

NATIONAL STANDARD METHOD

ISOLATION AND IDENTIFICATION OF *ACANTHAMOEBA* SPECIES

W 17

Issued by Standards Unit, Evaluations and Standards Laboratory
Specialist and Reference Microbiology Division

ISOLATION AND IDENTIFICATION OF *ACANTHAMOEBA* SPECIES

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Issue no: 2.3 Issue date: 03.05.05 Issued by Standards Unit, Evaluations and Standards Laboratory on behalf of the Water Working Group and the Environmental Surveillance Unit, CDSC. Page 2 of 12

Reference no: W 17i2.3

This SOP should be used in conjunction with the series of SOPs from the Health Protection Agency

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Email: standards@hpa.org.uk

INDEX

- STATUS OF NATIONAL STANDARD METHODS 2**
- INDEX..... 3**
- AMENDMENT PROCEDURE 4**
 - SCOPE..... 5
 - BACKGROUND 5
- 1.0 DEFINITIONS 6**
- 2.0 PRINCIPLE..... 6**
- 3.0 SAFETY CONSIDERATIONS..... 6**
 - 3.1 SPECIMEN COLLECTION 6
 - 3.2 SPECIMEN TRANSPORT AND STORAGE 6
 - 3.3 SPECIMEN PROCESSING..... 6
- 4.0 EQUIPMENT 6**
- 5.0 CULTURE MEDIA AND REAGENTS 7**
 - 5.1 PREPARATION OF NON-NUTRIENT AGAR *E. COLI* PLATES 7
- 6.0 SAMPLE PROCESSING..... 7**
 - 6.1 SAMPLE PREPARATION 7
 - 6.2 FILTRATION 8
 - 6.3 CENTRIFUGATION 8
 - 6.4 DIRECT CULTURE..... 8
 - 6.5 SUBCULTURE TO AGAR MEDIA 8
 - 6.6 RECOGNITION OF *ACANTHAMOEBA* SPECIES..... 9
 - 6.7 IDENTIFICATION 9
- 7.0 QUALITY CONTROL 10**
- 8.0 REPORTING OF RESULTS 10**
- 9.0 REFERENCE FACILITIES..... 10**
- FLOWCHART SHOWING THE PROCESS FOR THE ISOLATION AND IDENTIFICATION OF ACANTHAMOEBA SPECIES..... 11**
- REFERENCES 12**

ISOLATION AND IDENTIFICATION OF ACANTHAMOEBA SPECIES

Issue no: 2.3 Issue date: 03.05.05 Issued by Standards Unit, Evaluations and Standards Laboratory on behalf of the Water Working Group and the Environmental Surveillance Unit, CDSC. Page 3 of 12

Reference no: W 17i2.3

This SOP should be used in conjunction with the series of SOPs from the Health Protection Agency

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AMENDMENT PROCEDURE

Controlled document reference	W 17
Controlled document title	Standard Operating Procedure for isolation and identification of <i>Acanthamoeba</i> species

Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@hpa.org.uk.

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
4/ 03.05.05	2.2	2.3	1	Front page	Redesigned
			2	Status of document	Reworded
			4	Amendment page	Redesigned

ISOLATION AND IDENTIFICATION OF ACANTHAMOEBIA SPECIES

Issue no: 2.3 Issue date: 03.05.05 Issued by Standards Unit, Evaluations and Standards Laboratory on behalf of the Water Working Group and the Environmental Surveillance Unit, CDSC. Page 4 of 12

Reference no: W 17i2.3

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STANDARD OPERATING PROCEDURE FOR THE ISOLATION AND IDENTIFICATION OF ACANTHAMOEBA SPECIES

INTRODUCTION

Scope

The method described is suitable for the detection of *Acanthamoeba* species in samples of potable and non-potable water and in mud and soil¹.

Background

Free-living amoebae (FLA) are unicellular protozoa found in most soil and water habitats. They are characterised by a feeding and dividing trophozoite stage which, in many species, can form a resistant cyst². Certain species of FLA are pathogenic and *Acanthamoeba*, *Naegleria fowleri* and *Balamuthia mandrillaris* are known to infect humans, in some instances with fatal consequences. Detection and isolation of these organisms in water and other environmental samples may therefore be required in the interests of preventative public health microbiology.

The taxonomic classification of the *Acanthamoeba* is derived from observations of the trophozoite and cyst morphology. The most detailed study to date is that of Pussard and Pons³ who concluded that 18 species existed which could be assigned to three distinct morphological groups labelled I-III.

No human pathogenic species are found in morphological group I. Within group II, *Acanthamoeba castellanii*, *Acanthamoeba hatchetti*, *Acanthamoeba polyphaga* and *Acanthamoeba rhysodes* have been incriminated in human disease. Of group III, only *A. culbertsoni* is known to infect man.

Reports on the pathogenicity of *Acanthamoeba* strains are often conflicting and it is unclear whether all *Acanthamoeba* capable of growth at or above 37°C are potential pathogens. Members of morphological group II may or may not grow at 37°C, and this may include ocular pathogenic forms. Incubation of plates at 32°C and 37°C is advisable. *A. culbertsoni* is highly pathogenic for laboratory animals and is characterised by a temperature tolerance of 42°C.

Acanthamoeba cysts are highly resistant, withstanding extremes of temperature, disinfection and desiccation⁴. This accounts for the presence of *Acanthamoeba* in virtually all soil and aquatic environments and even the atmosphere². Members of morphological group II are most commonly encountered in the environment, with *A. polyphaga* and *A. castellanii* being most frequently identified.

Acanthamoeba species cause two distinct infections in man affecting the central nervous system and the cornea. These are granulomatous amoebic encephalitis (GAE) which is a chronic disease of the immunosuppressed host (HIV, chemical immunosuppression and alcoholism) and *Acanthamoeba* keratitis which is a severe and potentially blinding infection of the cornea that affects previously healthy persons.

Approximately 200 cases of *Acanthamoeba* keratitis have occurred in the United Kingdom. Contact lens wearers are most at risk from infection accounting for approximately 95% of cases³. Poor hygiene practices, notably the rinsing or storing of lenses in non-sterile solutions or tap water, are recognised risk factors. *Acanthamoeba* keratitis in non-contact lens wearers usually follows trauma to the eye and contamination with environmental matter.

ISOLATION AND IDENTIFICATION OF ACANTHAMOEBA SPECIES

Issue no: 2.3 Issue date: 03.05.05 Issued by Standards Unit, Evaluations and Standards Laboratory on behalf of the Water Working Group and the Environmental Surveillance Unit, CDSC. Page 5 of 12

Reference no: W 17i2.3

This SOP should be used in conjunction with the series of SOPs from the Health Protection Agency

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1.0 DEFINITIONS

Acanthamoeba species are free living amoebae which grow on a lawn of *Escherichia coli* as trophozoites, within a few days producing cysts. The characteristics of the trophozoites and cysts define the genus.

Trophozoites are 25 - 40 µm in diameter, produce numerous needle-like projections (acanthopodia) and demonstrate slow movement. The trophozoites possess a contractile vacuole discharging at approximately 2 minute intervals. Cysts are 10 - 30 µm in diameter, are double walled and often polygonal in shape.

2.0 PRINCIPLE

The detection of *Acanthamoeba* in lightly contaminated water samples involves concentration by membrane filtration, with elution of captured particles from the membrane, or by centrifugation and re-suspension of the pellet in a suitable diluent. In each case, a portion of the suspension is sub-cultured to a 'lawn' of *Escherichia coli* (NCTC 9001) and a set of plates incubated at both 32°C and 37°C. The trophozoite plaques are sub-cultured to Page's saline in a microtitre plate. The plate is incubated at 32°C for 1 – 3 hours and examined under a microscope.

Samples of heavily contaminated water are not subjected to a concentration step and are plated directly to the lawn of *E. coli*.

3.0 SAFETY CONSIDERATIONS⁵⁻¹⁴

Normal microbiology laboratory precautions apply.

3.1 Specimen collection

N/A

3.2 Specimen transport and storage

Compliance with current postal and transportation regulations is essential.

3.3 Specimen processing

- Laboratory acquired infections from pathogenic FLA have not been recorded. However, environmental samples should be handled and processed according to the recommendations of the Advisory Committee on Dangerous Pathogens¹⁴ where *Acanthamoeba* are classified as a Hazard Group 2 organism

The above guidance should be supplemented with local COSHH and risk assessments

4.0 EQUIPMENT

Usual laboratory equipment and in addition:

- Centrifuge (optional)
- Membrane filtration manifold (optional)
- Filter funnels (optional)

ISOLATION AND IDENTIFICATION OF ACANTHAMOEBIA SPECIES

Issue no: 2.3 Issue date: 03.05.05 Issued by Standards Unit, Evaluations and Standards Laboratory on behalf of the Water Working Group and the Environmental Surveillance Unit, CDSC. Page 6 of 12

Reference no: W 17i2.3

This SOP should be used in conjunction with the series of SOPs from the Health Protection Agency

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- Vacuum pump with moisture trap or protective filter to prevent carryover of water vapour, or alternative vacuum source (optional)
- Pyrex vacuum flask with protective jacket large volume or equivalent e.g. 5 L (optional)
- Stainless steel flat-tipped forceps (optional)
- Boiling waterbath (instrument steriliser) – optional
- Vortex mixer
- Incubator at 37°C ± 1°C
- Incubator at 32°C ± 1°C
- Petri dishes
- Membrane filters - 47mm diameter 0.45 µm pore size cellulose acetate (optional)
- Automatic pipettors and associated sterile pipette tips capable of delivering 1-10 mL volumes (optional)
- Pipettes (sterile total delivery) 1 mL and 10 mL graduated in 0.1 mL volumes (optional)
- Microtitre plates – flat-bottomed

5.0 CULTURE MEDIA AND REAGENTS

Page's saline

Sodium chloride	120 mg
Magnesium sulphate pentahydrate	4 mg
Calcium chloride dihydrate	4 mg
Disodium hydrogen phosphate	142 mg
Potassium dihydrogen phosphate	136 mg
Water	1 L
pH 6.8 ± 0.1 at 25°C	

Non – nutrient agar E. coli plates

Agar	15.0 g
Page's saline	1 L
pH 7.0 ± 0.2	

5.1 Preparation of non-nutrient agar *E. coli* plates

- Spread a single colony of a routine laboratory strain of *E. coli* NCTC 9001 onto a nutrient agar plate and incubate overnight at 37°C. Store in refrigerator and re-new each week.
- Recover all the bacteria with a sterile swab and suspend in 2 mL of sterile Page's saline or distilled water.
- Using a sterile plastic Pasteur pipette, inoculate 3 drops of the *E. coli* suspension on to the centre of a non-nutrient agar (NNA) plate. Spread the *E. coli* evenly over the surface of the agar using a sterile hockey stick or swab. Store NNA-*E. coli* plates at 4°C and use within 1 week.

6.0 SAMPLE PROCESSING

6.1 Sample preparation

Samples should be received and handled as described in SOP W1 section 5, although samples should be transported and stored at ambient temperature instead of at 2°C – 10°C. Samples should be analysed as soon as is practicable on the day of collection. In exceptional circumstances, if there is a delay, storage under the above conditions should not exceed 24 hours before the commencement of analysis.

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Issue no: 2.3 Issue date: 03.05.05 Issued by Standards Unit, Evaluations and Standards Laboratory on behalf of the Water Working Group and the Environmental Surveillance Unit, CDSC. Page 7 of 12

Reference no: W 17i2.3

This SOP should be used in conjunction with the series of SOPs from the Health Protection Agency

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Samples of mud, soil, aquatic plants and bathing pool filter deposits should be collected in sterile glass or polypropylene containers.

Samples of water may be processed by filtration, centrifugation or by direct plating to the isolation medium. Samples of solid materials such as mud are analysed by direct culture.

6.2 Filtration

The volume of water processed for the detection of pathogenic FLA is determined by the nature of the sample site and the purpose of the examination.

Mix the sample well to resuspend any deposit and filter slowly (approximately 30 seconds per 100 mL). Do not allow the membrane to run dry.

Stop filtration when 3-5 mL of water remains above the membrane and record the amount of sample processed. Transfer all the residual water into a sterile universal container and measure the volume. Add the membrane so that the upper sample surface is facing inwards from the walls of the container.

Vortex for 5 seconds or shake vigorously by hand for 15 seconds.

6.3 Centrifugation

Centrifugation can also be used to concentrate water samples, although recovery efficiencies may be lower than by membrane filtration. The volume of water processed will depend on the nature of the sample site.

Mix the sample well to resuspend any deposit and add to 50 mL conical-bottomed polypropylene centrifuge tubes.

Centrifuge at 750 xg for 20 minutes at room temperature using a swing-out rotor.

Carefully remove the supernatant by aspiration with a pipette leaving approximately 1 mL covering the pellet. Re-suspend the pellet in the supernatant by thorough mixing (pool the pellets if more than one tube is used per sample).

6.4 Direct culture

Direct culture may be used for samples likely to contain large numbers of the test organism.

6.5 Subculture to agar media

6.5.1 Filter concentrate

Distribute a measured amount of the filter concentrate evenly over the surface of NNA-*E. coli* plates and leave at room temperature for 2 - 4 hours. During this time the amoebae settle and adhere to the *E. coli* lawn.

Gently pipette off any excess liquid and place the plates in incubators at 32°C and 37°C (base downwards) for 18 – 24 hours. By the next day any residual liquid will have evaporated and the plates should be placed, base upwards, into polythene bags, sealed with a rubber band and re-incubated at 32°C and 37°C for up to 7 days.

Note: To ensure maximum recovery of amoebae, remove the membrane from the universal container, divide in half (use scissors and forceps, flamed with alcohol) and place face down

ISOLATION AND IDENTIFICATION OF ACANTHAMOEBA SPECIES

Issue no: 2.3 Issue date: 03.05.05 Issued by Standards Unit, Evaluations and Standards Laboratory on behalf of the Water Working Group and the Environmental Surveillance Unit, CDSC. Page 8 of 12

Reference no: W 17i2.3

This SOP should be used in conjunction with the series of SOPs from the Health Protection Agency

www.evaluations-standards.org.uk

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on to the surface of an NNA-*E. coli* plate. Seal plates (base upwards) inside a polythene bag and place in an incubator at 32°C and 37°C for up to 7 days.

6.5.2 Pellet from centrifugation

Inoculate on to NNA-*E. coli* medium. If a significant pellet is present, the resuspended material should be inoculated directly on to the surface of NNA-*E. coli* plates either as 5 discrete spots or three lines. If no pellet is visible, the concentrate should be gently spread over NNA-*E. coli* medium and left to absorb to dryness. Place in an incubator at 32°C and 37°C for up to 7 days.

6.5.3 Direct culture

Untreated, natural waters may contain large numbers of free-living amoebae. This can result in failure to obtain discrete isolation of amoebae through overcrowding on the NNA-*E. coli* plates with samples processed by membrane filtration or centrifugation alone. To avoid this possibility, additional small sample volumes should be inoculated directly on to the surface of NNA-*E. coli* plates.

Mix the sample well to resuspend any deposit.

Pipette 10 mL and 1 mL volumes directly on to NNA-*E. coli* plates and leave at room temperature for 2 - 4 hours.

Carefully pipette off the excess fluid and incubate plates base downward in an incubator at 32°C and 37°C overnight. Any residual liquid will have evaporated by the next day.

Seal plates and place in an incubator at 32°C and 37°C for up to 7 days.

6.5.4 Solid material

Vigorously shake sample to resuspend material in any accompanying water (add sterile Page's solution or distilled water if necessary).

Draw sample into a Pasteur pipette and inoculate on to the surface of an NNA-*E. coli* plate either as 5 discrete spots or 3 lines.

Allow the sample to dry into the agar and seal plates (base upwards) inside a polythene bag. Place in an incubator at 32°C and 37°C for up to 7 days.

6.6 Recognition of *Acanthamoeba* species

Using an inverted microscope with a x10 objective, examine the plates daily, for up to 7 days for the appearance of discrete growths of trophozoites. Because cysts are present in the air, care must be taken to prevent accidental contamination of the culture plates. *Acanthamoeba* species should be apparent after 48-72 hours incubation although plates should be kept for 7 days before being discarded as negative.

6.7 Identification

Environmental samples may contain many genera of free-living amoeba as well as *Acanthamoeba*. A simple screening method is to inoculate isolates from NNA-*E. coli* plates into wells of a flat-bottomed microtitre plate containing quarter strength Page's solution. Trophozoite plaques or out-growths from deposit inocula are gently scraped from the plate with a disposable 1 µL bacteriological loop and inoculated into wells of a 96 place microtitre plate containing 100 µL of Page's solution. The plate is sealed with clear film, place in an incubator at 32°C and, using an inverted microscope with a x20 objective, examined after 1, 2 and 3 hours. The morphological appearance of the trophozoites or cysts can then be recorded.

ISOLATION AND IDENTIFICATION OF ACANTHAMOEBA SPECIES

Issue no: 2.3 Issue date: 03.05.05 Issued by Standards Unit, Evaluations and Standards Laboratory on behalf of the Water Working Group and the Environmental Surveillance Unit, CDSC. Page 9 of 12

Reference no: W 17i2.3

This SOP should be used in conjunction with the series of SOPs from the Health Protection Agency

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Acanthamoeba trophozoites are approximately 25 - 40 µm in length and show numerous needle-like projections from the trophozoite body termed "acanthopodia"¹. Trophozoite movement is slow and poly-directional with a hyaline pseudopodium that slowly stretches out and widens. The cysts (10 – 30 µm in diameter) of Acanthamoeba frequently show pores at the angles of the polygonal cysts which are sealed internally by caps or opercula.

The characteristic features of the cysts enable Acanthamoeba strains to be differentiated in to the morphological groups I-III. However, identification of species within the groups by morphological criteria is subjective and prone to error. Molecular and biochemical studies have shown that *Acanthamoeba* is a genetically diverse genus and demonstrates poor correlation with morphological species identification based on cyst morphology¹⁵. The revision of the genus through the development of non-morphological identification techniques is currently being studied in several laboratories.

Unlike other waterborne pathogenic protozoa such as Cryptosporidium and Giardia, reagents for the identification of pathogenic FLA are not commercially available. Differentiation of the species requires biochemical or molecular analysis that may not be available to routine water analytical laboratories. However, provisional identification may be made by morphological observations of the trophozoite and cysts forms according to Page's taxonomic criteria¹.

7.0 QUALITY CONTROL

Media and techniques may be validated using the Neff strain of *Acanthamoeba castellanii* CCAP 1501

8.0 REPORTING OF RESULTS

Presence of trophozoites should be reported as:

'a trophozoites in b ml of sample'

Absence of trophozoites should be reported as:

'No trophozoites in b ml of sample'

9.0 REFERENCE FACILITIES

Isolates of *Acanthamoeba* species or other amoeba requiring identification may be sent to Dr David Warhurst, Malaria Reference Laboratory, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT.
Tel: 0207 927 2341; Fax: 0207 637 0248;
e-mail: d.warhurst@lshtm.ac.uk

ISOLATION AND IDENTIFICATION OF ACANTHAMOEBA SPECIES

Issue no: 2.3 Issue date: 03.05.05 Issued by Standards Unit, Evaluations and Standards Laboratory on behalf of the Water Working Group and the Environmental Surveillance Unit, CDSC. Page 10 of 12

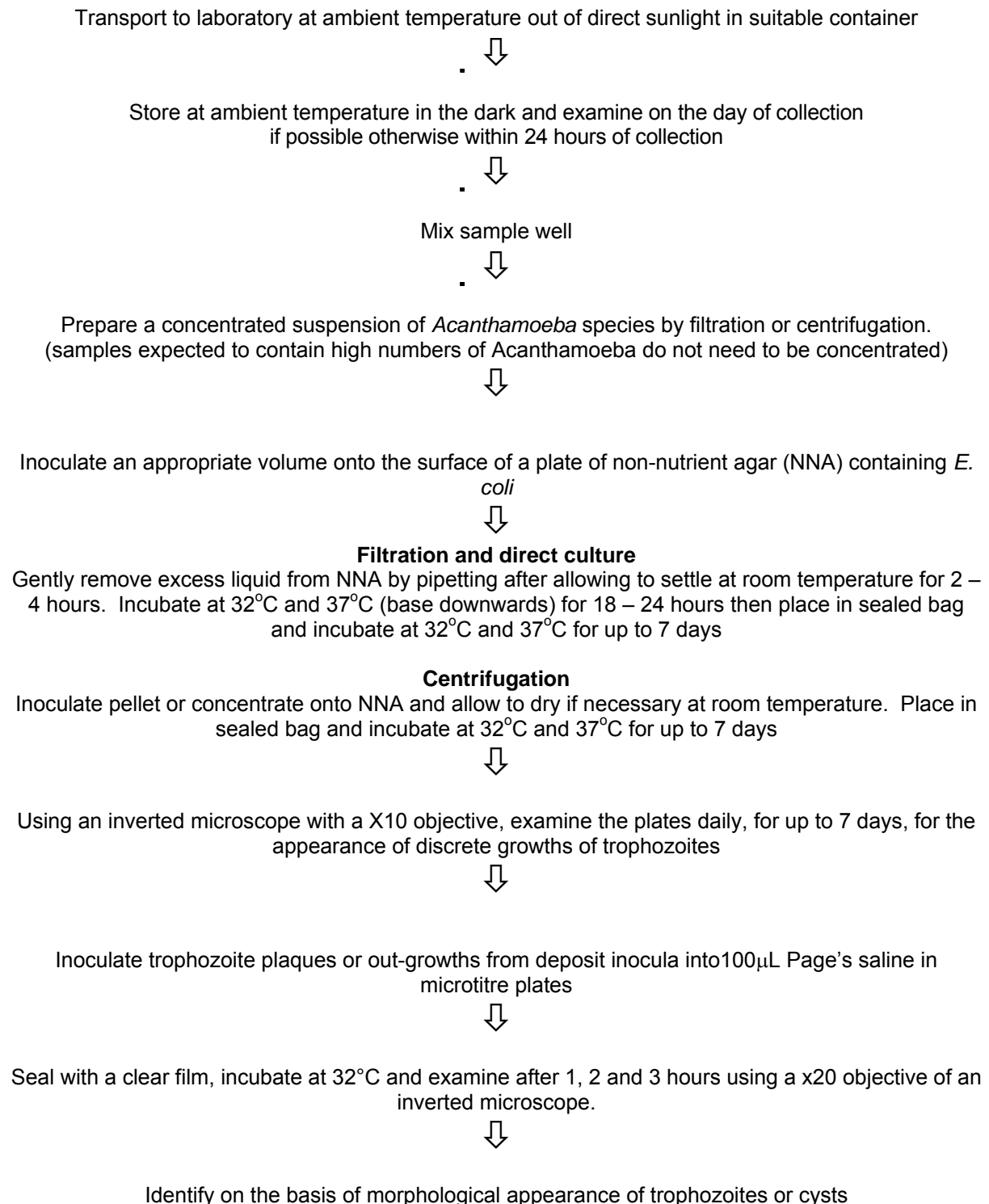
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Flowchart showing the process for the isolation and identification of *Acanthamoeba* species



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Issue no: 2.3 Issue date: 03.05.05 Issued by Standards Unit, Evaluations and Standards Laboratory on behalf of the Water Working Group and the Environmental Surveillance Unit, CDSC. Page 11 of 12

Reference no: W 17i2.3

This SOP should be used in conjunction with the series of SOPs from the Health Protection Agency

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ISOLATION AND IDENTIFICATION OF ACANTHAMOEBA SPECIES

Issue no: 2.3 Issue date: 03.05.05 Issued by Standards Unit, Evaluations and Standards Laboratory on behalf of the Water Working Group and the Environmental Surveillance Unit, CDSC. Page 12 of 12

Reference no: W 17i2.3

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