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Ophthalmic optics — Contact lens care products — Method for evaluating Acanthamoeba encystment by contact lens care products

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National foreword

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
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Foreword

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT), see the following URL: Foreword — Supplementary Information.

The committee responsible for this document is ISO/TC 172, *Optics and photonics*, Subcommittee SC 7, *Ophthalmic optics and instruments*.

Introduction

Acanthamoeba is a genus of small free-living amoeba common to most soil and aquatic habitats.[1],[2] The organism is characterized by a life cycle of a feeding and dividing trophozoite which, in response to adversity, can transform into a resistant cyst stage.[1],[2] *Acanthamoeba* cysts have been shown to resist extremes of temperature, pH, desiccation, and most chemical disinfectants at normal concentration for use.[1],[2],[3],[4]

Recently, it has been observed that a contact lens disinfecting solution associated with a significant number of *Acanthamoeba* keratitis cases was able to induce trophozoite encystment.[5],[6],[7] Such a phenomenon is of important concern as *Acanthamoeba* cysts can be resistant to contact lens disinfection systems and this can increase the risk of acquiring *Acanthamoeba* keratitis.[3],[4],[7],[8],[9]

This International Standard provides a methodology for assessing the capability of a contact lens disinfecting solution to induce *Acanthamoeba* trophozoite encystment. This method does not describe methodology to assess the efficacy of a contact lens disinfecting product against *Acanthamoeba* spp.

Ophthalmic optics — Contact lens care products — Method for evaluating Acanthamoeba encystment by contact lens care products

1 Scope

This International Standard specifies a method for evaluating the potential of products for contact lens disinfection to induce encystment of *Acanthamoeba* species. This method excludes the evaluation of oxidative systems that require a special lens case for use. This International Standard does not address the evaluation of disinfection efficacy of contact lens disinfecting products.

2 Terms and definitions

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

2.1
trophozoite
motile, feeding amoeboid form of *Acanthamoeba*

2.2
encystment
phase in the life cycle of *Acanthamoeba* where the trophozoite stage transforms into the cyst stage

2.3
mature cyst
dormant form of *Acanthamoeba* composed of an inner and outer cell wall, typically more resistant to a range of challenges than *trophozoites* (2.1)

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

2.4
immature cyst
cyst comprised only of the inner cell wall

2.5
room temperature
temperature defined as 18 °C to 25 °C

2.6
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, can occur.

Note 2 to entry: This term is synonymous with the term “subculture”.[10]

2.7
passage number
number of times cells in the culture have been subcultured or *passaged* (2.6)[10]

3 Principle

3.1 General

The assay tests the capability for a solution to induce *Acanthamoeba* trophozoite encystment as this physiological event can afford the organism protection from disinfection.

3.2 Encystment test

The encystment test is used to measure a disinfecting solution's potential for inducing trophozoite encystment to either the immature or mature cyst form. Assessment of this phenomenon is considered important as *Acanthamoeba* cysts can be resistant to many disinfecting systems at operating conditions.

In the encystment test, contact lens disinfecting solutions are exposed to *Acanthamoeba* trophozoites. Following detergent treatment and calcofluor white staining to lyse remaining trophozoites and stain the inner cell wall, the organisms are observed microscopically for the production of immature and mature cysts.

4 Encystment test method

4.1 General

Prior to conducting encystment studies, personnel should be trained and experienced in the following:

a) culturing and manipulating *Acanthamoeba*;

b) recognizing immature and mature cyst forms;

c) calculating the level of cyst formation as described in this International Standard.

4.2 Test organism

4.2.1 *A. castellanii* (ATCC 50370).

4.3 Culture media and reagents

4.3.1 Ac#6 axenic semi-defined *Acanthamoeba* growth medium (see Annex A).

4.3.2 1/4 strength Ringer's solution (see Annex B).

4.3.3 Sarkosyl-Calcofluor White (see Annex C).

4.3.4 Encystment positive and negative control solutions (see Annex D).

4.4 Test materials

4.4.1 Sterile 50 ml and 14 ml/15 ml polypropylene centrifuge tubes.

4.4.2 Sterile 12 well flat bottomed plasma treated microtitre plates of material compatible with the test material.

4.4.3 Calibrated pipettes (fixed and adjustable volume and multichannel) to deliver: 10 ml disposable, 20 µl, 100 µl, and 1 000 µl.

4.4.4 3 ml sterile disposable plastic Pasteur pipettes.

4.4.5 Fluorescence microscope with ×10, ×20, and ×40 phase contrast and fluorescence objectives with a UV-2A filter, excitation 330 nm–380 nm, and emission greater than 420 nm.

4.4.6 An inverted microscope with ×10, ×20, and ×40 objectives.

4.4.7 28 °C ± 2 °C and 32,5 °C ± 2,5 °C incubators.

4.4.8 Centrifuge.

4.4.9 Vortex mixer.

4.4.10 Cell counting chamber (e.g. Modified Fuchs Rosenthal INCYTO disposable hemocytometer).

4.4.11 Optional: Pivoting blade cell scraper.

4.4.12 Sterile 75 cm^2 and 150 cm^2/180 cm^2 flat polystyrene tissue culture flasks.

4.5 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.

4.6 Culture maintenance

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

4.6.2 Maintenance of stock cultures (see E.1).

4.6.3 Scaling up cultures for testing (24 h prior to test) (see E.2).

4.7 Preparation of microbial challenge

4.7.1 Grow trophozoites as described in 4.6.2 and 4.6.3.

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

4.7.2 Vigorously shake flasks to dislodge adherent trophozoites (rinse with a pipette if necessary).

NOTE Scrape the bottom of the flask with a cell scraper if necessary.

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

4.7.4 Resuspend one tube pellet in 10 ml of 1/4 strength Ringer's solution (see Annex B) and use to resuspend the other pellets if additional inoculum is required.

4.7.5 Wash ×3 with 10 ml of 1/4 strength Ringer's solution by centrifugation at 500 × *g* for 2 min at room temperature.

4.7.6 Resuspend pellet by vortexing in 1 ml to 2 ml of 1/4 strength Ringer's solution.

4.7.7 Enumerate trophozoite numbers using a cell counting chamber (make a 1:10 to 1:100 dilution in 1/4 strength Ringer's solution to assist) and record number/ml. A volume of 20 µl is used for cell counting using the hemocytometer.

NOTE A 1:100 dilution can be prepared by two 1:10 serial dilutions of 100 µl into 900 µl.

4.7.8 Adjust the stock concentration to $1,0 \times 10^7$/ml to $1,5 \times 10^7$/ml in 1/4 strength Ringer's solution and use immediately for testing.

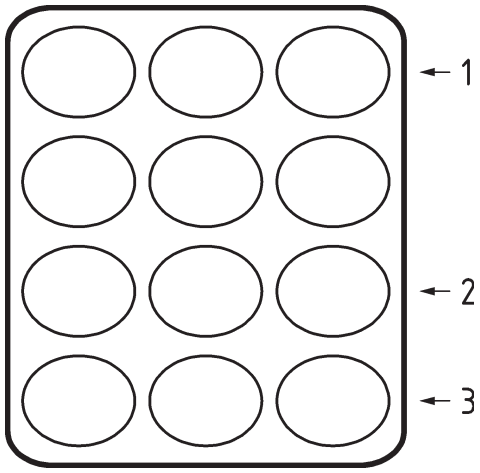
4.8 Encystment procedure

4.8.1 General

The encystment procedure consists of pipetting 3,0 ml ± 0,1 ml or weighing 3,0 g ± 0,1 g of control and/or test solutions into a well of a 12 well microtitre plate.

4.8.2 Control plate

4.8.2.1 Dispense the encystment positive control solution into three wells and dispense the negative control solution (see Annex D) into six wells each of a 12 well microtitre plate as shown in Figure 1.



Key

1 positive control
2 negative control T_{24}
3 negative control T_0

Figure 1 — 12 well microtitre plate for controls

4.8.2.2 Add 30 µl of $1,0 \times 10^7$/ml trophozoites in 1/4 strength Ringer's solution to all wells. This is designed to yield a final concentration of $1,0 \times 10^5$/ml in the test and control wells. If the stock trophozoite concentration is found to be $>1,0 \times 10^7$/ml, then add an appropriate volume to the wells to yield the required inoculum concentration of 1×10^5 trophozoites/ml (e.g. 20 µl if the stock suspension was counted as $1,5 \times 10^7$/ml).

NOTE Test solutions can be assayed on separate plates if the same trophozoite inoculum preparation is used and one plate has the appropriate encystment positive and negative controls.

4.8.2.3 Mix contents of wells by gently pipetting up and down three times with a 1 000 µl pipettor set to deliver 500 µl or a 3 ml disposable pipette.

4.8.2.4 Immediately perform cell chamber counts using the hemocytometer on each of the three T_0 encystment negative control wells and record the individual and averaged total cell count/ml in Table 1.

4.8.2.5 Measure the background cyst count in the inoculum by adding 50 µl of the Sarkosyl-Calcofluor White solution (see Annex C) to each of the three T_0 encystment negative control wells pipetting vigorously to mix and leaving at room temperature for 5 min.

NOTE It has been observed that 5 min is critical for optimal assessment. Longer exposure times can cause the cysts to lyse and become difficult to recognize.

4.8.2.6 Again, mix vigorously by pipetting up and down using a 1 000 µl pipettor set to deliver 500 µl or a 3 ml disposable pipette.

4.8.2.7 Immediately perform cell chamber counts using the hemocytometer on the Sarkosyl-Calcofluor White treated cells (under UV fluorescence with appropriate filter for Calcofluor white detection) to determine the background cyst level. Switch between UV fluorescence and white light to confirm observation of cysts. Count the number of refractile and fluorescent cells/ml and record in Table 1. These represent immature and mature cyst forms and give the background levels for the experiment (see Figure 2 for representative images of encysted trophozoites).

4.8.2.8 The test is invalid if the background cyst count in the challenge inoculum is greater than 5,0 %. If greater than 5,0 %, prepare fresh inoculum and repeat the procedure.

4.8.3 Test samples

4.8.3.1 Mix all solutions vigorously immediately before dispensing. Dispense each test solution directly from the product bottles into three wells in the microtitre plates as in 4.8.1.

4.8.3.2 Add 30 µl of $1{,}0 \times 10^7$/ml trophozoites in 1/4 strength Ringer's solution to all wells. This is designed to yield a final concentration of 1×10^5 trophozoites/ml in the test wells. If the stock trophozoite concentration is found to be $>1{,}0 \times 10^7$/ml, then add an appropriate volume to the wells to yield the required inoculum concentration of 1×10^5 trophozoites/ml (e.g. 20 µl if the stock suspension was counted as $1{,}5 \times 10^7$/ml).

4.8.3.3 Mix contents of wells by gently pipetting up and down three times with a 1 000 µl pipettor set to deliver 500 µl or a 3 ml disposable pipette.

4.8.3.4 Incubate test and control plates at 18 °C to 25 °C for 20 h to 24 h.

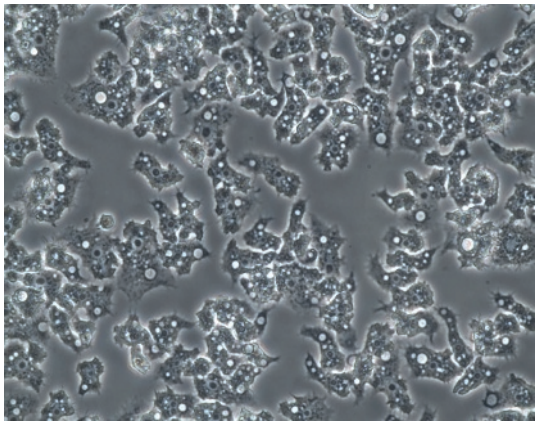
4.8.3.5 Measure the cyst count in the encystment positive control, T_{24} encystment negative control, and test wells by adding 50 µl of Sarkosyl - Calcofluor White solution (see Annex C) to each well pipetting vigorously to mix and leaving at room temperature for 5 min.

NOTE 1 Count the encystment positive control wells first and use as a basis for comparison for interpreting cyst formation in subsequent wells.

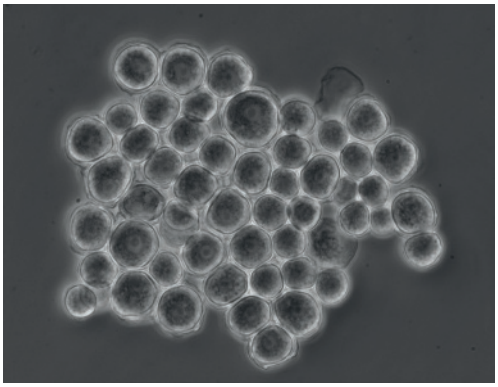
NOTE 2 It has been observed that 5 min is critical for optimal assessment. Longer exposure times can cause the cysts to lyse and become difficult to recognize.

NOTE 3 It can be necessary to stagger the addition of the Sarkosyl-Calcofluor White solution to the wells in order to avoid exposure for greater than 5 min.

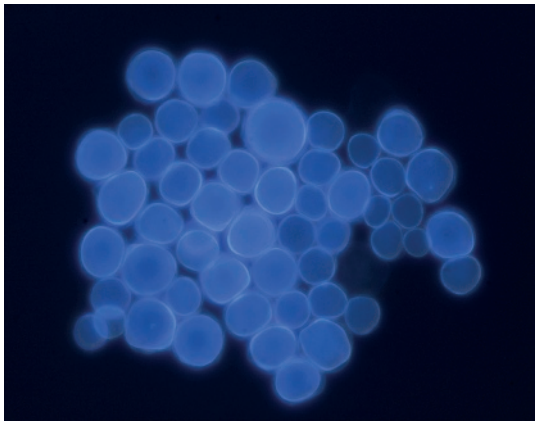
NOTE 4 Verify microscopically that the cysts are not attached to the well. Scrub with a swab if cysts are attached to the wells.



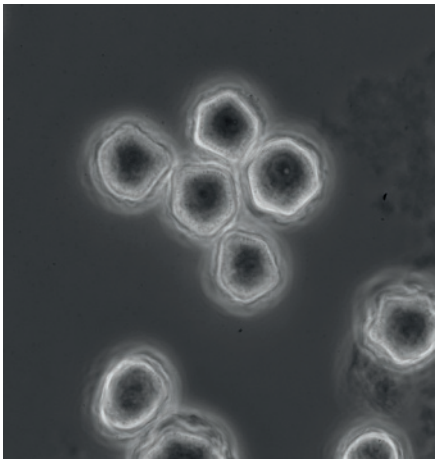
a) Trophozoites



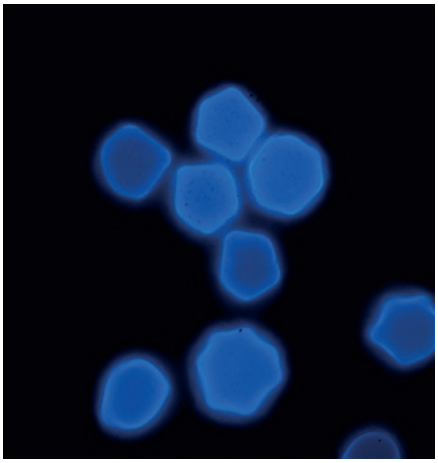
b) Immature cysts (phase contrast)



c) Immature cysts (calcofluor white staining)



d) Mature cysts (phase contrast)



e) Mature cysts (calcofluor white staining)

Figure 2 — Appearance of *A. castellanii* (50370) trophozoites, immature cysts, and mature cysts

4.8.3.6 Again, mix vigorously by pipetting up and down using a 1 000 µl pipettor set to deliver 500 µl or a 3 ml disposable pastette.

4.8.3.7 Immediately perform cell chamber counts using the hemocytometer on the Sarkosyl-Calcofluor White treated cells (under UV fluorescence with appropriate filter for Calcofluor white detection) to determine the cyst level. Switch between UV fluorescence and white light to confirm observation of cysts. Count the number of refractile and fluorescent cells representing immature and mature cysts for all test and control wells and record cyst count per ml in Table 2.

4.8.3.8 Count only cells that show the characteristic fluorescence as shown in Figure 2 d) and e). These are encysted trophozoites.

4.9 Encystment calculation

4.9.1 The background level of cysts present from the challenge inoculum at time zero of the experiment is calculated as shown in Table 1.

4.9.2 The level of trophozoite encystment after 20 h to 24 h incubation for both test and control solutions is calculated as shown in Table 2.

Table 1 — Calculating *Acanthamoeba* trophozoite challenge inoculum and background cyst level at time zero

	Prior to Sarkosyl-CW treatment				**Addition of Sarkosyl-CW**			
	Amoeba count/ml (0 h)				**Background cyst count/ml (0 h)**			
Solution	Well 1	Well 2	Well 3	Mean/ml	Well 1	Well 2	Well 3	Mean/ml
Negative control	a	b	c	D	e	f	g	H

Table 2 — Calculating the % trophozoite encystment level after incubation

	Cyst count /ml (24 h)			
Solution	Well 1	Well 2	Well 3	Mean/ml
Test or control solution	i	j	k	L

4.9.3 The mean amoeba inoculum count/ml: $(a+b+c)/3=D$.

4.9.4 The mean background cyst count/ml in the inoculum: $(e+f+g)/3=H$.

4.9.5 The mean background % encystment: $(H/D)\times 100$.

The background cyst count in the challenge inoculum should be ≤5,0 %.

4.9.6 The % trophozoite encystment obtained for a given test positive or negative control solution is calculated below.

4.9.7 The mean cyst count/ml for a given test solution: $(i+j+k)/3=L$.

4.9.8 The encystment level/ml, corrected for the background cyst level (H), for a given solution = L-H.

4.9.9 The % encystment for a given test solution: $\left[(L-H)/D\right]\times 100$.

5 Controls

5.1 Positive and negative encystment control solutions are run with each encystment test.

5.2 Details of the procedure are given in the encystment procedure (see 4.8).

5.3 Background encystment: The challenge trophozoite inoculum in the negative control wells shall show ≤5 % encystment at the start of the experiment.

5.4 Encystment positive control solution: After 20 h to 24 h, incubation shall give an encystment rate of >50 %.

5.5 Encystment negative control solution: After 20 h to 24 h, incubation shall give an encystment rate of <5 %.

Annex A
(normative)

Acanthamoeba growth medium (Ac#6)

A.1 General

This Annex details the preparation of culture medium Ac#6.[11]

A.2 Intended use

The axenic culture of *Acanthamoeba* trophozoites.

A.3 Composition

Biosate Peptone (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
Vitamin B12 stock solution[a](100 µg/ml: e.g. Sigma, B4051 or EMD, 1.11988.0100)	100 µl
L-Methionine stock solution[b] (5 mg/ml: e.g. Fluka, 64319 or Calbiochem, 4500)	3 ml
Deionised or nanopure water	to 1 000 ml

[a] Preparation of B12 stock solution (100 µg/ml): Dissolve 10 mg vitamin B12 in 100 ml of deionised or nanopure H_2O, aliquot 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 4 °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 in 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 4 °C for use within 1 month.

A.4 Method of preparation

A.4.1 Dissolve ingredients in a suitably sized clean glass container with gentle warming.

A.4.2 Adjust to pH 6,5 to 6,6 with 1N NaOH or 1N HCl.

A.4.3 Aliquot in suitable volumes (e.g. 250 ml) in borosilicate glass bottles and autoclave at 121 °C for 15 min.

A.4.4 Store autoclaved medium at room temperature for use within two months.[12]

Annex B
(informative)

1/4 strength Ringer's Solution

B.1 Intended use

Washing and dilution of *Acanthamoeba* trophozoites.

B.2 Composition

1/4 strength Ringer's tablet (e.g. Oxoid BR 0052G)	one tablet
Deionised or nanopure water	500 ml

B.3 Method of preparation

B.3.1 Add one 1/4 strength Ringer's tablet to 500 ml of deionised or nanopure water in a suitably sized borosilicate glass bottle.

B.3.2 Filter sterilize or autoclave at 121 °C for 15 min.

B.3.3 Measure pH of an aliquot of the solution (should be 7,0 ± 0,2).

B.3.4 Store at room temperature for use within six months.

Annex C
(informative)

Sarkosyl-Calcofluor White solution

C.1 Intended use

Detection of encysted trophozoites.

C.2 Composition

Sarkosyl (N-Lauroylsarcosine sodium salt; Sigma, Product Code L5125)	3 g
Calcofluor White (Fluorescent Brightener; Sigma, Product Code F3543)	200 mg
Deionised water	Make up to 20 ml

C.3 Method of preparation

C.3.1 Dissolve Sarkosyl in approximately 15 ml of deionised water. Add Calcofluor White powder, vortex to dissolve and make up to 20 ml.

C.3.2 Store in light-proof container at room temperature for use within one month.

Annex D
(informative)

Preparation of encystment control solutions

D.1 Encystment positive control solution

D.1.1 Intended use

Positive control solution which promotes *Acanthamoeba* trophozoite encystment.

D.1.2 Composition

NaCl	0,55 %
KCl	0,14 %
$NaH_2PO_4.H_2O$	0,01 %
$Na_2HPO_4.7H_2O$	0,12 %
EDTA	0,05 %
Propylene glycol	0,5 %
PHMB	0,25 ppm

D.1.3 Method of preparation

D.1.3.1 All components are % (w/w) or ppm in deionized water.

D.1.3.2 Filter sterilize using a compatible filter (e.g. cellulose acetate) and store in a compatible container (not glass) at room temperature for use within six months.

D.1.3.3 The final pH should be 7,0 ± 0,10.

D.2 Encystment negative control solution

D.2.1 Intended use

Negative control solution which does not promote *Acanthamoeba* trophozoite encystment.

D.2.2 Composition

NaCl	0,55 %
$NaH_2PO_4.H_2O$	0,01 %
$Na_2HPO_4.7H_2O$	0,12 %
EDTA	0,05 %
PHMB	0,25 ppm

D.2.3 Method of preparation

D.2.3.1 All components are % (w/w) or ppm in deionized water.

D.2.3.2 Filter sterilize using a compatible filter (e.g. cellulose acetate) and store in a compatible container at room temperature for use within six months.

D.2.3.3 The final pH should be 7,0 ± 0,10.

Annex E
(informative)

Maintenance of *Acanthamoeba* trophozoites and preparation for testing

E.1 Maintenance of stock cultures

E.1.1 *Acanthamoeba* castellanii (ATCC 50370) to be grown on Ac#6 medium.

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

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

E.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 three to four days at 28 °C ± 2 °C.

E.2 Scaling up cultures for testing

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

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

E.2.3 Refill with approximately 30 ml of fresh *Acanthamoeba* growth medium. Use the same growth medium as used in propagation of the trophozoites (see E.1.1).

E.2.4 Shake the flask to dislodge the trophozoites.

NOTE Scrape the bottom of the flask with a cell scraper if necessary.

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

E.2.6 Perform a trophozoite count using a hemocytometer and record cell number/ml in centrifuge tube.

E.2.7 Add 5×10^6 trophozoites from the centrifuge tube into a 150/180 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.

E.2.8 Make up the volume in the flask to 50 ml with appropriate *Acanthamoeba* growth medium.

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

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

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