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Ophthalmic optics — Contact lens care products —

Part 2: Method for evaluating disinfecting efficacy by contact lens care products using trophozoites of *Acanthamoeba* species as the challenge organisms

Optique ophtalmique — Produits d'entretien de lentilles de contact —

Partie 2: Méthode d'évaluation de l'efficacité désinfectante des produits d'entretien des lentilles de contact utilisant des trophozoïtes de l'espèce Acanthamoeba *comme organismes pour l'épreuve microbienne*

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Foreword

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This document was prepared by Technical Committee ISO/TC 172, *Optics and photonics,* Subcommittee SC 7, *Ophthalmic optics and instruments*.

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Ophthalmic optics — Contact lens care products —

Part 2: Method for evaluating disinfecting efficacy by contact lens care products using trophozoites of *Acanthamoeba* species as the challenge organisms

1 Scope

This document specifies a test method to be used in evaluating the antimicrobial activity of products for contact lens disinfection by chemical methods using the trophozoite form of *Acanthamoeba* species as the challenge organism.

This document is not applicable to the evaluation of oxidative systems that require a special lens case for use.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
contact lens disinfection
chemical or physical process to reduce the number of viable microorganisms

Note 1 to entry: This is specified in the performance requirement clauses of ISO 14729 or ISO 18369-1.

3.2
trophozoite
motile, feeding amoeboid form of *Acanthamoeba*

[SOURCE: ISO 19045:2015, 2.1]

3.3
encystment
phase in the life cycle of *Acanthamoeba* where the trophozoite stage transforms into the cyst stage

3.4
mature cyst
dormant form of *Acanthamoeba,* composed of an inner and outer cell wall, typically more resistant to a range of challenges than *trophozoites* (3.2)

Note 1 to entry: Challenges include heat, dehydration, chemical, etc.

3.5
immature cyst
cyst comprised only of the inner cell wall

3.6
room temperature
temperature between 18 °C to 25 °C

3.7
refrigerator temperature
temperature between 2 °C to 8 °C

3.8
passage
transfer or transplantation of cells, with or without dilution, from one culture vessel to another

Note 1 to entry: It is understood that any time cells are transferred from one vessel to another, a certain portion of the cells may be lost and, therefore, dilution of cells, whether deliberate or not, may occur.

Note 2 to entry: This term is synonymous with the term “subculture”.

3.9
passage number
number of times cells in the culture have been subcultured or passaged[10]

4 Principle

This assay challenges a contact lens disinfecting product with a standard inoculum of trophozoites of the specified *Acanthamoeba* species and establishes the extent of their viability at pre-determined time intervals comparable with those during which the product may be used.

5 *Acanthamoeba* trophozoite disinfecting test method

5.1 Organisms

5.1.1 *A. castellanii* (ATCC 50370), *A. polyphaga* (ATCC 30461).

5.1.2 Do not use *Acanthamoeba* trophozoites beyond passage number 5.

5.1.3 *Escherichia coli* (ATCC 8739).

NOTE *E. coli* is used for preparation of agar overlays for recovery of challenge organisms for recovery method one (5.9.1) and for inoculation of microtitre wells for recovery of challenge organisms for recovery method two (5.9.2).

5.2 Culture media and reagents

5.2.1 **Ac#6 axenic semi-defined *Acanthamoeba* growth medium** (in accordance with Annex A).

5.2.2 **¼ strength Ringer’s solution** (see Annex B).

5.2.3 **Page’s saline non-nutrient agar** (see Annex D) – recovery method one (see 5.9.1).

5.2.4 **Trypticase soy broth (TSB)** – (for use in Annex E).

5.2.5 **Neutralising Broth for both recovery methods** (see Annex G).

5.3 Test materials

5.3.1 Sterile 50 ml polypropylene centrifuge tubes.

5.3.2 Sterile 15 ml round-bottomed tubes (polystyrene, polypropylene or glass, depending on the formulations to be tested).

5.3.3 Sterile 12-well flat bottom opto-mechanical- or plasma-treated microtitre plates.

5.3.4 Sterile 96-well flat bottom opto-mechanical- or plasma-treated microtitre plates.

5.3.5 Calibrated pipettes (fixed and adjustable volume and multichannel) to deliver: 20 µl, 50 µl, 100 µl, 180 µl, 200 µl and 1 000 µl.

5.3.6 Sterile, disposable transfer pipets, capable of pipetting 3 ml and 10 ml.

5.3.7 Inverted microscope, with ×10, ×20 and ×40 phase contrast objectives.

5.3.8 (28 ± 2) °C incubator.

5.3.9 Centrifuge.

5.3.10 Vortex mixer.

5.3.11 Cell counting chamber (haemocytometer), with a depth of 0,2 mm; e.g. an appropriate reusable or disposable Fuchs or modified Fuchs Rosenthal haemocytometer.

5.3.12 Sterile 75 cm^2 and 175 cm^2 flat polystyrene tissue culture flasks.

5.3.13 Orbital shaker.

5.3.14 Refrigerator, with a temperature of 2 °C to 8 °C.

5.4 Test samples

Aliquots of the product to be tested shall be representative of the product to be marketed. The product should be taken directly from the final product container immediately prior to testing. Three lots of product shall be tested. Each lot of product shall be tested with a separate inoculum preparation.

5.5 Culture maintenance

5.5.1 The strain should not be subcultured more than five passages as per American Type Culture Collection (ATCC) protocols.

5.5.2 Maintenance of stock cultures and scaling up cultures for testing (see Annex C).

5.6 Growth and harvest of microbial challenge (trophozoite)

5.6.1 Grow trophozoites as described in Annex C using *Acanthamoeba* growth medium (Ac#6, Annex A).

Prepare a sufficient number of flasks based on the size of the experiment and the number of trophozoites required.

5.6.2 After the 24 h scale up, dislodge the adherent trophozoites. Trophozoites may be dislodged by vigorously shaking, by scraping the bottom of the flask with a cell scraper or by striking the flask with moderate force.

5.6.3 Decant trophozoites into 50 ml polypropylene centrifuge tubes and centrifuge at 500 × *g* for 5 min at room temperature.

5.6.4 Resuspend one tube pellet in 10 ml of ¼ strength Ringer's solution as specified in Annex B. If more inoculum is required, resuspend additional pellets using this same method.

5.6.5 Wash 3 times with 10 ml of ¼ strength Ringer's solution by centrifugation at 500 × *g* for 2 min at room temperature.

5.6.6 Resuspend pellet by vortexing in 1 ml to 2 ml of ¼ strength Ringer's solution.

5.7 Preparation of *Acanthamoeba* stock solution

5.7.1 Enumerate trophozoite numbers in the stock solution using a cell counting chamber (make a 1:10 to 1:100 dilution in ¼ strength Ringer's solution or appropriate diluent to assist) and record number cells/ml.

5.7.2 Adjust the *Acanthamoeba* stock concentration in ¼ strength Ringer's Solution to 5 × 10^6 cells/ml to 5 × 10^7 cells/ml based on the value obtained using the haemocytometer; this solution shall be called the standardized *Acanthamoeba* stock solution.

Inoculate 10 ml of 1/4 strength Ringer's solution with 0,1 ml of the standardized *Acanthamoeba* stock solution to result in 5 × 10^4 cells/ml and 5 × 10^5 cells/ml for the inoculum control solution.

5.8 Stand-alone procedure – inoculation

5.8.1 If the product is sensitive to light, protect it from light during the period of the test.

5.8.2 Prepare a set of three round-bottomed tubes (for each lot tested) with each tube containing 10 ml of test product solution per challenge organism. Tubes that are compatible with the test solution shall be used.

5.8.3 Inoculate the sample tube of the product to be tested with 0,1 ml of a suspension of the standardized *Acanthamoeba* stock solution providing the cell concentration range (4 × 10^4 cells/ml to 6 × 10^5 cells/ml) specified in 5.7.2 Ensure that the volume of inoculum does not exceed 1 % of the sample volume.

5.8.4 Mix contents of tubes using a vortex mixer (until a vortex forms). Ensure complete dispersion of the inoculum by adequate mixing.

5.8.5 Store the inoculated product at room temperature. The temperature shall be monitored using a calibrated device and the temperature documented.

5.9 Recovery procedures

Use at least four replicates in any recovery procedure. All recovery wells shall be observed at 14 days. Please see Annex J for representative photographic images of positive and negative wells.

5.9.1 Stand-alone procedure – recovery method one (12 well plate method)

5.9.1.1 Take 1,0 ml aliquots of the inoculated product for determination of viable count at the disinfecting time of interest following mixing using vortex mixer until a vortex forms. Recommended time points include:

25 % and 100 % of the minimum recommended disinfecting time for all organisms. If overnight contact lens disinfection is recommended, use a soaking time of 8 h.

5.9.1.2 At the specified time intervals remove 1,0 ml aliquot from the test article and add to 9,0 ml of validated neutralising broth (see Annex G) (10^{-1} dilution). Mix the suspension well using the vortex mixer until vortex forms. Allow to sit for appropriate time to allow neutralisation to be completed.

5.9.1.3 Perform a further five (5) 10-fold serial dilutions in ¼ strength Ringer's solution (see Annex B) (dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}).

5.9.1.4 Determine the viable count of organisms in appropriate dilutions by removing 1 ml of each dilution and placing it into the corresponding well of a 12-well tissue culture plate containing NNA as specified in Annex D with a lawn of *E. coli* (see Annex F). Plate each dilution in quadruplicate.

5.9.1.5 Incubate plates at 28 ± 2 °C and inspect microscopically for growth. All recovery wells shall be observed at 14 days. Please see Annex J for representative photographic images of positive and negative wells.

5.9.1.6 The absence of growth per well shall be documented, e.g. by recording a "-" (no recovery), the observance of growth per well shall be documented, e.g. by recording a "+" (recovery).

5.9.1.7 Determine log reduction values by using the most-probable number method using the Reed and Muench computation as specified in Annex H or the Spearman-Karber computation specified in Annex I. For recovery method one, the Reed and Muench spreadsheet will indicate 1 ml per well.

5.9.2 Stand-alone procedure – recovery method two (96 well plate method)

5.9.2.1 Take 20 µl aliquots of the inoculated product for determination of viable count at the disinfecting time of interest following mixing using vortex mixer until a vortex forms. Recommended time points include: 25 % and 100 % of the minimum recommended disinfecting time for all organisms. If overnight contact lens disinfection is recommended, use a soaking time of 8 h.

5.9.2.2 At the specified time intervals remove 20 µl from the test article and add to at least four outer wells of a 96-well microtitre plate (A1 to A4) containing 180 µl of validated neutraliser broth (see Annex G) (10^{-1} dilution). Allow to sit for appropriate time to allow neutralization to be completed. Refer to Figure 1 for an example of a 96 well microtiter plate layout.

5.9.2.3 Mix the contents of the outer wells by pipetting gently up and down six times and make five serial 10-fold dilutions across the microtitre plate by transferring 20 µl to the next well, mixing and transferring another 20 µl, etc. (wells B1-B4, C1-C4, D1-D4, E1-E4 and F1-F4). Discard the final 20 µl. The following dilutions will therefore be prepared in this step: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}.

For recovery method two, the Reed and Muench spreadsheet will indicate 0,2 ml per well.

5.9.2.4 Add 50 µl of *E. coli* (see Annex E) to each well.

5.9.2.5 Cover and incubate the plates at 28 ± 2 °C and inspect microscopically for growth. All recovery wells must be observed at 14 days. Please see Annex J for representative photographic images of positive and negative wells. Trophozoites may undergo encystment and so the wells may contain immature and mature cysts

5.9.2.6 The absence of growth per well shall be documented, e.g. by recording a "-" (no recovery), the observance of growth per well shall be documented, e.g. by recording a "+" (recovery).

5.9.2.7 Determine log reduction values by using the most-probable number method using the Reed and Muench computation (see Annex H) or the Spearman-Karber computation (see Annex I).

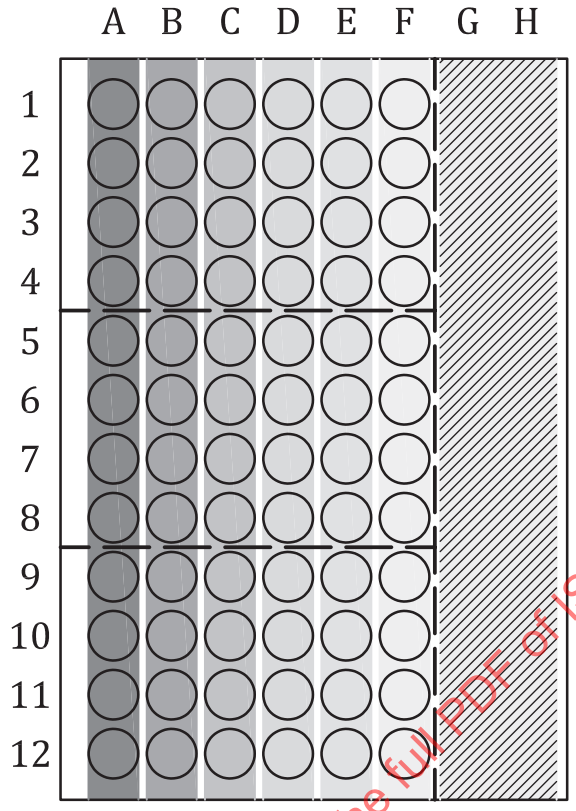

Figure 1 — Layout of the 96-well Plate for Method 2

Divide 96-well flat bottomed microtitre plates as shown in Figure 1:

Add 180 µl of validated neutralising broth (Annex G) to outer wells (column A) and 180 µl of ¼ strength Ringer's solution to the rest of the wells (columns B-F).

6 Controls

6.1 Inoculum control

6.1.1 The inoculum control shall be conducted at each trial using the same materials and methods employed in the assay substituting ¼ Ringer's for the test solution. Prepare an inoculum control by dispersing 0,1 ml of the standardized *Acanthamoeba* stock solution (5.7.2) into 10 ml of the ¼ Ringer's as used in 5.8.3. Execute 5.8.4 and 5.8.5 and either 5.9.1 or 5.9.2 depending upon the recovery method to be used for the product evaluation. The inoculum concentration shall be confirmed by haemocytometer count of the cells/ml in the inoculated ¼ Ringer's solution and the value recorded. For the purpose of determining log reductions, the inoculum concentration and cell concentrations challenged in the test solution shall be measured using the Reed and Muench spreadsheet or the Spearman-Karber spreadsheet.

6.2 Recovery medium control

6.2.1 Mix a 1/10 dilution (1 ml into 9 ml) of the disinfecting product in validated neutraliser broth using a vortex mixer and let it stand for the appropriate time to allow neutralisation to be completed. Inoculate the tube using 0,1 ml of the standardised *Acanthamoeba* stock solution (5.7.2) into the neutralised disinfection product. Execute 5.8.4 and 5.8.5 and either 5.9.1 or 5.9.2 depending upon the recovery method to be used for the product evaluation.

6.2.2 Ensure that the recovery from the neutraliser broth is at least 50 % of the inoculum control.

7 Performance criteria

If the average concentration of the cells on the inoculum control plates is below $1,0 \times 10^4$ cells/ml or above $5,0 \times 10^5$ cells/ml, the experiment is considered invalid and the test must be repeated.

Annex A
(normative)

Preparation of *Acanthamoeba* growth medium (Ac#6)

A.1 Intended use

Acanthamoeba growth medium (Ac#6) is used for axenic culture of *Acanthamoeba* trophozoites.

A.2 Composition

The composition of Ac#6 growth medium is given in Table A.1.

Table A.1 — Composition of Ac#6 growth medium

Material	Amount
Biosate (e.g. BBL: BD-211862)	20,0 g
Glucose (e.g. Sigma, G7021)	5,0 g
KH_2PO_4 (anhydrous: e.g. Fluka, 60219 or EMD, PX1565-1)	0,3 g
[a]Vitamin B12 stock solution (100 µg/ml: e.g. Sigma, B4051 or EMD,1.11988.0100)	100 µl
[b]L-Methionine stock solution (5 mg/ml: e.g. Fluka, 64319 or Calbiochem, 4500)	3 ml
Deionised or Nanopure™ water	to 1 000 ml

[a] Preparation of vitamin B12 stock solution (100 µg/ml):

Dissolve 10 mg vitamin B12 in 100 ml of deionised or Nanopure H_2O, aliquot into 10 ml volumes and autoclave at 121 °C for 15 min. Assign batch number and store at (-20 °C) for use within 12 months. Thaw an aliquot and store at 2 °C - 8 °C for use within 1 month.

[b] Preparation of L-Methionine stock solution (5 mg/ml):

Dissolve 500 mg L-Methionine in 100 ml of deionized or Nanopure H_2O, aliquot into 20 ml volumes and autoclave at 121 °C for 15 min. Assign batch number and store at (-20 °C) for use within 12 months. Thaw an aliquot and store at 2 °C - 8 °C for use within 1 month.

A.3 Method of preparation

A.3.1 Dissolve ingredients in a suitably sized clean glass container with gentle warming.

A.3.2 Adjust to pH 6,5 to pH 6,6 with 1 M hydrochloric acid or 1 M sodium hydroxide.

A.3.3 Adjust the final volume to 1 000 ml with Deionised or Nanopure™[1)] water using a measuring/graduated cylinder.

A.3.4 Aliquot in suitable volumes (e.g. 250 ml) into borosilicate glass bottles and autoclave at 121 °C for 15 min.

A.3.5 Store autoclaved medium at room temperature for use within 2 months.

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Annex B
(normative)

Preparation of ¼ strength Ringer's solution

B.1 Intended use

One quarter strength Ringer's solution is used for washing and diluting of *Acanthamoeba* trophozoites.

B.2 Composition

The composition of ¼ strength Ringer's solution is given in Table B.1.

Table B.1 — Composition of ¼ strength Ringer's solution

Material	Amount
¼ Strength Ringer's tablet (e.g. Oxoid BR 0052G)	1 tablet
Deionised or Nanopure™ water	500 ml

B.3 Method of preparation

B.3.1 Add one ¼ strength Ringer's tablet to 500 ml of deionised or Nanopure™[2)] water in a suitably sized borosilicate glass bottle.

B.3.2 Measure the pH of an aliquot of the solution. The pH should be 7,0 ± 0,2.

B.3.3 If required adjust to pH 6,8 to pH 7,2 with 1 M hydrochloric acid or 1 M sodium hydroxide.

B.3.4 Filter sterilise or autoclave at 121 °C for 15 min.

B.3.5 Store sterilised medium at room temperature for use within 6 months.

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Annex C
(normative)

Maintenance of *Acanthamoeba* trophozoites and preparation for testing

C.1 Maintenance of stock cultures

C.1.1 *Acanthamoeba castellanii* (ATCC 50370) and *Acanthamoeba polyphaga* (ATCC 30461) are to be grown separately on *Acanthamoeba* growth medium (Ac#6, see Annex A for preparation).

C.1.2 Obtain a 1 ml culture cryogenically stored at approximately (1×10^6) cells/ml (<3 passages from ATCC);

C.1.3 Thaw the culture by placing the cryogenic vial in a 37 °C ± 2 °C water bath;

C.1.4 Add the thawed culture to 30 ml of *Acanthamoeba* growth medium in a 75 cm^2 (medium sized) tissue culture flask and incubate the culture for 3 days to 4 days at 28 °C ± 2 °C.

C.2 Scaling up cultures for testing

C.2.1 The stock culture flask (C.1.4) will be used to scale up cultures to provide the inoculum for testing.

C.2.2 Carefully decant the culture medium so as not to dislodge the trophozoites.

C.2.3 Refill with approximately 30 ml of fresh *Acanthamoeba* growth medium (Ac#6, see Annex A for preparation).

C.2.4 Shake the flask to dislodge the trophozoites;

C.2.5 Decant trophozoites into a 50 ml polypropylene centrifuge tube (should be approximately 5×10^5 to 1×10^6 cells/ml).

C.2.6 Perform a trophozoite count from the centrifuge tube using a haemocytometer and record cell number /ml.

C.2.7 Add 5×10^6 trophozoites from the centrifuge tube into a 175 cm^2 (large) flat tissue culture flask to give a cell density of 1×10^5 /ml when made up to 50 ml with *Acanthamoeba* growth medium.

C.2.8 Make up the volume in the flask to 50 ml with Ac#6.

C.2.9 Gently mix the contents of the flask and then incubate the cultures for approximately 24 h at 28 °C ± 2 °C.

C.2.10 Under these growth conditions, one flask should yield approximately 1×10^7 to 2×10^7 trophozoites.

Annex D
(normative)

Preparation of non-nutrient agar (NNA)

D.1 Intended use

The medium in 12-well plates for recovery of challenged trophozoites in recovery method one.

D.2 Composition

The composition of stock solution 1, stock solution 2 and Page's saline non-nutrient agar is given in Table D.1, D.2 and D.3.

Table D.1 — Composition of stock solution 1

Material	Amount
NaCl	12,0 g
$MgSO_4.7H_2O$	0,40 g
$CaCl_2.6H_2O$	0,60 g
Deionized or Nanopure™ water	to 500 ml

Table D.2 — Composition of stock solution 2

Material	Amount
Na_2HPO_4	14,20 g
KH_2PO_4	13,60 g
Deionized or Nanopure™ water	to 500 ml

Table D.3 — Page's saline non-nutrient agar

Material	Amount
Stock solution 1	5 ml
Stock solution 2	5 ml
Deionized or Nanopure™ water	to 1 000 ml
Bacteriological agar	15,0 g

D.3 Method of preparation of stock solutions

D.3.1 Dissolve ingredients for each stock solution separately in suitably sized clean glass containers with gentle warming.

D.3.2 Autoclave at 121 °C for 15 min.

D.3.3 Store sterilised stock solutions in the refrigerator for up to 6 months.

D.4 Method of preparation of Page's saline non-nutrient agar (NNA)

D.4.1 Aseptically remove 5 ml aliquots of each stock solution, 1 and 2, and adjust to 1 000 ml using deionized or Nanopure™[3)] water. Disperse and mix the 15 g agar in the resulting solution and dispense into an appropriately sized clean glass container and then slowly bring to a boil. Transfer the agar solution to suitable vessels and autoclave at 121 °C for 15 min.

D.4.2 Store the agar in the refrigerator at a temperature of 2 °C to 8 °C for up to 6 months.

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Annex E
(informative)

Preparation of *E. coli* suspension

a) Grow *E. coli* ATCC 8739 in a 250 ml sterile disposable flask containing 25 ml of TSB and incubate on an orbital shaker at 100 rpm at 30 °C - 35 °C overnight.

b) Remove the flask containing the bacteria from the incubator and pour the bacterial suspension into 2 × 50 ml centrifuge tubes. Centrifuge for no longer than the equivalent of 10 min at 4 000*g* or less at 20 °C-25 °C.

c) Decant the supernatant and add 25 ml of ¼ strength Ringer's solution to each centrifuge tube. Vortex each tube until pellet is resuspended. This should provide a bacterial suspension of approximately 1 × 10^{10} cells/ml. This *E. coli* stock suspension may be stored in the refrigerator for up to 30 days.

d) Dilute the stock suspension above 1:8 in ¼ strength Ringer's solution to make a working stock suspension for inoculating 50 µl to each microtitre well for recovery method two.

Annex F
(informative)

Preparation of non-nutrient agar (NNA) plates with *E. coli*

a) Melt non-nutrient agar (NNA) at >85 °C;

b) Add 2 ml of non-nutrient agar (NNA) to each well of a 12-well tissue culture plate for recovery method one and allow it to solidify. Inoculate each well with 0,1 ml of the bacterial stock suspension.

c) Place the plates on the lab bench at ambient temperature overnight. For longer storage, NNA 12-well plates may be prepared and stored without *E. coli* for up to a month at 2 °C-8 °C. NNA 12-well plates containing *E. coli* can be stored for up to a week at 2 °C-8 °C. Plates stored for longer than a week should be reinoculated with fresh *E. coli*. Plates should not be allowed to dry out. Plates with dried/cracked NNA should be discarded.

Annex G
(informative)

Preparation of neutraliser broth for recovery methods

G.1 Intended use

To neutralise the activity of polyhexamethylene biguanide hydrochloride (PHMB), chlorhexidine, alexidine, myristamidopropyl dimethylamine (MAPD) and polyquaternium-1 in multipurpose contact lens solutions.

NOTE 1 Alternative neutraliser broths can be needed depending on the active ingredient of the contact lens care solution.

NOTE 2 This neutraliser broth is not appropriate for oxidative care systems.

G.2 Composition

The Composition of neutraliser broth is given in Table G.1.

Table G.1 — Composition of neutraliser broth

Material	Amount
Lecithin	3,5 g
Polysorbate 80	1,5 g
¼ Strength Ringer's tablet (e.g. Oxoid BR 0052G)	2 tablets
Deionised or Nanopure™ water	to 1 000 ml

G.3 Method of preparation

G.3.1 Dissolve ingredients in a suitably sized clean glass container with gentle warming.

G.3.2 Adjust to pH 7,4 ± 0,2 with 1 M hydrochloric acid or 1 M sodium hydroxide.

G.3.3 Adjust the final volume to 1 000 ml with Deionised on Nanopure™[4)] water using glass measuring/graduated cylinder.

G.3.4 Aliquot in suitable volumes (e.g. 250 ml) in borosilicate glass bottles and autoclave at 121 °C for 15 min.

G.3.5 Store autoclaved medium at room temperature for use within 3 months.

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Annex H
(normative)

Reed and Muench computation method for calculation of the 50 % endpoint titre

H.1 Intended use

This method determines the concentration of the trophozoite inoculum and the trophozoite concentration following test solution challenge.

H.2 Principle and example of using Reed and Muench computation method

H.2.1 Principle of method

Reed and Muench published this method in 1938 to determine 50 % endpoints in experimental biology. Their objective was to determine the dilution of sera or viruses, which when dispensed into test animals would result in a certain proportion of test animals reacting or dying (LD_{50}). In biological quantitation, the best method of determining the endpoint is the use of large numbers of animals at dilutions near the value for the 50 percent reaction. The reason for using 50 % endpoints is that many dose-response relationships in biology follow a function that flattens out as it approaches the minimal and maximal responses; therefore, it is easier to measure the concentration of the test substance that produces a 50 % response.

Their method is applicable primarily to a complete titration series: i.e., the whole reaction range, from 0 % to 100 % mortality (or infectivity, cytopathic effect, or survivors). The method can be utilized even if this condition is not met as long as the reactions occur in a uniform manner over the range of dilutions employed. If results are erratic (e.g. deaths scattered irregularly over a number of dilutions or survivors scattered irregularly over a number of dilutions), the endpoint will be inaccurate.

H.2.2 Typical example of method

Example 1 demonstrates use of the method using data representative of a study of virus inoculation of animals in which mortality of the animals is recorded based on ten-fold dilutions of the virus in order to determine the 50 % concentration resulting in death.

The arrows in columns c and d contain accumulated values for the total number of animals that died or survived. The accumulated mortality ratio (column g) represents the accumulated number of dead animals (column e) over the accumulated total number: for example, at the 10^{-3} dilution, there occurred the equivalent of 5 deaths out of a total of 7 animals.

In Example 1, the mortality in the 10^{-3} dilution is above 50 %; that in the next lower dilution, 10^{-4}, is considerably below 50 %. Therefore, the 50 % endpoint lies somewhere between the 10^{-3} and 10^{-4} dilution of the inoculated virus. The necessary proportionate distance of the 50 % mortality endpoint, which obviously lies between these two dilutions, is obtained from column h as follows:

EXAMPLE 1

$$\frac{\text{\% mortality at dilution next above 50 \% } - \text{ 50 \%}}{\text{\% mortality at dilution next above 50 \% } - \text{ \% mortality at dilution next below 50 \%}} = \text{proportionate distance}$$

or

$$\frac{71-50}{71-13} = \frac{21}{58} = 0{,}36 \text{ (or } 0{,}4)$$

Since the distance between any two dilutions is a function of the incremental steps used in preparing the series, e.g. 10-fold, it is necessary to correct (multiply) the proportionate distance by the dilution factor, which is the logarithm of the dilution steps employed. In the case of serial 10-fold dilutions, the factor is 1 (log of 10 = 1) and so is disregarded. In the procedure that follows, the factor is understood to be negative. Therefore, the negative log of LD50 endpoint titer equals the negative log of the dilution above 50 % mortality plus the proportionate distance factor (calculated above). In the example given, the following values are obtained:

Negative log of lower dilution (next above 50 % mortality) = -3,0

Proportionate distance (0,4) × dilution factor (log 10) = -0,4

Log LD50 titre = -3,4

LD50 titre = $10^{-3,4}$

Table H.1 — Example 2 – Example of endpoint titration

				Accumulated values			
						Mortality	
Virus dilution (a)	Mortality ratio (b)	Died (c)	Survived (d)	Total dead - (e)	Total survived (f)	Ratio (g)	% (h)
10^{-1}	6/6	^ 6	v 0	17	0	17/17	100
10^{-2}	6/6	^ 6	v 0	11	0	11/11	100
10^{-3}	4/6	^ 4	v 2	5	2	5/7	71
10^{-4}	1/6	^ 1	v 5	1	7	1/8	13
10^{-5}	0/6	^ 0	v 6	0	13	0/13	0

H.2.3 Adaptation of Reed and Muench method to determining concentrations of organisms

Based on +/- Survivors in Multiple Dilutions The following example shows the method as applied to calculating the approximate concentration of *Acanthamoeba* cells in the inoculum control (7.1.1), in the recovery medium control (7.2.1) and in each challenged test product (6.9.1 and 6.9.2).

Calculation Formulae are given in Table H.2.

Table H.2 — Calculation formula

Accumulated values					
- Log_{10} dilution V ml/well **(a)**	**+ Wells with growth (b)**	**- Wells no growth (c)**	**Total wells with growth + (d)**	**Total wells no growth - (e)**	**% wells with growth (f)**
1	D1	R-D1	D1+D2+D3+D4+D5+D6	(R-D1)	100x[d/(d+e)]
2	D2	R-D2	D2+D3+D4+D5+D6	(R-D1) + (R-D2)	100x[d/(d+e)]
3	D3	R-D3	D3+D4+D5+D6	(R-D1) + (R-D2) +(R-D3)	100x[d/(d+e)]
4	D4	R-D4	D4+D5+D6	(R-D1) + R-D2) + (R-D3) + (R-D4)	100x[d/(d+e)]
5	D5	R-D5	D5+D6	(R-D1) + (R-D2) + (R-D3) + (R-D4) + (R-D5)	100x[d/(d+e)]
6	D6	R-D6	D6	(R-D1) + (R-D2) + (R-D3) + (R-D4) + (R-D5) + (R-D6)	100x[d/(d+e)]
D1 to D6 = number of wells with growth at each dilution R = number of replicates per dilution V = ml/well					

$$d_p = \frac{\%\ Next\ above\ 50\ \% - 50}{\%\ Next\ above\ 50\ \% - \%\ Next\ below\ 50\ \%}$$

Log_{10} (50 % cell concentration) = Log_{10} dilution Next above 50 % (a) + PD = X

50 % endpoint titer per volume per well = 10^X cells/(volume/well) = 10^x cells/V

50 % endpoint titer per cells per ml in 10^0 test tube= [(10^x cells/V) × (1/V)] = 10^x × (1/V)

NOTE The 50 % endpoint is cells per volume per well in the 10^0 test tube: if 0,2 ml of each dilution is added per well, the 50 % endpoint calculates the number of cells per 0,2 ml in the 10^0 test tube. Therefore, 50 % endpoint shall be multiplied by (1/V) or 1 divided by the volume of each dilution placed per well. If 0,2 ml is added per well, multiply by 1/0,2 or 5.

50 % endpoint 10^x cells/ml = approximate 10^x cells/ml

Log reduction = $Log_{10}(cells/ml)_{inoculum} - Log_{10}(cells/ml)_{test}$

If the 50 % recovery is observed in a particular dilution, that is an endpoint dilution and proportional calculation is not required. Therefore, the 50 % endpoint is the dilution at which the 50 % recovery occurs.

H.2.4 Example of calculation using sample data

Example of calculation using representative data are given in Table H.3.

Table H.3 — Example of calculation using representative data

Accumulated values					
- Log_{10} dilution V ml/well **(a)**	**+ Wells with growth (b)**	**- Wells no growth (c)**	**Total wells with growth + (d)**	**Total wells no growth - (e)**	**% wells with growth (f)**
1	4	0	16	0	100
2	4	0	12	0	100
3	4	0	8	0	100
4	3	1	4	1	80
5	1	3	1	4	20
6	0	4	0	8	0
# replicates = 4 ml/well = 1 (method 1) ml/well = 0,2 (method 2)					

% Next above 50 % growth = 80

% Next below 50 % growth = 20

Proportionate distance [PD] = $\frac{80-50}{80-20}=0,5$

-Log_{10} dilution Next above 50 % [D] = 4

Sum PD + D (Log_{10}) = 4,5

$[10^{(\text{Sum PD + D})}]$ = $10^{4,5}$ = 3,16E+04 cells

$[10^{(\text{Sum PD + D})}] \times (1/(\text{ml/well}))$ = 3,16E+04 cells/ml in 10^0 tube of test solution

Annex I
(normative)

Spearman-Karber computation method for calculation of the 50 % endpoint titre

I.1 Spearman-Karber formula

Formula I.1 shows the Spearman-Karber formula.

$$\text{Log value} = X_0 - d/2 + d\sum r_i/n_i \quad \text{(I.1)}$$

where

X_0 $\log_{10}$ of the reciprocal of the highest dilution at which wells are positive

d $\log_{10}$ of the dilution factor

n_i number of wells used in each individual dilution

r_i number of positive wells (out of n_i)

Summation is started at dilution X_0

Table I.1 shows an example of spreadsheet and calculation Formula are given in Table I.2.

Table I.1 — Example spreadsheet

	A	B	C	D	E	F
1	SPEARMAN-KARBER CALCULATION				X_0 - (d/2) + d(Sum r_i/n_i)	
2	Dilution	Number of wells (n_i)	Positive Wells (r_i)	$P = (r_i/n_i)$	Calculation	Value
3	-1	4	4	1	Low dilution (X_0) = 1,00	1
4	-2	4	4	1	log10 dilution factor (d)	1
5	-3	4	4	1	d/2	0,5
6	-4	4	0	0	Sum(P) from lowest dilution	3
7	-5	4	0	0		
8	-6	4	0	0	Recovery	Cells/ml
9				3	3,5	3 162,278

Table I.2 — Calculation formula

	A	B	C	D	E	F
1	SPEARMAN-KARBER CALCULATION				X_0 - (d/2) + d(Sum r_i/n_i)	
2	Dilution	Number wells (n_i)	Positive wells (r_i)	$P = (r_i/n_i)$	Calculation	Value
3	-1	4	4	=C3/B3	Low dilution (X_0) = 1,00	=abs(A3)
4	-2	4	4	=C4/B4	log10 dilution factor (d)	=log10*(A3*10)
5	-3	4	4	=C5/B5	d/2	=F4/2
6	-4	4	0	=C6/B6	Sum (P) from lowest dilution	=D9
7	-5	4	0	=C7/B7		
8	-6	4	0	=C8/B8	Recovery	Cells/ml
9				=SUM(D3:D8)	=F3-F5+F4*F6	=POWER(10,E9)

Example based on data above:

$X_0 = 1$

$d = 1$

$d/2 = 0,5$

$n_i = 4$

$r_i = 4$

Log value = 1 – ½ + 1 ∑(4/4)+(4/4)+(4/4)+(0/4)+(0/4)+(0/4)

Log value = 3,5