Technical Paper Report

Prepared For :	**Jiangsu SOHO International Group Yangzhou Co., Ltd.** 8th/F.,Tower 4, West City Superior Building Plaza, No. 303 WenHui West Road, Yangzhou 225001, Jiangsu, China.
Product Name :	**Hand Sanitizer**
Trade Mark :	**N/A**
Model(s) :	**5ML,** 30ML, 50ML, 60ML, 100ML, 200ML, 236ML 300ML, 310ML, 350ML, 400ML, 500ML, 1L, 4L, 5L
Report No. :	**CCT20031607VRS**

CE

Technician by:

Date: 27 March 2020

Jiangsu SOHO International Group Yangzhou Co., Ltd.

8th/F.,Tower 4, West City Superior Building Plaza, No. 303 WenHui West Road, Yangzhou 225001, Jiangsu, China.

TEST REPORT EN 14476:2013 - VIRUCIDAL ACTIVITY EN1276:2010 Chemical disinfectants and antiseptics— Test method and requirements	
Reference No. ..... :	CCT20031607VRS
Contents ..... :	36 pages
Client	
Name ..... :	Jiangsu SOHO International Group Yangzhou Co., Ltd.
Address..... :	8th/F.,Tower 4, West City Superior Building Plaza, No. 303 WenHui West Road, Yangzhou 225001, Jiangsu, China.
Test specification	
Standard ..... :	EN 14476:2013 &EN1276:2010
Test procedure ..... :	CE
Procedure deviation ..... :	N.A.
Non-standard test method..... :	N.A.
Test item	
Description..... :	Hand Sanitizer
Trademark ..... :	N/A
Model and/or type reference ..... :	5ML
Manufacturer ..... :	Jiangsu Oppeal Daily Cosmetics Corp., Ltd.
Address..... :	South Huatong Road, Yangshou Town, Hanjiang District, Yangzhou 225001, Jiangsu, China

Test case verdicts

Test case does not apply to the test object ..... : N(.A.)

Test item does meet the requirement..... : P(ass)

Test item does not meet the requirement..... : F(ail)

General remarks

This test report shall not be reproduced except in full without the written approval of the testing laboratory.

The test results presented in this report relate only to the item tested.

"(see remark #)" refers to a remark appended to the report.

"(see appended table)" refers to a table appended to the report.

Throughout this report a comma is used as the decimal separator.

Remark :

The EUT complies with the standard EN 14476:2013 &EN1276:2010 requirement.

-This technical report is only used for internal reference of the company, and not for any other legal basis and use.

Copy of marking plate:

Hand Sanitizer
Model： **5ML**  Jiangsu Oppeal Daily Cosmetics Corp., Ltd. Made in China

Note: because of the sameness of labels, only above label is listed

EN 14476:2013

- VIRUCIDAL ACTIVITY

4. Principe of the test

The study consists in determining the virucidal activity of product within test –organism Influenza virus AH1N1. The product is according to the EN 14476 if, in one of the test conditions, it demonstrates at least a 4 log reduction after:

- 60 minutes of contact time for the instrument and surface disinfectants
- 1 minute or 30 s of contact time for the hygienic handrub and handwash

According to European Standard 14476, time additional can be chosen and tested in the conditions of the try.

The realization of this standard comprises several stages, which are :

- Proportion titration of the virus suspension.
- Preliminary test consist in determining the level of disinfecting cytotoxicity according to the cellular line necessary to the detection of the virus. This stage allows defining the cytotoxic concentration of the product, corresponding to the sensitivity of the cells to the tested virus.
- Virus inactivation test by Ethanol 25 % (v/v). The stage is a control of the test system by the test method.
- Virucidal testing.
- Realization of the virus control.

5. Test conditions:

Product diluent :	ppi water
Concentrations of the product tested :	80 %
Test mixtures:	homogeneous
Contact time (obligatory):	60 minutes
Contact time (additionnal) :	/
Test temperature :	20°C + 1°C
Incubation temperature:	37°C + 1°C +5% CO2
Neutralization metodology:	Detoxification on Microspin TH S-400.columms
High level soiling condition:	0,3g/l SAB
Monolayer cell :	MDCK

Test-organisms :

Influenza virus type A- H1N1- ATCC-UR1469 (10^7 to 10^8 UI/ml).

6. Materiel and method:

6–1 – Determination of infectivity (virus titration)

Infectivity was determinate by means of end point dilution in a micro procedure.

The suspension viral untreated (control and stock suspension) and treated by the product at the test concentration of 80 %, are diluted in series of 10^{-2} to 10^{-10} maximum, in MEM + 2% FCS frozen. 0,1 ml of each diluted is transferred in 8 wells of the micro titration plate containing the confluent cells, from starting with the highest dilution. After one hour of incubation at 37°C in presence of CO_2 (5 %), 0,1 ml of culture medium is added in each well. The reading of the ECP is realized under the inverted microscope daily after 2 days and 5 days of incubation. Calculation of infectivity titer is determined by the Spearman-Karber method.

6-2 – Determination of subcytotoxic dilution of the disinfectant

The aim of this test is to determine the concentration of chemical desinfectant inducing no sign of toxicity with respect to cellular line allowing the description of the virus to be tested.

For elimination of cytotoxicity, two techniques are described later, and are selected according to following conditions :

The dilution method is the first one tested. It is validated if the difference between the log $TCID_{50}$ of virus titre of the stock suspension and the level non cytotoxic level's is > at 5 log. If it's not the case, the molecular sieving is carried out. The validation of the conditions method is similar at the first method. If the results are not satisfactory some is the technique employed, the virus test on disinfecting is unrealizable.

1- Dilution method :

The test solution of product, added with 1/5 of water for injectable preparation, ppi water, is diluted in series of 10^{-2} to 10^{-6} in MEM 2 % FCS frozen. Then 0,1 ml of each dilution is transferred in 8 wells of micro titration plate, we start with the highest dilution. After one hour of incubation at 37°C in presence of CO_2 (5 %), 0,1 ml of culture medium is added in each well. The subcytotoxic effect is assessed after an incubation not exceeding the longest virus culture period cultivated on the system studied (5 days).

2- Molecular sieving technique, with a molecular on Microspin™S-400 HR columns :

Test solution of the product added with water for injectable preparation, ppi water, is filtered on Microspin™S-400 HR columns . Then, filtrate is diluted in series of 10^{-2} to 10^{-6} in MEM + 2 % FCS frozen. 0,1 ml of each dilution is transferred in 8 wells of micro titration plate. After one hour of incubation at 37°C, with 5 % of CO_2, 0,1 ml of culture medium is added in each well. The subcytotoxicity effect is assessed after an incubation not exceeding the longest virus culture period cultivated on the system studied.

6-3 – Cell sensitivity to virus

The aim of this test is to make sure that MDCK cells with test solution (at the subcytotoxic concentrations) don't alter behavior of virus with the cells. Sensitivity of cells compared to the virus is appreciated by comparison of the virus titre of the stock virus suspension obtained on a cell monolayer treated with the subcytotoxic dilution of disinfectant, with the cell monolayer untreated.

Treatment of cells (with the subcytotoxic concentration of disinfectant)

0,1 ml of the lowest apparently non cytotoxic dilution of the test solution are distributed on to each 8 wells established cell cultures in microtitre plates. Plates are incubated at 37°C for 1 h with 5 % CO_2. In the same time, the stock virus suspension is diluted to 10^{-2} to 10^{-10}. Then, 0,1 ml of each dilution is added in each wells. Plates are again incubated at 37°C for 1 h with 5 % CO_2. 0,1 ml of cell media is added in each wells. The reading of ECP is realized thanks to calculation of infectivity titer are determined by the Spearman-Karber method.

Cells untreated with the subcytotoxic concentration of disinfectant

It's the same procedure as cells treated. Only difference is that the dilution subcytotoxic is removed by MEM 2 % foetal calf serum. Only these dilutions of the product can be used for the determination of the residual infectivity which produces a titer reduction of the virus of < 1 log.

Validation of test :

Difference between log DICT50 and titrate of cells treated and untreated must be < 1 log for the test of cells sensibility of virus will be valided.

6-4 – Control of efficiency for suppression of disinfectant activity

Dilution in ice cold medium

Immediately after preparation of the test mixture (virus + interfering substance + test solution) (d = 10^{-1}), reaction is stopped : 0.5 ml of the test mixture is placed into 4.5 ml of ice cold MEM + 2 % foetal calf serum (d = 10^{-2}).

Dilutions are realized in the same media from 10^{-3} to 10^{-8}.

The Influenza virus A H1N1 is titrated as described in 6-1.

Filtration technique

Just after the preparation of the test mixture, reaction is stopped putting 1 ml on Microspin™S- 400 HR columns.

After a centrifugation, filtrate is diluted in ice cold MEM + 2 % foetal calf serum from 10-2 to 10^{-8}. The Influenza virus A H1N1 is titrated as described in 6-1.

6-5 – Virucidal testing

The aim of a virucidy test for a disinfectant is to put in contact the viral suspension with an interfering substance and a test solution.

Reaction is stopped after the time of contact notified :

- By dilution on ice cold medium (MEM)
- By filtration technique on Microspsin^{TM}S-400HR columns. Filtrate is diluted form 10 to 10 in a ice cold medium.

Titer of each test is determined as described in 6-1.

6-6 – Inactivation test of the virus at Ethanol of 25 % (v/v)

Virucidy tests are realized on the stock suspension of Influenza virus A H1N1 with Ethanol in order to control the behavior of our strain with chemical agents.

This control requires realization of the following procedures :

- Control of the Ethanol cytotoxicity opposite our cell line
- Control of efficiency of the stopped activity
- Virucidal testing of ethanol (5.15, 30 and 60 minutes)

6-7 – Titration of the virus standard

The infectivity of the test virus suspension shall be determined under test conditions at contact times 0 min and 60 min.

The product test solution is substituted by water.

The Influenza virus A H1N1 is titred as described in 6-1.

7. RESULTS

7.1 Virus titration:

The infectivity titer of the suspension viral is: 7.625 in logUI/ml

7.2 Cytotoxicity of product

Examinations showed that, without treatment, the disinfectant had a toxicity of 3.5 logCD50/ml For elimination of cytotoxicity, the technique by Detoxification on Microspin TH S-400.columms is choice (see table 1)

Table 1	Level of cytotoxicity (log)
dilution technical C1	3.5
ultrafiltration on microspinTM S-400 columns C2	2.5

7.3 Sensibility of cells

Difference between the virus titrations on treated cell (A) and on untreated cell (B) is lower than 1 (see table2), consequently the test is valided.

Table 2	Treated cell : A	Untreated cell : B
Log DICT50	7.75	8.00

7.4 Virucidal activity of product

The table 3 will gather the results obtained by the following points:

- contol of efficiency for suppression o disinfectant activity
- Virucidal testing
- Inactivation test of the virus
- Titration of the virus

Product		NOCOLYSE	Ethanol	Virus control	Virus control test
Concentrations		80 %	25%	N.A.	N.A.
interfering substance		0,3g/l SAB	PBS	PBS	0,3g/l SAB
Level of ……. min Cytotoxicity Log DICT50		2.5	1.5	N.A.	N.A.
neutralization control Log DICT50		7,825	N.A.	N.A.	N.A.
Log DICT50 after ……. min	0	< 2.5	8,125	8.00	8,125
	5	N.A.	7.75	N.A.	N.A.
	15	N.A.	7.5	N.A.	N.A.
	30	N.A.	6.625	N.A.	N.A.
	60	< 2.5	5.75	7.75	7,875
> 4 log reduction after ……. min		Reduction superior of 5,375 log in 60 min	Reduction inferior of 4 log in 60 min	N.A.	N.A.

Calcul of virucidal activity : Reduction = LogDICT 50 test - LogDICT 50 viral control

8. CONCLUSION

The product NOCOLYSE, batch n° 2200709, is active at 80 % after a contact time of 60 minutes in accordance with NF EN 14476 under clean conditions on Influenza virus type A-H1N1.

EN1276:2010

Chemical disinfectants and antiseptics— Testmethod and requirements

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 with the exception of handwash products whose first dilution is done in hard water (5.4.2)) is added to a test suspension of bacteria in a solution of an interfering substance. The mixture is maintained at the chosen test temperature for the adopted contact time. At the end of this contact time, an aliquot is taken, and the bactericidal and/or the bacteriostatic activity in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found, membrane filtration is used. The numbers of surviving bacteria in each sample are determined and the reduction is calculated.

5.1.2 The test is performed using Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Enterococcus hirae as test organisms. For temperatures ⩾ 40 ° C only Enterococcus faecium shall be used.

For testing of hand hygiene products, Pseudomonas aeruginosa, Escherichia coli K12, Staphylococcus aureus and Enterococcus hirae are used as test organisms.

5.1.3 Additional test organisms can be used.

5.2 Materials and reagents

5.2.1 Test organisms

The bactericidal activity shall be evaluated using the following strains as test organisms:

— Pseudomonas aeruginosa ATCC 15442;

— Escherichia coli ATCC 10536;

— Staphylococcus aureus ATCC 6538;

— Enterococcus hirae ATCC 10541;

— Escherichia coli K12 NCTC 10538;

— Enterococcus faecium ATCC 6057.

If required for specific applications, additional strains may be chosen, for example from:

— Salmonella Typhimurium ATCC 13311;

— Lactobacillus brevis DSM 6235;

— Enterobacter cloacae DSM 6234.

Refer to Annex A for strain references in some other culture collections.

The required temperature for growing these test organisms is (36 ± 1) °C or (37 ± 1) °C (5.3.2.3). The same temperature (either 36 °C or 37 °C) shall be used for all incubations for growing microorganisms performed during a test and its control and validation. If additional test organisms are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere, media) noted in the test report. If the additional test organisms selected do not correspond to the specified strains, their suitability for supplying the required inocula shall be verified. If these additional test organisms are not classified at a reference centre, their identification characteristics shall be stated. In addition, they shall be held by the testing laboratory or national culture collection under a reference for five years.

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 microbiological purposes. They shall be free from substances that are toxic or inhibitory to the test organisms.

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 are to be rigorously followed.

NOTE For each culture medium and reagent, a limitation for use is to be fixed.

5.2.2.2 Water

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

Sterilize in the autoclave (see 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 If distilled water of adequate quality is not available, water for injections can be used.

See 5.2.2.7 for the procedure to prepare hard water.

5.2.2.3 Tryptone Soya Agar (TSA)

Tryptone soya agar, consisting of:

Tryptone, pancreatic digest of casein	15.0 g
Soya peptone, papaic digest of soybean meal	5.0 g
Sodium chloride (NaCl)	5.0 g
Agar	15.0 g

Water (5.2.2.2) to 1000.0 ml

Sterilize in the autoclave (5.3.2.1 a). After sterilization the pH of the medium shall be equivalent to 7,2 ± 0,2 when measured at (20 ± 1) °C.

NOTE In the case of encountering problems with neutralization (5.5.1.2 and 5.5.1.3), it can be necessary to add neutralizer to the TSA. Annex B gives guidance on the neutralizers that can be used.

5.2.2.4 Diluent

Tryptone sodium chloride solution, consisting of:

Tryptone, pancreatic digest of casein 1.0 g

Sodium chloride (NaCl) 8.5 g

Water (5.2.2.2) to 1000.0 ml

Sterilize in the autoclave (5.3.2.1 a). After sterilization, the pH of the diluent shall be equivalent to 7.0 ± 0,2 when measured at (20 ± 1) °C.

5.2.2.5 Neutralizer

The neutralizer shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.2. It shall be sterile.

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

5.2.2.6 Rinsing liquid (for membrane filtration)

The rinsing liquid shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.3. It shall be sterile, compatible with the filter membrane and capable of filtration through the filter membrane under the test conditions described in 5.5.3.

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

5.2.2.7 Hard water for dilution of products

For the preparation of 1000 ml 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 1000 ml. Sterilize by membrane filtration (5.3.2.7) or in the autoclave (5.3.2.1 a). Autoclaving – if used - may cause a loss of liquid. In this case, make up to 1000 ml with water (5.2.2.2) under aseptic conditions. Store the solution in the refrigerator (5.3.2.8)

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 1000 ml. Sterilize by membrane filtration (5.3.2.7). Store the solution in the refrigerator (5.3.2.8) for no longer than one week;

— place 600 ml to 700 ml of water (5.2.2.2) in a 1000 ml volumetric flask (5.3.2.12) and add 6.0 ml (5.3.2.9) of solution A, then 8.0 ml of solution B. Mix and dilute to 1000 ml with water (5.2.2.2). The

pH of the hard water shall be 7.0 ± 0,2, when measured at (20 ± 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.

When preparing the product test solutions (5.4.2), 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 (CaCO3) in the test tube.

5.2.2.8 Interfering substance

5.2.2.8.1 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.

5.2.2.8.2 Clean conditions (bovine albumin solution – low concentration)

Dissolve 0,3 g of bovine albumin fraction V (suitable for microbiological purposes) in 100 ml of water

(5.2.2.2).

Sterilize by membrane filtration (5.3.2.7), keep in the refrigerator (5.3.2.8) and use within one month.

The final concentration of bovine albumin in the test procedure (5.5) is 0,3 g/l.

5.2.2.8.3 Dirty conditions (bovine albumin solution – high concentration)

Dissolve 3.0 g of bovine albumin fraction V (suitable for microbiological purposes) in 100 ml of water

(5.2.2.2).

Sterilize by membrane filtration (5.3.2.7), keep in the refrigerator (5.3.2.8) and use within one month.

The final concentration of bovine albumin in the test procedure (5.5) is 3.0 g/l.

5.2.2.8.4 Milk (dairies, etc.)

Skimmed milk, guaranteed free of antibiotics and additives and reconstituted at a rate of 100 g powder per litre of water (5.2.2.2), shall be prepared as follows:

— prepare a solution of 10.0 % (v/v) in water (5.2.2.2) by adding 10 parts of reconstituted milk to 90 parts of water. Heat for 30 min at (105 ± 3) °C [or 5 min at (121 ± 3) °C].

The final concentration of reconstituted milk in the test procedure (5.5) is 1.0 % (v/v) of reconstituted milk.

5.3 Apparatus and glassware

5.3.1 General

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

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

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

5.3.2 Usual microbiological laboratory equipment 1) and, in particular, the following

5.3.2.1 Apparatus for sterilization:

a) for moist heat sterilization, an autoclave capable of being maintained at 121~124 ° C for a minimum holding time of 15 min;

b) for dry heat sterilization, a hot air oven capable of being maintained at 180~185° C for a minimum holding time of 30 min, at 170~175° C for a minimum holding time of 1 h or at 160~165° C for a minimum holding time of 2 h.

5.3.2.2 Water baths, capable of being controlled at (20 ± 1) °C, at (45 ± 1) °C (to maintain meltedTSA in case of pour plate technique) and at additional test temperatures ± 1 °C (5.5.1).

5.3.2.3 Incubator, capable of being controlled either at (36 ± 1) °C or (37 ± 1) °C (5.2.1).

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

NOTE A puncture electrode or a flat membrane electrode are to be used for measuring the pH of the agarmedia (5.2.2.3).

5.3.2.5 Stopwatch

5.3.2.6 Shaker

a) Electromechanical agitator, e.g. Vortex ® mixer 2) .

b) Mechanical shaker

5.3.2.7 Membrane filtration apparatus, constructed of a material compatible with the substancesto be filtered.

The apparatus shall have a filter holder of at least 50 ml volume. It shall be suitable for use with filters of diameter 47 mm to 50 mm and 0,45 µm pore size for sterilization of hard water (5.2.2.7), bovine albumin (5.2.2.8.2 and 5.2.2.8.3), and if the membrane filtration method is used (5.5.3).

The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of the microorganisms over the membrane and to prevent overlong filtration, the device shall be set so as to obtain the filtration of 100 ml of rinsing liquid in 20 s to 40 s.

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

5.3.2.9 Graduated pipettes, of nominal capacities 10 ml and 1 ml and 0,1 ml, or calibrated automatic pipettes.

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

5.3.2.11 Glass beads, 3 mm to 4 mm in diameter.

5.3.2.12 Volumetric flasks

5.4 Preparation of test organism suspensions and product test solutions

5.4.1 Test organism suspensions (test and validation suspension)

5.4.1.1 General

For each test organism, two different suspensions shall be prepared: the “test suspension” to perform the test and the “validation suspension” to perform the controls and method validation.

5.4.1.2 Preservation and stock cultures of test organisms

The test organisms and their stock cultures shall be prepared and kept in accordance with EN 12353.

5.4.1.3 Working culture of test organisms

In order to prepare the working culture of the test organisms (5.2.1), prepare a subculture from the stock culture (5.4.1.2) by streaking onto TSA slopes (5.2.2.3) or plates (5.3.2.10) and incubate (5.3.2.3).

After 18 h to 24 h prepare a second subculture from the first subculture in the same way and incubate for 18 h to 24 h. From this second subculture, a third subculture may be produced in the same way. The second and (if produced) third subcultures are the working cultures.

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 in the incubator (5.3.2.3) during the 48 h period.

Never produce and use a fourth subculture.

For additional test organisms, any departure from this method of culturing the test organisms or preparing the suspensions shall be noted, giving the reasons in the test report.

5.4.1.4 Test suspension (“N”)

a) Take 10 ml of diluent (5.2.2.4) and place in a 100 ml flask with 5 g of glass beads (5.3.2.11). Take the working culture (5.4.1.3) and transfer loopfuls of the cells into the diluent (5.2.2.4). The cells should be suspended in the diluent by rubbing the loop against the wet wall of the flask to dislodge the cells before immersing in the diluent. Shake the flask for 3 min using a mechanical shaker (5.3.2.6 b). Aspirate the suspension from the glass beads and transfer to another tube.

Adjust the number of cells in the suspension to (1.5×10^8) cfu/ml [3]) to (5×10^8) cfu/ml using diluent (5.2.2.4), estimating the number of cfu by any suitable means. Maintain this test suspension in the water bath at the test temperature $\theta \pm 1$ °C (5.5.1.1) and use within 2 h.

The use of spectrophotometer for adjusting the number of cells is highly recommended (approximately 620 nm wavelength - cuvette 10 mm path length). Therefore each laboratory produces calibration data for each test organism knowing that suitable values of optical density are generally found between 0,150 and 0,460. A colourimeter is a suitable alternative.

b) For counting, prepare 10 −6 and 10 −7 dilutions of the test suspension using diluent (5.2.2.4). Mix (5.3.2.6 a). Take a sample of 1.0 ml of each dilution in duplicate and inoculate using the pour plate or the spread plate technique.

1) When using the pour plate technique, transfer each 1.0 ml sample into separate Petri dishes and add 15 ml to 20 ml melted TSA (5.2.2.3), cooled to (45 ± 1) °C.

2) When using the spread plate technique, spread each 1.0 ml sample – divided into portions of approximately equal size – on an appropriate number (at least two) of surface dried plates containing TSA (5.2.2.3).

For incubation and counting, see 5.4.1.6.

5.4.1.5 Validation suspension (“Nv”)

a) To prepare the validation suspension, dilute the test suspension (5.4.1.4) with the diluent (5.2.2.4) to obtain the bacterial count of (3.0×10^2) cfu/ml to (1.6×10^3) cfu/ml [about one fourth (1 + 3) of the 10 −5 dilution].

b) For counting, prepare a 10^{-1} dilution with diluent (5.2.2.4). Mix (5.3.2.6 a). Take a sample of 1.0 ml in duplicate and inoculate using the pour plate or the spread plate technique (5.4.1.4).

For incubation and counting, see 5.4.1.6.

5.4.1.6 Incubation and counting of the test and the validation suspensions

For incubation and counting of the test and validation suspension, the procedure is as follows:

a) Incubate (5.3.2.3) the plates for 20 h to 24 h. Discard any plates that are not countable for any reason. Count the cfu on the plates to determine the total number of cfu. Incubate the plates for further 20 h to 24 h. Do not recount plates that no longer show well-separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

b) Note for each plate the exact number of colonies but record “> 330” for any counts higher than 330 and determine the Vc values according to 5.6.2.2.

c) Calculate the numbers of cfu/ml in the test suspension “N” and in the validation suspension “Nv” using the methods given in 5.6.2.3 and 5.6.2.5. Verify according to 5.7.

5.4.2 Product test solutions

The concentration of a 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 (5.5.2 or 5.5.3). Product test solutions shall be prepared in hard water (5.2.2.7) at a minimum of three different concentrations to include one concentration in the active range and one concentration in the non-active range (5.8.2). The product 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 that are not diluted when applied, shall be prepared in water (5.2.2.2).

Handwash products shall be tested at 50 % concentration as highest concentration (1:1 dilution, in order to simulate real use conditions) and therefore shall be pre-diluted in hard water (5.2.2.7) at 62.5 %

concentration. Such a product is nevertheless regarded as a ready-to-use product and the subsequent dilutions shall be performed with water (5.2.2.2).

For solid products, dissolve the product as received by weighing at least 1.0 g ± 10 mg of the product in a volumetric flask and filling up with hard water (5.2.2.7). Subsequent dilutions (lower concentrations) shall be prepared in volumetric flasks (5.3.2.12) on a volume/volume basis in hard water (5.2.2.7).

For liquid products, dilutions of the product shall be prepared with hard water (5.2.2.7) on a volume/volume basis using volumetric flasks (5.3.2.12).

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

NOTE Counting microorganisms embedded in a precipitate or flocculent is difficult and unreliable.

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 bactericidal activity of the product

5.5.1 General

5.5.1.1 Experimental conditions

For general purpose disinfection and hand hygiene, the experimental conditions in Table 1 and Table 2

apply:

The recommended contact time for the use of the product is within the responsibility of the manufacturer.

For contact times equal or below 1 min, 5 s tolerance shall apply. For longer contact times.10 s tolerance shall apply.

5.5.1.2 Choice of test method (dilution-neutralization or membrane filtration)

The method of choice is the dilution-neutralization method (5.5.2). To determine a suitable neutralizer, carry out the validation of the dilution neutralization method (5.5.2.3, 5.5.2.4 and 5.5.2.5 in connection with 5.5.2.6) using a neutralizer, chosen according to laboratory experience and published data.

If this neutralizer is not valid, repeat the validation test using an alternative neutralizer taking into

account the information given in Annex B.

If both neutralizers are found to be invalid, the membrane filtration method (5.5.3) may be used.

NOTE In special circumstances, it can be necessary to add neutralizer to TSA (5.2.2.3).

5.5.1.3 General instructions for validation and control procedures

The neutralization and/or removal of the bactericidal and/or bacteriostatic activity of the product shall be controlled and validated - only for the highest product test concentration - for each of the used test organisms and for each experimental condition (interfering substance, temperature, contact time).

These procedures (experimental condition control, neutralizer or filtration control and method validation) shall be performed at the same time with the test and with the same neutralizer – or rinsing liquid – used in the test.

In the case of ready-to-use-products, use water (5.2.2.2) instead of hard water.

If because of problems with neutralization, a neutralizer has been added to TSA (5.5.1.2) used for the validation and control procedures, the TSA used for the test shall contain the same amount of this neutralizer as well.

5.5.1.4 Equilibration of temperature

Prior to testing, equilibrate all reagents (product test solutions (5.4.2), test suspension (5.4.1.4), validation suspension (5.4.1.5), diluent (5.2.2.4), hard water (5.2.2.7) and interfering substance (5.2.2.8)) to the test temperature (θ ± 1) °C (5.5.1.1) using the water bath (5.3.2.2) controlled at (θ ± 1)°C.

Check that the temperature of the reagents is stabilized at (θ ± 1) °C.

The neutralizer (5.2.2.5) or the rinsing liquid (5.2.2.6) and water (5.2.2.2) shall be equilibrated at (20 ± 1) °C.

In the case of ready-to-use-products, water (5.2.2.2) shall be additionally equilibrated to (20± 1) °C.

5.5.1.5 Precautions for manipulation of test organisms

Do not touch the upper part of the test tube sides when adding the test or the validation suspensions (5.4.1).

5.5.2 Dilution-neutralization method 4)

5.5.2.1 General

The test and the control and validation procedures (5.5.2.2 through 5.5.2.5) shall be carried out in parallel and separately for each experimental condition (5.5.1.1).

5.5.2.2 Test “Na” – determination of bactericidal concentrations

The procedure for determining bactericidal concentrations is as follows.

a) Pipette 1.0 ml of the interfering substance (5.2.2.8) into a tube. Add 1.0 ml of the test suspension (5.4.1.4). Start the stopwatch (5.3.2.5) immediately, mix (5.3.2.6 a)) and place the tube in a water bath controlled at the chosen test temperature θ ± 1°C (5.5.1.1) for 2 min ± 10 s.

At the end of this time, add 8.0 ml of one of the product test solutions (5.4.2). Restart the stopwatch at the beginning of the addition. Mix (5.3.2.6 a) and place the tube in a water bath controlled at θ for the chosen contact time t (5.5.1.1). Just before the end of t, mix (5.3.2.6 a) again.

b) At the end of t, take a 1.0 ml sample of the test mixture “Na” and transfer into a tube containing 8.0 ml neutralizer (5.2.2.5) and 1.0 ml water (5.2.2.2). Mix (5.3.2.6a)) and place in a water bath controlled at the chosen temperature. After a neutralization time of 5 min ± 10 s (in case of contact times of 10 min or shorter only (10 ± 1)s), mix and immediately take a sample of 1.0 ml of the neutralized test mixture “Na” (containing neutralizer, product test solution, interfering substance and test suspension) in duplicate and inoculate using the pour plate or spread plate technique.

1) When using the pour plate technique, pipette each 1.0 ml sample into separate Petri dishes and add 15 ml to 20 ml of melted TSA (5.2.2.3), cooled to (45 ± 1) °C.

2) When using the spread plate technique, spread each 1.0 ml sample – divided into portions of approximately equal size – on an appropriate number (at least two) of surface dried plates containing TSA (5.2.2.3).

For incubation and counting, see 5.5.2.6.

c) Perform the procedures a) and b) using the other product test solutions at the same time.

d) Perform the procedures a) to c) applying the chosen experimental conditions (5.5.1.1).

For handwashes, two additional decimal dilution shall be plated.

5.5.2.3 Experimental conditions control “A” – validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions To validate the selected experimental conditions and/or verify the absence of any lethal effect in the test conditions, the procedure is as follows.

a) Pipette 1.0 ml of the interfering substance used in the test (5.5.2.2) into a tube. Add 1.0 ml of thevalidation suspension (5.4.1.5). Start the stopwatch immediately, mix (5.3.2.6 a)) and place the tubein a water bath controlled at the chosen temperature (θ ± 1) °C for 2 min ± 10 s.

At the end of this time, add 8.0 ml of hard water (5.2.2.7) [In the case of ready-to-use products:water (5.2.2.2) instead of hard water]. Restart the stopwatch at the beginning of the addition. Mix(5.3.2.6 a)) and place the tube in a water bath controlled at the chosen temperature (θ ± 1) °C for t.Just before the end of t, mix (5.3.2.6 a)) again.

b) At the end of t, take a sample of 1.0 ml of this mixture “A” in duplicate and inoculate using the pourplate or the spread plate technique (5.5.2.2 b)).

For incubation and counting, see 5.5.2.6.

5.5.2.4 Neutralizer control “B” – verification of the absence of toxicity of the neutralizer

To verify the absence of toxicity of the neutralizer, the procedure is as follows.

a) Pipette 8.0 ml of the neutralizer – used in the test (5.5.2.2) — and 1.0 ml of water (5.2.2.2) into a tube. Add 1.0 ml of the validation suspension (5.4.1.5). Start the stopwatch at the beginning of the addition, mix (5.3.2.6 a)), and place the tube in a water bath controlled at the chosen temperature (θ ± 1) °C for 5 min ± 10 s (10 s ± 1 s for contact times equal to or shorter than 10 min). Just before the end of this time, mix (5.3.2.6 a)).

b) At the end of this time, take a sample of 1.0 ml of this mixture “B” in duplicate and inoculate using the pour plate or the spread plate technique (5.5.2.2 b)).

For incubation and counting, see 5.5.2.6.

5.5.2.5 Method validation “C” – dilution-neutralization validation

To validate the dilution neutralization method, the procedure is as follows.

a) Pipette 1.0 ml of the interfering substance used in the test (5.5.2.2) into a tube. Add 1.0 ml of the diluent (5.2.2.4) and then, starting a stopwatch, add 8.0 ml of the product test solution only of the highest concentration used in the test (5.5.2.2). Mix (5.3.2.6 a)) and place the tube in a water bath

controlled at the chosen temperature (θ ± 1) °C for t. Just before the end of t, mix (5.3.2.6 a)) again.

b) At the end of t transfer 1.0 ml of the mixture into a tube containing 8.0 ml of neutralizer (used in 5.5.2.2). Restart the stopwatch immediately at the beginning of the addition. Mix (5.3.2.6 a)) and place the tube in a water bath controlled at the chosen temperature (θ ± 1) °C for 5 min ± 10 s ((10 ± 1) s for contact times equal or shorter than 10 min). Add 1.0 ml of the validation suspension (5.4.1.5). Start a stopwatch at the beginning of the addition and mix (5.3.2.6 a)). Place the tube in a water bath controlled at (20 ± 1) °C for (30 ± 1) min. Just before the end of this time, mix (5.3.2.6 a)) again. At the end of this time, take a sample of 1.0 ml of the mixture “C” in duplicate and inoculate using the pour plate or the spread plate technique (5.5.2.2 b)).

For incubation and counting, see 5.5.2.6.

5.5.2.6 Incubation and counting of the test mixture and the control and validation mixtures

For incubation and counting of the test mixture and the control and validation mixtures, the procedure is as follows.

a) Incubate (5.3.2.3) the plates for 20 h to 24 h. Discard any plates that are not countable (for any reason). Count the cfu on the plates to determine the total number of colony forming units.

Incubate the plates for a further 20 h to 24 h. Do not recount plates that no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

b) Note for each plate the exact number of colonies but record > 330 for any counts higher than 330 and determine the Vc values according to 5.6.2.2.

c) Calculate the numbers of cfu/ml in the test mixture “Na” and in the validation mixtures “A”, “B” and “C” using the method given in 5.6.2.4 and 5.6.2.6. Verify according to 5.7.

5.5.3 Membrane filtration method 5)

5.5.3.1 General

The test and the control and validation procedures (5.5.3.2 through 5.5.3.5) shall be carried out in parallel and separately for each experimental condition (5.5.1.1).

Each membrane filtration apparatus shall be equipped with a membrane of 0,45 µm pore size and 47 mm to 50 mm diameter (5.3.2.7) and filled with 50 ml of the rinsing liquid (5.2.2.6). The time required for filtering — if longer than one minute in exceptional cases — shall be recorded in the test report. When transferring the membranes to the surface of an agar plate, care should be taken to ensure that the test organisms are on the upper side of the membrane when placed on the plate, and to avoid trapping air between the membrane and agar surface.

5.5.3.2 Test “Na” – determination of the bactericidal concentrations

The procedure for determining the bactericidal concentrations is as follows:

a) See 5.5.2.2 a).

b) At the end of t, take a sample of 0,1 ml of the test mixture “Na” in duplicate and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.5.3.1). Filter immediately. Filter through at least 150 ml but no more than 500 ml of rinsing liquid (5.2.2.6). If the rinsing liquid is not water, complete the procedure by filtering 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of

separate TSA plates.

For handwash products at the end of t take a sample of 0,1 ml of the test mixture Na and dilute in 100-fold with 9,9 ml of rinsing liquid (5.2.2.6). Mix and transfer 0,1 ml sample in duplicate into two separate membrane filtration apparatus (5.5.3.1). Filter immediately. Filter through at least 150 ml but no more than 500 ml of rinsing liquid (5.2.2.6). If the rinsing liquid is not water, complete the procedure by filtering 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate TSA plates. Validation of non-toxicity of the neutralizing agent shall be performed according to 5.5.3.4 for handwash products.

For incubation and counting, see 5.5.3.6.

c) See 5.5.2.2 c).

d) See 5.5.2.2 d).

5.5.3.3 Experimental conditions control “A” – validation of the selected experimental conditions and/or verification of the absence of any lethal effect in the test conditions

To validate the selected experimental conditions and/or verify the absence of any lethal effect in the test conditions, the procedure is as follows:

a) See 5.5.2.3 a).

b) At the end of t, take a sample of 1.0 ml of this mixture “A” in duplicate and transfer each 1.0 ml sample into a separate membrane filtration apparatus (5.5.3.1). Filter immediately and additionally with 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate TSA plates (5.2.2.3).

For incubation and counting, see 5.5.3.6.

5.5.3.4 Filtration control “B” – validation of the filtration procedure To validate the filtration procedure, proceed as follows.

Take 0,1 ml of the validation suspension (5.4.1.5) in duplicate (suspension for control “B”) and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.5.3.1).

For handwash products take a sample of 0,1 ml of the validation suspension (5.4.1.5) in duplicate and dilute 100-fold with 9,9 ml of rinsing liquid (5.2.2.6). Mix and filter each sample into a separate membrane filtration apparatus (5.5.3.1).

Filter immediately. Filter through the rinsing liquid (5.2.2.6) the same way as in the test (5.5.3.2 b)). If the rinsing liquid is not water, complete the procedure by filtering 50 ml of water (5.2.2.2). Then transfer each of the membranes to the surface of separate TSA plates (5.2.2.3).

For incubation and counting, see 5.5.3.6.

5.5.3.5 Method validation “C” – validation of the membrane filtration method or counting of the bacteria on the membranes which have previously been in contact with the mixture of product and interfering substance

For validation of the membrane filtration method or counting of the bacteria on the membranes that have previously been in contact with the mixture of product and interfering substance, the procedure is

as follows.

a) See 5.5.2.5 a).

b) At the end of t, take 0,1 ml of the validation mixture “C” in duplicate and transfer each 0,1 ml sample into a separate membrane filtration apparatus (5.5.3.1). Filter immediately. Filter through the rinsing liquid (5.2.2.6) the same way as in the test (5.5.3.2 b)), then cover the membranes with 50 ml of the rinsing liquid (5.2.2.6) and add 0,1 ml of the validation suspension (5.4.1.5). Filter immediately again and additionally with 50 ml of water (5.2.2.2), then transfer each of the membranes to the surface of separate TSA plates (5.2.2.3).

For incubation and counting, see 5.5.3.6.

5.5.3.6 Incubation and counting of test mixture and the control and the validation mixtures

For incubation and counting of the test mixture and the control and validation mixtures, the procedure is as follows.

a) Incubate (5.3.2.3) the plates for 20 h to 24 h. Discard any plates that are not countable (for any reason). Count the colonies on the membranes. Incubate the plates for a further 20 h to 24 h. Do not recount plates that no longer show well separated colonies. Recount the remaining plates. If the number has increased use only the higher number for further evaluation.

b) Note for each plate the exact number of colonies but record “> 165” for any counts higher than 165 and determine the Vc values according to 5.6.2.2.

c) Calculate the numbers of cfu/ml in the test mixture “Na” and in the validation mixtures “A”, “B” and “C” using the method given in 5.6.2.4 and 5.6.2.6. Verify according to 5.7.

5.6 Experimental data and calculation

5.6.1 Explanation of terms and abbreviations

5.6.1.1 Overview of the different suspensions and test mixtures

N and Nv represent the bacterial suspensions, Na represents the bactericidal test mixture, A (experimental conditions control), B (neutralizer or filtration control), C (method validation) represent the different control test mixtures.

N, Nv, N_0, Nv_0, Na and A, B and C represent the number of cells counted per ml in the different test mixtures in accordance with Table 3.

5.6.1.2 Vc values

All experimental data are reported as Vc values:

— in the dilution-neutralization method (test and controls), a Vc value is the number of colony- forming units counted per 1.0 ml sample;

— in the membrane filtration method, a Vc value is the number of colony-forming units counted per 0,1 ml sample of test mixture “Na” and per 1.0 ml sample in the controls. 1 ml for “A” and 0,1 ml for “B” and “C”.

5.6.2 Calculation

5.6.2.1 General

The first step in the calculation is the determination of the Vc values, the second the calculation of N, N o , Na, Nv, Nv_o , A, B and C. The third step is the calculation of the reduction R (5.8).

5.6.2.2 Determination of Vc values

The Vc values are determined as follows.

a) The usual limits for counting bacteria on agar plates are between 15 and 300. In this standard, a deviation of 10 % is accepted, so the limits are 14 and 330. On membranes, the usual upper limits are different: 150, therefore with the 10 % deviation, the limit is 165.

NOTE 1 The lower limit (14) is based on the fact that the variability is increasing the smaller the number counted in the sample (1 ml or 0,1 ml) is, and therefore subsequent calculations can lead to wrong results. The lower limit refers only to the sample (and not necessarily to the counting on one plate), e.g. three plates per 1 ml sample with 3 cfu, 8 cfu and 5 cfu give a Vc value of 16. The upper limits (330.165) reflect the imprecision of counting confluent colonies and growth inhibition due to nutriment depletion. They refer only to the counting on one plate and not necessarily to the sample.

b) For counting the test suspension “N” (5.4.1.6), the validation suspension “Nv” (5.4.1.6) and for all countings of the dilution-neutralization method (5.5.2.6), determine and record the Vc values according to the number of plates used per 1 ml sample (5.6.1.2).

If more than one plate per 1 ml sample has been used to determine the Vc value, the countings per plate are to be noted.

If the count on one plate is higher than 330, report the number as “> 330”. If more than one plate per 1 ml sample has been used and at least one of them shows a number higher than 330, report this Vc value as “> sum of the counts” (e.g. for “> 330, 310, 302”, report “> 942”).

If a Vc value is lower than 14, report the number but substitute by “< 14” for further calculation (in the case of Na).

For the membrane-filtration method (5.5.3), the counts on the membranes are the Vc values (5.6.1.2). Report the Vc values below the lower limit (14) or above the upper limit (165) as described above.

c) Only Vc values within the respective counting limits are taken into account for further calculation, except in the case of Na (5.6.2.4).

5.6.2.3 Calculation of N and N_0

N is the number of cells per ml in the test suspension (5.4.1.4, 5.6.1.1).

Since two dilutions of the test suspension (5.4.1.4 in connection with 5.4.1.6) are evaluated, calculate

the number of cfu/ml as the weighted mean count using the following Formula (1):

$$N = \frac{C}{\left(n_1 + 0,1n_2\right)10^{-6}} \qquad (1)$$

where

c is the sum of Vc values taken into account;

n_1 is the number of Vc values taken into account in the lower dilution, i.e. 10^{-6};

n_2 is the number of Vc values taken into account in the higher dilution, i.e. 10^{-7};

10^{-6} is the dilution factor corresponding to the lower dilution.

Round off the results calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is more than 5, the preceding figure is increased by one unit; 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 cfu/ml is expressed by a number between 1.0 and 9,9 multiplied by the appropriate power of 10.

$$\frac{168 + 215 + 14 + 25}{(2 + 0{,}1 \times 2)\ 10^{-6}} = \frac{422}{2{,}2 \times 10^{-6}} = 1{,}9182 \times 10^{8} = 1{,}9\ x\ 10^{8}\ \text{(in cfu/ml)}$$

N_0 is the number of cells per ml in the test mixture (5.5.2.2 a) at the beginning of the contact time (time "zero" = 0). It is one-tenth of the weighted mean of N due to the tenfold dilution by the addition of the product and interfering substance.

5.6.2.4 Calculation of Na

Na is the number of survivors per ml in the test mixture (5.5.2.2 a) or (5.5.3.2 a) at the end of the contact time and before neutralization or membrane filtration. It is tenfold higher than the Vc values due to the addition of neutralizer and water (5.5.2.2 b) or the sample volume of 0,1 ml (5.5.3.2 b) in the membrane filtration method.

Calculate Na using the following Formula (2):

$Na = 10\ c/n$ (2)

where

c is the sum of Vc values taken into account;

n is the number of Vc values taken into account.

If one or both of the duplicate Vc values are either below the lower or above the upper limit, express the results as "less than" or "more than".

EXAMPLE

a) duplicate Vc values: 2.16

$$Na = \frac{(<14 + 16)\ \text{x}\ 10}{2} \leq 150$$

b) duplicate Vc values (membrane filtration): > 165, > 165

$$Na = \frac{(>165 + >165)\ \text{x}\ 10}{2} \geq 1\ 650$$

c) duplicate Vc values (two spread plates per 1.0 ml sample): > 660, 600

$$Na = \frac{(>660 + 600)\,\text{x}\,10}{2} \geq 6\ 300$$

5.6.2.5 Calculation of Nv and Nv_o

Nv is the number of cells per ml in the validation suspension (5.4.1.5 a). It is tenfold higher than the counts in terms of Vc values due to the dilution step of 10 −1 (5.4.1.5 b).

Nv_0 is the number of cells per ml in the mixtures “A”, “B” and “C” at the beginning of the contact time (time 0) (5.6.1.1). It is one-tenth of the mean of the Vc values of Nv (5.4.1.6 c) taken into account.

Calculate Nv and Nv_0 using the following Formulae (3) and (4):

Nv = 10c/n (3)

Nv_0 = c/n (4)

where

c is the sum of Vc values taken into account;

n is the number of Vc values taken into account.

5.6.2.6 Calculation of A, B and C

A, B and C are the numbers of survivors in the experimental conditions control “A” (5.5.2.3 or 5.5.3.3), neutralizer control “B” (5.5.2.4) or filtration control “B” (5.5.3.4) and method validation “C” (5.5.2.5 or 5.5.3.5) at the end of the contact time t (A) or the defined times 5 min (B) and 30 min (C). They correspond to the mean of the Vc values of the mixtures “A”, “B” and “C” taken into account.

Calculate A, B and C using the following Formula (5):

A, B, C = c/n (5)

where

c is the sum of Vc values taken into account;

n is the number of Vc values taken into account.

5.7 Verification of methodology

5.7.1 General

A test is valid if:

— all results meet the criteria of 5.7.3 and

— the requirements of 5.8.2 are fulfilled.

5.7.2 Control of weighted mean counts

For results calculated by weighted mean of two subsequent dilutions (e.g. “N”), the quotient of the mean of the two results shall be not higher than 15 and not lower than 5. Results below the lower limit are taken as the lower limit number (14). Results above the respective upper limit (5.6.2.2 b) are taken as the upper limit number.

EXAMPLE

For N 10^{-6} dilution: 168 cfu/ml + 215 cfu/ml.10^{-7} dilution: 20 cfu/ml + < 14 cfu/ml;
(168 + 215) / (20 + 14) = 383/34 = 11.26 therefore between 5 and 15.

NOTE When the counts obtained on plates are out of limts fixed for the determination of Vc values (5.6.2.2 b),

check for the weighted mean as mentioned above but use only the Vc values within the counting limits for the calculation of N.

5.7.3 Basic limits

For each test organism check that:

a) N is between 1.5×10^8 and 5.0×10^8 ($8{,}17 \leqslant \lg N \leqslant 8.70$)

N_0 is between 1.5×10^7 and 5.0×10^7 ($7{,}17 \leqslant \lg N\,0 \leqslant 7.70$)

b) Nv is between 300 and 1600 (3.0×10^2 and 1.6×10^3)

(Nv_0 is between 3.0 × 10 1 and 1.6×10^2)

c) A,B,C are equal to or greater than $0.5 \times Nv_0$.

d) control of weighted mean counts (5.7.2): quotient is not lower than 5 and not higher than 15.

5.8 Expression of results and precision

5.8.1 Reduction

The reduction (R = No /Na) is expressed in logarithm.

For each test organism record the number of cfu/ml in the test suspension N (5.6.2.3) and the test Na (5.6.2.4). Calculate N 0 (5.6.2.3).

For each product concentration and each experimental condition, calculate and record the decimal log reduction (lg) separately using the following Formula (6):

$$\lg R = \lg No - \lg Na \qquad (6)$$

For the controls and validation of the dilution-neutralization method or membrane filtration method, record Nv 0 (5.6.2.5), the results of A, B and C (5.6.2.6) and their comparison with Nv 0 (5.7.3 c).

5.8.2 Control of active and non-active product test solution (5.4.2)

At least one concentration per test (5.5.2.2 a - c or 5.5.3.2 a - c) shall demonstrate a 5 lg or more

reduction and at least one concentration shall demonstrate a lg reduction of less than 5.

Handwashes shall demonstrate only a 3 lg or more reduction at in test concentration of 50 % or less and at least one concentration shall demonstrate a lg reduction of less than 3.

5.8.3 Limiting test organism and bactericidal concentration

For each test organism, record the lowest concentration of the product which passes the test. (lg R ⩾ 5 or ⩾ 3 for handwashes). Record as the limiting test organism the test organism requiring the highest of these concentrations (it is the least susceptible to the product in the chosen experimental conditions).

The lowest concentration of the product active on the limiting test organism is the bactericidal concentration determined according to this standard.

5.8.4 Precision, replicates

Taking into account the precision of the methodology determined by a statistical analysis based on data provided by a collaborative study, replication of the test (nine replicates for a precision of ± 1 lg in reduction) is recommended (Annex E). The number of replicate tests shall be decided according to the required level of precision, taking into account the intended use of the test results.

Replication means the complete test procedure with separately prepared test - and validation suspensions. The replicate of the test may be restricted to the limiting test organism. The mean of the results of the replicates - not each single result - shall demonstrate at least a 5 lg reduction (3 lg for handwashes) and shall also be calculated and recorded.

5.9 Interpretation of results - conclusion

5.9.1 General

According to the chosen experimental conditions, the bactericidal concentrations determined according to this standard can differ (Clause 4).

5.9.2 Bactericidal activity for general purposes

The product shall be deemed to have passed the EN 1276 standard if it demonstrates in a valid test at least a 5 lg reduction within the adopted test conditions as described in Tables 1 and 2 with the chosen interfering substance simulating clean or dirty conditions defined by this standard when the test organisms are Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Enterococcus hirae (E. faecium when the test temperature is ⩾ 40 ° C).

5.9.3 Bactericidal activity for hand hygiene

The bactericidal concentration for hand hygiene is the concentration of the tested product for which at least a 5 lg reduction for hygienic handrub and 3 lg reduction for hygienic handwash (at 50 % in test

concentration or less) is demonstrated in a valid test under the chosen test conditions. The product shall have passed the EN 1276 standard under the adopted test conditions as described in Tables 1 and 2.

The bactericidal concentration is the concentration active on the limiting strain.

5.10 Test report

The test report shall refer to this document (EN 1276).

The test report shall state, at least, 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 receipt at the test laboratory;

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) test method and its validation:

1) if the dilution-neutralization method is used, full details of the test for validation of the neutralizer shall be given;

2) if the membrane filtration method is used, full details of the procedure which was carried out in order to justify the use of the membrane filtration method shall be given;

e) 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 5.4.2);

4) appearance product dilutions;

5) contact time(s);

6) test temperature(s);

7) interfering substance(s);

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

9) temperature of incubation;

10) neutralizer or rinsing liquid;

11) identification of the bacterial strains used;

f) test results:

1) controls and validation;

2) evaluation of bactericidal activity;

3) number of replicates per test organism;

g) special remarks;

h) conclusion;

i) locality, date and identified signature.

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

Table B.1 — Examples of neutralizers of the residual antimicrobial activity of chemical disinfectants and antiseptics and of rinsing liquids

Antimicrobial agent	Chemical compounds able to neutralize residual antimicrobial activity	Examples of suitable neutralizers and of rinsing liquids (for membrane filtration methods) [a]
Quaternary ammonium compounds and fatty amines Amphoteric compounds	Lecithin, Saponin, Polysorbate 80, Sodium dodecyl sulphate, Ethylene oxide condensate of fatty alcohol (non-ionic surfactants) [b]	- Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. - Polysorbate 80, 30 g/l + sodium dodecyl sulphate, 4 g/l + lecithin, 3 g/l. - Ethylene oxide condensate of fatty alcohol, 3 g/l + lecithin, 20 g/l + polysorbate 80, 5 g/l. *Rinsing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*
Biguanides and similar compounds	Lecithin [c], Saponin, Polysorbate 80	- Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. *Rinsing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*
Oxidizing compounds (Chlorine, iodine, hydrogen peroxide, peracetic acid, hypochlorites, etc.)	Sodium thiosulphate [d] Catalase [for hydrogen peroxide or products releasing hydrogen peroxide]	- Sodium thiosulphate, 3 g/l to 20 g/l + polysorbate 80, 30 g/l + lecithin, 3 g/l. - Polysorbate 80, 50 g/l + catalase 0,25 g/l + lecithin 10 g/l. *Rinsing liquid: sodium thiosulphate, 3 g/l.*

Antimicrobial agent	Chemical compounds able to neutralize residual antimicrobial activity	Examples of suitable neutralizers and of rinsing liquids (for membrane filtration methods) [a]
Aldehydes	L – histidine Glycine	- Polysorbate 80, 30 g/l + lecithin, 3 g/l + L-histidine, 1 g/l (or + glycine, 1 g/l). - Polysorbate 80, 30 g/l + saponin, 30 g/l + L-histidine, 1 g/l (or + glycine, 1 g/l). *Rinsing liquid: polysorbate 80, 5 g/l + L-histidine, 0,5 g/l (or + glycine, 1 g/l).*
Phenolic and related compounds: orthophenylphenol, phenoxyethanol, triclosan, phenylethanol, etc. Anilides	Lecithin Polysorbate 80 Ethylene oxide condensate of fatty alcohol [b]	- Polysorbate 80, 30 g/l + lecithin, 3 g/l. - Ethylene oxide condensate of fatty alcohol, 7 g/l + lecithin, 20 g/l, + polysorbate 80, 4 g/l. *Rinsing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*
Alcohols	Lecithin, Saponin, Polysorbate 80 [e]	- Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. *Rinsing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*

[a] According to the pH of the tested product, the pH of the neutralizer or the rinsing liquid can be adjusted at a suitable value or prepared in phosphate buffer [ex: phosphate buffer 0,25 mol/l: potassium dihydrogen phosphate (KH_2PO_4) 34 g; distilled water (500 ml); adjusted to pH 7,2 ± 0,2 with sodium hydroxide (NaOH) 1 mol/l; distilled water up to 1 000 ml].

[b] The carbon chain-length varies from C_{12} to C_{18} carbon atoms.

[c] Egg and soya; egg is preferable.

[d] The toxic effect of sodium thiosulphate differs from one test organism to another.

[e] For the neutralization of short chain alcohols (less than C5), simple dilution can be appropriate. Care is to be taken if the alcohol-based -products contain additional antimicrobial agents.

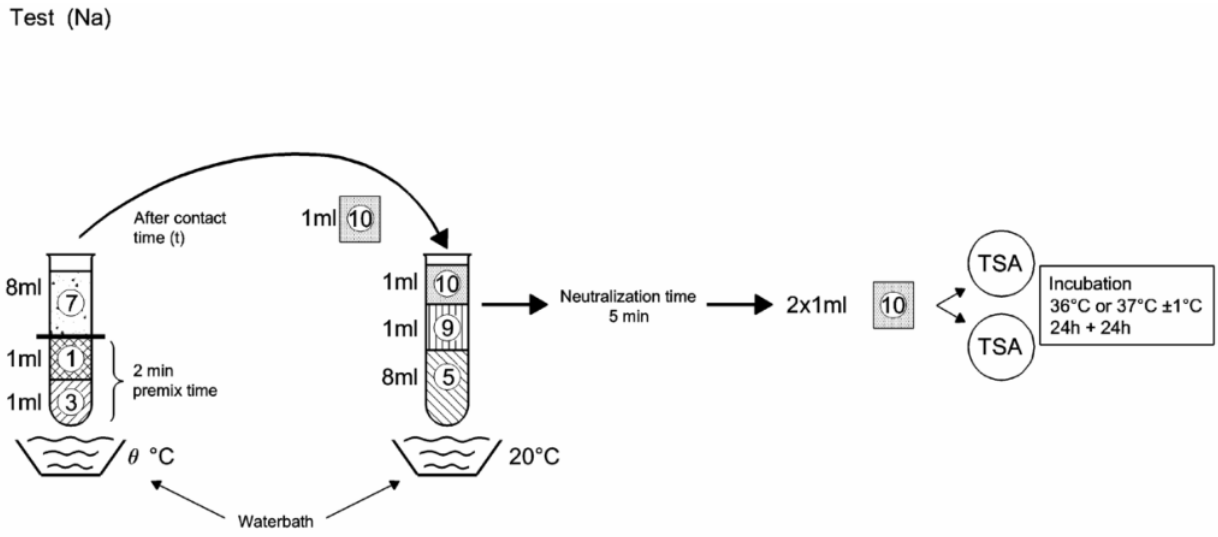
Other neutralizer mixtures can be required for products containing more than one antimicrobial agent.

The concentrations of the various neutralizing compounds or of the neutralizer as such cannot be adequate to neutralize high concentrations of the products.

Table 3 — Number of cells counted per ml in the different test mixtures

	Number of cells per ml in the bacterial suspensions	Number of cells per ml in the test mixtures at the beginning of the contact time (time = 0)	Number of survivors per ml in the test mixtures at the end of the contact time t (*A*) or 5 min or 10 s (*B*) or 30 min (*C*)
Test	N Test suspension	N_0 (= $N/10$)	Na (before neutralization or filtration)
Controls	Nv Validation suspension	Nv_0 (= $Nv/10$)	A, B, C

Graphical representations of dilution neutralization method and membrane filtration method



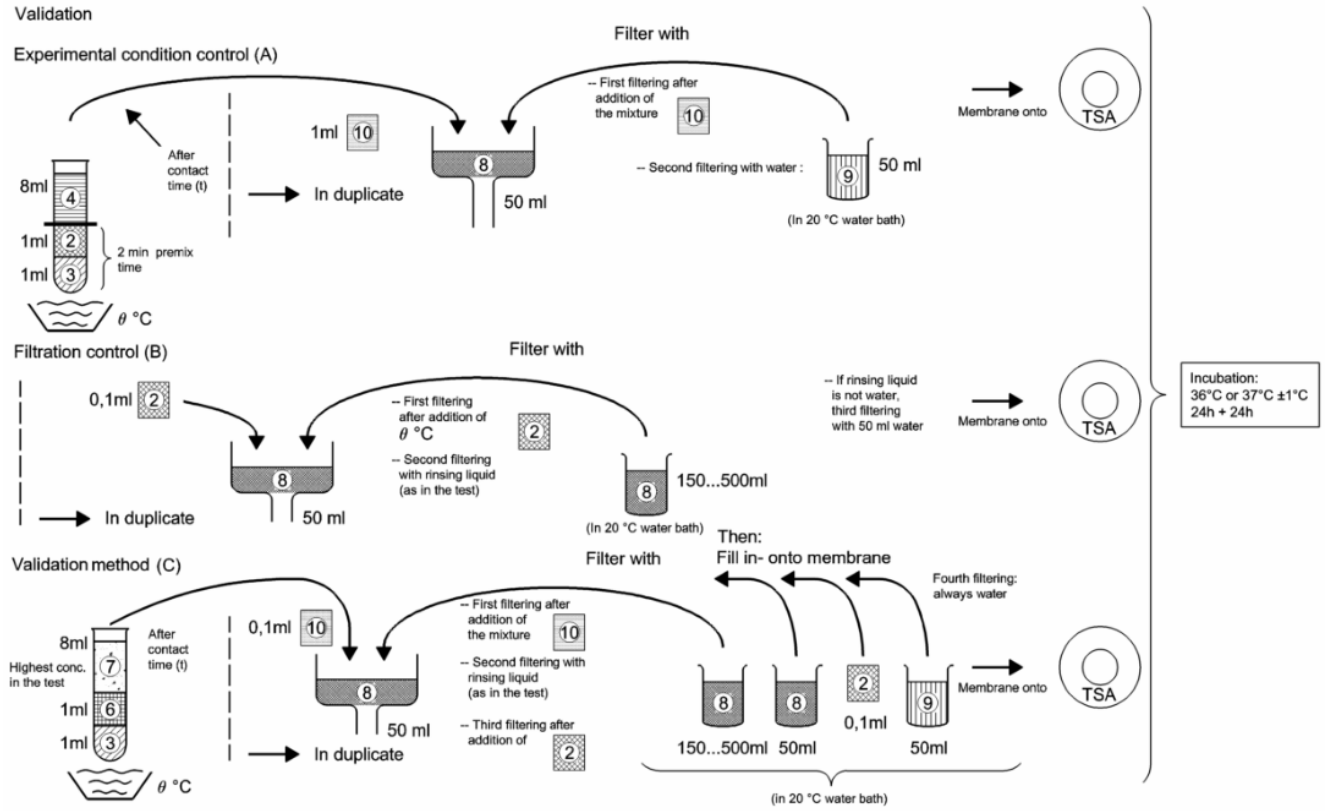
Key

1	Test Suspension (N)	3	Interfering substance	5	Neutralizer (20 °C)	7	Product test solution
2	Validation Supension (Nv)	4	Hard water	6	Diluent	8	Rinsing liquid (20 °C)

9	Water
10	Mixture

NOTE In case of contact times of 10 min or shorter, neutralization time is only (10 ±1) s.

Figure C.1 — Dilution neutralization method test



Key

1	Test Suspension (*N*)	3	Interfering substance	5	Neutralizer (20 °C)	7	Product test solution
2	Validation Supension (*Nv*)	4	Hard water or distilled water	6	Diluent	8	Rinsing Liquid (20 °C)
9	Water	10	Mixture				

NOTE For handwash products, 0,1 ml of test mixture (Na) and pre-diluted in 9,9 ml of rinsing liquid (5.5.3.2).

Figure C.4 — Membrane filtration method validation

Validation and controls

Validation suspension (Nv_0)			Experimental Conditions control (A)			Neutralizer or filtration control (B)			Method validation (C) Product conc.: *10 ml/l*		
*Vc*1	*86 (40 + 46)*	$\bar{x}$ =	*Vc*1	*79 (43 + 36)*	$\bar{x}$ =	*Vc*1	*86 (42 + 44)*	$\bar{x}$ =	*Vc*1	*75 (35 + 40)*	$\bar{x}$ =
*Vc*2	*92 (47 + 45)*	89	*Vc*2	*84 (39 + 45)*	*81,5*	*Vc*2	*91 (43 + 48)*	*88,5*	*Vc*2	*87 (41 + 46)*	*81*
30 ≤ $\bar{x}$ of Nv_0 ≤ 160 ? ☒ *yes* ☐ *no*			$\bar{x}$ of A is ≥ 0,5 x $\bar{x}$ of Nv_0 ? ☒ *yes* ☐ *no*			$\bar{x}$ of B is ≥ 0,5 x $\bar{x}$ of Nv_0 ? ☒ *yes* ☐ *no*			$\bar{x}$ of C is ≥ 0,5 x $\bar{x}$ of Nv_0 ? ☒ *yes* ☐ *no*		

Test suspension and Test

Test-suspension (*N* and N_0):	***N***	*Vc*1	*Vc*2	$\bar{x}$ wm = *193,64* x 10^6 ; lgN = 8,29
	10^{-6}	*168*	*213*	No = N/10 ; lg No = 7,29
	10^{-7}	*20*	*25*	7,17 ≤ lgN_0 ≤ 7,70? ☒ *yes* ☐ *no*

Conc. of the product %	*Vc*1	*Vc*2	***Na*** = $\bar{x}$ x10	**lg*Na***	**lg*R*** *(N_0 = 7,29)*	Contact-time (min)
0,50	*> 660*	*> 630*	*> 6 450*	*> 3,81*	*< 3,48*	*5 min*
0,75	*122*	*154*	*1 380*	*3,14*	*4,15*	*5 min*
1,00	*7*	*0*	*< 140*	*< 2,15*	*> 5,14*	*5 min*

Remarks:

Counting per plate for;

(*N*) 10^{-6}: 80 + 88; 105 + 108 **(*Na*)** 0,75 %: 66 + 56; 71 + 83

10^{-7}: 9 + 11; 15 + 10 1,00 % *Vc*1: 1 + 6

Explanations:

Vc = count per ml (one plate or more) $\bar{x}$ wm = weighted mean of $\bar{x}$

$\bar{x}$ = average of *Vc*1 and Vc2 (1. + 2. duplicate) *R* = reduction (lg *R* = lgN_0 – lg*Na*)

Attachments: real photos

Photo 1

********** END OF THE REPORT **********