.

A.2 Preparation of diluted phage suspension

To prepare the diluted phage suspension, dilute the bacteriophage test suspension (see 5.5.3) with SM-buffer (see 5.2.2.7) to obtain the phage titer of 2 x 10^4 PFU/ml to 1 x 10^5 PFU/ml.

For counting of the diluted phage suspension, take a sample of 0,1 ml in duplicate from a 10^{-1} serial dilution step and from a 10^{-2} serial dilution step prepared in phage diluent (see 5.2.2.6) and transfer each 0,1 ml sample into a separate test tube. Add 0,3 ml of a 16 h to 24 h bacterial working culture grown in M17-broth (see 5.4.2). Add 0,1 ml from a 50 mmol/l $CaCl_2$ solution (see 5.2.2.8), mix briefly (see 5.3.2.6) and incubate for 10 min at room temperature to allow the phage to absorb to the host bacterial cells.

Add 2,5 ml to 3 ml of M17-overlay agar (see 5.2.2.5) melted before in a boiling water bath or in a microwave oven and subsequently cooled down in a 47 °C ± 1 °C water bath (see 5.3.2.2). Mix briefly (see 5.3.2.6) and pour the samples evenly onto M17-bottom agar plates (see 5.2.2.4). For homogeneous repartition of the samples, the agar plates shall be tilted slowly by hand before hardening of the overlay-agar.

Extreme care shall be taken to avoid uncontrolled overheating in the microwave oven which may cause uncontrolled overboiling of liquefied agar. Lids shall be unscrewed loosely covered on the containers to avoid the hazard of explosion.

Incubate the plates at 30 °C ± 1 °C (see 5.3.2.3) for at least 6 h or overnight for 16 h to 24 h. Discard any plates which are not countable (for any reason). Count the phage-derived plaques and determine the plaque forming units per ml. Calculate the number of PFU/ml in the diluted phage suspension (N_v) using the method given in 5.8.1.

A.3 Preparation of product test solution

For validation, prepare a product test solution (see 5.6) of the highest actual strength of the product used in the test.

A.4 Test for validation

A.4.1 Dilution-neutralization method (see Figure A.2)

Perform the following procedure for each experimental condition (bacteriophage strain, interfering substance, temperature, contact time).

Prior to testing, equilibrate all reagents (products test solutions, diluent [i.e. SM-buffer, see 5.2.2.7], diluted phage suspension, interfering substances, hard water) to the test temperature of ± 1 °C using the water bath (see 5.3.2.2) controlled at ± 1 °C. Check that the temperature of the reagents is stabilized at ± 1 °C. The neutralizer and the water (see 5.2.2.2) shall be equilibrated at 20 °C ± 1 °C:

a) validation of selected experimental conditions (or verification of the absence of any lethal effect in the test conditions):

- place in a test tube 1,0 ml of the selected interfering substance (see 5.2.2.14) and 1,0 ml of the diluted phage suspension prepared in clause A.2. Mix (see 5.3.2.6) for a few second and leave in the water bath at ± 1 °C for 2 min ± 10 s. At the end of this time, add 8,0 ml of hard water (see 5.2.2.13). Start the stopwatch at the beginning of the addition and mix (see 5.3.2.6) for a few seconds;

- leave in the water bath at ± 1 °C for the time *t* ± 10 s. Immediately before the end of the contact time, mix again (see 5.3.2.6). At the end of the contact time, take a sample of 0,1 ml in duplicate from the test mixture and from a 10^{-1} serial dilution prepared in phage diluent (see 5.2.2.6) and determine the phage titers (plaque-forming units per ml) (see 5.5.2 and 5.5.3);

- incubate the plates for at least 6 h or overnight for 16 h to 24 h at 30 °C ± 1 °C (see 5.3.2.3). Count the phage-derived plaques and determine the number of plaque-forming units for each plate. Discard any plates which are not countable (for any reason). Calculate the number of PFU/ml in the experimental conditions control (A) using the method given in 5.8.1;

b) neutralizer toxicity control:

- place in a test tube 9,6 ml of neutralizer (see 5.2.2.12 and Annex B) and 0,2 ml of water (see 5.2.2.2). Add 0,2 ml of the diluted phage suspension prepared in clause A.2;

- start the stopwatch at the beginning of the addition and mix (see 5.3.2.6), leave in contact in the water bath at 20 °C ± 1 °C for 5 min ± 10 s. Immediately before the end of the contact time, mix (see 5.3.2.6). At the end of the contact time, take a sample of 0,1 ml in duplicate from the test mixture and from a 10^{-1} serial dilution prepared in phage diluent, (see 5.2.2.6) and determine the phage titers (plaque-forming units per ml) (see 5.5.2 and 5.5.3);

- incubate the plates for at least 6 h or overnight for 16 h to 24 h at 30 °C ± 1 °C (see 5.3.2.3). Count the phage-derived plaques and determine the number of plaque-forming units for each plate. Discard any plates which are not countable (for any reason). Calculate the number of PFU/ml in the neutralizer toxicity control (B) using the method given in 5.8.1;

c) dilution-neutralization control:

- place in a test tube 1,0 ml of the selected interfering substance (see 5.2.2.14) and 1,0 ml of the phage buffer (i.e. SM-buffer, see 5.2.2.7) and finally, starting a stopwatch, 8,0 ml of the product dilution prepared in accordance with clause A.3. Leave in contact in the water bath for the time *t* ± 10 s at a temperature of ± 1 °C;

- then, transfer 0,2 ml of the mixture into a test tube containing 9,6 ml of neutralizer (see 5.2.2.12) previously equilibrated in the water bath at 20 °C ± 1 °C. Leave in contact in this water bath for 5 min ± 10 s;

- add 0,2 ml of the diluted phage suspension prepared in clause A.2;

- start a stopwatch at the beginning of the addition of phage and mix (see 5.3.2.6) for a few seconds. Leave in contact in the water bath at 20 °C ± 1 °C for 30 min ± 1 min;

- immediately before the end of the contact time, mix (see 5.3.2.6);

- at the end of the contact time, take a sample of 0,1 ml in duplicate from the test mixture and from a 10^{-1} serial dilution prepared in phage diluent (see 5.2.2.6) and determine the phage titers (plaque-forming units per ml) (see 5.5.2 and 5.5.3);

- incubate the plates for at least 6 h or overnight for 16 h to 24 h at 30 °C ± 1 °C (see 5.3.2.3). Count the phage-derived plaques and determine the number of plaque-forming units for each plate. Discard any plates which are not countable (for any reason). Calculate the number of PFU/ml in the dilution-neutralization control (C) using the method given in 5.8.

A.4.2 Molecular sieving method (see Figure A.4)

Perform the following procedure for each experimental conditions (test bacteriophage, interfering substance, temperature, contact time).

Prior to testing, equilibrate all reagents (products test solutions, diluted phage suspension, interfering substances, hard water) at the test temperature of °C ± 1 °C using the water bath (see 5.3.2.2) controlled at °C ± 1 °C. Check that the temperature of the reagents is stabilized at °C ± 1 °C.

a) validation of selected experimental conditions (or verification of the absence of any lethal effect in the test conditions):

- place in a test tube 1,0 ml of the selected interfering substance (see 5.2.2.14) and 1,0 ml of the diluted phage suspension prepared in A.2. Mix (see 5.3.2.6) for a few second and leave in the water bath at ± 1 °C for 2 min ± 10 s. At the end of this time, add 8,0 ml of hard water (see 5.2.2.13). Start the stopwatch at the beginning of the addition and mix (see 5.3.2.6) for a few seconds;

- leave in the water bath at ± 1 °C for the time *t* ± 10 s. Immediately before the end of the contact time, mix again (see 5.3.2.6). At the end of the contact time, transfer 2,0 ml of the test mixture on the top of a Sephadex®[11)] column (see 5.2.2.11) or equivalent. Centrifuge at 1 000 x *g* in a centrifuge (see 5.3.2.13) for 10 min. Take a sample of 0,1 ml in duplicate from the resulting filtrate and from a 10^{-1} serial dilution prepared in phage diluent (see 5.2.2.6) and determine the phage titers (plaque-forming units per ml) (see 5.5.2 and 5.5.3);

- incubate the plates for at least 6 h or overnight for 16 h to 24 h at 30 °C ± 1 °C (see 5.3.2.3). Count the phage-derived plaques and determine the number of plaque-forming units for each plate. Discard any plates which are not countable (for any reason). Calculate the number of PFU/ml in the experimental conditions control (A) using the method given in 5.8.1;

b) phage non retention control:

- place in a test tube 1,0 ml of the selected interfering substance (see 5.2.2.14) and 1,0 ml of the diluted phage suspension prepared in clause A.2. Add 8,0 ml of hard water (see 5.2.2.13). Mix (see 5.3.2.6) for a few seconds and transfer 2,0 ml of the test mixture on the top of a Sephadex®[12)] column (see 5.2.2.11) or equivalent. Centrifuge at 1 000 x *g* in a centrifuge (see 5.3.2.13) for 10 min;

- take a sample of 0,1 ml in duplicate from the resulting filtrate and from a 10^{-1} serial dilution prepared in phage diluent (see 5.2.2.6) and determine the phage titers (plaque-forming units per ml) (see 5.5.2 and 5.5.3);

- incubate the plates for at least 6 h or overnight for 16 h to 24 h at 30 °C ± 1 °C (see 5.3.2.3). Count the phage-derived plaques and determine the number of plaque-forming units for each plate. Discard any plates which are not countable (for any reason). Calculate the number of PFU/ml in the phage non retention control (B) using the method given in 5.8.1;

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11) Analytical quality of cross-linked dextran beads for molecular sieving (i.e. gel filtration). Sephadex® G-25 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.

c) product elimination control:

¾ place 1,0 ml of interfering substance (see 5.2.2.14) into a test tube, add 1,0 ml of the phage buffer (i.e. SM-buffer, see 5.2.2.7) and finally, starting a stopwatch, 8,0 ml of the product dilution prepared in clause A.3. Leave in contact in the water bath for the time t min ± 10 s at a temperature of ± 1 °C;

¾ immediately before the end of the contact time, mix (see 5.3.2.6). At the end of the contact time, transfer 2,0 ml of the test mixture on the top of a Sephadex column (see 5.2.2.11) or equivalent. Centrifuge at 1 000 x *g* in a centrifuge (see 5.3.2.13) for 10 min. Then transfer 0,9 ml of the filtrate into a test tube, add 0,1 ml of the diluted phage suspension prepared in clause A.2;

¾ start a stopwatch at the beginning of the addition of phage and mix (see 5.3.2.6) for a few seconds. Leave in contact in the water bath at 20 °C ± 1 °C for 30 min ± 1 min;

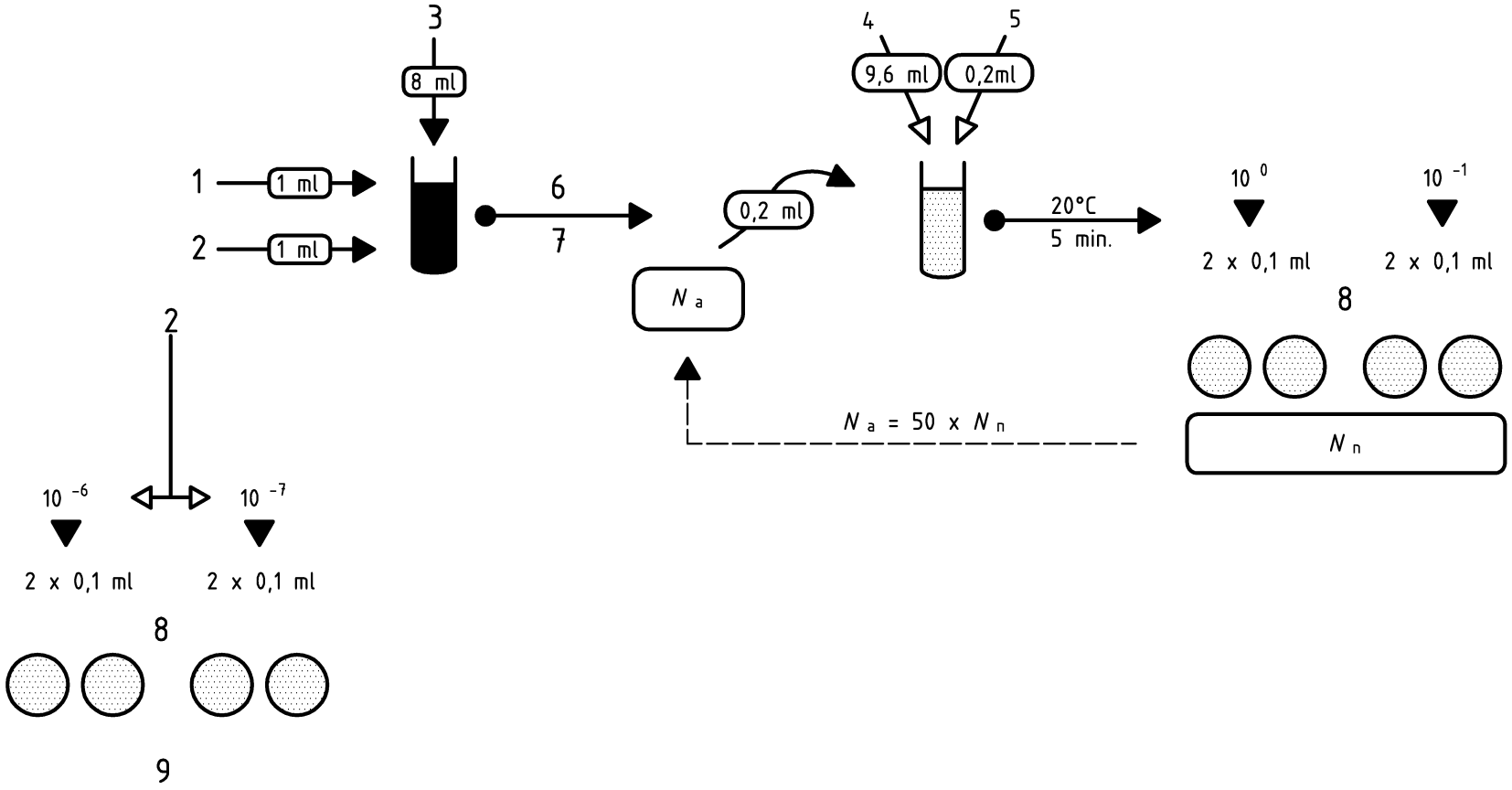
¾ immediately before the end of the contact time, mix (see 5.3.2.6);

¾ at the end of the contact time, take a sample of 0,1 ml in duplicate from the test mixture and from a 10^{-1} serial dilution prepared in phage diluent (see 5.2.2.6) and determine the phage titers (plaque-forming units per ml) (see 5.5.2 and 5.5.3);

¾ incubate the plates for at least 6 h or overnight for 16 h to 24 h at 30 °C ± 1 °C (see 5.3.2.3). Count the phage-derived plaques and determine the number of plaque-forming units for each plate. Discard any plates which are not countable (for any reason). Calculate the number of PFU/ml in the product elimination control (C) using the method given in 5.8.1.

A.5 Validation

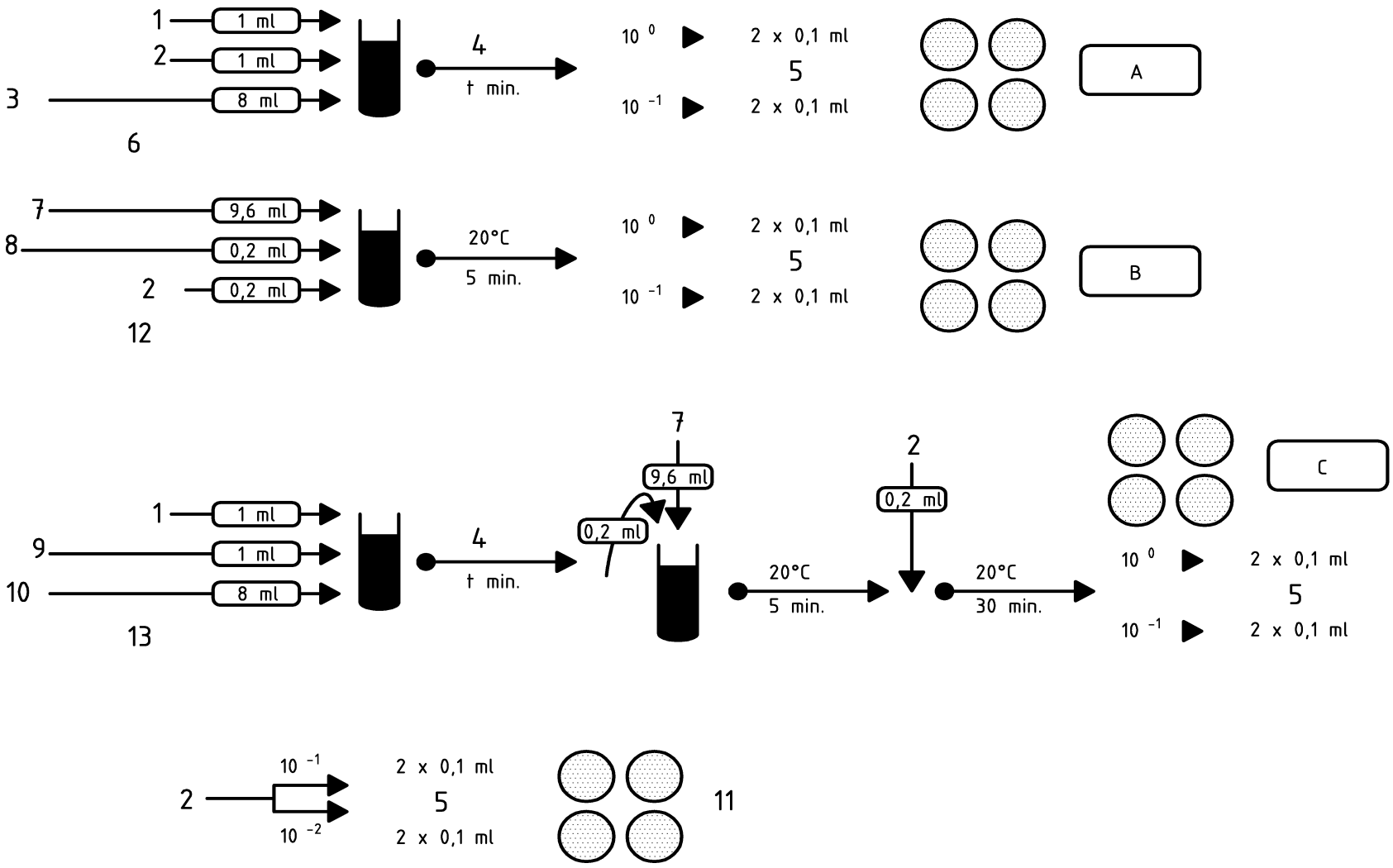
Check that the test results comply with the relevant requirements of 5.8.1 or 5.8.2.



Key

1 Interfering substance
2 Bacteriophage test suspension
3 Product
4 Neutralizer
5 Water
6 Test temperature
7 Contact time
8 Plaque test
9 N ($8 \times 10^8 - 3 \times 10^9$ PFU/ml)

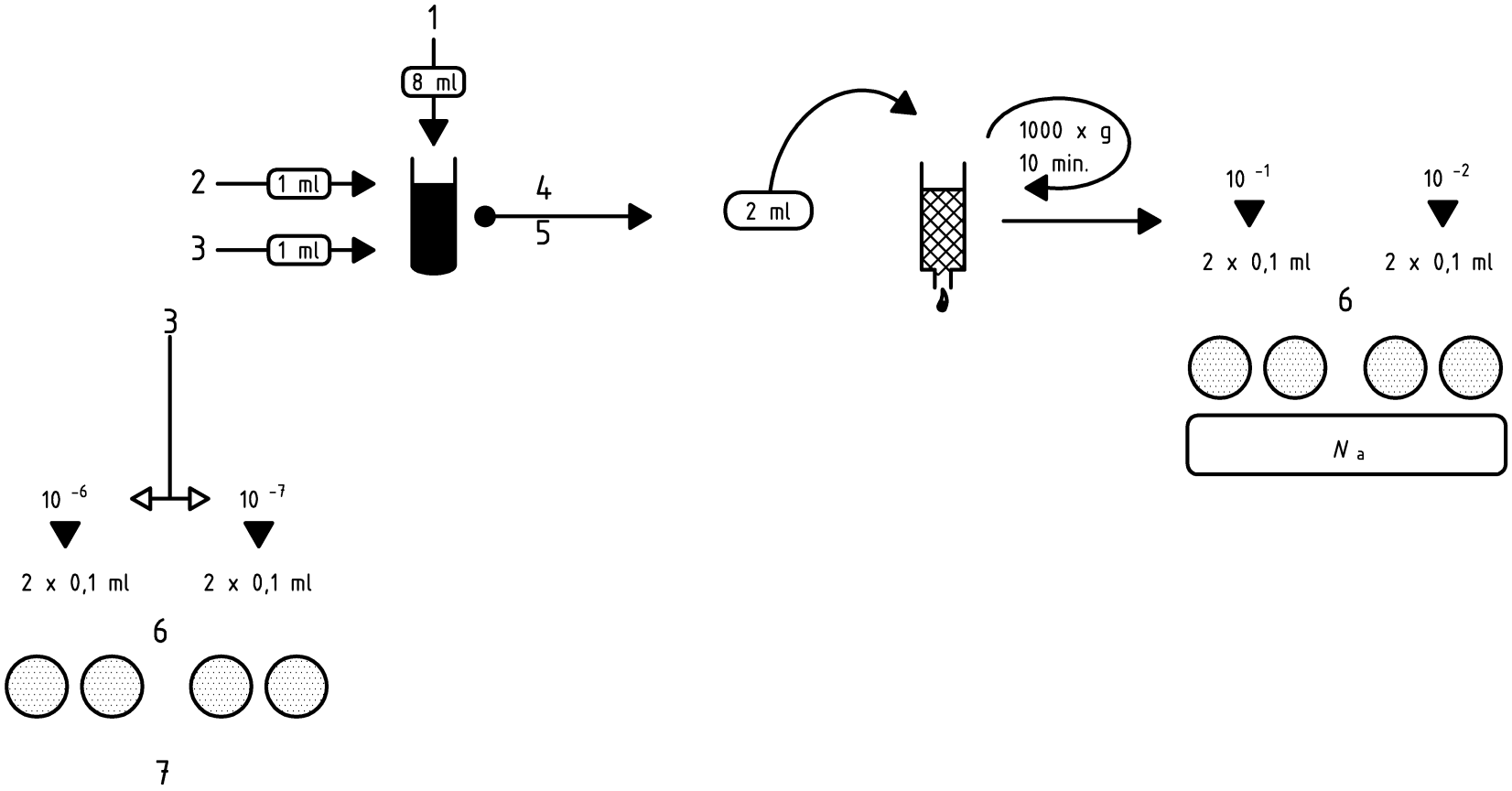
Figure A.1 — Dilution neutralization method (actual test)



Key

1 Interfering substance
2 Diluted phage suspension
3 Hard water
4 Test temperature
5 Plaque test
6 Experimental conditions control
7 Neutralizer
8 Water
9 SM-buffer
10 Product
11 N_v ($2 \times 10^4 - 1 \times 10^5$ PFU/ml)
12 Neutralizer toxicity control
13 Dilution neutralization control

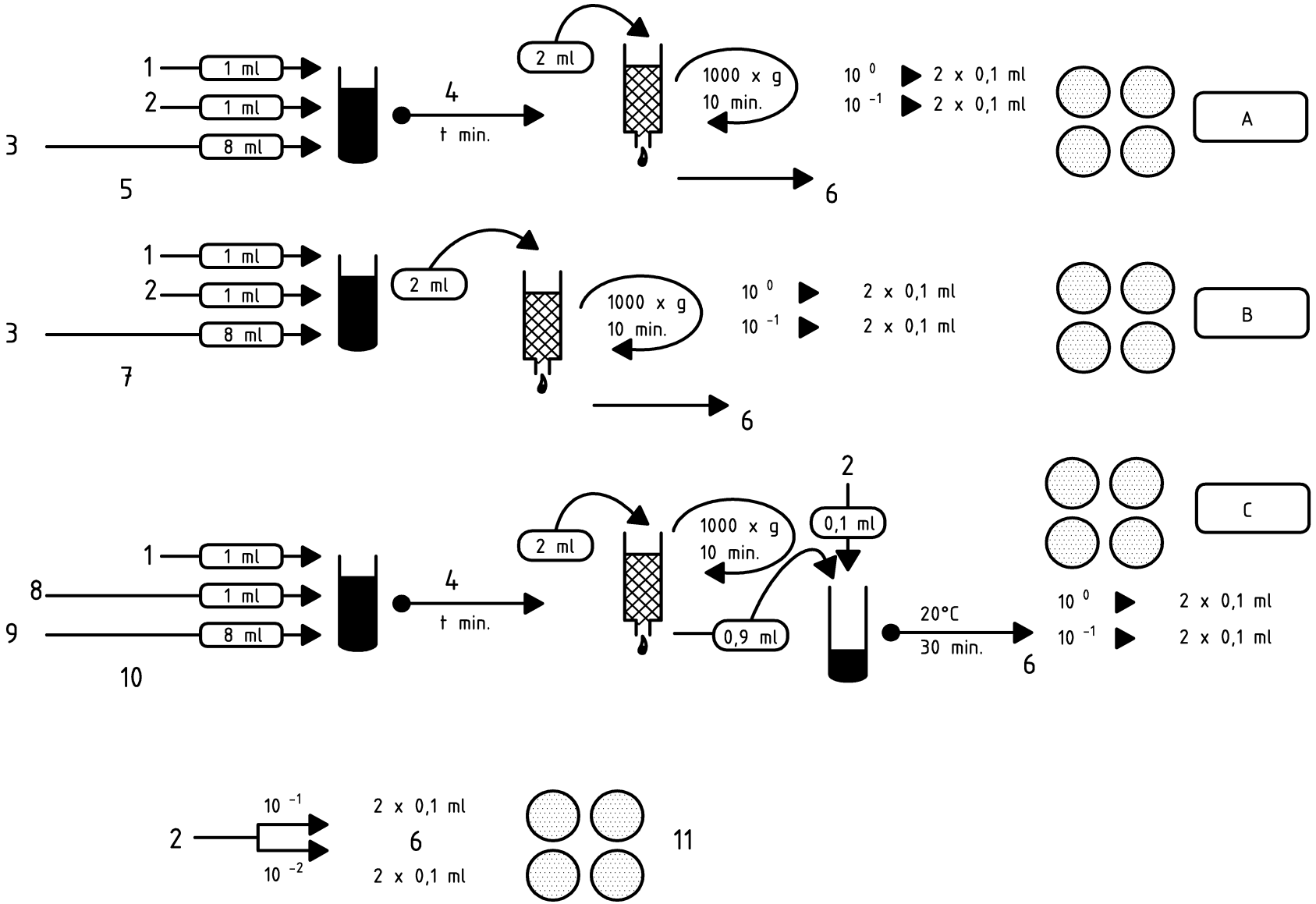
Figure A.2 — Dilution neutralization method (validation)



Key

1 Product
2 Interfering substance
3 Bacteriophage test suspension
4 Test temperature
5 Contact time
6 Plaque test
7 N ($8 \times 10^8 - 3 \times 10^9$ PFU/ml)

Figure A.3 — Molecular sieving method (actual test)



Key

1 Interfering substance
2 Diluted phage suspension
3 Hard water
4 Test temperature
5 Experimental conditions control
6 Plaque test
7 Phage non retention control
8 SM-buffer
9 Product
10 Product elimination control
11 N_v ($2 \times 10^4 - 1 \times 10^5$ PFU/ml)

Figure A.4 — Molecular sieving method (validation)

Annex B
(informative)

Neutralizers

It is recommended to use the following neutralizer first:

- M17-broth (see 5.2.2.3) supplemented with a volume fraction of 3 % polysorbate 80 [12], a mass fraction of 0,3 % sodium thiosulfate, a mass fraction of 0,3 % L-cysteine, and a mass fraction of 0,3 % L-histidine;
- the solution should be formulated with double-concentrated M17-broth (see 5.2.2.3) in the following way:
  - double-conc. M17-broth (see 5.2.2.3) 50 ml;
  - polysorbate 80 [13] 3,0 ml;
  - sodium thiosulfate 0,3 g;
  - L-cysteine 0,3 g;
  - L-histidine 0,3 g;
  - water (see 5.2.2.2) fill up to a volume of 100 ml in a volumetric flask;
  - sterilize by membrane filtration (0,45 µm pore size) (see 5.3.2.7).

Where a neutralizer is specified, any of the following may be used:

- lecithin 3 g/l, polysorbate 80 [13] 30 g/l, sodium thiosulfate 5 g/l, L-histidine 1 g/l, saponine 30 g/l in phage diluent (see 5.2.2.6) or in phosphate buffer 0,0025 mol/l (0,25 N to a volume fraction of 1 %);
- phosphate buffer 0,25 N:
  - KH_2PO_4 34 g;
  - water (see 5.2.2.2) 500 ml;
  - adjust to pH 7,2 ± 0,2 with 1 N NaOH;
  - water (see 5.2.2.2) fill up to 1 000 ml;
  - sterilize at $121\ ^{+3}_{0}$ °C for 15 min;
  - fresh egg yolk diluted to a volume fraction of 5 % or a volume fraction of 0,5 %;
  - 30 g/l polysorbate 80 [13] 4 g/l sodium lauryl sulfate, lecithin 3 g/l;
  - a volume fraction of 5 % fresh egg yolk, 40 g/l polysorbate 80;

12) Analytical quality, non-hydrolized in accordance with European Pharmacopoeia volume 1. TWEEN 80® 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.

- a volume fraction of 7 % ethylene oxide condensate of fatty alcohol, 20 g/l lecithin, a volume fraction of 4 % polysorbate 80;
- a volume fraction of 4 % ethylene oxide condensate of fatty alcohol, 4 g/l lecithin;
- 30 g/l polysorbate 80, lecithin 3 g/l, L-histidine 1 g/l;
- glycine as a function of concentration of product;
- 30 g/l polysorbate 80, lecithin 3 g/l;
- phospholipid emulsion 100 g/l at 50 mg/ml;
- sodium thioglycolate at 0,5 g/l or 5 g/l;
- L-cysteine at 0,8 g/l or 1,5 g/l;
- thiomalic acid at a volume fraction of 0,075 % (adjusted to pH 7 with NaOH);
- sodium thiosulfate at 5 g/l;
- catalase or peroxidase: One unit of these enzymes catalyses the decomposition of 1,4 moles of hydrogen peroxide per min at 25 °C and at pH 7;
- polysorbate 80 [13)] 30 g/l, saponin 30 g/l, L-histidine 1 g/l, L-cysteine 1 g/l.

NOTE The preceding list is not exhaustive and other media can be tried.

13) Analytical quality, non-hydrolized in accordance with European Pharmacopoeia volume 1. TWEEN 80® 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.

Annex C
(informative)

Example of a typical test report

Virucidal activity against bacteriophages in general use conditions:

a) identification of the test laboratory;

b) identification of the sample:

- ¾ name of the product: Z;
- ¾ batch number: 91-71-51;
- ¾ manufacturer: Centipede Formulations Inc;
- ¾ date of delivery: 1991-02-11;
- ¾ storage conditions: Room temperature and darkness;
- ¾ product diluent recommended by the manufacturer for use: Potable water;
- ¾ active substance(s) and its (their) concentration(s) (optional): Not indicated;

c) experimental conditions:

- ¾ period of analysis: 1991-02-20 to 1991-03-12;
- ¾ product diluent used during the test: hard water; 300 mg/kg $CaCO_3$;
- ¾ product test concentrations: a volume fraction of 0,5 ; 0,75 ; 1 %;
- ¾ appearance product dilutions: Colourless, clear product solution;
- ¾ contact time: t = 15 min ± 10 s;
- ¾ test temperature: = 20 °C ± 1 °C;
- ¾ interfering substance: a volume fraction of 1 % whey;
- ¾ stability of the mixture/interfering substance and product diluted in hard water: Precipitate absent throughout the test;
- ¾ temperature of incubation: 30 °C ± 1 °C;
- ¾ identification of the bacteriophage strains used:
  - bacteriophage P001 (DSM 4262);
  - bacteriophage P008 (DSM 10567);
  - both virulent for *Lactococcus lactis* subsp. *lactis* F7/2 (DSM 4366);

d) operation procedure:

¾ method used: dilution-neutralization;

neutralizer : Polysorbate 80[14)], 30 ml/l;

sodium thiosulfate, 3 g/l;

L-cysteine 3 g/l;

L-histidine, 3 g/l;

in M17-broth;

sterilized by membrane filtration (0,45 µm pore size);

e) test results (see Tables C.1 and C.2);

f) conclusion:

¾ according to EN xxx (date of edition), the batch 91.71.51 of product Z, when diluted at a volume fraction of …% in hard water, possesses virucidal activity against bacteriophage in 15 min at 20 °C in the presence of a volume fraction of 1 % acidic whey derived from low-fat skim milk for the reference bacteriophages P001 (DSM 4262) and P008 (DSM 10567), both lytic for Lactococcus lactis subsp. lactis F7/2 (DSM 4366);

g) locality, date and identified signature.

NOTE The test product, batch No. and manufacturer are given as imaginary examples only.

14) Analytical quality, non-hydrolized in accordance with European Pharmacopoeia volume 1. TWEEN 80® 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.

Table C.1 — Test results (for dilution neutralization method)

Test Phage (see 5.2.1)		**Validation test** (see A.4.1)				**Bacteriophage test suspension** (see 5.5.3)	**Product:**..... **Test procedure at concentration** (see 5.7.2.2.2) **Concentration given in**: ☑ % (v/v) ☐ ppm		
		Diluted phage suspension (see clause A.2)	**(A)**	**(B)**	**(C)**	**(N)**	**0,50**	**0,75**	**1,00**
P 0 0 1	P_c	(10^{-1}): = 200; = 200	(10^{0}): = 200; = 200	(10^{0}): 105; 95	(10^{0}): 103; 97	(10^{-6}): 96; 104	(10^{0}): = 200; = 200	(10^{0}): 19; 21	(10^{0}): 2; 1
	P_c	(10^{-2}): 52; 48	(10^{-1}): 51; 49	(10^{-1}): 11, 9	(10^{-1}): 10, 10	(10^{-7}): 9; 11	(10^{-1}):= 200; =200	(10^{-1}): 1; 3	(10^{-1}): 0; 0
		N_v : 5,0 x 10^4	**A : 5,0 x 10^3**	**B : 1,0 x 10^3**	**C : 1,0 x 10^3**	**N : 1,0 x 10^9**	**N_n : = 2,0 x 10^4**	**N_n : 2,0 x 10^2**	**N_n : = 1,5 x 10^2**
							N_a : = 1,0 x 10^6	**N_a : 1,0 x 10^4**	**N_a : = 7,5 x 10^3**
							R : = 1,0 x 10^2	**R : 1,0 x 10^4**	**R : = 1,3 x 10^4**
P 0 0 8	P_c	(10^{-1}): =300; =300	(10^{0}): =300; =300	(10^{0}): 102; 98	(10^{0}): 104; 96	(10^{-6}): 98; 102	(10^{0}): =300; = 300	(10^{0}): 24; 22	(10^{0}): 1; 0
	P_c	(10^{-2}): 51; 49	(10^{-1}): 55; 45	(10^{-1}): 12, 8	(10^{-1}): 11, 9	(10^{-7}): 10; 10	(10^{-1}): = 300; = 300	(10^{-1}): 1; 3	(10^{-1}): 0; 0
		N_v : 5,0 x 10^4	**A : 5,0 x 10^3**	**B : 1,0 x 10^3**	**C : 1,0 x 10^3**	**N : 1,0 x 10^9**	**N_n : = 3,0 x 10^4**	**N_n : 2,0 x10^2**	**N_n : = 1,5 x 10^2**
							N_a : = 1,5 x 10^6	**N_a : 1,0 x 10^4**	**N_a : = 7,5 x 10^3**
							R : = 6,6 x 10^1	**R : 1,0 x 10^4**	**R : = 1,3 x 10^4**

$N_a = 50 \cdot N_n$

$R = (N / N_a) \times 0,1$

P_c : Number of PFU per individual plate

N : Number of PFU/ml in the bacteriophage test suspension (see 5.5.3)

N_v : Number of PFU/ml of the diluted phage suspension (see A.2)

A : Number of PFU/ml in the experimental conditions control test mixture (see A.4.1)

B : Number of PFU/ml in the neutralizer toxicity control mixture (see A.4.1)

C : Number of PFU/ml in the dilution neutralization control mixture (see A.4.1)

N_n : Number of PFU/ml in the neutralized mixture (see A.4.1)

N_a : Number of PFU/ml in the test mixture (see 5.7.2.2.2)

R : Reduction in phage activity for validation

Table C.2 — Test results (for molecular sieving method)

Test phage (see 5.2.1)		**Validation test** (see A.4.1)				**Bacteriophage test suspension** (see 5.5.3)	**Product**: ..... **Test procedure at concentration** (see 5.7.2.2.2) Concentration given in: ☑ volume fraction in % ☐ 10^{-6}		
		Diluted phage suspension (see clause A.2)	**(A)**	**(B)**	**(C)**	**(N)**	**0,50**	**0,75**	**1,00**
P 0 0 1	P_c	(10^{-1}): = 200; = 200	(10^{0}): = 200, = 200	(10^{0}): = 200; = 200	(10^{0}): = 200; = 200	(10^{-6}): 95; 105	(10^{-1}): = 200; = 200	(10^{-1}): 190; 210	(10^{-1}): 8; 12
	P_c	(10^{-2}): 52; 48	(10^{-1}): 54; 46	(10^{-1}): 51; 49	(10^{-1}): 52; 48	(10^{-7}): 9; 11	(10^{-2}):= 200; = 200	(10^{-2}): 7; 13	(10^{-2}): 0; 0
		N_V : 5,0 x 10^4	***A*** **: 5,0 x 10^3**	***B*** **: 5,0 x 10^3**	***C*** **: 5,0 x 10^3**	***N*** **: 1,0 x 10^9**	**N_a : = 2,0 x 10^5**	**N_a : 2,0 x 10^4**	**N_a : = 1,5 x 10^3**
							R **: = 5,0 x 10^2**	***R*** **: 1,0 x 10^4**	***R*** **: = 6,7 x 10^4**
P 0 0 8	P_c	(10^{-1}): = 300; = 300	(10^{0}): = 300; = 300	(10^{0}): = 300; = 300	(10^{0}): = 300; = 300	10^{-6}): 99; 101	(10^{-1}): = 300; = 300	(10^{-1}): 105; 95	(10^{-1}): 19; 21
	P_c	(10^{-2}): 51; 49	(10^{-1}): 55, 45	(10^{-1}): 50; 50	(10^{-1}): 52; 48	(10^{-7}): 9; 11	(10^{-2}): = 300; = 300	(10^{-2}): 8; 12	(10^{-2}): 1; 2
		N_V : 5,0 x 10^4	***A*** **: 5,0 x 10^3**	***B*** **: 5,0 x 10^3**	***C*** **: 5,0 x 10^3**	***N*** **: 1,0 x 10^9**	**N_a : = 3,0 x 10^5**	**N_a : 1.0 x 10^4**	**N_a : 2,0 x 10^3**
							R : = 3,3 x 10^2	**R : 1,0 x 10^4**	**R : 5,0 x 10^4**

$R = (N / N_a) \cdot 0,1$

P_C : Number of PFU per individual plate

N : Number of PFU/ml in the bacteriophage test suspension (see 5.5.3)

N_V : Number of PFU/ml of the diluted phage suspension (see clause A.2) for validation

A : Number of PFU/ml in the experimental conditions control test mixture (see A.4.2)

B : Number of PFU/ml in the phage non retention control mixture (see A.4.2)

C : Number of PFU/ml in the product elimination control mixture (see A.4.2)

N_a : Number of PFU/ml in the test mixture (see 5.7.2.3.2)

R : Reduction in phage activity

Annex D
(informative)

Information on the application and interpretation of European standards on chemical disinfectants and antiseptics

CEN/TC 216 would like to draw the attention of the reader of this standard to the agreements which were reached concerning the relationship between this standard and future standards.

This information should be followed when using the European Standards on chemical disinfectants and antiseptics.

D.1 General guidelines for the application and interpretation of test methods in accordance with European Standards for chemical disinfectants and antiseptics

a) all "use recommendations" for chemical disinfectant and antiseptic products should be supported by results of bactericidal, fungicidal, sporicidal and virucidal European Standard tests which are appropriate to the intended field and method of application;

b) to achieve this, chemical disinfectant and antiseptic products should be subjected to a specified programme of testing which will include phase 1, phase 2 step 1 and phase 2 step 2 tests, except for situations as given in points e), f) and g);

c) "use recommendations" may be supported by results of phase 3 tests which are appropriate to the intended field and method of application;

d) the various steps and phases are defined as follows:

- ¾ phase 1 suspension tests for the basic activity of the product;
- ¾ phase 2 step 1 suspension tests under conditions representative of practical use;
- ¾ phase 2 step 2 other laboratory tests eg handwash, handrub and surface tests simulating practical conditions;
- ¾ phase 3 field tests under practical conditions;

e) it is accepted that for certain applications, the phase 2 step 1 and phase 2 step 2 tests may provide sufficient information for the particular application and that additional phase 1 tests may not be relevant.

For applications where phase 2 step 1 and phase 2 step 2 tests without phase 1 tests are used to support use recommendations, the justification for omitting phase 1 tests should be given. Such applications will be indicated either in the standard itself or in the additional standard which specifies guidelines for the application and interpretation of the tests;

f) it is accepted that for certain applications, the phase 2 step 1 suspension tests may provide sufficient information for the particular application and that additional phase 2 step 2 tests may not be relevant.

For applications where phase 2 step 1 tests without phase 2 step 2 tests are used to support use recommendations the justification for omitting phase 2 step 2 tests should be given. Such applications will be indicated either in the standard itself or in the additional standard which specifies guidelines for the application and interpretation of the tests;

g) its is accepted that for certain applications the phase 2 step 2 together with phase 1 tests may provide sufficient information for the particular application and that additional phase 2 step 1 tests may not be relevant.

   For applications where phase 2 step 2 tests without phase 2 step 1 tests are used to support product claims, the justification for omitting phase 2 step 1 tests should be given. Such applications will be indicated either in the standard itself or in the additional standard which specifies guidelines for the application and interpretation of the tests;

h) all bactericidal, fungicidal and sporicidal claims for "bioactive substances" should be supported by appropriate phase 1 tests.

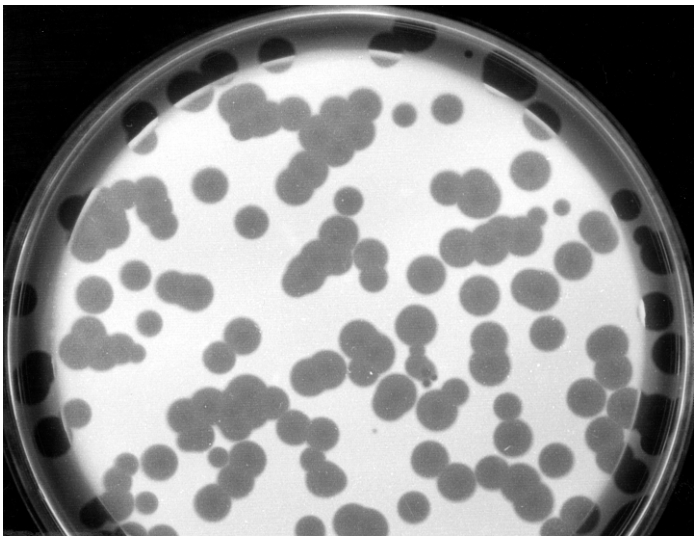
D.2 Guide to interpretation of tests for chemical disinfectants and antiseptics

A separate standard (or standards) which will be used as a "Guide to interpretation of tests for chemical disinfectants and antiseptics" will be prepared after the standard test methods have been agreed ; the purpose of this standard will be to specify in detail the relationship of the various tests to one another and to use recommendations.

Annex E
(informative)

Example of plaques from lysates of phages P001 and P008

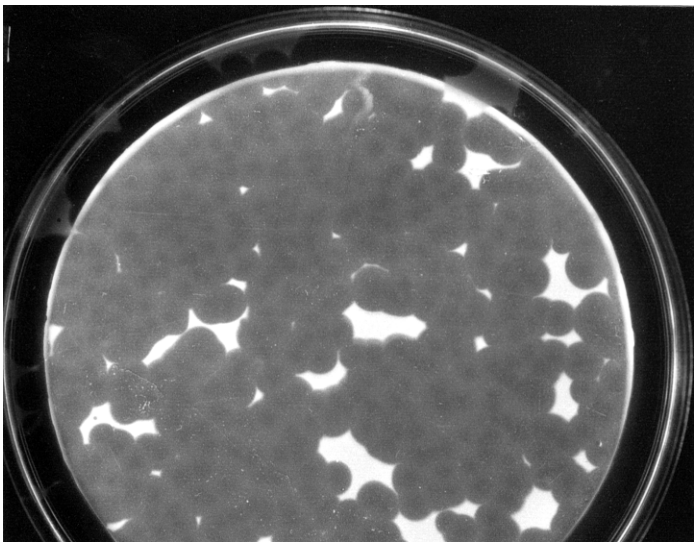
In 5.5.2 plaques showing confluent lysis are selected. This annex shows from which serial dilution step they are obtained.



0,1 ml from 10^{-5} serial dilution:

NOT GOOD for harvesting,

titer will be far too low.

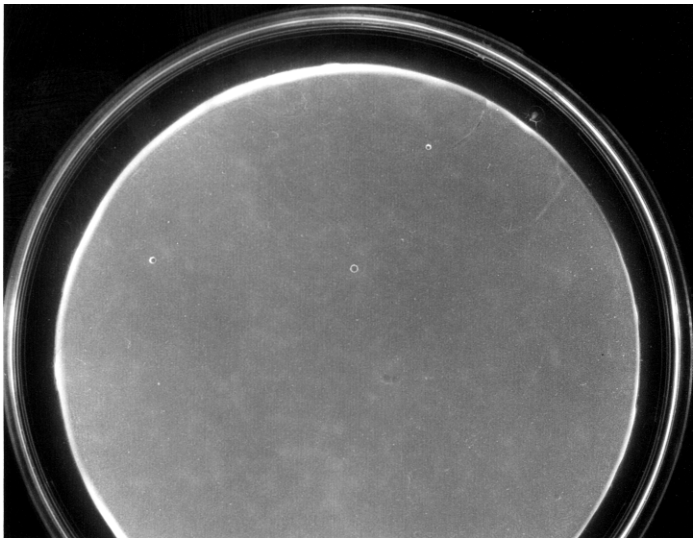


0,1 ml from 10^{-4} serial dilution:

CONFLUENT LYSIS, GOOD

for harvesting, titer will be

high (10^{10} / 10^{11} UFP per ml).

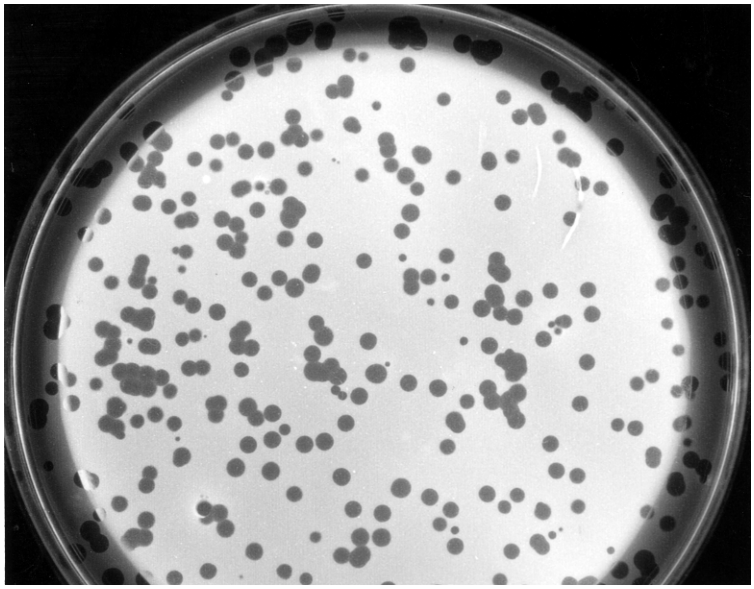


0,1 ml from 10^{-3} serial dilution:

NOT GOOD for harvesting,

titer will be far too low.

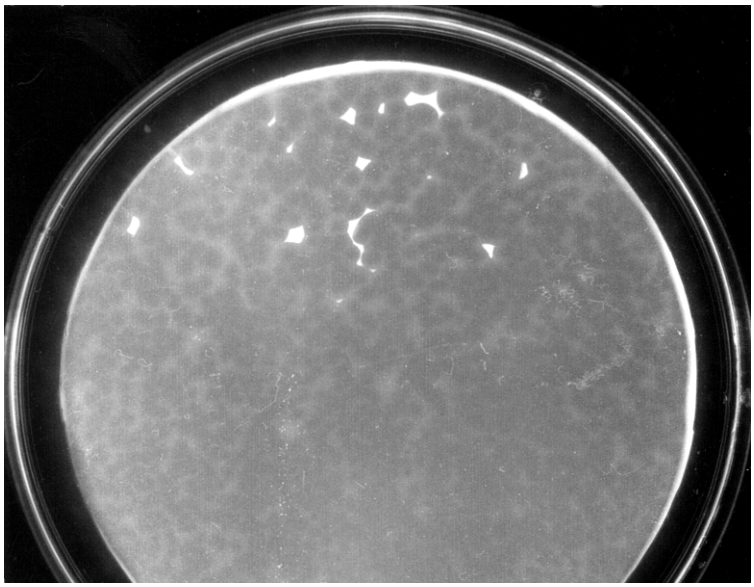
Figure E.1 — Example of plaques from the lysate of phage P001



0,1 ml from 10^{-5} serial dilution:

NOT GOOD for harvesting,

titer will be far too low.

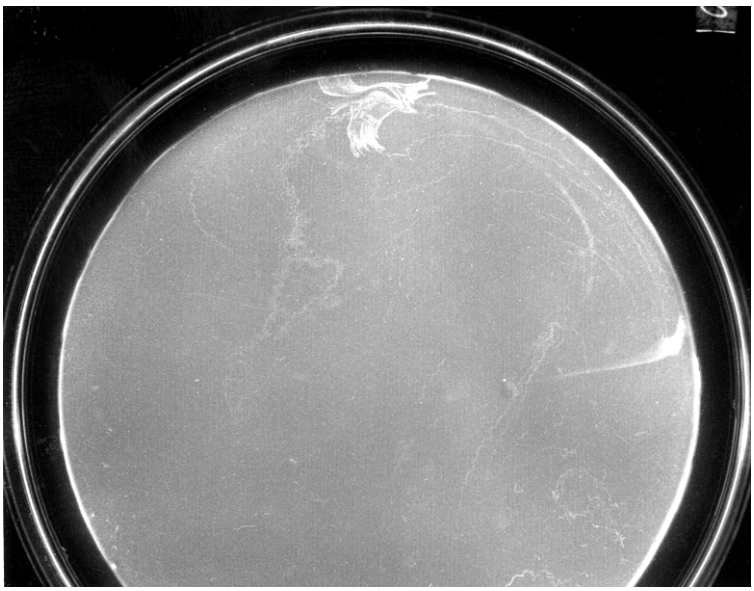


0,1 ml from 10^{-4} serial dilution:

CONFLUENT LYSIS, GOOD

for harvesting, titer will be

high (10^{10} / 10^{11} UFP per ml).



0,1 ml from 10^{-3} serial dilution:

NOT GOOD for harvesting,

titer will be far too low.

Figure E.2 — Example of plaques from the lysate of phage P008

Bibliography

[1] EN 1040:1997, *Chemical disinfectants and antiseptics – Basic bactericidal activity – Test method and requirements (phase 1).*

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