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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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Contents

Page

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).

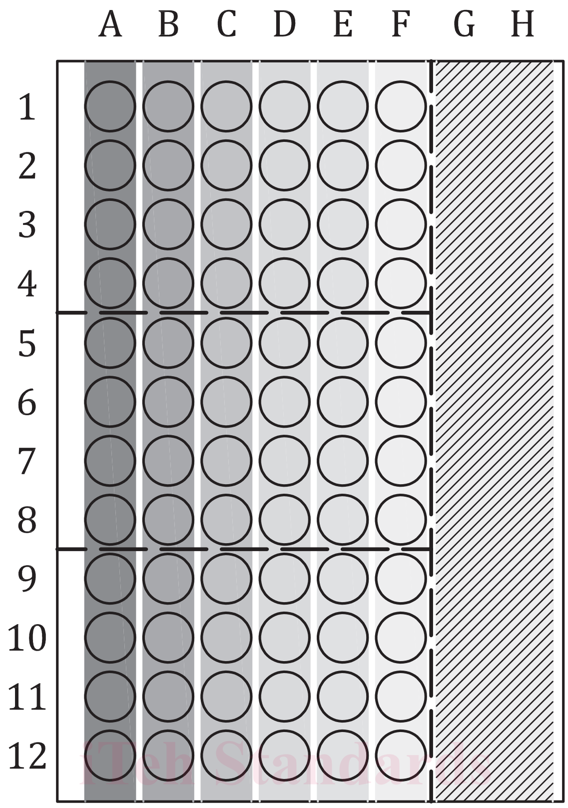

Figure 1 — Layout of the 96-well Plate for Method 2

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.