BS EN 14675:2015

BSI Standards Publication

Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of virucidal activity of chemical disinfectants and antiseptics used in the veterinary area — Test method and requirements (Phase 2, step 1)

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National foreword

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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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ISBN 978 0 580 79145 1

ICS 11.080.20; 11.220

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This British Standard was published under the authority of the Standards Policy and Strategy Committee on 30 April 2015.

Amendments issued since publication

Date	Text affected

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 14675

April 2015

ICS 71.100.35

Supersedes EN 14675:2006

English Version

Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of virucidal activity of chemical disinfectants and antiseptics used in the veterinary area - Test method and requirements (Phase 2, step 1)

Antiseptiques et désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité virucide des antiseptiques et des désinfectants chimiques utilisés dans le domaine vétérinaire - Méthode d'essai et prescriptions (phase 2, étape 1)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der viruziden Wirkung chemischer Desinfektionsmittel und Antiseptika für den Veterinärbereich - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

This European Standard was approved by CEN on 14 February 2015.

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Foreword

This document (EN 14675:2015) 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of an identical text or by endorsement, at the latest by October 2015 and conflicting national standards shall be withdrawn at the latest by October 2015.

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This document supersedes EN 14675:2006.

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Introduction

This European Standard specifies a suspension test for establishing whether a chemical disinfectant or antiseptic has or does not have a virucidal activity in the areas described in the scope.

This laboratory test takes into account practical conditions of application of the product including contact time, temperature, test organisms and interfering substances, i.e. conditions which may influence its action in practical situations.

The conditions are intended to cover general purposes and to allow reference between laboratories and product types. Each utilization concentration of the chemical disinfectant or antiseptic found by this test corresponds to defined experimental conditions. However, for some applications the recommendations of use of a product may differ and therefore additional test conditions need to be used.

1 Scope

This European Standard specifies a test method and the minimum requirements for virucidal activity of chemical disinfectant and antiseptic products that form a homogeneous, physically stable preparation when diluted with hard water or – in the case of ready-to-use-products – with water. Products can only be tested at a concentration of 80 % or less as some dilution is always produced by adding the test organisms and interfering substance.

This European Standard applies to products that are used in the veterinary area, i.e. in the breeding, husbandry, production, transport and disposal of all animals except when in the food chain following death and entry to the processing industry.

NOTE 1 The method described is intended to determine the virucidal activity of commercial formulations or active substances under the conditions in which they are used.

NOTE 2 This method corresponds to a phase 2 step 1.

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 and the following apply:

3.1
cytotoxicity
morphological alteration of cells and/or their destruction or their reduced sensitivity to virus multiplication caused by the product

3.2
plaque forming units
PFU
number of infectious virus particles per unit volume (ml)

3.3
reference test for virus inactivation
test with a defined reagent (e.g. formalin) instead of a product for the internal control of the test

Note 1 to entry: Results of reference virus inactivation test should be within limits for validating the method.

3.4
reference virus suspension
virus suspension of a defined virus strain which is not passaged more than 10 times, is maintained in national culture collection centres and kept in small volumes (less than 1 ml) at a temperature of -70°C or preferably at about -196 °C under liquid nitrogen

Note 1 to entry: Stock virus suspensions are prepared from reference virus suspensions.

3.5
stock virus suspension
virus suspension of a defined strain that is multiplied in a suitable cell line which produces high virus titers, to obtain a virus suspension of the same characteristics as the reference virus suspension and kept in a small volume at a temperature of below 70 °C or preferably at about -196 °C over liquid nitrogen

3.6
test virus suspension
virus suspension that is used in the virucidal testing of the disinfectant

3.7
tissue culture infectious dose
$TCID_{50}$
viral dose that induces a cytopathic effect (CPE) (3.8) in 50 % of inoculated cell culture

3.8
viral cytopathic effect
CPE
morphological alteration of cells and/or their destruction as a consequence of virus multiplication

3.9
viral plaque
area of lysis formed in a cell monolayer under semisolid medium due to infection by and multiplication of a single infectious virus particle

3.10
virus titre
amount of infectious virus per unit volume present in a cell culture lysate or in a solution

4 Requirements

The product when diluted with hard water (5.2.2.3) or – in the case of ready-to-use products – with water (5.2.2.2) and tested in accordance with Table 1 and Clause 5 shall demonstrate at least a lg reduction in virus titre of 4. It is possible to test also the product as delivered (highest test concentration is 80 %).

Table 1 — Obligatory and additional test conditions

	Obligatory conditions	Additional conditions
Test organism	*Bovine enterovirus* Type 1 (ECBO)	
Test temperature [a]	10 °C	4 °C, 20 °C or 40 °C
Contact time [b]	30 min	1 min, 5 min and 60 min
Interfering substance–low level soiling [c]	3,0 g/l bovine albumin	
Interfering substance-high level soiling [c]	10 g/l bovine albumin plus 10 g/l yeast extract	

[a] Allowed deviation ± 1 °C.

[b] The allowed deviation for each chosen contact time is ± 10 s, except for ≤1 min for which it is ± 5 s.

[c] To be chosen according to practical applications.

5 Test method

5.1 Principle

5.1.1 A sample of the product as delivered and/or diluted with hard water (or water for ready to use products) is added to a test suspension of virus in a solution of an interfering substance. The mixture is maintained at 10 °C ± 1 °C for 30 min ± 10 s (obligatory test conditions).

At the end of the contact time, 0,5 ml of virus/disinfectant mixture is taken. The virucidal activity is immediately suppressed by dilution in ice-cold diluent. A dilution series with a factor of ten is prepared in an ice-cold medium held in an ice bath for 10 min. Pipettes shall be changed after each dilution to avoid carry-over of virus.

The dilutions are transferred into cell culture units (wells of microtitre plates) containing suspended cells. Eight series units shall be inoculated with each dilution. After incubation, the titre of infectivity is calculated. The titration results of quantal tests shall show dilution steps with the percentage of positive results (presence of CPE or plaques) lying between 100 % and 0 %. The values are calculated according to Spearman and Kärber (see Annex C).

Values of virus inactivation are calculated from differences of virus titres before and after treatment with the product.

5.1.2 Additional and optional contact times and temperatures are specified.

5.2 Materials and reagents

5.2.1 Test virus

The virucidal activity shall be evaluated using the following strain:

— Bovine enterovirus Type 1 (Enteric Cytopathogenic Bovine Orphan Virus – ECBO) ATCC VR-248[1)].

NOTE 1 Bovine enterovirus Type 1, strain ECBO, is selected as the model virus for the large Genus Picornavirus. The Genus Picornavirus includes many clinically important virus species, for example Coxsackie A and B, and enteric cytopathogenic human orphan (ECHO). Some of these viruses are of primary importance and therefore a constant risk for animals in the veterinary area. Moreover, they have a high resistance to chemicals, are acid-stable (except inter alia rhinovirus, aphtovirus) and are unaffected by lipid solvents such as ether, and most detergents or quaternary ammonium products.

NOTE 2 It is the model virus for all applications namely for disinfection of instruments and surfaces and post-contamination treatment of post-mortem rooms, kennels and for animal accommodation.

NOTE 3 Due to large differences of resistance against physical and chemical influences between and within different virus groups, the testing of all viruses against any particular chemical disinfectant or antiseptic is financially impossible. Therefore, in this European Standard, testing is restricted to only one so called ‘model virus’ that has been selected on the basis of the present knowledge as a representative example of virus tenacity and of important clinical relevance in the veterinary area. If a chemical disinfectant or antiseptic shows virucidal activity according to the requirements of this European Standard, it can be considered for a phase 2 step 2 test.

If improvements in the methodology of virus multiplication, virus infectivity or cytoxicity reduction of products are elaborated, they may be used in parallel with the methodology described in this method to show the improvement.

1) ATCC VR-248, is a strain supplied by the American Type Culture Collections. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named. A corresponding strain supplied by other culture collections may be used if they can be shown to lead to the same results.

5.2.2 Culture media and reagents

5.2.2.1 General

All weights of chemical substances given in this European Standard refer to the anhydrous salts. 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 virological purposes. They shall be free from substances that are toxic or inhibitory to the test organism.

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

For each culture medium and reagent a time limitation for use shall be fixed.

Commercial ready-to-use products and culture media can be purchased, if they comply with the required specifications.

5.2.2.2 Water

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

Sterilize in the autoclave [5.3.2.1 a)].

NOTE 1 Sterilization is not necessary if the water is used, e.g. for preparation of culture media, and subsequently sterilized.

NOTE 2 See 5.2.2.3 for the procedure to prepare hard water.

If distilled water of adequate quality is not available, water for injections (see bibliographic reference [3]) can be used.

5.2.2.3 Hard water for dilution of products

For the preparation of 1 l of hard water, the procedure is as follows:

— prepare solution A: dissolve 19,84 g magnesium chloride ($MgCl_2$) and 46,24 g calcium chloride ($CaCl_2$) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration [5.3.2.1 c)] or in the autoclave [5.3.2.1 a)]. Autoclaving – if used – may cause a loss of liquid. In this case make up to 1 000 ml with water (5.2.2.2) under aseptic conditions. Store the solution in the refrigerator (5.3.2.16) for no longer than one month;

— prepare solution B: dissolve 35,02 g sodium bicarbonate ($NaHCO_3$) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration [5.3.2.1 c)]. Store the solution in the refrigerator (5.3.2.16) for no longer than one week;

— place 600 ml to 700 ml of water (5.2.2.2) in a 1 000 ml volumetric flask (5.3.2.13) and add 6,0 ml of solution A, then 8,0 ml of solution B. Mix and dilute to 1 000 ml with water (5.2.2.2). The pH of the hard water shall be 7,0 $\pm$ 0,2, when measured at 20 °C $\pm$ 1 °C (5.3.2.4). 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 product test solutions (5.4), the addition of the product to the hard water produces a different final water hardness in each test tube. In any case the final hardness is lower than 375 mg/l of calcium carbonate ($CaCO_3$) in the test tube.

5.2.2.4 Interfering substance

5.2.2.4.1 General

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 its final concentration in the test.

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

NOTE The term 'interfering substance' is used even if it contains more than one substance.

5.2.2.4.2 Low level soiling (Bovine albumin solution)

Dissolve 3 g of bovine albumin (Cohn fraction V for Dubos Medium) in 90 ml of water (5.2.2.2) in a 100 ml volumetric flask (5.3.2.13). Make up to the mark with water (5.2.2.2).

Sterilize by membrane filtration [5.3.2.1 c)]. Keep in a refrigerator (5.3.2.16) and use within one month.

The final concentration of bovine albumin in the test procedure (5.5) is 3 g/l.

5.2.2.4.3 High level soiling (mixture of bovine albumin solution with yeast extract)

Dissolve 50 g yeast extract powder in 150 ml of water (5.2.2.2) in a 250 ml volumetric flask (5.3.2.13) and allow foam to collapse. Make up to the mark with water (5.2.2.2). Transfer to a clean dry bottle and sterilize in the autoclave [5.3.2.1 a)]. Allow to cool to 20 °C ± 1 °C.

Pipette 25 ml of this solution into a 50 ml volumetric flask (5.3.2.13) and add 10 ml of water (5.2.2.2). Dissolve 5 g of the bovine albumin fraction V in the solution with shaking and allow foam to collapse. Make up to the mark with water (5.2.2.2) sterilize by membrane filtration [5.3.2.1 c)], keep in a refrigerator (at 2 °C to 8 °C) (5.3.2.16) and use within one month.

The final concentration in the test procedure (5.5) is 10 g/l yeast extract and 10 g/l bovine albumin.

5.2.2.5 Antibiotic suspension

Chemicals

50 million units penicillin-G	(e.g. Sigma PEN-K [2)])
50 g streptomycin sulphate (approx. equal to 750 i.u./mg)	(e.g. Sigma Cat: 56501 [2)])
500 000 units mycostatin	(e.g. Nystatin: E R Squibb 591502 [2)])
Water (5.2.2.2) to 2,5 l.	

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

Preparation

Dissolve vial contents in water (5.2.2.2) and make 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

5.2.2.6 Antibiotics-Trypsin-Versene® (ATV) 10 × Concentrate

Chemicals

Sodium chloride (NaCl)	80 g
Potassium chloride (KCl)	4 g
Glucose	10 g
Trypsin	5 g (e.g. Difco 1:250 Cat No: 0152-15-9 [2)])
Versene® (EDTA)	2 g (e.g. Koch-Light Cat No: 0012424-/B3 [2)])
0,2 % Phenol Red solution in water (5.2.2.2)	1 000 ml

Preparation

Make up solution to 1 l with water (5.2.2.2) [omitting the sodium bicarbonate and antibiotics (5.2.2.5 and 5.2.2.6)].

Filter through a membrane filter (0,22 μm pore size) using positive pressure.

Add 76,2 ml 7,5 % sodium bicarbonate solution in water (5.2.2.2) and 5 ml antibiotics (5.2.2.5) to the sterile filtrate.

Dispense aseptically into 50 ml aliquots and store at - 20 °C.

For use add 50 ml of thawed ATV 10 × concentrate to 450 ml of water (5.2.2.2).

The working strength solution contains trypsin 0,05 %, versene 0,02 %, pH = 7,8.

5.2.2.7 Dulbecco's Phosphate Buffered Saline pH 7,2 – 7,4 (PBS)

Sodium chloride (NaCl)	8,00 g
Potassium dihydrogen phosphate (KH_2PO_4)	0,12 g
Disodium hydrogen phosphate anhydrous (Na_2HPO_4)	0,91 g
Potassium chloride (KCl)	0,2 g
Water (5.2.2.2)	to 1 000 ml

The solution may be sterilized by autoclaving at 121 °C for 15 min.

5.2.2.8 Earle's balanced salt solution (BSS), 10 × concentrated

Sodium chloride (NaCl)	68,0 g
Magnesium sulphate heptahydrate ($MgSO_4$ $7H_2O$)	2,0 g
Sodium dihydrogen orthophosphate monohydrate (NaH_2PO_4 H_2O)	1,4 g
Potassium chloride (KCl)	4,0 g
Calcium chloride ($CaCl_2$)	2,0 g
Glucose	10,0 g

The calcium chloride ($CaCl_2$) 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 a final volume of 1 000 ml with water (5.2.2.2).

This 10x solution is sterilized by membrane filtration [5.3.2.1 c)] and shall be stored at 4 °C ± 1°C for 4 weeks. For use, the 10x solution is diluted 1:10 with sterile water (5.2.2.2) and buffered by the addition of 2,5 % of an 8,8 % sodium bicarbonate ($NaHCO_3$) solution.

5.2.2.9 Phenol red, 1 %

a) 10 g of alcohol-soluble phenol red is placed in a 100 ml beaker. Approximately 20 ml of 1N sodium hydroxide (NaOH) solution are added, mixed and allowed to stand for a few minutes;

b) the dissolved dye is transferred into a 1 000 ml volumetric flask (5.3.2.13);

c) additional 10 ml of 1N sodium hydroxide (NaOH) solution are added into the beaker. The dissolved material is transferred into the volumetric flask (5.3.2.13);

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

5.2.2.10 Eagle's minimum essential medium (MEM)

The following protocol is given as an example of procedure for the preparation of this cell culture nutrient medium. It may be also obtained from commercial sources. The concentration of amino acids and vitamins can be doubled to give "fortified" MEM.

The medium is prepared 10x concentrated and stored in the refrigerator (5.3.2.16). At the time of use glutamine and antibiotics (stored at - 20 °C) and Sodium Bicarbonate are added to the 1x solution.

a) Solution A

per litre 10x medium

L Arginine HCl	1,05 g
L-Histidine HCl	0,31 g
L Lysine HCl	0,58 g
L Tryptophane	0,10 g
L Phenylalanine	0,32 g
L Threonine	0,48 g
L Leucine	0,53 g

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

These amino acids are dissolved in 200 ml of 0.075N HCl [1,5 ml of commercial C.P. HCl (11,9 N) + 236,6 ml water (5.2.2.2) = 238,1 ml of 0.075N HCl] with gentle heating (80°C).

b) Solution B

per litre 10x medium

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 with heating at 80 °C.

c) Solution C

per litre 10x medium

Nìcotinamide	0,2 g
Pyridoxal	0,2 g
Thiamine	0,2 g
Pantothenic acid	0,2 g
Choline	0,2 g
l-Inositol	0,4 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 and stored at - 20 °C.

The solutions A, B and C are 10-fold concentrated preparations and can be stored in the refrigerator (5.3.2.16).

d) 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 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 °C.

e) Solution E

Dissolve 200 mg folic acid (crystalline) in 200 ml of 1x Earle’s BSS (5.2.2.8), pH = 7,4. The solution is dispensed in 10 ml amounts and stored at - 20 °C.

f) Glutamine solution, 3 %

Dissolve 12 g l glutamine in 400 ml of water (5.2.2.2) and sterilize by membrane filtration [5.3.2.1c)]. The solution is stored at - 20 °C, and 1 ml is added to each 100 ml of 1x MEM. This solution shall be added just at the time of use.

5.2.2.11 Preparation of the final mixture of 10x Eagle MEM

a) The following reagents are dissolved in Solution B:

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

1,4 g of sodium dihydrogen orthophosphate monohydrate (NaH_2PO_4 H_20) are dissolved in 55 ml of water (5.2.2.2) and added to the above pool.

b) Dissolve 10 g of glucose in 50 ml of water (5.2.2.2) and add 20 ml of a 1 % phenol red solution (5.2.2.9) to the pool.

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

Solution	per litre of 10x medium
C	10 ml
D	10 ml
E	10 ml

d) In a separate flask containing 160 ml of water (5.2.2.2), 2 g anhydrous calcium chloride are dissolved and then added to the pool with vigorous shaking.

e) 200 ml of Solution A are added to the pool and the volume is brought to approximately 950 ml with water (5.2.2.2); the mixture can be held in the refrigerator (5.3.2.16) overnight.

f) The total volume is brought to exactly 1 000 ml with water (5.2.2.2) and the solution is sterilized by membrane filtration [5.3.2.1 c)]. This 10x medium is stored at 4 °C.

5.2.2.12 Preparation of 1X Eagle MEM

For use, the 10x medium is diluted to 1x with sterile water (5.2.2.2), and 1 % of the 3 % glutamine solution (5.2.2.10, f) and 2,5 % of an 8,8 % sodium bicarbonate solution in water (sterilized by membrane filtration) are added.

a) A *growth medium* for cell multiplication is supplemented with 10 % FCS. Add 10 parts of FCS (5.2.2.13) to 90 parts of MEM.

b) A *maintenance medium* to maintain the cell culture metabolism without stimulation of cell proliferation is supplemented with 2 % FCS. Add 2 parts of FCS (5.2.2.13) to 98 parts of MEM.

5.2.2.13 Foetal calf serum (FCS)

FCS shall 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 Crystal violet solution

Mix 70 ml of methanol and 30 ml of water (5.2.2.2). Add 1 g of crystal violet and stir to dissolve. Filter with a 0,45 µ filter. Store at room temperature.

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 an autoclave [5.3.2.1 a)];

b) by dry heat in an hot air oven [5.3.2.1 b)].

5.3.2 Usual microbiological laboratory equipment[3] and, 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 $^{+5}_{0}$) °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;

c) for media sterilization, use suitable membrane filtration apparatus with filters of diameter 47 mm to 50 mm and membranes with 0,22 μ m pore size.

5.3.2.2 Water baths, capable of being controlled at 4 °C ± 1 °C and 10 °C ± 1 °C and at additional temperatures ± 1 °C (5.5.1).

5.3.2.3 CO_2 incubator, (95 % air, 5 % CO_2), capable of being controlled at either 36 °C ± 1 °C or 37 °C ± 1 °C.

5.3.2.4 pH-meter, having an inaccuracy of calibration of no more than ± 0,1 pH units at 20 °C ± 1 °C.

5.3.2.5 Inverted microscope, for reading cell cultures microscopically.

5.3.2.6 Stopwatch.

5.3.2.7 Shakers

a) Electromechanical agitator, e.g. Vortex® mixer[4]

b) Mechanical shaker

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

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

5.3.2.8 96-well microtitre plates, for cell culture use.

5.3.2.9 Containers: sterile test tubes, bottles or flasks of suitable capacity.

5.3.2.10 Magnetic stirrer, for keeping cells in suspension before seeding.

5.3.2.11 Petri dishes (plates), of size 30 mm to 100 mm.

5.3.2.12 Graduated sterile pipettes, of nominal capacities 10 ml and 1 ml and 0,1 ml or calibrated automatic pipette.

5.3.2.13 Volumetric flasks.

5.3.2.14 Biological safety cabinet, class II.

5.3.2.15 Centrifuge.

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

5.3.2.17 Freezer, for storage at temperature below or equal to -20 °C and -70 °C.

5.3.2.18 Six well plates for cell culture use.

5.4 Product test solutions

The concentration of the product test solution shall be 1,25 times the desired test concentration because it is diluted to 80 % during the test and the method validation (6.4). This does not apply to ready-to-use products.

Product test solutions shall be prepared in hard water (5.2.2.3) at a minimum of three different concentrations to include one concentration in the active range and one concentration in the non-active range. The product as received may be used as one of the product test solutions, in this case the highest tested concentration is 80 %.

Dilutions of ready-to-use products, i.e. products which are not diluted when applied, shall be prepared in water (5.2.2.2).

For solid products, dissolve the product as received by weighing at least 1,0 g $\pm$ 10 mg of the product in a volumetric flask (5.3.2.13) and filling up with hard water (5.2.2.3). Subsequent dilutions (lower concentrations) shall be prepared in volumetric flasks (5.3.2.13) on a volume/volume basis in hard water (5.2.2.3).

For liquid products, dilutions of the product shall be prepared with hard water (5.2.2.3) on a volume/volume basis using volumetric flasks (5.3.2.13).

The product test solutions shall be prepared freshly and used in the test within 2 h. They shall give a physically homogenous preparation, stable during the whole procedure. If during the procedure a visible inhomogenity appears due to the formation of a precipitate or flocculate (for example, through the addition of the interfering substances), it shall be recorded in the test report.

The concentration of the product stated in the test report shall be the desired test concentration. Record the test concentration in terms of mass per volume or volume per volume and details of the product sample as received.

5.5 Procedure for assessing the virucidal activity of the product

5.5.1 Experimental conditions (obligatory and additional)

Besides the obligatory temperature, contact time, interfering substances and test organisms, additional experimental conditions and test organisms can be selected according to the practical use considered for the product (Clause 4):

a) temperature (in °C):

   1) the obligatory and additional temperatures to be tested are specified in Clause 4, Table 1;

   2) the allowed deviation for each chosen temperature is ±1 °C;

b) contact time t (in min):

   1) the obligatory additional contact times to be tested are specified in Clause 4, Table 1;

   2) the allowed deviation for each chosen contact time is ± 10 s, except for 1 min for which it is ± 5 s;

c) interfering substance:

   the obligatory interfering substance to be tested is 3,0 g/l bovine albumin (5.2.2.4.2) for low level soiling or 10 g/l bovine albumin plus 10 g/l yeast extract (5.2.2.4.3) for high level soiling according to practical applications;

d) test organisms (5.2.1):

   the obligatory test organism is: *Bovine enterovirus* Type 1 (ECBO).

5.5.2 Preparation of the test virus suspension

The stock virus suspension is passaged to produce a test virus suspension. The virus is multiplied in a MDBK cell line which produces high titres of infectious virus particles. The cell debris shall then be separated by centrifugation (400 g for 15 min). This preparation is called ‘test virus suspension’. Other appropriate cell lines may be used if they can be shown to be equivalent to MDBK.

The test virus suspension is used undiluted for the test procedure.

It is suggested that the minimum titre of the virus suspension - determined by a quantal test (6.2) or by plaque test (6.3) - is at least $10^{7.5}$ $TCID_{50}$/ml. In any case, it shall be sufficiently high to at least enable a titre reduction of 4 lg to verify the method.

Stock virus suspensions are kept frozen at a temperature below –70 °C (5.3.2.17).

NOTE Reference virus suspensions may be obtained from national or international reference laboratories, e.g. American Type Culture Collection (ATCC).

5.5.3 Preparation of cell line

Cells for virus titration, if used as suspensions in quantal tests, shall be added to the dilutions of the test mixture in such a density as to enable the formation of a monolayer in at least two days in the cell control. Cell suspensions are prepared by trypsinizing (0,05 % trypsin (ATV) solution) (5.2.2.6) confluent cells and then making a cell suspension (3×10^5/ml) in medium (MEM 5.2.2.10) containing 10 % Foetal Calf Serum (FCS).

5.6 Infectivity assay

5.6.1 Predilution of viral suspension

These dilutions are performed in tubes or directly in microtitre plates by tenfold dilution steps with a volume of 0,2 ml of viral suspension plus a volume of 1,8 ml medium (MEM 5.2.2.10). Care shall be taken to change the pipettes for each dilution step.

5.6.2 Virus titration of cells in suspension on microtitre plates

Transfer 0,1 ml of each virus dilution into eight wells of a microtitre plate (5.3.2.8), beginning with the highest dilution. Add 0,1 ml of cell suspension containing a double concentration of foetal calf serum (FCS) (to obtain the final desired concentration) in such a density as to enable the formation of a monolayer (> 90 %) in at least two days in the cell control. Eight wells serve as the cell control and do not receive any viral suspension. Virus multiplication will induce appearance of CPE that can be read by using an inverted microscope (5.3.2.5) after appropriate incubation time.

5.6.3 Virus titration on monolayers of cells on microtiter plates

Transfer 0,1 ml of each virus dilution into eight wells of a microtiter plate (5.3.2.8) containing a confluent (> 90 %) cell monolayer without any medium. The last row of eight wells will receive 0,1 ml of culture medium and will serve as the cell control. After 1 h of incubation at 37 °C, 0,1 ml of MEM + 2 % FCS is added to each well. Change pipettes for tubes or wells when adding medium. Virus multiplication will induce appearance of CPE that can be read by using an inverted microscope (5.3.2.5) after appropriate incubation time.

5.6.4 Plaque assay

Virus titration can be carried out also by performing a plaque assay. Plastic tray wells (surface diameter 30 mm) with confluent cell sheets are washed once with Dulbecco's phosphate buffered saline (PBS) (5.2.2.7) and inoculated with 0,2 ml of serial dilutions of virus in MEM + 2 % FCS. Six wells are generally used per dilution. After absorption period of 1 h at 37 °C, during which the cell sheets are kept moist by tilting the dishes every 8 min to 10 min, the inoculum is removed and the cell sheets are washed once with PBS. Subsequently, the wells are overlaid with 3 ml of a mixture consisting of 2 % melted agarose or Oxoid[5)] purified agar and 2 times concentrated MEM (5.2.2.10) with 4 % FCS. The cultures are incubated for 5 d to 6 d at 37 °C in a CO2incubator (5.3.2.3). Plaques can be counted after addition of 2 ml of a second overlay with the same composition of the first and also containing 5 % of a 1:1 000 solution of neutral red and further incubation (in the dark) at 37 °C for 24 h to 48 h in a CO_2 incubator (5.3.2.3).

Counting can be performed also after addition of crystal violet (5.2.2.12). The cell sheets are fixed by adding 2 ml of 10 % trichloroacetic acid (TCA) over the agar overlay for 10 min to 15 min at room temperature. The agar overlay is then removed and 2 ml of crystal violet are added. After 10 min to 15 min at room temperature, the wells are extensively washed with water and the plaques (holes in the cell sheet) are counted (see C.2).

5) This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of this product. Corresponding products supplied by other manufacturers may be used if they can be shown to lead to the same results.

5.7 Virucidal test – preparation

5.7.1 General

Prior to testing, equilibrate all reagents (except the test virus suspension (5.5.2) which after thawing is kept on ice), i.e. interfering substance solutions (5.2.2.4), hard water (5.2.2.3), cell culture media, diluents and product test solution (5.4) to 10 °C ± 1 °C using the water bath (5.3.2.2). Check that the temperature of the reagents is stabilized.

Tubes (5.3.2.9) are filled with ice-cold MEM + 2 % FCS for the various titrations that shall be performed after the different contact times and placed in a water bath at 4 °C (5.3.2.2) or on crushed ice. Microtitre plates (5.3.2.8) are labelled for identification.

5.7.2 Test method

Pipette 1 ml of interfering substance (5.2.2.4) into a test tube. Add 1 ml of the test virus suspension (5.5.2).

Start a stopwatch immediately, mix [5.3.2.7a)] and place the test tube in a water bath (5.3.2.2) at (10 ± 1) °C for 2 min ± 10 s. At the end of this time, add 8 ml of the product test solutions (5.4). Restart the stopwatch, mix [5.3.2.7a)] and place the test tube in a water bath (5.3.2.2) at (10 ± 1) °C for 30 min ± 10 s.

Just before the end of the contact time, mix [5.3.2.7a)]. At the end of the contact time pipette 0,5 ml of the test mixture into 4,5 ml cold MEM (5.2.2.10) + 2 % FCS and put it immediately in a water bath (5.3.2.2) at (4 ± 1) °C or place on crushed ice. Prepare dilutions up to 10^{-8} by pipetting the diluted test mixture into a tube containing cold MEM + 2 % FCS in a water bath (5.3.2.2) at (4 ± 1) °C or placed on crushed ice. The dilutions shall be kept at 4 °C or on crushed ice until inoculation into microtitre plates (for cell culture inoculation).

The dilutions are transferred into cell culture units (wells of microtitre plates) containing suspended cells. Eight units shall be inoculated with each dilution. After incubation, the titre of infectivity is calculated. The titration results of quantal tests shall show dilution steps with the percentage of positive results (presence of CPE) lying between 100 % and 0 %. The values are calculated according to Spearman and Kärber (C.1).

The test can be also carried out by performing a plaque assay (5.6.4). The values will be calculated according to C.2.

5.8 Control of efficiency for suppression of disinfectant activity

5.8.1 Dilution in ice-cold medium

Immediately after preparation of the test mixture (5.7.2) mix and pipette 0,5 ml of the test mixture into 4,5 ml of ice-cold MEM + 2 % FCS. Mix and start the clock. Incubate the mixture in the ice bath for 30 min ± 10 s. Immediately prepare dilutions up to 10^{-6} (5.7.2), and titrate the virus (5.6 applies). This control is performed in parallel to the test (5.7.2)

The difference of titre with the test suspension shall be ≤ 0,5 lg.

5.8.2 Filtration technique

If the difference is > 0,5 lg a 1/100 ice-cold dilution can be tried or another technique shall be used, molecular sieving with SephadexTM LH 20 or ultrafiltration with Minicon® (Millipore) or with ready to use MicroSpinTM S 400 HR columns (GE Healthcare) (see B.4).

6 Calculation and expression of results

6.1 Protocol of the CPE result

The cell culture results are recorded as “0” for no CPE, and “1” (approximately 25 % of the cells show CPE) to “4” (all of cells with CPE) (quantal tests) or as number of plaques (plaque-forming units, PFU).

6.2 Calculation of infectivity titre ($TCID_{50}$)

Calculation of the virus titre shall be carried out by the Spearman-Kärber method. (A calculation example is given in C.1.)

6.3 Calculation of PFU

A practical way to calculate the number of plaque-forming units per millilitre (PFU/ml) is described in C.2.

Only replicas with non-confluent plaques shall be used for calculation. PFU should be calculated using three replicas. If replicas from 2 dilutions fall within this range, calculate the number of PFU/ml as the weighted mean count. If replicas from only one dilution fall within this range, calculate the arithmetic mean.

6.4 Verification of the methodology

A test is only valid if the following criteria are met:

a) the test virus suspension allows the determination of a lg reduction of 4 of the virus titre after treatment with the product, with respect to the virus titre before treatment with the product (virus control);

b) the difference of the logarithmic titre of the virus control minus the logarithmic titre of the virus in the reference inactivation test (B.2) is between -0,5 and -2,5 after 30 min;

c) the cytotoxicity (B.1) of the product solution does not affect cell morphology and growth or virus susceptibility in such a way as to compromise the demonstration of a lg reduction of 4 in the virus titre;

d) the comparative virus titration on cells inoculated with test mixture dilutions or with virus dilutions in MEM + 2 % FCS, result in a difference of lg < 1 of virus titre.

e) the difference to the test suspension in the control of efficiency for suppression of product’s activity (5.8.1) shall be ≤ 0,5 lg.

f) at least one concentration per test (5.7.2) shall demonstrate a 4 lg or more reduction and at least one concentration shall demonstrate a lg reduction of less than 4 (5.4).

6.5 Calculation of the virucidal activity of products

The virus inactivation induced by the virucidal activity of each product test solution is calculated for each exposure time. Then the lg reduction is determined by subtracting the logarithmic titre of each disinfectant dilution from the logarithmic titre of the virus control.

A lg reduction of 4 of the virus titre shall be achieved for the disinfectant to pass the test.

6.6 Expression of results

All results are tabulated as raw data and are presented in addition in negative logarithmic values of $TCID_{50}$.

In case no virus multiplication is observed (5.6.2) in the highest concentration, this value is denoted with the sign “≤”. In case virus multiplication is observed (5.6.2) in all dilutions, this value is denoted with the sign “≥”.

7 Conclusion

7.1 General

The product shall be deemed to have passed the EN 14675 standard if it demonstrates in a valid test a lg reduction of 4 or more in titre within 30 min or less at 10 °C, with the chosen interfering substance under the conditions defined by this European Standard when the test organism is *Bovine enterovirus* Type 1 (ECBO).

Virucidal activity against additional test viruses and/or additional contact times and temperatures (5.5.1) can be demonstrated by a lg reduction of 4 in titre.

7.2 Test report

The test report shall refer to this European Standard (i.e. EN 14675).

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

a) identification of the testing laboratory;

b) identification of the client;

c) identification of the sample:

   1) name of the product;

   2) batch number and – if available – expiry date;

   3) manufacturer – if not known: supplier;

   4) date of delivery;

   5) storage conditions;

   6) product diluent recommended by the manufacturer for use;

   7) active substance(s) and their concentration(s) (optional);

   8) appearance of the product;

d) experimental conditions:

   1) date(s) of test (period of analysis);

   2) diluent used for product test solution (hard water or distilled water);

   3) product test concentrations (= desired test concentrations according to 5.4);

   4) appearance of product dilutions;

   5) contact time(s);

   6) test temperature(s);

7) interfering substances;

8) stability and appearance of the mixture during the procedure (note the formation of any precipitate or flocculent);

9) temperature of incubation;

10) identification of the viral strain used;

e) test results:

1) validation of test results;

2) titre of virus suspension;

3) maximum detectable virus inactivation;

4) virus inactivation of the reference virus inactivation test after 30 min;

5) Table of results (raw data)

f) special remarks;

g) conclusion;

h) locality, date and identified signature.

NOTE An example of a typical test report is given in Annex D.

Annex A
(informative)

Referenced strains of national collections

Bovine enterovirus	Type 1	(ECBO)	
		ATCC	VR-248

Annex B
(normative)

Cytotoxicity, reference inactivation test, test virus titration and detoxification of test mixtures

B.1 Cytotoxicity caused by product solutions

a) To check for possible structural alteration of cells by the product, prepare solutions as for disinfectant test, replacing virus with distilled water (5.2.2.2). Serial dilutions (dilution step: 1:10) are prepared in the culture medium and are inoculated into cell cultures (monolayers or suspensions cells). This shall be done in parallel with the test. Any microscopic changes in the cells are recorded when reading the tests for CPE.

b) Comparative virus titrations are performed on cells that have or have not been treated with disinfectants to control the reduction of the sensitivity to viruses as follows:

   1) treatment of cells: 0,1 ml of the lowest apparently non-cytotoxic dilution (no microscopic alteration) of the disinfectant or MEM + 2 % FCS are distributed onto each of eight confluent cell cultures in microtitre plates with 96 wells. After one hour at 37 °C the supernatant is discarded;comparative titration of virus: the virus is diluted from 10^{-2} to 10^{-8} and inoculated on the treated or untreated cell cultures in parallel;

   2) only those dilutions of the product solution which a) show a low degree of cell destruction (< 25 % of monolayer) or b) produce a titre reduction of the virus of lg < 1 can be used for the determination of the virucidal activity.

c) If the cytotoxicity is so great that the reduction of the residual infectivity titre cannot be followed over the range of 4 lg $TCID_{50}$, special techniques shall be used, such as:

   1) molecular sieving with a molecular sievefilter, i.e. Sephadex™ LH 20[6)] (see B.4.1);

   2) ultrafiltration with Minicon®[6)] (Millipore) or with ready to use i.e. MicroSpinTM S 400 HR columns[6)] (GE Healthcare) (see B.4.2).

Appropriate controls are required to demonstrate that the procedure does not reduce the infectivity of the virus. Use according to the instructions of the manufacturer.

B.2 Reference inactivation test

A control of the test system shall be included using formalin (37 % formaldehyde solution).

Two parts of the virus suspension shall be mixed with eight parts of MEM + 2 % FCS and 10 parts of 1,4 % formalin (w/v) for the control of the system's validity. The contact time shall be 30 min.

Immediately at the end of the chosen contact time, mix [5.3.2.7a)] and pipette 0,2 ml of the test mixture into a tube containing 1,8 ml of cold MEM + 2 % FCS in a water bath at 4 °C ± 1 °C, or place it on crushed ice,

6) SephadexTM LH 20, Minicon® and MicroSpinTM S 400 HR are examples of suitable products available commercially. This information is given for the convience of user of this standard and does not constitute an endorsement by CEN of these products.

followed by a further 1:10 dilution. Leave the mixture in the water bath (5.3.2.2). Dilutions up to 10^{-6} are prepared by pipetting the diluted test mixture into a next tube containing cold MEM + 2 % FCS in the water bath at 4 ° C ± 1 °C or on crushed ice. The dilution factor shall be 10.

B.3 Titration of test virus suspension

To determine whether the virus titre is adequate, the infectivity of the test virus suspension shall be determined for each test.

The infectivity of the test virus suspension shall be determined under test conditions but without exposure to the product at a contact time of 30 min. The $TCID_{50}$ (3.8) of the virus suspension is determined by substitution of the product test solution (5.4) by hard water (5.2.2.3).

5.7.2 applies with the following modification:

8 ml of hard water (5.2.2.3) are added instead of the product test solution.

B.4 Detoxification of test mixtures by molecular sieving

B.4.1 Molecular sieving with SephadexTM LH 20[7)]

B.4.1.1 Principle

The samples are sieved through Sephadex™ LH 20 which retains the product and allows the virus to pass through. The viral titre of the filtrate is compared with that of the initial viral suspension treated under the same conditions.

The efficiency for suppression of disinfectant shall be demonstrated under the same conditions as described in 5.7.2, after incubation of the mixture at 10 °C for 30 min ± 10 s and addition of ice-cold MEM + 2 % FCS.

B.4.1.2 Sephadex suspension

Prepare the suspension of Sephadex™ LH 20 mixing 22 g in 100 ml of PBS. It is sterilized for 30 min at 120 °C. On removal from the autoclave, it is left at room temperature before being dispensed at the rate of 25 ml per previously sterilized stainless steel bottle or 20 ml per sterile syringe placed in a previously sterilized centrifuge bottle.

Centrifuge at 1 000 g for 10 min.

After discarding the centrifuging liquid, the column is ready for use (see Figure B.1).

B.4.1.3 Procedure

The samples are deposited, each at the rate of 1 ml, on the top of a Sephadex™ column. The whole of the device is centrifuged at 1 000 g for 10 min in a centrifuge which is preferably cooled to 4 °C. Collect the filtrate in a sterile manner. The volume collected shall be about 1 ml.

Two similar operations are carried out beforehand for 1 ml of the solution of the product under test and for the viral suspension.

The virus filtrates are titrated.

7) Sephadex™ LH 20 is an example of a suitable product available commercially. This information is given for the convenience of user of this standard and does not constitute an endorsement by CEN of this product.

The titre of the viral suspension obtained by sieving is compared with the titre of the initial unsieved viral suspension. The difference of titre shall shall not exceed 0,5 lg.

The virucidal activity is measured by comparing the titres of the treated and untreated viral suspensions.

B.4.2 Molecular sieving with Microspin column

B.4.2.1 Principle

The samples are sieved through the Microspin S 400 HR column (GE Healthcare), which retains the product and allows the virus to pass through (see Figure B.2)

B.4.2.2 Column preparation

Re-suspend the resin of the column by vortexing. Loosen the cap of the tube and twist off the bottom closure. Place the column in the supplied collection tube and centrifuge at 735 g for 1 min. The column shall be used immediately after centrifugation. Remove the buffer sieved.

B.4.2.3 Procedure

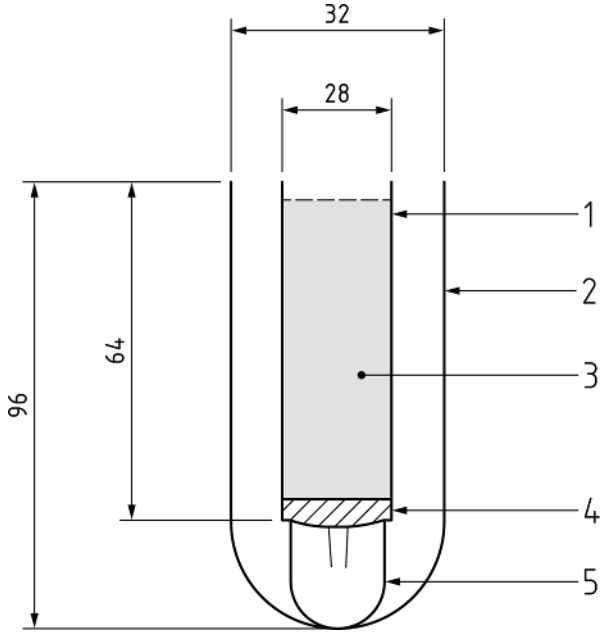
Just after centrifugation, inject 1 ml of the sample in the top centre of the column. Beware, to not touch the resin bed of the column with the pipette tip. Recap and centrifuge at 735 g for 2 min. After centrifugation, collect the filtrate and discard the column. The sample is ready to use.

Two similar operations are carried out beforehand for 1 ml of the solution of the product under test and for the viral suspension.

The filtrates are titrated.

The titre of the viral suspension obtained by sieving is compared with the titre of the initial unsieved viral suspension. The difference of titre shall not exceed 0,5 lg.

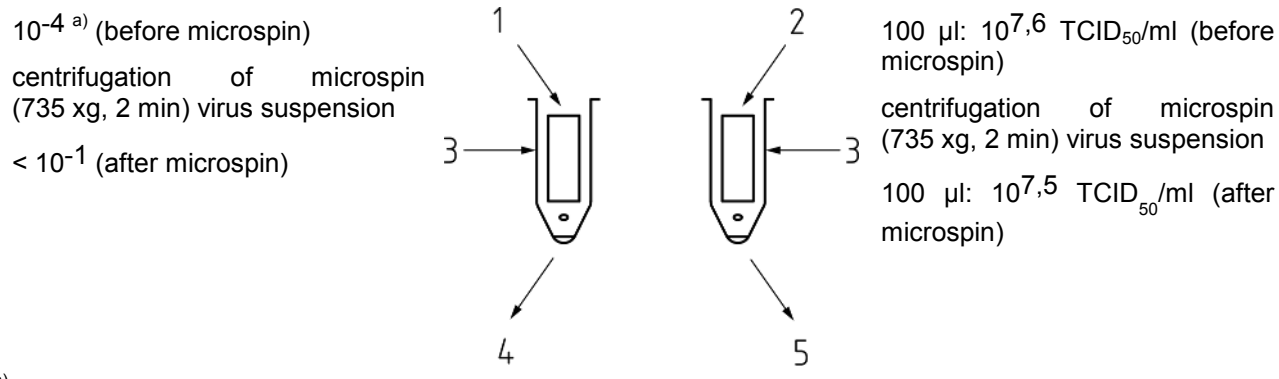
Dimensions in millimetres



Key

1 plastic (polypropylene) syringe
2 45 ml (Jouan) glass centrifuge tube
3 SEPHADEXTM LH-20 gel
4 glass fibre or polyester fabric
5 tube made of neutral glass

Figure B.1 — Molecular sieving device with syringe



a) Highest dilution toxic to freshly seeded Vero cells

b) Herpes virus was selected to demonstrate that:

1) no spontaneous inactivation occurs with this very unstable virus and

2) larger viruses pass the sieve.

Figure B.2 — Molecular sieving using MicroSpinTM S 400 HR

Annex C
(informative)

Calculation of the viral infectivity titre

C.1 Quantal tests - Example of $TCID_{50}$ determination by the Spearman-Kärber method

The Spearman-Kärber method is most simple and gives results most compatible with other calculations. Prerequisite of the evaluation is the use of several dilutions which cover infection of all cell culture units to those in which no virus multiply.

For calculation of the negative logarithm of $TCID_{50}$ 50% endpoint titre:

Negative lg of $TCID_{50}$ = negative lg of the lowest dilution step – {[Σ (% positive/dilution)/100] – 0,5} × lg of dilution factor

Table C.1 — Example of titration results

Dilution (lg)	Result [a]	% positive
4	444 444	100
5	444 344	100
6	443 003	66,7
7	400 020	33,3
8	000 000	0
Sum of % positive cultures		300

[a] 1 to 4 = virus present, degree of CPE in six cell culture units (tubes, microtitre plates)
0 = no virus present

Negative lg $TCID_{50}$ = – 4 – (300/100 – 0,5) ×1 = - 6,5

Therefore, the 50 % end point is $10^{-6,5}$ or lg $TCID_{50}$ = 6,5

C.2 Plaque test

$$\text{PFU} / t = \frac{\Sigma C_1 + C_2 ..... C_n}{\left(n_1 + n_2 \times v_2 + n_n \times v_n\right) \times d}$$

where

t is the test volume that was added per dilution step to a plate;

C_1 is the PFU number of all plates of the first dilution step (lowest dilution step) with non-confluent plaques;

C_2 is the PFU number of all plates of the second dilution step with non-confluent plaques;

C_n is the PFU of all plates of the last dilution (highest dilution step);

n_1 is the number of all plates of the first dilution step (lowest dilution step) with non-confluent plaques to which C_1 corresponds;

n_2 is the number of all plates of the second dilution step with non-confluent plaques (C_2);

v_2 is the dilution factor between n_1/n_2 (e.g. $n_1 = 10^{-3}$ and $n_2 = 10^{-4}$, then $v_2 = 0,1$);

n_n is the number of all plates of the last dilution for which PFUs were counted (Cn);

v_n is the dilution factor between n_1/n_n (e.g. $n_1 = 10^{-3}$ and $n_n = 10^{-6}$, then $v_n = 0,001$);

d is the dilution step of C_1.

Round off the calculated PFU/t to one decimal number. If the last number is under 5, the preceding number remains unchanged, if the last number is equal to or over 5, one is added to the preceding number. Proceed from the last decimal number.

$$PFU\ /\ t = \frac{\left(52 + 48 + 49\right) + \left(25 + 27 + 31\right)\left(5 + 6 + 7\right)}{\left(3 + \left(3 \times 0,1\right) + \left(3 \times 0,01\right)\right) \times 10^{-3}} = \frac{250}{3,33 \times 10^{-3}} = 75\,075,075$$

$$\lg 75\,075,075 = 4,875 \approx 4,9$$

If the test volume that was added per dilution to a plate was 0,2 ml, the lg PFU/ml = 5,6

Annex D
(informative)

Example of a typical test report

NOTE 1 All names and examples in Annex D are fictitious apart from those used in this European Standard.

NOTE 2 Only the test result of one replicate for Bovine enterovirus Type 1 is given as an example:

HHQ Laboratories

Antiseptville/Euroland

Tel. ++011-57 83 62-0

Fax ++011-57 83 62-19

e-mail: h.h.Q.lab@net.com

TEST REPORT

EN 14675, VIRUCIDAL ACTIVITY

(obligatory and additional conditions)

Client: Centipede Formulations Inc., Markkleeberg / Euroland

Disinfectant-sample

Name of the product: Z **Batch number:** 05-36-025

Manufacturer or – if not known – **supplier:** Centipede Formulations Inc. (manufacturer)

Storage conditions (temp. and other): Room temperature, darkness

Appearance of the product: Liquid, clear, yellowish

Active substance(s) and their concentration(s): Not indicated

Product diluent recommended by the manufacturer for use: Potable water

Period of testing

Date of delivery of the product: 2005-06-08 **Dates of tests**: see “Test results“ (Annex E)

Experimental conditions

Product diluent: hard water; concentrations of the product tested: see "Test results" (Annex E)

Obligatory conditions: test-organisms: *Bovine enterovirus* Type 1 (ECBO) ATCC VR-248;
test temperature: 10 °C;
contact time: 30 min;
interfering substance: 10 g/l yeast extract + 10 g/l bovine albumin;
incubation temperature: 37 °C

Special remarks regarding the results:

At least one concentration of the product demonstrated a lg reduction in virus titre of less than 4, and at least one concentration of the product demonstrated at least a lg reduction in virus titre of 4.

No precipitate during the test procedure (the test mixture was homogeneous)

Conclusion:

For the product Z (batch 05-36-025), the virucidal concentration for general purposes determined according to the EN 14675 standard (obligatory conditions) under high-level soiling is:

1 % (v/v);

Antiseptville, 2005-07-21 Alexandra May, MD, PhD, Scientific Director

Annex E
(informative)

Presentation of test results of one active concentration

Table E.1 — Table of results of product XYZ and ECBO

Product	Concentration	Interfering substance	Level of cytotoxicity	lg $TCID_{50}$ after ... min					> 4 lg reduction after ... min
				0	5	15	30		
XYZ	1 % (v/v)	3 g/l BSA	3,5	7,9	6,7	5,2	< 3,5		30
XYZ	1 % (v/v)	10 g/l BSA + yeast extract	3,5	8,1	7,2	5,8	< 3,5		30
Formaldehyde	0,7 % (w/v)	MEM + 2 % FCS	3,5	7,6	7,5	5,7	4,5		30
virus control	n.a.	MEM + 2 % FCS	n.a.	7,6	n.d.	n.d.	7,8		n.a.
virus control	n.a.	3 g/l BSA	n.a.	7,9	n.d.	n.d.	7,7		n.a.
virus control	n.a.	10 g/l BSA + yeast extract	n.a.	8,1	n.d.	n.d.	8		n.a.
n.a. not applicable. n.d. not done.									

Table E.2 — Raw data for product XYZ with 0,3 g/l BSA tested against ECBO (quantal test)

Product	Concentration	Interfering substance	Contact Time	Dilutions (lg)[a]							
				2	3	4	5	6	7	8	9
XYZ	1 % (v/v)	0,3 g/l BSA	5	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4444 4444	4000 0000	4000 0000
			15	4444 4444	4444 4444	4334 4000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000
			30	4444 4444	4444 4444	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000
XYZ cyto-toxicity	1 %	0,3 g/l BSA	n.a.	4444 4444	4444 4444	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
Form-aldehyde	0,7 (w/v)	MEM + 2 % FCS	15	4444 4444	4444 4444	4444 4334	3344 2344	4300 0000	0000 0000	0000 0000	0000 0000
			30	4444 4444	4444 4444	3344 4324	0000 0000	0000 0000	0000 0000	0000 0000	0000 0000
Form-aldehyde cytotoxicity control	0,7 (w/v)	MEM + 2 % FCS	n.a.	4444 4444	4444 4444	0000 0000	0000 0000	n.d.	n.d.	n.d.	n.d.
Virus control	n.a.	0,3 g/l BSA	0	4444 4444	4444 4444	4444 4444	4444 4444	4334 3244	3324 4322	4000 0000	0000 0000
			30	4444 4444	4444 4444	4444 4444	4434 4334	3244 4444	3444 3420	4400 0000	0000 0000

a

1 to 4 virus present, degree of CPE in 8 cell culture units (tubes, wells of microtitre plates).

0 no virus present.

n.a. not applicable.

n.d. not done.

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