ENVIRONMENT AGENCY

The Microbiology of Drinking Water (2010) - Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

Methods for the Examination of Waters and Associated Materials

The Microbiology of Drinking Water (2010) - Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

Methods for the Examination of Waters and Associated Materials

This booklet contains details of the procedures that may be used for taking and analysing aqueous and non-aqueous samples for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts.

This bluebook updates and replaces the previous version published in 2009.

Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products but serves only as illustrative examples of the type of products available. Equivalent products may be available and it should be understood that the performance of the method might differ when other materials are used and all should be confirmed by validation of the method.

Within this series there are separate booklets, each dealing with different topics concerning the microbiology of drinking water. Booklets include

The Microbiology of Drinking Water (2002)
Part 1 - Water quality and public health
Part 3 - Practices and procedures for laboratories (currently undergoing revision)
Part 10 - Methods for the isolation and enumeration of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment.

The Microbiology of Drinking Water (2004)
Part 11 - Taste, odour and related aesthetic problems
Part 12 - Methods for micro-organisms associated with taste, odour and related aesthetic problems.

The Microbiology of Drinking Water (2006)
Part 9 - The isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube-most probable number techniques

The Microbiology of Drinking Water (2007)
Part 7 - Methods for the enumeration of heterotrophic bacteria (currently undergoing revision)
Part 13 - The isolation and enumeration of aerobic spore-forming bacteria by membrane filtration

The Microbiology of Drinking Water (2009)
Part 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)

The Microbiology of Drinking Water (2010)
Part 2 - Practices and procedures for sampling
Part 5 - The isolation and enumeration of enterococci by membrane filtration
Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration
Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

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About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments and biota.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

In the procedures described in each method any reference to the tolerances to be adopted with respect to, for example the amount or volume of reagents to be used is left to the discretion of the laboratory. These tolerances should be as low as possible in order to satisfy stringent performance criteria. Tolerances of between 1 - 5 % have been shown to be satisfactory for most purposes. Lower tolerances should result in improved precision.

In the methods described, for example for wavelengths, storage conditions, concentrations of the same or similar reagents, etc, differences may be noted. This information is provided by individual laboratories operating under their own management systems and is dependent on specific conditions pertaining to each laboratory. It is assumed this information is supported by sufficient data to justify its inclusion. Users intending to use or vary the quoted wavelengths, storage conditions, concentrations, etc, should ensure they are appropriate to their own laboratory and verify their application to demonstrate appropriate performance of the method. In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Environment Agency.

Methods are produced by panels of experts in the appropriate field, often in co-operation with working groups and the main committee. The names of those members principally associated with these methods are listed at the back of this booklet. A report describing all SCA activities for the period 1 July to 30 June is produced annually and is available from the Agency's web-page (www.environment-agency.gov.uk/nls).

Users should ensure they are aware of the most recent version of the draft they seek. If users wish to receive copies or advance notice of forthcoming publications, or obtain details of the index of methods then contact the Secretary on the Agency's internet web-page or by post, see address listed at the back of this booklet.

Great efforts are made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
Secretary
February 2010

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such publications are; "Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

Glossary

ACDP	Advisory Committee on Dangerous Pathogens
ANOVA	analysis of variance
bar	unit of pressure equal to 1 atmosphere (15 lb in^{-2}, 101.325 kPa, 760 mm of mercury.
break-through	penetration of a filter medium, for example a membrane filter, by particulate material that would otherwise be trapped by the filter medium.
by-pass	passage of sample water through a filter apparatus such that the water does not pass through the filtration module itself but leaks to waste, past for example an O-ring, allowing (oo)cysts to by-pass the unit.
cpm	cycles per minute
Cryptosporidium	a protozoan parasite
Cryptosporidium oocyst	the environmentally resistant transmissive life cycle stage in which *Cryptosporidium* occurs in the environment. The oocyst (which is shed in the faeces of an infected person or animal) contains 4 sporozoites capable of causing infection.
DAPI	4',6-diamidino-2-phenylindole
DIC	differential interference contrast (microscopy)
DNA	deoxyribonucleic acid
excystation	process by which the infective bodies contained in cysts or oocysts are released
FITC	fluorescein isothiocyanate
Giardia	a protozoan parasite
Giardia cyst	the resting life cycle stage in which *Giardia* occurs in the environment, and which is capable of causing infection
hydrophobic / hydrophobicity	the tendency of a surface to repel wetting by water
IMS	immuno-magnetic separation
MAb	monoclonal antibody
(oo)cyst	this term signifies either *Cryptosporidium* oocyst or *Giardia* cyst or both, as appropriate, depending on the context
OLBs	(oo)cyst-like bodies, i.e. organisms that resemble (oo)cysts but are not.
PCR	polymerase chain reaction
protoplasmic "residual body"	protoplasmic material remaining within an oocyst after sporozoites have excysted.
rcf	relative centrifugal force, equivalent to force of gravity (g)
RFLP	restriction fragment length polymorphism
V_{PV}	pellet volume
V_{SV}	final mixture volume

Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

Introduction

Cryptosporidium is a genus of coccidian protozoan parasites, found worldwide in a variety of vertebrate hosts, including humans. Some species of this parasite, notably *Cryptosporidium hominis* and *Cryptosporidium parvum*, cause disease (cryptosporidiosis) usually manifested as diarrhoea in humans (*Cryptosporidium hominis* and *Cryptosporidium parvum*) and young livestock (*Cryptosporidium parvum*).

Giardia is a genus of flagellated protozoan parasites. *Giardia duodenalis* (sometimes referred to as *Giardia intestinalis* and *Giardia lamblia*) is recognised as one of the most common worldwide protozoan parasites causing diarrhoea (giardiasis), infesting the small intestine of humans, and other vertebrates.

Transmission of infection can occur by any route where infective *Cryptosporidium* oocysts or *Giardia* cysts are ingested. Both oocysts and cysts can survive for prolonged periods of time in cool, moist environments, with *Cryptosporidium* oocysts being more resistant to chlorination than *Giardia* cysts.

Information on the biology, transmission and public health significance of *Cryptosporidium* and *Giardia* is given in Appendix 1.

Increasingly, molecular typing of environmental and outbreak isolates is being conducted to better understand the transmission and epidemiology of *Cryptosporidium*. The techniques employed are outside the scope of this document, but an overview of the rationale and principles of such investigations is presented in Appendix 2.

Definitions

Cryptosporidium oocysts are the environmentally resistant transmissive life cycle stages in which *Cryptosporidium* occurs in the environment. The oocyst (which is shed in the faeces of an infected person or animal) contains 4 sporozoites capable of causing infection. When ingested, disease is transmitted and a new cycle of infection set up. The oocysts are resistant to adverse conditions in the environment and can remain dormant but viable for months in waters, sediments and soils.

Limitations

Environmental monitoring is problematical owing to the low numbers and uneven distribution of *Cryptosporidium* oocysts and *Giardia* cysts normally found in many waters, and sand filter materials. The lack of *in-vitro* culture methods for increasing (oo)cyst numbers also causes difficulties in detection, which currently relies on examination by microscopy. Consequently, it can be difficult to accurately identify (oo)cysts from other particulate material and debris found in concentrated suspensions obtained from waters.

The procedures used for the separation, recovery and identification of (oo)cysts are labour intensive and time consuming. The presence of particulate material in a sample may interfere with every stage of the sample collection and analytical processes, including the microscopic examination.

The failure to detect (oo)cysts in a sample does not ensure or guarantee that the sample is indeed *Cryptosporidium*- or *Giardia*-free. No *Cryptosporidium* or *Giardia* method is capable of achieving 100 % recovery, and indeed actual recoveries are much lower. Reported recovery data for waters obtained using procedures described in this booklet are presented in Appendix 3.

Health and safety

The analytical procedures described in this booklet should only be carried out by competent, trained persons with adequate supervision where necessary.

Reagents and organisms used in this method are covered by the Control of Substances Hazardous to Health Regulations 2002[1] and appropriate risk assessments should be carried out before using this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2].

Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted. Field operations should be conducted with due regard for possible local hazards, and appropriate safety equipment should be used when required. *Cryptosporidium* and *Giardia* are classified as ACDP Hazard Group 2[3] organisms, i.e. the organisms can cause human disease and may be hazardous to laboratory staff. Laboratory procedures should only be performed in properly equipped laboratories within at least a category 2 containment facility[3]. Filters that have been used to separate (oo)cysts from water samples, may contain significant numbers of (oo)cysts and other potentially pathogenic micro-organisms, and the ova and cysts of other parasites. Filters should therefore only be processed in an appropriate laboratory.

If used in a viable state, *Cryptosporidium* oocysts and *Giardia* cysts used for seeding or quality assurance purposes may pose a risk of infection to humans. A non-viable form of (oo)cysts may be produced by the irradiation or heat treatment of viable (oo)cysts. If non-viable (oo)cysts are used, consideration should be given to the possibility that the inactivation treatment used may affect, for example the surface characteristics of the (oo)cyst and potentially affect recoveries. Comparison work may therefore be advisable depending on the type of inactivation used and the information being sought. Consistent use of one type of treatment should be used within a laboratory.

In addition, equipment (for example, mercury bulbs) should be treated cautiously and handled appropriately if damaged. Mercury vapour lamps have a limited safe working life of 100 - 200 hours depending on their specification. Beyond this period the fluorescence output may fade and bulbs may explode, damaging the lamp housing and posing a risk of exposure to mercury vapour. Bulbs should therefore be changed at regular intervals and their fluorescence output calibrated using a fluorescence calibration control slide.

References

1. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No 2677. See also Approved Code of Practice and Guidance L5 (Fourth edition) HSE Books 2002 ISBN 0 7176 2534 6.

2. The management, design and operation of microbiological containment laboratories. Guidance HSE Books 2001 ISBN 0 7176 2034 4.

3. Advisory Committee on Dangerous Pathogens, The Approved List of Biological Agents. Health and Safety Executive 2004.

4. Drinking Water Inspectorate. Guidance on assessing risk from *Cryptosporidium* oocysts in treated water supplies to satisfy the Water Supply (Water Quality) regulations 1999. (Available from: http://www.dwi.gov.uk). These regulations have now been revoked.

5. Standing Committee of Analysts, The Microbiology of Drinking Water (2010) - Part 2 - Practices and procedures for sampling, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

6. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency. (currently undergoing revision)

7. Some Observations on Factors which affect Recovery Efficiency in *Cryptosporidium* Analysis, A P Walker in "*Cryptosporidium* The Analytical Challenge" Eds. M Smith and K C Thompson, Royal Society of Chemistry, 2001.

Practices and procedures for sampling

1 Rationale for sampling

This section describes general guidance for sampling. For detailed information on filtration and flocculation procedures and on elution and concentration techniques for the analysis of waters, see methods A and B. For the analysis of sand filter samples, see method C.

The principal purposes for which a sample of water may be taken for the examination of *Cryptosporidium* oocysts or *Giardia* cysts include

- Water treatment process control:
- Monitoring of catchment areas and water sources:
- Risk assessment:
- Incident management:
- Water-borne or swimming pool outbreak investigations:
- Regulatory or statutory purposes.

The introduction of a new treatment process, or the modification of an existing one, may require water quality monitoring to assess the effectiveness of the new or modified process to remove *Cryptosporidium* oocysts and/or *Giardia* cysts.

Hydrological catchment areas can be contaminated with *Cryptosporidium* oocysts and *Giardia* cysts arising from, for example surface run-off, direct faecal contamination by animals with access to water sources, sewage discharge, or the spillage or spreading of slurry. These events can introduce large numbers of (oo)cysts to water sources in a relatively short period of time. Water quality monitoring of raw water, sewage effluents and catchment source waters, particularly during periods of heavy rainfall, can help to identify where contamination may have occurred. Subsequent remedial action can then be taken to prevent (oo)cysts gaining access to water supplies.

Sampling and analysis within a public supply system may be important to help locate or confirm the source around which a cluster of cases of disease has been identified. Data generated by such monitoring, and used with other water quality parameters and information, for example epidemiological evidence, can be used to ascertain whether the water is a potential source of infection.

Where drinking water has been shown to pose a risk that (oo)cysts may be present in the water following treatment, or may subsequently enter the distribution system, then water quality monitoring should be undertaken. Risk assessments should take into account all activities within a catchment area that have the potential to allow (oo)cysts to gain access to raw water used for drinking water abstraction, and should be subject to regular review and audit. Further guidance on carrying out such risk assessments can be found elsewhere[4].

For swimming pool waters, faecal release (often associated with sub-optimal treatment) is probably more likely to be the cause of an outbreak. In the investigation of suspected swimming pool outbreaks, the examination of the pool water, sand samples from filters, back-wash waters and materials from strainer baskets can facilitate and support the outbreak investigation and help demonstrate whether the pool presents a risk to the public.

The purpose for which a sample is taken and the manner in which the results are to be used and reported should be considered when sampling plans and strategies are developed, and before any sample is taken for examination.

2 Sample collection

The sampling procedure used will depend on the sample type, purpose of the analysis, sensitivity required, and to some extent, on how quickly this and related information needs to be provided. The volume of sample taken, see Table A, may be small, i.e. 10 - 50 litres (which may be collected on site, typically as a grab sample) or large, for example up to 1000 litres (which usually requires on-site filtration and which may take up to 24 hours to collect). During emergencies or outbreak investigations it may be advantageous to change large volume sample filters at more frequent intervals than 24 hours. Small volumes of samples may provide an indication of water quality at the time the sample is taken. However, due to the varying recovery efficiencies of the methods used, it may be very difficult to reliably determine low numbers of (oo)cysts in small volumes of samples. Large volumes of samples should provide an overall indication of water quality covering a longer period of time. When suspected water-borne outbreaks are investigated, the volume of sample taken may need to be a compromise between competing factors, for example the time taken for sample collection versus the urgency of requiring the results. This may require strategic sampling of large volumes of water, together with more frequent sampling of smaller volumes of water (so that results may be obtained more rapidly in suspect areas).

Table A Typical sample volumes for *Cryptosporidium* and *Giardia* monitoring

Sampling environment	Sample range* (litres)
Distribution system waters	10 to 1000
Post-filter and final waters	10 to 1000
Backwash waters	1 to 10**
Surface waters of low turbidities	10 to 100**
Other surface waters	10 to 50**
Ground waters	10 to 1000**
Swimming pool waters	100 to 1000**
Treated wastewater effluents	10 to 20
Untreated wastewater	1 to 5
Solid samples (for example filter sand)	100 to 1000***

* Whilst it is desirable to use large volumes of sample, small volumes may need to be taken to facilitate management of out-break situations where results are required quickly.
** Depending on the particulate loading of the sample.
*** Amount in grams

The quality of water being sampled may also influence the volume of sample to be taken. For example, turbid water when processed and concentrated will often result in the production of a large quantity, or pellet, of suspended particulate material. The treatment of this material may prolong the analysis time, or decrease the overall recovery efficiency of the method. High turbidity in drinking water and swimming pool water samples may be indicative of significant contamination. Where high turbidities are recorded, this may merit immediate attention and analysis for *Cryptosporidium*.

Appropriate information, such as the recovery efficiencies of the methods used to detect and enumerate (oo)cysts, the purpose for which the sample is to be taken and the

expected number of (oo)cysts to be enumerated, may be used to estimate the most suitable amount of sample that needs to be taken.

2.1 Small volumes of samples

Small-volume grab samples of up to 10 or 50 litres (Table A) may be collected, for example in clean, appropriately-sized polyethylene jerry cans and transported to the laboratory for processing. Low cost disposable containers, or non-disposable containers that are thoroughly washed between use, may also be used. Consideration should be given to segregating containers that are used for storing the samples of raw and treated waters. Prior to the collection of the sample, the container should be rinsed with the water that is to be sampled.

Volumes of water of up to 50 litres may be filtered on-site using appropriate methods. This may be useful for sampling surface waters, ground waters or post-filter waters with relatively high turbidities. Filter systems, such as Idexx Filta-Max®, Pall Envirochek™ or Idexx Filta-Max *xpress*™ filters have been found suitable. Alternatively, flat-bed membrane filtration may be used.

Samples from water supply distribution systems should be collected with the same considerations given for flushed, chemical or bacteriological samples, but tap disinfection is not required for *Cryptosporidium* and *Giardia*. Further guidance can be found elsewhere[5] in this series. Unless any debris present in the water in a sample line or pipe that is to be sampled is also to be analysed, then the sample line or pipe should be flushed with water in the pipe until the debris is removed, prior to taking the sample.

2.2 Large volumes of samples

Because the collection and transport of large volumes of water can be both time consuming and costly, an alternative approach may be to filter the sample on-site using an appropriate filtration device. Large volumes of samples should be filtered on-site using appropriate methods involving membrane filters or appropriate filters enclosed in a suitable housing system. These procedures have been found suitable for clean water sources, such as ground waters, post-filter and final waters, distribution samples and good quality surface waters. Filter systems such as Idexx Filta-Max®, Pall Envirochek™ HV or Idexx Filta-Max *xpress*™ filters have been found suitable. Alternatively, flat-bed membrane filtration may be used. The basic principle of all these procedures is based on the separation of particulate matter (including any (oo)cysts that may be present) from the bulk of water sampled. For optimum separation of (oo)cysts, this will depend on the volume of water sampled, the amount of particulate matter (including (oo)cysts) present in the water, and the ability to recover (oo)cysts from the filter.

The basic apparatus required for all filtration systems for filtering water under pressure (for example, drinking water distribution systems or swimming pool recirculation systems) consists of an inlet and outlet hose, a filter holder, a flow control device and a water meter. Filter housings should be checked before being used to filter the sample, to ensure leaks do not occur. The specific filter chosen should be connected in line with the water supply ensuring the flow is through the filter, water meter and flow control device as recommended by the manufacturer. The water meter should be included and the readings of the meter recorded at the beginning and end of the filtration period. During filtration, the flow control device valve should be used to give a flow rate appropriate to the type of filter being used. Pressure meters fixed to both the sample inlet and outlet can be useful, as

significant drops in pressure (i.e. head-loss) over the sampling period can suggest filter blockage. This may be due to turbidity, indicating poor water quality and possible risk of break-through, i.e. penetration of the membrane filter by particulate material that should otherwise have been trapped by the filter. A suitable pump (for example peristaltic type) may need to be used for un-pressurised sources. Sample housings for treated and untreated sources should, ideally, be kept segregated and consideration should be given to dedicating specific housing systems to individual sources.

When the required volume of water has been filtered or the specified sampling time has been reached, turn off the supply. The whole filter housing can be removed and, after ensuring the sampling ports are sealed, transferred to the laboratory.

2.3 Sample details

Appropriate sample details should be recorded, including the following information:

(i) sample location,
(ii) date and time filtration begins and ends,
(iii) date and time of sample collection,
(iv) volume of sample,
(v) name of sampling officer etc,
(vi) flow rate.

Any additional information that may affect the generation and interpretation of the results reported should also be recorded, for example, the turbidity of the water. Details on the reason for sampling can also be helpful if it becomes necessary to prioritise the analyses within the laboratory.

2.4 Other materials to be sampled

Other types of samples, such as sand from sand filters, sediments from service pipes, etc may also need to be analysed. The amount of material required will depend on the extraction and clean-up procedures used and the amount and nature of interfering substances present in the material. The material submitted should be as representative as possible of the material at the location from where the material is to be taken. Care should be taken when eluting material from filter sand and similar samples as any (oo)cysts present may become damaged by excessive agitation.

A Isolation of *Cryptosporidium* oocysts and *Giardia* cysts by filtration

A1 Introduction

The procedures described in this section are applicable to drinking waters and may be applicable (with suitable adaptation) for the analysis of other matrices, see method C. The Filta-Max *xpress*™ system is new at the time of publishing this booklet. This technique has only undergone a phase 2 study (see Appendix 4) as, following discussion with the regulator, a phase 1 study was not deemed necessary in this case. Data, from the phase 2 study, submitted to the Standing Committee of Analysts is summarised in Annex 3. Information on the routine use of this method, and others, would be welcomed to assess their full capabilities as details are included in this booklet for information purposes only as examples of the types of procedures that are available. Users are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on performances.

Several filtration techniques are described, namely

AA *IDEXX Filta-Max® filtration*,
AB *Pall Life Sciences Envirochek™ filtration*,
AC *Flat-bed membrane filtration*, and
AD *IDEXX Filta-Max xpress™ filtration*

followed by specific elution and concentration procedures. Procedures for the concentration of particulate matter from the filter washings are then described.

Whilst details of procedures are included in this booklet, if a specific manufacturer's product is used, then the manufacturer's instructions should be followed and strictly adhered to.

The analysis is then completed using immuno-magnetic separation and microscopic examination (section D).

A2 Scope

The methods of filtration described in this booklet are suitable for the isolation and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts in all types of waters. The methods may be used to facilitate identification of the routes of contamination of water supplies for

(i) catchment control purposes (including raw water monitoring),
(ii) evaluating the effectiveness of treatment practices, and
(iii) investigating potential or actual outbreak situations.

Users wishing to employ any of the methods outlined in this section should verify its performance for their own specific matrices and under their own laboratory conditions.

A3 Principle

Oocysts and cysts may be separated from water samples by one of four filtration methods. From the filtered material, a pellet of particulate matter, V_{PV} ml, is produced, which when

held in suspension, V_{SV} ml, is then ready for the IMS stage and microscopic examination (section D).

A4 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[6] in this series.

Some of the following procedures are based on proprietary products, and if used, manufacturer's instructions should be followed. Inclusion in this document does not constitute an endorsement of these products but serves only as illustrative examples of the type of products that are available. Users should decide which product or procedure is appropriate for their own requirements and should verify its performance under their own laboratory conditions with appropriate samples.

A4.1 *Apparatus for sampling by filtration*

See the appropriate section for each of the filtration techniques.

A4.2 *Apparatus for elution and concentration*

See the appropriate section for each of the elution and concentration techniques.

A5 Reagents

Commercial or alternative formulations of these reagents are available, but may possess minor variations to their formulation. The performance of all reagents should be verified prior to their use in the method. Further guidance on assessing the performance of methods or parts of methods used for drinking water analysis is given in Appendix 4. Variations in the preparation and storage of reagents should also be verified. Commercially available reagents should be used and stored according to manufacturer's instructions. Water for reagents should be distilled, deionised or of similar quality that is (oo)cyst-free. Unless otherwise stated chemical constituents should be added as anhydrous salts. Where reagents are stored in a refrigerator they should be allowed to reach room temperature before use.

A5.1 *Reagents for elution and concentration*

See the appropriate section for each of the elution and concentration techniques.

A6 Analytical procedure

A6.1 *Procedures for filtration methods*

Four filtration techniques are available. See the appropriate section in AA, AB, AC or AD for each of the filtration techniques.

A6.1.1 *Transport of samples*

Filters or grab samples may be transported to the laboratory at ambient temperature in the dark. Samples that cannot be processed immediately should be stored in the range

5 ± 3 °C in the dark and should not be allowed to freeze. Ideally, analysis should begin within 48 hours of collection, and preferably on the day of receipt within the laboratory. Allow stored samples to reach room temperature before commencing the analysis.

All sample details (see section 2.3) should be recorded and the sample allocated a unique reference code, which may be used subsequently to identify the sample.

A6.2 *Elution and concentration procedures*

See the appropriate section in AA, AB, AC or AD for each of the elution and concentration techniques.

When all the particulate material has been centrifuged in a single 50 ml centrifuge tube, measure and record the pellet volume V_{PV} ml of particulate material. Remove the supernatant liquid to within 5 ml of the pellet and add water to the centrifuge tube to bring the total volume V_{SV} ml, typically to 9 ml and vortex to re-suspend the particulate material. The suspension is now ready to proceed to the immuno-magnetic separation (IMS) stage and microscopic examination (section D) or may be stored in the range 5 ± 3 °C for up to 3 days before this process begins. However, delay in continuing the analysis at this stage may adversely affect the results of analysis.

The following sections, AA, AB, AC and AD describe procedures for individual manufacturer's products, each terminating at a stage that is then ready for the IMS stage and microscopic examination (section D).

AA Procedures using the IDEXX Filta-Max® system

AA1 Apparatus for IDEXX Filta-Max® filtration

In addition to standard laboratory equipment, specific Filta-Max® items include:

Filta-Max® filter modules (see Figure AA1).
Filta-Max® filter housings (see Figure AA2).
Sampling rig consisting of water meter, flow control device (1 litre per minute), inlet and outlet pressure meters (optional) tubing and connectors.

Figure AA1 Filta-Max® filter modules

Figure AA2 Filta-Max® filter housing with connectors and tightening tools

Membrane filters, 7[illegible]mm diameter. The nominal pore size should be sufficient to ensure filtration of the (o)cysts.
Manual (or automated) wash station with plunger heads (see Figure AA3).
Elution set, consisting of an elution tube, elution base and stainless steel tube (see Figure AA4).
Concentrator set, consisting of a concentrator tube, concentrator base with filter support (see Figure AA4) and a magnetic stirrer assembly (see Figure AA5).
Re-sealable plastic bags for washing membranes.
Vacuum set, including pump with vacuum gauge and catch-bottle (see Figure AA5) or a dedicated vacuum system connected to the concentrator.
Magnetic stirrer plate.
Counter (optional).
Centrifuge, capable of operating to at least 1100 rcf.
Centrifuge tubes (conical, plastic and graduated) 50 ml capacity.
Forceps,
Rubber bungs (for closing off the elution stainless steel tube).

Figure AA3 **Filta-Max® manual wash station** (with assembled tube set and filter in place)

Figure AA4 **Elution tube** (standing - threaded at one end) **elution base** (with stainless steel tube attached) **concentrator tube** (laying - threaded at both ends) **and concentrator base** (with tap)

Figure AA5 Filta-Max® concentration tube set with stirrer assembly, on a magnetic stirrer and connected to a catch-bottle and vacuum pump

AA2 Reagents for IDEXX Filta-Max® filters

AA2.1 *Phosphate buffered saline solution* (PBS)

Sodium chloride	8.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	1.15 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g
Potassium chloride	0.2 g
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH of the solution to 7.3 ± 0.2 with either 1M hydrochloric acid solution or 1M sodium hydroxide solution. The reagent may be stored in the dark in the range 20 ± 5 °C for up to three months. Commercially available buffer solutions may contain preservatives which may extend the shelf life. Discard the solution if there is any sign of turbidity.

AA2.2 *Elution buffer solution*

Polyoxyethylene(20)sorbitan monolaurate (for example Tween 20)	1 ml
Phosphate buffered saline solution (AA2.1)	10 litres

Dispense 1 ml of polyoxyethylene(20)sorbitan monolaurate into a 50 ml container and dissolve the detergent in approximately 10 ml of warm (typically, about 40 °C) water. Add the contents of the container to approximately 8 litres of phosphate buffered saline solution (AA2.1) in a 10 litre vessel. Rinse the container with 10 ml of water, and add the rinsings to the vessel. Repeat this process with a further 10 ml of water. Make to 10 litres with phosphate buffered saline solution. The reagent may be stored in the dark in the range 20 ± 5 °C for up to one month. Commercially available buffer solutions may contain preservatives which may extend the shelf life. Discard the solution if there is any sign of

turbidity, or if the solution shows any resistance to filtration (for example due to bacterial growth).

AA3 Filtration using Filta-Max® filters

The Filta-Max filtration system has been found suitable for the examination of samples of surface, ground and treated waters, including swimming pool waters, where, depending on the turbidity of the sample, the volume of water filtered ranges between 10 - 1000 litres (or more). A filter module consists of a set of open-cell reticulated foam rings compressed between two plates so as to produce a filter with a nominal pore size of 1 µm. A known volume of water (at a controlled flow rate) is filtered through the filter, housed in a reusable housing. Large volumes of sample may be filtered on-site and the filter and housing returned to the laboratory for analysis; smaller volumes of sample may be filtered at the laboratory. It may be appropriate to flush or retain any residual debris from the sampling tap or pipe before it is connected to the filter housing. This will depend on whether the debris needs analysing, or whether the debris is to be analysed with the water to be filtered.

Ensure that all the O-rings are located correctly in the housing and lightly lubricated, for example with silicone vacuum grease. Place a filter module (rounded end-plate down) into the housing and align the lid onto the base. Tighten the lid firmly to ensure the filter module is properly placed within the housing so filter by-passing of the water being sampled does not occur. However, it is important not to over-tighten the housing.

Attach the filter housing to the water supply under investigation, ensuring that the direction of water flow is as indicated on the housing. Large volumes of sample should be filtered using a flow meter in line with the housing to measure the volume of water filtered. Small volumes of sample may be measured directly. Typically, a flow rate of up to a maximum of 4 litres per minute is used. A pump, capable of operating at up to 5 bar, may be required to achieve a suitable flow rate. Small volumes of samples may be filtered using a peristaltic pump.

Following filtration, the filter housing should be sealed, for example with rubber bungs or self-sealing quick-connect couplings, and then transferred to the laboratory for processing.

AA4 Elution and concentration from Filta-Max® filters

For the concentration and elution of the trapped particulate material on the Filta-Max® filter, the module is removed from the housing and the compressed foam rings released by removing the retaining bolt. A wash station is then used to elute any trapped material into an elution buffer solution (AA2.2). The system is so designed that the mixture is stirred to ensure any particulate matter, including (oo)cysts is held in suspension, while at the same time elution buffer is removed through a membrane filter, thus concentrating the suspension of particulate matter. The number of membrane filters required will depend on the amount of particulate material and whether any of the filters become blocked during the concentration stage.

The resulting concentrated suspension, of up to 50 ml should then be transferred to a 50 ml conical centrifuge tube and centrifuged at 1100 rcf for 15 minutes. Most of the supernatant liquid should then be carefully removed. A small volume (typically, 5 ml) of the supernatant liquid should be left covering the pellet of particulate material. If this volume is too small, (oo)cysts might be lost. The volume, V_{PV} ml, of particulate matter should be

measured and recorded. Water should then be added to this mixture, i.e. the pellet volume and the small volume of supernatant liquid, and made to a known volume, V_{SV} ml, typically 9 ml. This volume should be such so as to give, for example a final volume suitable for immuno-magnetic separation (IMS) to be carried out. The final volume, V_{SV} ml, of the suspension will depend on the amount of solid material present and collected and the IMS kit used subsequently. The pellet volume, V_{PV}, is critical in that a larger pellet volume (i.e. greater than 0.5 ml) may have a detrimental effect on (oo)cyst recoveries and the volume is often specified by IMS manufacturers. In addition, the final volume, V_{SV}, is also important in that the reagent volumes used in IMS procedures are dependent on this volume.

Where samples from different sources are examined routinely, and in order to minimise the risk of cross contamination, it may be advantageous to set aside separate equipment, such as plunger head, elution set and concentrator set, dedicated for use with each source.

AA4.1 *First elution*

The elution apparatus consists of an elution set, a wash station and a concentrator set which is designed to produce a concentrated suspension of particulate matter of less than 50 ml. The following process highlights the procedures for manual wash stations. Where automatic wash stations are used manufacturer's guidance should be followed.

Attach a plunger head to the wash station and lightly lubricate the O-ring, usually, with silicone vacuum grease.

Place a membrane filter (rough surface uppermost) onto the filter support in the concentrator base and screw the concentrator tube into the base. Ensure the membrane filter is held securely and that the tap on the base is closed.

Unscrew the housing top, remove the Filta-Max® module from its housing and screw it into the plunger head of the wash station. Pour any residual water contained in the filter housing into the assembled concentrator set.

Place the elution base into the jaws of the wash station, locate the elution tube into the base and lower the filter module through the tube. Remove the retaining screw from the filter module. This should allow the Filta-Max® filter to expand. Screw the stainless steel tube into the underside of the elution base.

Add approximately 600 ml of elution buffer solution (AA2.2) into the concentrator assembly, tilt the tube to an angle of approximately 45 °, with the tap upwards, and open the tap briefly to release any trapped air. Attach the concentrator assembly to the underside of the elution base (see Figure AA3).

Pump the plunger up and down (typically, five times) to facilitate expansion of the Filta-Max® filter. This procedure should be carried out smoothly in order to minimise foaming. If there is no visible expansion of the filter, leave it to soak (typically, up to 5 minutes). Extending this period is unlikely to significantly increase the recovery of (oo)cysts. Wash the filter module by moving the wash station plunger up and down (typically, a further 20 times) to complete the first elution stage.

Detach the concentrator assembly and hold it under the open end of the stainless steel tube, keeping the end of the tube above the surface of the eluate. Press the plunger up and down (typically, a further 5 times) to remove any residual eluate from the foam rings. Plug the end of the stainless steel tube, for example with a small rubber bung.

AA4.2 *First concentration*

Place the concentrator assembly on a magnetic stirrer. Locate the magnetic stirring bar assembly into the top of the concentrator tube and set the stirrer so that the whole of the suspension in the tube rotates and mixes. Any particulate material should be kept in suspension while elution buffer is filtered through the membrane filter. The design of this action is such that the majority of the particulate matter is held in suspension while elution buffer is filtered through the membrane filter. Thus, the intention is not to capture or trap the particulate matter on the filter, but to keep it in suspension. Connect the vacuum pump (with a vacuum gauge) via a catch-bottle, and open the tap on the concentrator base.

Apply the minimum vacuum, as necessary, to filter the elution buffer. For samples of low turbidity, the catch-bottle can simply be placed below the level of the concentrator tube, whereupon the elution buffer should flow by gravity through the membrane filter. For samples with high turbidities, apply a vacuum of no greater than 40 kPa (300 mm of mercury) to filter the elution buffer through the membrane filter. Excessive vacuum may adversely affect (oo)cyst recovery.

Particulate material may accumulate on the membrane filter and block the membrane filter. If this occurs, decant the suspension of particulate matter into a clean container. Dismantle the concentrator assembly and remove the membrane filter and place it in a plastic bag, taking care not to lose any particulate matter suspension from the surface of the membrane filter. This filter is then retained for further processing. Place a second membrane filter onto the concentrator base and reassemble the unit. The fresh membrane filter should be used smooth-side uppermost, as this side may be less prone to blockage and might be easier to wash. Pour the retained suspension back into the concentrator, rinsing the container, and continue the filtration process.

This process may need to be repeated depending on the number of membrane filters required. Each membrane filter should be placed in its own, separate plastic bag (taking care not to lose any particulate matter from the surface of the membrane filter) and then retained for further processing.

When the volume of suspension decreases, i.e. to a level approximately half way down the stirrer bar (about 30 ml) close the tap and disconnect the vacuum set. It is important not to filter off all of the elution buffer. Any (oo)cysts present in the suspension should be kept suspended in the elution buffer, retained above the membrane filter. Remove the stirrer assembly, rinse it with elution buffer and combine the rinsings with the suspension, and pour the suspension from the concentrator into a 50 ml centrifuge tube. The membrane filter may now removed and placed in a plastic bag (taking care not to lose any particulate matter from the surface of the membrane filter) and then retained for further processing. For certain samples, where the membrane filter is clearly still functional, the original filter may be retained in place for the second elution.

AA4.3 *Second elution*

A new membrane filter (if required) is now placed on the concentrator base and the concentrator reassembled, to undertake a second elution of the Filta-Max® filter. Add a further 600 ml of elution buffer to the concentrator assembly and attach it to the underside of the elution base as before. Repeat the elution procedure by pumping the plunger (for example at least a further 10 times). Detach the concentrator assembly and hold it under the open end of the stainless steel tube, keeping the end of the tube above the suspension. Press the plunger up and down (for example a further 5 times) to remove any suspension of particulate matter from the foam rings and use elution buffer to rinse the outside of the stainless steel tube into the concentrator.

AA4.4 *Second concentration*

Place the concentrator assembly on a magnetic stirrer. Locate the magnetic stirring bar assembly into the top of the concentrator tube and set the stirrer so that the whole of the suspension in the tube rotates and mixes. Connect the vacuum pump (with a vacuum gauge) via a catch-bottle, and open the tap on the base of the concentrator.

Apply the minimum vacuum, as necessary, to filter the elution buffer. For samples of low turbidity, the catch-bottle can simply be placed below the level of the concentrator tube, whereupon the elution buffer should flow by gravity through the membrane filter. For samples with high turbidities, apply a vacuum of no greater than 40 kPa (300 mm of mercury) to filter the elution buffer through the membrane filter.

Some particulate material may accumulate on the membrane filter, and if this occurs sufficiently to block the membrane filter, decant the suspension of particulate matter into a clean container. Dismantle the concentrator assembly and remove the membrane filter and place it in a plastic bag. Place a fresh membrane filter onto the concentrator base and reassemble the unit. The fresh membrane filter may be used smooth-side uppermost, as this side may be less prone to blockage and might be easier to wash. Pour the retained suspension back into the concentrator, rinsing the container, and continue the filtration process.

This process may need to be repeated depending on the number of membrane filters required. Each membrane filter should be placed in its own, separate plastic bag and retained for further processing.

Concentrate the suspension to approximately 50 ml. Add all of the contents of the centrifuge tube from the first elution and continue filtration, reducing the volume until the remaining suspension is again at a level half way down the stirrer bar. Remove the stirrer assembly, rinse and pour the liquid (approximately 30 ml) into the centrifuge tube used previously. Detach the concentrator tube from its base and carefully remove the membrane filter using fine forceps. Transfer the membrane filter to a plastic bag, taking care not to lose any remaining suspension from the surface.

If at any time during the elution and concentration stages a membrane filter ruptures or becomes displaced, then proceed in a similar manner as described for replacing a blocked membrane. If this occurs, decant the suspension of particulate matter into a clean container. Dismantle the concentrator assembly and remove the membrane filter and place it in a plastic bag, taking care not to lose any particulate matter suspension from the

surface of the membrane filter. This filter is then retained for further processing. Any liquid in the catch-bottle should be added to the suspension transferred to the container. Place a second membrane filter onto the concentrator base and reassemble the unit. The fresh membrane filter should be used smooth-side uppermost, as this side may be less prone to blockage and might be easier to wash. Pour the retained suspension back into the concentrator, rinsing the container, and continue the filtration process.

AA4.5 *Washing of membrane filters*

Add 5 ml of elution buffer solution (AA2.2) to each of the plastic bags containing a membrane filter. Seal each bag. Wash the filters, i.e. for about 60 - 120 seconds, for example by rubbing each filter between fingers and thumb with sufficient force to ensure optimum recovery of (oo)cysts. Transfer the washings to the centrifuge tube. Repeat the washing and transfer procedure, as necessary and transfer the washings to the centrifuge tube. A second centrifuge tube should be used if necessary.

AA4.6 *Concentration of particulates from filter washings from Filta-Max®,*

Centrifuge tubes used for this stage are typically screw capped 50 ml graduated plastic tubes with a conical base and graduations down to 0.5 ml. Some applications require larger tubes. Centrifuge tubes should be “balanced” before centrifuging.

Cap the centrifuge tubes containing the filter washings and centrifuge typically at 1100 rcf for 15 minutes. The deceleration phase should be as smooth as possible to minimise the risk of re-suspending the particulate material and the application of any braking systems should be avoided. When completed, remove the centrifuge tubes taking care not to re-suspend any particulate material. The supernatant liquid may be removed by using a pipette and a vacuum source not exceeding 0.2 bar and keeping the pipette just below the meniscus of the supernatant liquid.

If 250 ml or larger centrifuge tubes have been used, carefully remove the supernatant liquid to within 30 - 40 ml of the pellet of particulate material taking care not to lose any particulate material. Re-suspend the particulate material, for example by vortexing for 10 - 15 seconds until all visible aggregates are dispersed, then quantitatively transfer the suspension to a 50 ml centrifuge tube, rinsing the tube as necessary and adding the rinsing to the 50 ml centrifuge tube and centrifuge as above.

If more than one 50 ml centrifuge tube is used, carefully remove the supernatant liquid to approximately 5 ml above the pellet of particulate matter, taking care not to lose any of the particulate material. Re-suspend the particulate material, for example by vortexing for 10 - 15 seconds, until all visible aggregates are dispersed, then combine the suspensions from each tube into one tube, i.e. all of the particulate material should be contained in one 50 ml tube. Rinse each tube with 1 - 2 ml of water, vortex and transfer the washings to the tube containing the particulate material. Vortex the resulting suspension and centrifuge as above.

Once all the particulate material has been centrifuged in a single 50 ml centrifuge tube, measure and record the pellet volume V_{PV} ml of particulate material. Remove the supernatant liquid to within 5 ml of the pellet and add water to the centrifuge tube to bring the total volume V_{SV} ml, typically to 9 ml and vortex to re-suspend the particulate material. The suspension is now ready to proceed to the IMS stage and microscopic examination (section D) or may be stored in the range 5 ± 3 °C for up to 3 days before this process

begins. However, delay in continuing the analysis at this stage may adversely affect the results of analysis.

If the total pellet volume V_{PV} exceeds that specified by the IMS kit manufacturer, re-suspend the pellet by vortexing and transfer the suspension to another centrifuge tube. This tube should be such that an accurate measure of the pellet volume can be made from volume graduations on the base of the tube. Centrifuge the suspension as above and measure the pellet volume. Re-suspend the pellet and divide the suspension into suitable aliquot volumes such that each aliquot represents no more than the maximum pellet volume specified by the IMS kit manufacturer. Add water to each aliquot, bring the total volume, V_{SV} ml, typically to 9 ml and vortex to re-suspend the particulate material. Each aliquot of the suspension is now ready to proceed to the IMS stage and microscopic examination (see section D).

AB Procedures using the Pall Life Sciences Envirochek™ system

AB1 Apparatus for Pall Life Sciences Envirochek™ filtration

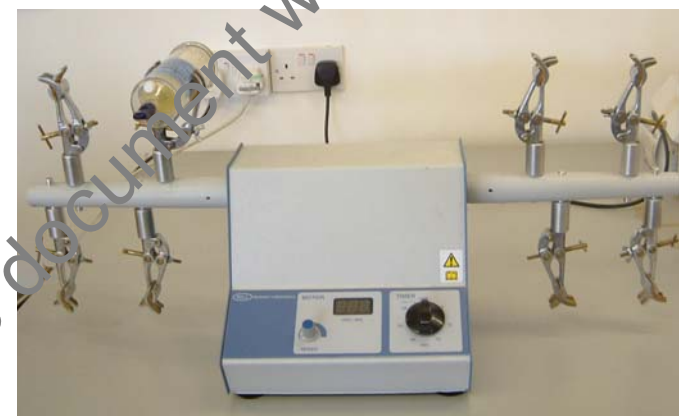
In addition to standard laboratory equipment, specific Envirochek™ items include:

Envirochek™ HV sampling capsules (see Figure AB1).
Sampling rig typically consisting of water meter, flow control device, inlet and outlet pressure meters (optional) tubing and connectors.

Figure AB1 Envirochek™ HV sampling capsule

Wrist action shaker, with arms for agitating or shaking the Envirochek™ capsule (see Figure AB2).
Centrifuge, capable of operating to at least 1100 rcf.
Centrifuge bottles (conical, plastic, screw-top and graduated) 250 ml capacity.
Centrifuge tubes (conical, plastic and graduated) 50 ml capacity.

Figure AB2 Wrist shaker with Envirochek™ HV capsule *in situ*



AB2 Reagents for Pall Life Sciences Envirochek™ filters

AB2.1 *Tris buffer solution*

Tris(hydroxymethyl)aminomethane	121.1 g
Water	1000 ml

Dissolve the tris(hydroxymethyl)aminomethane in 700 ml of water and adjust the pH to

7.4 ± 0.2 with 1M hydrochloric acid solution or 1M sodium hydroxide solution. Make to 1000 ml with water. This buffer solution may be stored in the range 20 ± 5 °C but should be used within three months. Commercially available buffer solutions may contain preservatives which may extend the shelf life.

AB2.2 *Ethylenediaminetetraacetic acid solution*

Ethylenediaminetetraacetic acid, disodium salt, dihydrate	186.1g
Water	1000 ml

Dissolve the ethylenediaminetetraacetic (EDTA) acid salt in 800 ml of hot (about 80 °C) water. Cool the solution (to about 25 °C) and, initially, adjust the pH to approximately 8.0 with 6M sodium hydroxide solution. Finally, adjust the pH to 8.0 ± 0.2 with 1M hydrochloric acid solution or 1M sodium hydroxide solution. Make to 1000 ml with water and mix well. This solution may be stored in the range 20 ± 5 °C but should be used within three months. Commercially available EDTA acid salt solutions may contain preservatives which may extend the shelf life.

AB2.3 *Elution buffer solution*

Polyethylene glycol (12) lauryl ether (Laureth-12)	1 g
Tris buffer solution (AB2.1)	10 ml
EDTA solution (AB2.2)	2 ml
Anti-foaming agent A	0.15 ml
Water	to 1000 ml

Weigh the polyethylene glycol (12) lauryl ether into a glass beaker, add 100 ml of warm (typically, about 50 °C) water and stir to dissolve. Transfer the solution to a suitable container, rinsing the beaker several times with water to ensure the transfer of all the detergent to the container. Add 10 ml of tris buffer solution (AB2.1) and 2 ml of EDTA solution (AB2.2). Mix well and add 0.15 ml of anti-foaming agent A. Make to 1000 ml with water and mix well. This solution may be stored in the dark in the range 20 ± 5 °C but should be used within 2 months.

AB2.4 *Pre-treatment buffer solution*

Sodium polyphosphate $(NaPO_3)_n$	5g
Water	1000 ml

Dissolve the sodium polyphosphate in the water. This solution may be stored in the range 20 ± 5 °C but should be used within 1 week.

AB3 Filtration using Envirochek™ filters

The Envirochek™ HV capsule has been found suitable for sampling surface and treated waters including swimming pool waters.

Envirochek™ HV filters consist of pleated membranes (of suitable pore size) contained in a polycarbonate housing capsule, which indicates the direction of water flow. Each capsule is supplied with two caps which are used to seal the inlet and outlet ports (see Figure AB3). This facilitates the transport of the filter to the laboratory after filtration of the volume of water is complete, and ensures that losses of (oo)cysts are minimised. The Envirochek™

HV filter consists of a pleated polyester membrane filter with a nominal pore size of 1 µm. The membrane is directly laminated to a polypropylene support and is suitable for filtering large volumes of water (over 1000 litres). The effective filtration area of the Envirochek™ HV membrane is about 1300 cm^2 and the capsule operates with a differential pressure of about 4 bar.

Figure AB3 Envirochek™ HV capsule with end caps

Attach the filter capsule to the water supply under investigation, ensuring that the direction of water flow is as indicated on the capsule. It may be appropriate to flush or retain any residual debris from the sampling tap or pipe before it is connected to the filter housing. This will depend on whether the debris needs analysing, or whether the debris is to be analysed with the water to be filtered.

Large volumes of sample should be filtered using a flow meter in line with the capsule to measure the volume of water filtered. Small volumes of sample may be measured directly. Typically, a flow rate of up to a maximum of 3.4 litres per minute is used for the HV filter. For regulatory sampling, it may be necessary to use a defined maximum flow rate through the filter. The differential pressure across the Envirochek™ HV filter should not exceed 4.1 bar. A pump, capable of operating at up to 5 bar, may be required to achieve a suitable flow rate. Small volumes of samples may be filtered using a peristaltic pump. Large amounts of particulate material present in water may cause the filter to become blocked. This will limit the volume of water that can be filtered.

Following filtration of the desired volume of sample, the filter capsule should be sealed and then transferred to the laboratory for processing.

AB4 Elution and concentration from Envirochek™ HV filters

Envirochek™ HV filters should undergo a pre-elution treatment step using sodium polyphosphate solution (AB2.4) before being eluted with elution buffer solution (AB2.3). In addition, warming both reagents (AB2.3 and AB2.4) to about 37 °C facilitates the drainage of the filters and the removal of particulate material.

AB4.1 *Envirochek™ HV filter elution*

Support the capsule vertically with the inlet port positioned uppermost. Remove the two end caps and allow any water in the capsule to drain out through the outlet port. Replace the bottom cap and fill the capsule through the inlet port with pre-treatment buffer solution (AB2.4) until it covers the pleated filter. The depth of solution above the pleated filter

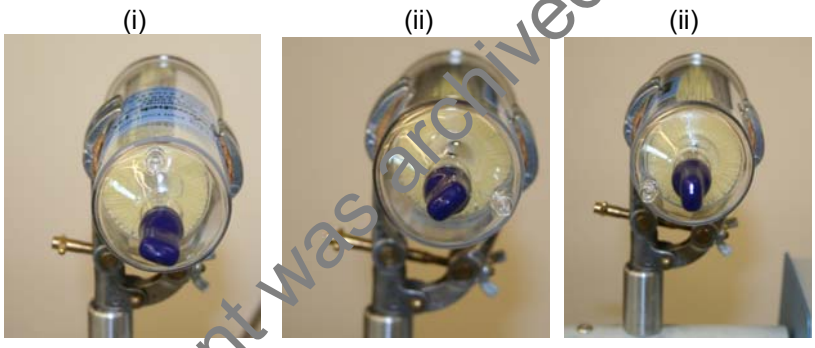
should be approximately 10 mm. Typically, 125 ml of pre-treatment buffer solution may be required. Replace the upper end cap and secure the capsule horizontally in the jaws of the wrist shaker, see Figure AB4(i). Set the timer and shake at 600 ± 25 cpm for 5 minutes.

Remove the capsule from the shaker. Support the capsule vertically with the inlet port positioned uppermost. Remove both end caps and allow the pre-treatment buffer in the capsule to drain out through the outlet port. Replace the bottom end cap and fill the capsule through the inlet port with water. Replace the upper end cap and rinse the filter membrane by gently rotating the capsule for 30 seconds. Support the capsule vertically with the inlet port uppermost. Remove both end caps and allow the water to drain out of the capsule through the outlet port.

Replace the bottom cap and fill the capsule with elution buffer solution (AB2.3). The depth of the solution should be approximately 10 mm above the pleated filter. Typically, 125 ml of elution buffer solution may be required. Replace the upper end cap and secure the capsule horizontally in the jaws of the wrist shaker, see Figure AB4(i). Set the timer and shake at 600 ± 25 cpm for 5 minutes.

Remove the capsule from the shaker. Remove the upper end cap and pour the eluate (containing any associated particulate material) into a 250 ml conical centrifuge tube.

Figure AB4 Position of the Envirochek™ HV filter during the elution process



Alternatively, distribute the suspension equally into three 50 ml centrifuge tubes. Add a further aliquot of elution buffer to the capsule, cap the inlet end and repeat the shaking procedure. The second elution should be shaken for 5 minutes such that the capsule is in the 4 o'clock position or is rotated through 120 ° in relation to the first elution, see Figure AB4(ii) followed by 5 minutes in the 8 o'clock position, see Figure AB4(iii). After shaking, remove the upper end cap and add the eluate (with any associated particulate material) to the 250 ml centrifuge tube. Alternatively, pour the suspension into a further series of three 50 ml centrifuge tubes. The suspension is now contained in either one 250 ml centrifuge tube, or in two series of three 50 ml centrifuge tubes.

If necessary or appropriate, removal of particulate material from the filter may be improved by warming all the solutions (AB2.3 and AB2.4) used to about 37 °C and increasing the shaker speed to 850 ± 25 cpm.

AB4.2 *Concentration of particulates from filter washings from Envirochek™ HV*

Centrifuge tubes used for this stage are typically screw capped 50 ml graduated plastic tubes with a conical base and graduations down to 0.5 ml. Some applications require larger tubes. Centrifuge tubes should be "balanced" before centrifuging.

Cap the centrifuge tubes containing the filter washings and centrifuge typically at 1100 rcf for 15 minutes. The deceleration phase should be as smooth as possible to minimise the risk of re-suspending the particulate material and the application of any braking systems should be avoided. When completed, remove the centrifuge tubes taking care not to re-suspend any particulate material. The supernatant liquid may be removed by using a pipette and a vacuum source not exceeding 0.2 bar and keeping the pipette just below the meniscus of the supernatant liquid.

If 250 ml or larger centrifuge tubes have been used, carefully remove the supernatant liquid to within 30 - 40 ml of the pellet of particulate material taking care not to lose any particulate material. Re-suspend the particulate material, for example by vortexing for 10 - 15 seconds until all visible aggregates are dispersed, then quantitatively transfer the suspension to a 50 ml centrifuge tube, rinsing the tube as necessary and adding the rinsing to the 50 ml centrifuge tube and centrifuge as above.

If more than one 50 ml centrifuge tube is used, carefully remove the supernatant liquid to approximately 5 ml above the pellet of particulate matter, taking care not to lose any of the particulate material. Re-suspend the particulate material, for example by vortexing for 10 - 15 seconds, until all visible aggregates are dispersed, then combine the suspensions from each tube into one tube, i.e. all of the particulate material should be contained in one 50 ml tube. Rinse each tube with 1 - 2 ml of water, vortex and transfer the washings to the tube containing the particulate material. Vortex the resulting suspension and centrifuge as above.

Once all the particulate material has been centrifuged in a single 50 ml centrifuge tube, measure and record the pellet volume V_{PV} ml of particulate material. Remove the supernatant liquid to within 5 ml of the pellet and add water to the centrifuge tube to bring the total volume V_{SV} ml, typically to 9 ml and vortex to re-suspend the particulate material. The suspension is now ready to proceed to the IMS stage and microscopic examination (section D) or may be stored in the range 5 ± 3 °C for up to 3 days before this process begins. However, delay in continuing the analysis at this stage may adversely affect the results of analysis.

If the total pellet volume V_{PV} exceeds that specified by the IMS kit manufacturer, re-suspend the pellet by vortexing and transfer the suspension to another centrifuge tube. This tube should be such that an accurate measure of the pellet volume can be made from volume graduations on the base of the tube. Centrifuge the suspension as above and measure the pellet volume. Re-suspend the pellet and divide the suspension into suitable aliquot volumes such that each aliquot represents no more than the maximum pellet volume specified by the IMS kit manufacturer. Add water to each aliquot, bring the total volume, V_{SV} ml, typically to 9 ml and vortex to re-suspend the particulate material. Each aliquot of the suspension is now ready to proceed to the IMS stage and microscopic examination (see section D).

AC Procedures using the flat-bed membrane system

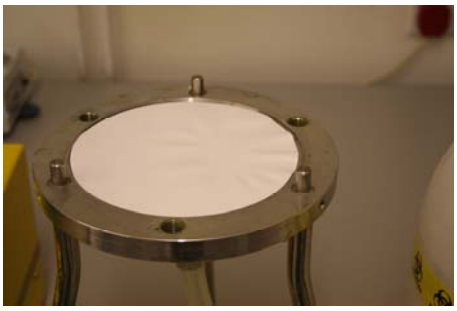
AC1 Apparatus for flat-bed membrane filtration

In addition to standard laboratory equipment, specific flat-bed membrane filtration items include:

Large diameter membrane filters made of, for example cellulose acetate or polycarbonate, of nominal pore size between 1 - 2 µm; typically, 142 mm or larger diameter membrane filters have been found suitable.
Filter housing suitable for membrane filters of choice (see Figure AC1).
Filter housing stand (optional).
Polyethylene, nylon or silicone tubing, hose clips and connectors.
Flow control device for positive pressure filtration.
Water meter (optional).
Suitable pump and power source for remote filtration (or pressure vessel) compressor, hoses and connectors.

Figure AC1 Apparatus for concentration of samples by membrane filtration





Forceps, labels, water-proof marker pens, disposable gloves.
Re-sealable plastic bags for washing membranes. Alternatively, flat-bottomed deep-sided stainless steel tray and rubber bladed scraper.
Centrifuge, capable of operating to at least 1100 rcf.
Centrifuge tubes (conical, plastic and graduated) 50 ml capacity.

AC2 Reagents for flat-bed membrane filters

AC2.1 *Polyoxyethylene sorbitan monooleate solution*

Polyoxyethylene sorbitan monooleate (for example Tween 80)	1 g
Water	to 1000 ml

Dissolve the polyoxyethylene sorbitan monooleate in approximately 900 ml of water. Mix well and make to 1000 ml with water. The solution may be stored in the range 20 ± 5 °C but should be used within one month. Discard the solution if there is any sign of turbidity.

AC3 Filtration using flat-bed membrane filters

Known volumes (whether small or large) of samples of relatively low turbidity may be filtered by flat-bed membrane filtration. Samples may be filtered on-site and the membrane filters transported to the laboratory for analysis. Alternatively, small volumes of sample, i.e. grab samples, may be transported to the laboratory for filtration and analysis.

This procedure is suitable for the examination of most types of waters from all stages of treatment and distribution, and source waters with relatively low turbidities. The method is not suitable for waters with high turbidities, as during the filtration process, these waters tend to block the membrane filter. The volume of sample that can be filtered will therefore depend on the turbidity of the water.

It may be appropriate to flush or retain any residual debris from the sampling tap or pipe before it is connected to the filter housing. This will depend on whether the debris needs analysing, or whether the debris is to be analysed with the water to be filtered.
Filtration through the membrane may be achieved using a variable speed peristaltic pump or a pressure vessel and compressor. The filtration assembly unit should comprise inlet and outlet hoses, filter holder and membrane filter (see Figure AC1). The flow through the filter is controlled by a variable speed peristaltic pump or may require a flow control device if a pressure vessel is used.

New filter holders should be checked to ensure they can withstand the applied pressure so that by-pass flow and leakage do not occur. The flow control valve, where required, should be set to a flow rate appropriate to the type of filter being used, to prevent damage occurring to the filter and to provide optimum filtration of (oo)cysts from filtered water. A peristaltic pump or pressure vessel and compressor will be required to filter the samples.

The filter holder assembly unit should be thoroughly cleaned before use. If contaminated water passes through the unit, the unit should be dismantled and the individual components washed in hot (about 50 °C) detergent solution. Clean tap water should be flushed through the assembled unit and hoses. If a pressure vessel is used, this should be rinsed several times with clean water, and then re-filled with hot tap water (at about 50 °C) before being connected to the filter unit. The assembly unit should then be flushed with hot water (at about 50 °C). To avoid any air gaining access to the unit, ensure that any bleed valves operate correctly during the flushing process.

Separate filtration units should be used for treated and untreated waters.

Using either positive pressure or a peristaltic pump, a known volume of water is filtered through a membrane filter located in a flat-bed filter housing. The flow rate through the filter should be controlled according to the type of filter being used, which is then removed from the housing.

Unscrew the filter housing and carefully locate the membrane filter centrally on the support screen. Ensure that the filter is correctly orientated with regard to the direction of water flow. Some filters may require pre-moistening before placement to ensure correct location and to avoid damage to the filter.

If there is an upper support grid located in the filter housing, this should be removed. Ensuring that the membrane filter is correctly sited and that the housing ring seal is placed correctly in position, carefully tighten the filter housing sufficiently to prevent any leakage occurring. Turn on the tap, or pump, and commence the filtration process, ensuring that the flow rate is adjusted to that appropriate for optimum filtration of (oo)cysts and the type of membrane filter being used. Typically, a water flow rate of between 1 - 1.5 litres per minute may be used. At the start of the filtration process operate the valve on the filter housing to bleed away any trapped air.

If a pressure vessel is used to filter water through the membrane filter, pressurise the head-space above the sample using a compressor. Typically, a maximum head-space pressure of up to 2 bar may be used. Using a flow control valve on the filter housing outlet, adjust the flow rate through the membrane filter.

Filter the required volume of water (typically 10 - 100 litres). When the required volume of water has been filtered, turn off the supply of water to the filter housing. It will be necessary to purge the excess water through the filter by pumping a small amount of air into the inlet manifold. This will reduce the amount of standing water on the surface of the membrane and minimise the risk of losing (oo)cysts upon opening the manifold. Open the housing and use forceps to carefully lift the edge of the membrane filter and remove the filter from the filter housing. Place the membrane filter into a plastic sample bag and seal the bag. The bag should then be placed into a second protective bag and sealed, before being transferred to the laboratory. Alternatively, the housing ports should be sealed and the filter housing (containing the membrane filter and any particulate material, including (oo)cysts) disconnected before being transferred to the laboratory.

AC4 Elution and concentration from flat-bed membrane filters

Particulate material on the surface of the membrane filter is eluted using a detergent solution and an abrasive physical process such that (oo)cysts are not damaged. The eluate is then concentrated by centrifugation and a concentrated suspension or pellet of particulate matter produced.

AC4.1 *Elution*

Add approximately 25 ml of polyoxyethylene sorbitan monololeate solution (AC2.1) to the plastic bag containing the membrane filter. Seal the bag, excluding as much air as possible in order to minimise foaming during the elution process.

Remove the particulate matter from the surface of the membrane filter by rubbing the outside surface of the bag, taking care not to damage the filter, plastic bag or any (oo)cysts. Repeat this process with 25 ml of polyoxyethylene sorbitan monooleate solution (AC2.1) as necessary, until the surface of the membrane filter appears clear of particulate matter. Pour the resulting suspension into one or more 50 ml conical centrifuge tubes. Rinse the plastic bag with 5 - 10 ml of polyoxyethylene sorbitan monooleate solution (AC2.1) and transfer the washings to one of the centrifuge tubes. Centrifuge tubes should be “balanced” before centrifuging.

Alternatively, the membrane filter may be placed directly onto the base of a deep-sided stainless steel tray and processed directly. Saturate the membrane filter with approximately 10 ml of polyoxyethylene sorbitan monooleate solution (AC2.1) and tilt the tray slightly. Using careful downward sweeping movements, use a rubber bladed scraper, and allow the particulate matter to collect at the bottom edge of the tray. Repeat this process several times until the surface of the membrane filter appears clear of particulate matter. Pour the resulting suspension into a 50 ml conical base centrifuge tube.

Rinse the membrane filter, rubber bladed scraper and inner tray surface with more polyoxyethylene sorbitan monooleate solution (AC2.1) and transfer the washings to the centrifuge tube. If required, repeat the procedure to remove all particulate matter from the surface of the membrane filter, decanting the washings to a further centrifuge tube, if necessary.

AC4.2 *Concentration of particulates from filter washings from flat-bed membrane filters*

Centrifuge tubes used for this stage are typically screw capped 50 ml graduated plastic tubes with a conical base and graduations down to 0.5 ml. Some applications require larger tubes. Centrifuge tubes should be "balanced" before centrifuging.

Cap the centrifuge tubes containing the filter washings and centrifuge typically at 1100 rcf for 15 minutes. The deceleration phase should be as smooth as possible to minimise the risk of re-suspending the particulate material and the application of any braking systems should be avoided. When completed, remove the centrifuge tubes taking care not to re-suspend any particulate material. The supernatant liquid may be removed by using a pipette and a vacuum source not exceeding 0.2 bar and keeping the pipette just below the meniscus of the supernatant liquid.

If 250 ml or larger centrifuge tubes have been used, carefully remove the supernatant liquid to within 30 - 40 ml of the pellet of particulate material taking care not to lose any particulate material. Re-suspend the particulate material, for example by vortexing for 10 - 15 seconds until all visible aggregates are dispersed, then quantitatively transfer the suspension to a 50 ml centrifuge tube, rinsing the tube as necessary and adding the rinsing to the 50 ml centrifuge tube and centrifuge as above.

If more than one 50 ml centrifuge tube is used, carefully remove the supernatant liquid to approximately 5 ml above the pellet of particulate matter, taking care not to lose any of the particulate material. Re-suspend the particulate material, for example by vortexing for 10 - 15 seconds, until all visible aggregates are dispersed, then combine the suspensions from each tube into one tube, i.e. all of the particulate material should be contained in one 50 ml tube. Rinse each tube with 1 - 2 ml of water, vortex and transfer the washings to the tube containing the particulate material. Vortex the resulting suspension and centrifuge as above.

Once all the particulate material has been centrifuged in a single 50 ml centrifuge tube, measure and record the pellet volume V_{PV} ml of particulate material. Remove the supernatant liquid to within 5 ml of the pellet and add water to the centrifuge tube to bring the total volume V_{SV} ml, typically to 9 ml and vortex to re-suspend the particulate material. The suspension is now ready to proceed to the IMS stage and microscopic examination (section D) or may be stored in the range 5 ± 3 °C for up to 3 days before this process begins. However, delay in continuing the analysis at this stage may adversely affect the results of analysis.

If the total pellet volume V_{PV} exceeds that specified by the IMS kit manufacturer, re-suspend the pellet by vortexing and transfer the suspension to another centrifuge tube. This tube should be such that an accurate measure of the pellet volume can be made from volume graduations on the base of the tube. Centrifuge the suspension as above and measure the pellet volume. Re-suspend the pellet and divide the suspension into suitable aliquot volumes such that each aliquot represents no more than the maximum pellet volume specified by the IMS kit manufacturer. Add water to each aliquot, bring the total volume, V_{SV} ml, typically to 9 ml and vortex to re-suspend the particulate material. Each aliquot of the suspension is now ready to proceed to the IMS stage and microscopic examination (see section D).

AD Procedures using the IDEXX Filta-Max xpress™ system

AD1 Apparatus for IDEXX Filta-Max xpress™ filtration

In addition to standard laboratory equipment, specific Filta-Max *xpress*™ items include:

Filta-Max *xpress*™ filter modules (see Figure AD1).
Filta-Max filter housings (see Figure AD2) with Swagelock QC and QF quick-connect fittings.
Flow meter (optional).
Sampling rig consisting of water meter, flow control device (1 litre per minute), inlet and outlet pressure meters (optional), tubing and connectors.

Figure A5 Filta-Max *xpress*™ filter modules

Figure AD2 Filta-Max® filter housing with connectors and tightening tools

Automated pressure elution station (see Figure AD3).
Air compressor or source of compressed air.
Centrifuge, capable of operating at 2000 rcf and taking 500 ml centrifuge tubes.
Centrifuge tubes, conical, plastic, graduated, 500 ml capacity.
Peristaltic pump.

Figure AD3 Filta-Max *xpress*™ pressure elution station

AD2 Reagents for IDEXX Filta-Max xpress™ filters

AD2.1 *Phosphate buffered saline solution* (PBS)

Sodium chloride	8.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	1.15 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g
Potassium chloride	0.2 g
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH of the solution to 7.3 ± 0.2 with either 1M hydrochloric acid solution or 1M sodium hydroxide solution. The reagent may be stored in the dark in the range 20 ± 5 °C for up to three months. Commercially available buffer solutions may contain preservatives which may extend the shelf life. Discard the solution if there is any sign of turbidity.

AD2.2 *Elution buffer solution*

Polyoxyethylene(20)sorbitan monolaurate (for example Tween 20)	1 ml
Phosphate buffered saline solution (AD2.1)	10 litres

Dispense 1 ml of polyoxyethylene(20)sorbitan monolaurate into a 50 ml container and dissolve the detergent in approximately 10 ml of warm (typically, about 40 °C) water. Add the contents of the container to approximately 8 litres of phosphate buffered saline solution (AD2.1) in a 10 litre vessel. Rinse the container with 10 ml of water, and add the rinsings to the vessel. Repeat this process with a further 10 ml of water. Make to 10 litres with phosphate buffered saline solution. The reagent may be stored in the dark in the range 20 ± 5 °C for up to one month. Commercially available buffer solutions may contain preservatives which may extend the shelf life. Discard the solution if there is any sign of turbidity, or if the solution shows any resistance to filtration (for example due to bacterial growth).

AD3 Filtration using IDEXX Filta-Max xpress™ filters

The Filta-Max *xpress*™ filtration system has been found suitable for the examination of large volume samples of treated waters for *Cryptosporidium* and from small volume samples of source waters for *Cryptosporidium* and *Giardia*. A filter module consists of a set of alternating 55 mm diameter and 40 mm diameter open-cell reticulated polyurethane rings compressed between two plates so as to produce a filter with a nominal pore size of 1 µm. A known volume of water (at a controlled flow rate) is filtered through the filter, housed in a re-usable housing. Large volumes of sample may be filtered on-site and the filter and housing returned to the laboratory for analysis; smaller volumes of sample may be filtered at the laboratory. It may be appropriate to flush or retain any residual debris from the sampling tap or pipe before it is connected to the filter housing. This will depend on whether the debris needs analysing, or whether the debris is to be analysed with the water to be filtered.

Ensure that all the O-rings are located correctly in the housing and lightly lubricated, for example with silicone vacuum grease. Place a filter module (rounded end-plate down) into the housing and align the lid onto the base. Tighten the lid firmly to ensure the filter module is properly placed within the housing so no filter by-pass of the water being sampled occurs. However, it is important to not over-tighten the housing.

Attach the filter housing to the water supply under investigation, ensuring that the direction of water flow is as indicated on the housing. Large volumes of sample should be filtered using a flow meter in line with the housing to measure the volume of water filtered. Typically, a flow rate of 1 - 2 litres per minute is used. A pump, capable of operating at up to 5 bar, may be required to achieve a suitable flow rate. Small volumes of samples may be filtered using a peristaltic pump.

Following filtration, the filter housing should be sealed, for example with rubber bungs or self-sealing quick-connect couplings, and then transferred to the laboratory for processing.

AD4 Elution and concentration from Filta-Max xpress™ filters

For the elution and concentration of the trapped particulate material on the Filta-Max *xpress*™ filter, the module remains in the housing and is processed in a Filta-Max *xpress*™ pressure elution station.

AD4.1 Elution

Ensure that the buffer reservoir of the pressure elution station has sufficient elution buffer (AD2.2) for processing the filter(s). Use 400 ml of elution buffer for each filter being processed. Remove the cap of a 500 ml centrifuge tube and place the tube in the collection vessel holder. Attach the outlet diverter to the Swagelock QF fitting on the top of the filter housing. Turn the housing upside down, placing the outlet diverter over the centrifuge tube. Connect the Swagelock QC fitting (on the bottom of the upturned housing) to the pressure elution station, close the door and start the filter elution cycle. The filter is then eluted with the eluate being deposited into the centrifuge tube. On completion of the elution cycle, remove the housing from the pressure elution station and disconnect the outlet diverter. The filter can be removed from the housing and discarded. Cap the centrifuge tube containing the eluate, which is then further processed, see section AD4.2.

AD4.2 *Concentration of particulates from filter washings from Filta-Max xpress™ filters*

Centrifuge the 400 ml of eluate from the filter at 2000 rcf for 15 minutes. The deceleration phase should be as smooth as possible to minimise the risk of re-suspending the particulate material and the application of any braking systems should be avoided. When completed, remove the centrifuge tubes taking care not to re-suspend any particulate material. The supernatant liquid may be removed by using a wide bore pipette (for example a 5 ml serological pipette) and a peristaltic pump or alternative vacuum source with the vacuum restricted to a maximum of 0.5 psig (0.033 bar) and keeping the pipette just below the meniscus of the supernatant liquid. The flow rate for removal of the supernatant liquid should be approximately 200 ml per minute. Remove the supernatant liquid leaving 7 - 8 ml in the tube with the pellet of particulate material. Re-suspend the particulate material, for example by vortexing for 20 seconds until all visible aggregates are dispersed, then quantitatively transfer the suspension to a Leighton tube, rinsing the centrifuge tube with 1 - 1.5 ml of water and adding the rinsings to the suspension in the Leighton tube. Rinse the centrifuge tube again with 1 - 1.5 ml of water and add the rinsings to the Leighton tube. The volumes used for rinsing the tube are chosen dependent on the volume of supernatant transferred and so as to make a final volume of typically, 9 ml. Alternatively, the supernatant liquid and rinsings can be added to a small centrifuge tube (for example a 50 ml tube) and centrifuged typically at 1100 rcf for 15 minutes so that the pellet volume (V_{PV}) can be recorded (see AD4.1). Vortex to re-suspend the particulate material. The suspension is now ready to proceed to the IMS stage and microscopic examination (section D) or may be stored in the range 5 ± 3 °C for up to 3 days before this process begins. However, delay in continuing the analysis at this stage may adversely affect the results of analysis.

If the total pellet volume (V_{PV}) exceeds that specified by the IMS kit manufacturer, re-suspend the pellet by vortexing and transfer the suspension to another centrifuge tube. This tube should be such that an accurate measure of the pellet volume can be made from volume graduations on the base of the tube. Centrifuge the suspension as above (see AD4.1) and measure the pellet volume. Re-suspend the pellet and divide the suspension into suitable aliquot volumes such that each aliquot represents no more than the maximum pellet volume specified by the IMS kit manufacturer. Add water to each aliquot, bring the total volume, V_{SV} ml, typically to 9 ml and vortex to re-suspend the particulate material. Each aliquot of the suspension is now ready to proceed to the IMS stage and microscopic examination (section D).

B Isolation of *Cryptosporidium* oocysts and *Giardia* cysts by chemical flocculation

B1 Introduction

The procedures described in this booklet may be applicable to drinking waters and may be applicable (with suitable adaptation) for the analysis of other matrices. As the method has been validated in only one laboratory, details are included for information purposes only, as an example of the type of procedures that are available. Information on the routine use of this method would be welcomed to assess its full capabilities.

B2 Scope

The chemical flocculation method described in this booklet may be suitable for the isolation and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts in small volumes, typically about 10 litres, of all types of waters. Flocculation techniques are most suited to small volumes of samples that possess high turbidities, which may compromise the performance of other techniques, especially filtration techniques, and which tend to block the filters.

The method may be used to facilitate identification of the routes of contamination of water supplies for

(i) catchment control purposes (including raw water monitoring),
(ii) evaluating the effectiveness of treatment practices, and
(iii) investigating potential or actual outbreak situations.

Users wishing to employ this method should verify its performance for their own specific matrices and under their own laboratory conditions.

B3 Principle

Chemical flocculation techniques involve the addition of chemical flocculating agents to small volumes of samples, typically 10 litres. The flocculating agents combine with suspended particulate matter contained within the aqueous sample to produce a precipitate which is allowed to settle. The result of this combination is an increase in the size and mass of suspended matter, leading to quicker settlement of the particulate material in the water. Following settlement, the resulting supernatant liquid is discarded leaving a concentrate of sediment material. The flocculating agents of the combined precipitate are then dissolved, leaving the original particulate matter as suspended material, which is then further concentrated using centrifugation. A pellet of particulate matter, V_{PV} ml, is then produced which when held in suspension, V_{SV} ml, is ready for the IMS stage and microscopic examination (section D).

Chemical flocculation techniques are limited, practically, by the volume of sample that can be easily handled within the laboratory.

B4 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[6] in this series.

Generally, only normal laboratory equipment is required and include:

10 litre barrels for sample collection and subsequent flocculation.
10 litre barrels with taps for dispensing reagents.
Measuring cylinders, 100 ml, 250 ml, 500 ml and 1000 ml capacity.
Aspiration tubes with 10 ml open-ended disposable pipettes.
Vacuum source, catch bottles/reservoirs.
Centrifuges, capable of accommodating 1000 ml centrifuge bottles and/or 50 ml centrifuge tubes and operating at 7200 rcf and 1050 rcf.
1 litre centrifuge bottles.
50 ml centrifuge tubes.
Vortex mixer.
Wash bottles.
pH meter with calibration buffers (pH 10.0, pH 7.0 and pH 4.0).
Balances.
Calibrated timers.

B5 Reagents

Commercial or alternative formulations of these reagents are available, but may possess minor variations to their formulation. The performance of all reagents should be verified prior to their use in the method. Further guidance on assessing the performance of methods or parts of methods used for drinking water analysis is given in Appendix 4. Variations in the preparation and storage of reagents should also be verified. Commercially available regents should be used and stored according to manufacturer's instructions. Water for reagents should be distilled, deionised or of similar quality that is (oo)cyst-free. Unless otherwise stated chemical constituents should be added as anhydrous salts. Where reagents are stored in a refrigerator they should be allowed to reach room temperature before use.

B5.1 *Calcium chloride dihydrate solution*[7]

Calcium chloride dihydrate	1470 g
Water	to 10000 ml

Dissolve the calcium chloride dihydrate in 9000 ml of water. Make to 10000 ml with water. Mix well. The solution may require warming (for example, at about 40 °C) to achieve complete dissolution. The solution may be stored in the range 20 ± 5 °C for up to one month.

B5.2 *Sodium hydrogen carbonate solution*[7]

Sodium hydrogen carbonate	840 g
Water	to 10000 ml

Dissolve the sodium hydrogen carbonate in 9000 ml of water. Make to 10000 ml with water. The solution may be stored in the range 20 ± 5 °C for up to one month.

B5.3 *Sodium hydroxide solution (1M)*

Sodium hydroxide	400 g
Water	to 10000 ml

Dissolve the sodium hydroxide in 9000 ml of water. Cool the solution. Make to 10000 ml with water. The solution may be stored in the range 20 ± 5 °C for up to one month.

B5.4 *Sodium hydroxide solution (0.1M)*

Sodium hydroxide	40 g
Water	to 10000 ml

Dissolve the sodium hydroxide in 9000 ml of water. Cool the solution. Make to 10000 ml with water. The solution may be stored in the range 20 ± 5 °C for up to one month.

B5.5 *Sulphamic acid solution*[7]

Sulphamic acid	1000 g
Water	to 10000 ml

Dissolve the sulphamic acid in 9000 ml of water. Make to 10000 ml with water. The solution may be stored in the range 20 ± 5 °C for up to one month.

B5.6 *Stock solution of polyoxyethylene(20)sorbitan monooleate*

Polyoxyethylene(20)sorbitan monooleate (for example Tween 80)	10 g
Water	to 1000 ml

Dissolve the polyoxyethylene(20)sorbitan monooleate in 900 ml of water. Make to 1000 ml with water. Mix well. The solution may be stored in the range 20 ± 5 °C for up to one month. Discard the solution if there is any sign of turbidity.

B5.7 *Polyoxyethylene(20)sorbitan monooleate*

Stock solution of polyoxyethylene(20)sorbitan monooleate (B5.6)	10 ml
Water	to 1000 ml

Add the stock solution of polyoxyethylene(20)sorbitan monooleate (B5.6) to 900 ml of water. Make to 1000 ml with water. The solution may be stored in the range 20 ± 5 °C for up to one month. Discard the solution if there is any sign of turbidity.

B6 Analytical procedure

Add 100 ± 10 ml of calcium chloride solution (B5.1) to 10000 ± 200 ml of well-shaken aqueous sample. Mix well. To this mixture, add 100 ± 10 ml of sodium hydrogen carbonate solution (B5.2). Mix well. Raise the pH of this mixture to approximately 9.0 by adding 100 ± 10 ml of sodium hydroxide solution (B5.3) to the mixture. Mix well. Allow the precipitate of calcium carbonate sufficient time to settle, for example a minimum of 4 hours, but no longer than 24 hours. After the precipitated calcium carbonate has settled, carefully discard the supernatant liquid, for example using a gentle vacuum source. Care should be taken not to discard or lose any of the precipitate.

Carefully, add sufficient sulphamic acid solution (B5.5) typically no more than 250 ml, to completely dissolve the calcium carbonate precipitate. The sulphamic acid solution should be added slowly in approximately 50 ml aliquots, to avoid excessive effervescence. At the

same time, the mixture should be gently shaken to dissolve all the calcium carbonate precipitate. The container may need to be tilted and rotated to ensure that any calcium carbonate precipitate that adheres to the side of the container also dissolves.

When the calcium carbonate has dissolved, transfer the resulting well-shaken mixture into a 1000 ml centrifuge bottle. Add 100 ± 10 ml of detergent solution (B5.7) to the sample container and shake vigorously to ensure any particulate matter becomes suspended in the solution and does not adhere to the sides of the container. Transfer this mixture into the 1000 ml centrifuge bottle. Repeat this process with a further 100 ± 10 ml quantity of detergent solution (B5.7) ensuring all particulate matter is transferred to the 1000 ml centrifuge bottle.

Using 1M sodium hydroxide solution (B5.3) carefully, adjust the pH of the mixture in the 1000 ml centrifuge bottle to a pH value between 2.5 - 3.5. Finally, adjust the pH of the mixture with 0.1M sodium hydroxide solution (B5.4) to a value between 5.5 - 6.5. Ensure that the mixture is continuously mixed throughout this process.

If excess 0.1M sodium hydroxide solution (B5.4) is added, and the pH of the mixture is raised above 7.0, then calcium carbonate will precipitate within the centrifuge bottle and the process of dissolving the calcium carbonate precipitate and pH-adjustment will need to be repeated.

After pH adjustment to a value between 5.5 - 6.5, centrifuge the mixture at 7200 rcf for 12 minutes at room temperature. The deceleration phase should be as smooth as possible to minimise the risk of re-suspending the particulate material and the application of any braking system should be avoided. Immediately after centrifugation, remove the tube from the centrifuge, and carefully, discard the supernatant liquid, for example using a gentle vacuum source, but avoid removing any particulate matter. Leave sufficient liquid to just cover the resulting pellet of particulate matter. Shake the tube vigorously to re-suspend the particulate matter and transfer the suspension to a 50 ml centrifuge tube. Add sufficient detergent solution (B5.7) i.e. approximately 20 ml, to the 1000 ml centrifuge bottle and rinse the tube to re-suspend any remaining particulate matter. Transfer the rinsings to the 50 ml centrifuge bottle. Make to approximately 50 ml with water.

Centrifuge this suspension at 1050 rcf for 10 minutes at room temperature. Immediately after centrifugation, remove the 50 ml tube from the centrifuge, carefully discard supernatant liquid, ensuring particulate matter is not removed or discarded. Estimate and record the volume, V_{PV} ml, of particulate material in the tube.

Water should then be added to the centrifuge tube and made to a known total volume, V_{SV} ml, typically 9 ml. Vortex the tube to re-suspend the pellet of particulate material. The suspension is now ready to proceed directly to the IMS stage and microscopic examination (section D) or may be stored in the range 5 ± 3 °C for up to 3 days. However, delay in continuing the analysis at this stage may adversely affect the results of analysis.

If the total pellet volume (V_{PV}) exceeds that specified by the IMS kit manufacturer, re-suspend the pellet by vortexing and transfer the suspension to another centrifuge tube. This tube should be such that an accurate measure of the pellet volume can be made from volume graduations on the base of the tube. Centrifuge the suspension as above (see AD4.1) and measure the pellet volume. Re-suspend the pellet and divide the suspension into suitable aliquot volumes such that each aliquot represents no more than the maximum pellet volume specified by the IMS kit manufacturer. Add water to each aliquot, bring the

total volume, V_{SV} ml, typically to 9 ml and vortex to re-suspend the particulate material. Each aliquot of the suspension is now ready to proceed to the IMS stage and microscopic examination (section D).

C Isolation of *Cryptosporidium* oocysts and *Giardia* cysts from filter sand

C1 Introduction

The procedures described in this section have not been fully validated and details are included for information purposes only, as examples of the type of procedures that are available. Information on the multi-laboratory use of these procedures would be welcomed to assess their full capabilities.

C2 Scope

The elution procedures described in this section may be suitable for the isolation of *Cryptosporidium* oocysts and *Giardia* cysts from samples of sand taken from water treatment works or swimming pool filters. The method may be suitable for the examination of sand

- before or during its use in water treatment processes;
- from filters associated with outbreaks of cryptosporidiosis from swimming pools.

Users wishing to employ this method, particularly for the analysis of other solid samples, should verify its performance under their own laboratory conditions.

C3 Principle

Filter sand, typically 100 g is added to a container and 500 ml of elution buffer added. The sand is gently swirled with the elution buffer for approximately 5 minutes. Whilst the sand is allowed to settle, other particular matter is held in suspension and the supernatant liquid is decanted into a second container. The sand is again gently swirled with elution buffer, the sand allowed to settle and the supernatant liquid transferred to the second container. The combined elution buffer is then centrifuged and excess supernatant liquid discarded, leaving sufficient elution buffer covering the centrifuged (oo)cysts. From the centrifuged material, a pellet of particulate matter, V_{PV} ml, is then produced which when held in suspension, V_{SV} ml, is ready for the IMS stage and microscopic examination (section D).

Sand may be dirty and contain significant amounts of particulate material and it may not be possible to analyse large amounts of sand. Care should be taken to ensure that the samples of sand submitted to the laboratory are representative of the total amount of sand undergoing sampling. Vigorous or prolonged shaking of sand samples with the elution buffer may result in the destruction of (oo)cysts and the elution process should be restricted to minimum swirling.

C4 Apparatus

1 litre borosilicate glass bottle with cap.
Measuring cylinder, 500 ml capacity.
Centrifuge, capable of operating at 1100 rcf.
Centrifuge bottles (conical, plastic, screw-top and graduated) 250 ml capacity.
Centrifuge tubes (conical, plastic and graduated) 50 ml capacity.
(Centrifugation tubes should be “balanced” before use).

C5 Reagents

Commercial or alternative formulations of these reagents are available, but may possess minor variations to their formulation. The performance of all reagents should be verified prior to their use in the method. Further guidance on assessing the performance of methods or parts of methods used for analysis is given in Appendix 4. Variations in the preparation and storage of reagents should also be verified. Commercially available reagents should be used and stored according to the manufacturers instructions. Water for reagents should be distilled, deionised or of similar quality that is (oo)cyst-free. Unless otherwise stated chemical constituents should be added as anhydrous salts. Where reagents are stored in a refrigerator they should be allowed to reach room temperature before use.

C5.1 E*lution buffer*

C5.1.1 *Tris buffer solution*

Tris(hydroxymethyl)amino-methane	121.1 g
Water	1000 ml

Dissolve the tris(hydroxymethyl)amino-methane in 700 ml of water and adjust the pH to 7.4 ± 0.2 with 1M hydrochloric acid solution or 1M sodium hydroxide solution. Make to 1000 ml with water. This buffer solution may be stored in the range 20 ± 5 °C but should be used within 3 months. Commercially available buffer solutions may contain preservatives which may extend the shelf life.

C5.1.2 *Ethylenediaminetetraacetic acid solution*

Ethylenediaminetetraacetic acid, disodium salt, dihydrate (EDTA)	186.1g
Water	1000 ml

Dissolve the ethylenediaminetetraacetic acid in 800 ml of hot (about 80 °C) water. Cool the solution to about 25 °C and, initially, adjust the pH to approximately 8.0 with 6M sodium hydroxide solution. Finally, adjust the pH to 8.0 ± 0.2 with 1M hydrochloric acid solution or 1M sodium hydroxide solution. Make to 1000 ml with water and mix well. This solution may be stored in the range 20 ± 5 °C but should be used within 2 months.

C5.1.3 *Combined solution*

Polyethylene glycol (12) lauryl ether (Laureth-12)	1 g
Tris buffer solution (C5.1.1)	10 ml
EDTA solution (C5.1.2)	2 ml
Anti-foaming agent A	0.15 ml
Water	to 1000 ml

Weigh the polyethylene glycol (12) lauryl ether into a glass beaker, add 100 ml of warm (typically about 50 °C) water and stir to dissolve. Transfer the solution to a suitable container, rinsing the beaker several times with water to ensure the transfer of all the detergent to the container. Add 10 ml of tris buffer solution (C5.1.1) and 2 ml of EDTA solution (C5.1.2). Mix well and add 0.15 ml of antifoaming agent A. Make to 1000 ml with water and mix well. This solution may be stored in the dark in the range 20 ± 5 °C but should be used within 2 months.

C5.2 *Polyoxyethylene sorbitan monooleate solution*

Polyoxyethylene sorbitan monooleate (for example Tween 80)	1 g
Water	to 1000 ml

Dissolve the polyoxyethylene sorbitan monooleate in approximately 900 ml of water. Mix well and make to 1000 ml with water. The solution may be stored in the range 20 ± 5 °C but should be used within one month. Discard the solution if there is any sign of turbidity.

C6 Analytical procedure

C6.1 Sand washing

Carefully thoroughly mix the sample of sand. For example, gently invert the container of sand. Alternatively, gently stir the sand in the container with a clean spatula. Weigh out an amount of well mixed sand, typically 100 g into a 1-litre container and add a volume (typically, 500 ml) of elution buffer (C5.1.3 or C5.2). Mix the sand and elution buffer by gently swirling the contents of the container to completely suspend the sand in the elution buffer. Continue this process for 5 minutes. Stop swirling the contents and allow the sand to settle. This should take no more than 1 minute.

Carefully decant the supernatant liquid equally into ten 50 ml centrifuge tubes. Cap the centrifuge tubes and centrifuge typically at 1100 rcf for 15 minutes. The deceleration stage should be as smooth as possible to minimise the risk of re-suspending the particulate material and the application of any braking systems should be avoided. When completed, remove the centrifuge tubes taking care not to re-suspend any particulate material. The supernatant liquid may be removed (and discarded or kept for further processing as required) using a pipette and a vacuum source not exceeding 0.2 bar and keeping the pipette just below the meniscus of the supernatant liquid. The supernatant liquid should be removed to within 5 ml of the pellet of centrifuged material.

The sand is washed again with a further volume of elution buffer as used previously, typically 500 ml, and the second washings added to the centrifuge tubes used previously. The tubes are centrifuged a second time and the supernatant liquid removed as described in the preceding paragraph. Re-suspend the particulate material, for example by vortexing for between 10 - 15 seconds, until all visible aggregates are dispersed. Combine the suspensions from each tube into a single centrifuge tube, i.e. all of the particulate material should be contained in one 50 ml centrifuge tube. Rinse each centrifuge tube with 1 - 2 ml of water, vortex and transfer the washings to the tube containing the particulate material. An additional tube should be used if required. Vortex the resulting suspension and centrifuge as above. If two tubes are required, remove the supernatant liquid from each tube, transfer the particulate material to one tube. Centrifuge this suspension at 1100 rcf for 15 minutes at room temperature. Immediately after centrifugation, remove the 50 ml tube from the centrifuge, carefully discard supernatant liquid, ensuring particulate matter is not removed or discarded. Estimate and record the volume, V_{PV} ml, of particulate material in the tube.

Water should then be added to the centrifuge tube and made to a known total volume, V_{SV} ml, typically 9 ml. Vortex the tube to re-suspend the pellet of particulate material. The suspension is now ready to proceed directly to the IMS stage and microscopic examination (section D) or may be stored in the range 5 ± 3 °C for up to 3 days. However, delay in continuing the analysis at this stage may adversely affect the results of analysis.

If 250 ml centrifuge tubes have been used in place of 50 ml centrifuge tubes, carefully remove the supernatant liquid to within 30 - 40 ml of the pellet of particulate material, taking care not to lose any particulate material. Re-suspend the particulate material, for example by vortexing for between 10 - 15 seconds, until all visible aggregates are dispersed, then quantitatively transfer the suspension to 50 ml centrifuge tubes, rinsing each 250 ml tube as necessary and adding the rinsings to the 50 ml centrifuge tubes. Centrifuge as described in the preceding paragraph. Remove the supernatant liquid from each 50 ml centrifuge tube, re-suspend the particulate material and combine the particulate material from each tube. Rinse each tube with 1 - 2 ml of water and combine the rinsings into the single 50 ml centrifuge tube. Centrifuge this suspension at 1100 rcf for 15 minutes at room temperature. Immediately after centrifugation, remove the 50 ml tube from the centrifuge, carefully discard supernatant liquid, ensuring particulate matter is not removed or discarded. Estimate and record the volume, V_{PV} ml, of particulate material in the tube.

Water should then be added to the centrifuge tube and made to a known total volume, V_{SV} ml, typically 9 ml. Vortex the tube to re-suspend the pellet of particulate material. The suspension is now ready to proceed directly to the IMS stage and microscopic examination (section D) or may be stored in the range 5 ± 3 °C for up to 3 days. However, delay in continuing the analysis at this stage may adversely affect the results of analysis.

If the total pellet volume (V_{PV}) exceeds that specified by the IMS kit manufacturer, re-suspend the pellet by vortexing and transfer the suspension to another centrifuge tube. This tube should be such that an accurate measure of the pellet volume can be made from volume graduations on the base of the tube. Centrifuge the suspension as above (see AD4.1) and measure the pellet volume. Re-suspend the pellet and divide the suspension into suitable aliquot volumes such that each aliquot represents no more than the maximum pellet volume specified by the IMS kit manufacturer. Add water to each aliquot, bring the total volume, V_{SV} ml, typically to 9 ml and vortex to re-suspend the particulate material. Each aliquot of the suspension is now ready to proceed to the IMS stage and microscopic examination (section D).

D Isolation of *Cryptosporidium* oocysts and *Giardia* cysts by immuno-magnetic separation and enumeration by microscopic examination

D1 Introduction

Once (oo)cysts and any associated particulate material are concentrated into a small volume, i.e. a pellet volume, V_{PV} (see sections A - C) the (oo)cysts are then separated from other particulate matter using para-magnetic beads coated with specific antibodies which bind to the surface of the (oo)cysts. Following separation of the beaded-(oo)cyst complex from the particulate matter, the complex is broken down with subsequent separation of the beads and (oo)cysts. The (oo)cysts are then stained sequentially with specific FITC conjugated MAb and DAPI stains and examined microscopically.

D2 Definitions

In the context of this booklet, presumptive *Cryptosporidium* oocysts are defined as organisms which stain with a fluorescein isothiocyanate (FITC) conjugated monoclonal antibody (MAb) stain specific for *Cryptosporidium* oocysts (which are round to slightly oval in shape) and which exhibit an apple-green fluorescence in far blue light (wavelength of 488 nm). This fluorescence is normally more intense at the periphery of the oocyst than at the centre. The size of an oocyst may range between 3.0 to 8.5 µm depending on the species (see Appendix 1, Table 1) although the size range of 4.0.to 6.0 µm contains those species most commonly associated with human infections.

Cryptosporidium oocysts are confirmed if oocyst measurements are verified at 1000x magnification, and 1 to 4 sporozoites (with oval nuclei of not more than 1.5 µm) are observed. Observation of nuclei is aided by staining with 4',6-diamidino-2-phenylindole (DAPI) and examining for sky-blue staining in UV light. Additional confirmation is by observation of sporozoites with possibly apically-positioned nuclei using Normarski differential interference contrast (DIC) microscopy. Sporozoites should be contained either within intact oocysts or be found adjacent to ruptured oocysts. For reporting for drinking water compliance purposes, it will be necessary to observe national reporting requirements.

In the context of this booklet, presumptive *Giardia* cysts are defined as organisms which stain with a FITC conjugated MAb stain specific for *Giardia* cysts (which are elliptical or round in shape) and which exhibit apple-green fluorescence in far blue light (wavelength of 488 nm). This fluorescence is normally more intense at the periphery of the cyst than at the centre. The size of a *Giardia* cyst ranges between 8 to 18 µm by 5 to 15 µm. The species most commonly associated with human infection are generally within the size range of 11 to 14 µm by 7 to 10 µm.

Giardia cysts are confirmed if cyst measurements are verified at 1000x magnification, and 2 to 4 nuclei are observed by DAPI staining and examining for sky-blue staining in UV light. Additional confirmation is by observation of nuclei and organelles (including flagellar axonemes) using DIC microscopy.

When the concentrated suspensions obtained from waters are examined by immuno-fluorescence microscopy, other (oo)cyst-like bodies (OLBs) such as algal cells and fungal spores, may not easily be distinguished from (oo)cysts, and in these cases, may initially be mistaken for *Cryptosporidium* oocysts and/or *Giardia* cysts. When this occurs, false-positive identification may result, leading to incorrect reporting. In addition, cross-reaction

of the fluorescent antibodies with non-target organisms may also occur leading to false-positive identification and incorrect reporting. Examination using DIC microscopy should confirm their identities and remove this doubt.

D3 Apparatus

D3.1 *Apparatus for immuno-magnetic separation (IMS)*

Rotating mixer.
Tubes for incubating beads and particulate material, for example, Leighton tubes possessing a flat sided surface, with magnetic particle concentrator for these tubes.
Tubes for dissociating (oo)cysts from beads, for example, Eppendorf micro-centrifuge tubes, with magnetic particle concentrator for these tubes.
Incubator, capable of operating at temperatures up to 42 °C.
High speed vortex mixer.
Pipettes, capable of dispensing quantities of 10 ml, 1 ml and between 5 - 200 µl.
Microscope slides possessing ground glass areas (for labelling purposes) and having a clear well (for example 9 mm in diameter) in the centre of the slide surrounded by a well wall (with a hydrophobic coating). Slides with wells that are surrounded by polytetrafluoroethylene have been found suitable.

D3.2 *Apparatus for staining and mounting*

Cover slips, typically 22 x 22 mm, sufficiently thin to prevent interference with focussing using the 100x microscope objective.
Micro-pipettes of various volumes with plastic disposable tips.
Pasteur pipettes (plastic and disposable).
Incubator (fan assisted) for incubating stains, maintained in the range 37 ± 2 °C, with humidity chamber (for example, a plastic tray containing a layer of damp paper towel and fitted with a clip-on sealing lid).
Incubator (fan assisted) for slide drying, temperature not exceeding 42 °C.

D3.3 *Apparatus for microscopic examination*

Standard epifluorescence microscope with suitable light supply capable of excitation at 490 nm for FITC fluorescence and 350 nm for DAPI fluorescence. Suitable excitation sources may include 50 - 100 watt high pressure mercury vapour lamps, or metal halide lamps with appropriate bandpass filters, or pulsed light emitting diode sources. Mercury vapour lamps have a limited safe working life of 100 - 200 hours depending on their specification. Beyond this period the fluorescence output may fade and bulbs may explode, damaging the lamp housing and posing a risk of exposure to mercury vapour. Bulbs should therefore be changed at regular intervals and their fluorescence output calibrated using a fluorescence calibration control slide.
Band pass filter blocks (dichroic mirrors) to enable

(i) far blue light excitation wavelength of 490 nm for FITC fluorescence which also permits red-fluorescing counter stain to be seen, and
(ii) ultraviolet excitation wavelength of 350 nm for DAPI fluorescence.

Eyepiece graticule graduated in 100 units.
Calibrated slide micrometer 1 mm in length, graduated in 100 units, each unit of 10 µm length.
Fluorescence calibration slide containing fluorescent beads over a range of FITC fluorescence intensities.

D4 Reagents

D4.1 *Reagents for immuno-magnetic separation*

D4.1.1 Para-magnetic beads coated with either *Cryptosporidium* or *Giardia* antibodies, available separately as individual tests, or combined in one test.

D4.1.2 IMS Reagents A (detergent solution) and B (protein-blocking solution) each reagent supplied as a 10x concentrated solution (available from IMS kit manufacturers).

D4.1.3 Hydrochloric acid solution (0.1M). Add 0.9 ml of concentrated hydrochloric acid (SG 1.18) to 900 ml of water. Mix well. Make to 1000 ml with water. The solution may be stored in the range 20 ± 5 °C for up to one month.

D4.1.4 Sodium hydroxide solution (1M). Dissolve 400 g of sodium hydroxide in 9000 ml of water. Cool the solution. Make to 10000 ml with water. The solution may be stored in the range 20 ± 5 °C for up to one month.

D4.1.5 Methanol. Analytical grade reagent.

D4.2 *Reagents for staining and mounting*

D4.2.1 Fluorescein isothiocyanate (FITC) conjugated monoclonal antibody (MAb) stain specific for *Cryptosporidium* oocysts. This solution may contain Evans Blue, a staining reagent used in some commercial formulations to quench background fluorescence (causing background material to fluoresce red in contrast to the green fluorescence of FITC). This solution should be stored and used according to manufacturer's instructions.

D4.2.2 Fluorescein isothiocyanate (FITC) conjugated monoclonal antibody (MAb) stain specific for *Giardia* cysts. This solution may contain Evans Blue, a staining reagent used in some commercial formulations to quench background fluorescence (causing background material to fluoresce red in contrast to the green fluorescence of FITC). This solution should be stored and used according to manufacturer's instructions.

D4.2.3 Combined stain formulation specific for both *Cryptosporidium* oocysts and *Giardia* cysts. This solution may contain Evans Blue, a staining reagent used in some commercial formulations to quench background fluorescence (causing background material to fluoresce red in contrast to the green fluorescence of FITC). This solution should be stored and used according to manufacturer's instructions.

D4.2.4 4',6-diamidino-2-phenylindole (DAPI) stock solution. Dissolve 1 mg of 4',6-diamidino-2-phenylindole in 500 µl of methanol. This solution may be stored in the range 5 ± 3 °C for up to one month.

D4.2.5 DAPI-PBS stain. Dilute one volume of DAPI stock solution (D4.2.4) in 5000 volumes of phosphate buffered saline solution (D4.2.7) for example, 5 µl to 25 ml. This solution should be used on the day of preparation.

D4.2.6 Mounting fluid. The following mounting fluid has been found suitable, although alternatives are available. Warm 95 ml of glycerol in a beaker to approximately 50 °C and add 2 g of 1,4-diazabicyclo[2.2.2]octane (DABCO). Stir until dissolved. When cool, the pH

of this solution should be 8.0 ± 0.2. This solution may be stored in the range 20 ± 5 °C but should be used within six months.

D4.2.7 *Phosphate buffered saline solution* (PBS)

Sodium chloride	8.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	1.15 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g
Potassium chloride	0.2 g
Water	1000 ml

Dissolve the ingredients in the water and adjust the pH of the solution to 7.3 ± 0.2 with either 1M hydrochloric acid solution or 1M sodium hydroxide solution. The reagent may be stored in the dark in the range 20 ± 5 °C for up to three months. Commercially available buffer solutions may contain preservatives which may extend the shelf life. Discard the solution if there is any sign of turbidity.

D5 Immuno-magnetic separation

Water and other environmental samples may contain a significant quantity of particulate material, which may comprise organic and/or inorganic material and include algal cells and other cellular material, all of which may interfere with the detection of (oo)cysts during immuno-magnetic separation (IMS) and subsequent microscopic examination. All of the pellet volume, V_{PV} ml should be examined. This may require one or more slides. *Cryptosporidium* oocysts and *Giardia* cysts can be separated from other particulate material found in the sample using a process known as immuno-magnetic separation (IMS). This process separates the (oo)cysts from other particulate material whereby unwanted material is then discarded. Generally, IMS techniques reduce the volume of suspended material, V_{SV} ml, from typically 9 ml to 50 µl, and this volume is easily manageable when dried onto a microscope slide.

Individual manufacturers of IMS kits usually specify the maximum volume of particulate material (i.e. the pellet volume, V_{PV} ml) that should be used for individual tests. It is important therefore that the amount of particulate material recovered from a sample is measured to ensure that the specified maximum pellet volume for an IMS kit is not exceeded.

Particulate material present in waters and related samples which has been concentrated by filtration and centrifugation, is suspended in (oo)cyst-free water, and is then added to a mixture of buffer solutions, one containing detergent to keep the particulate material in suspension, and the other containing proteins to minimise non-specific materials binding to the beads. To this mixture is added antibody-coated para-magnetic beads. These beads are coated with either anti-*Cryptosporidium* antibody or anti-*Giardia* antibody. The resulting solution is mixed (2 - 5 minutes) at room temperature. During this period any (oo)cysts present in the suspension form a complex with the beads. Using a magnet, the beads (and complexed (oo)cysts) are magnetically “attracted” to one side of the glass tube and thus are separated from the remaining particulate material, which is subsequently discarded. The beads are re-suspended in a buffer solution and transferred to a smaller tube. The beads may then be rinsed to remove further un-wanted particulate material adhering to the beads. Any (oo)cysts bound or complexed to the beads are then dissociated from the beads using vortexing and acid, for example hydrochloric acid, and the beads and (oo)cysts separated using a second magnet. The beads are retained in the tube by

magnetic attraction, and the (oo)cysts in the acid solution transferred to a microscope slide. The slide contains a small amount of alkali (to neutralise the acid solution) before being dried, fixed and stained for microscopic examination.

Several manufacturers produce a range of IMS products, each product possessing its own specific instructions for use. Whilst a general overview of the processes involved is described in this section, users should follow specific manufacturer's instructions, especially with reference to specific details of, for example reagents and their volumes and incubation times.

D5.1 *Preparation of the sample*

Buffer solutions which have been stored in a refrigerator should be allowed to reach ambient temperature, i.e. room temperature. Any crystals present in either buffer solution should be completely dissolved before the solutions are used. Aliquots (typically, 1 ml) of IMS reagent solutions A and B (D4.1.2) are added to, and mixed in, a suitable tube, i.e. a Leighton tube. A suitable volume, V_{PV} ml or V_{SV} ml, or appropriate dilution of suspension should be added to the same tube. The amount of material contained in the Leighton tube, from which (oo)cysts are to be processed should represent a known volume of particulate material from the sample and be within the volume of particulate material specified by the individual IMS manufacturer.

D5.2 *Immuno-capture*

The centrifuge tube containing the pellet sample is rinsed with 1 ml of water and this is added to the Leighton tube. The beads, which are usually provided as a suspension, are vortexed-mixed for about 10 seconds and the container inverted to ensure that the beads are well mixed. Finally, a suspension of the beads, typically contained in 100 µl are added to the Leighton tube.

Separate aliquots can be used and beads that are either specific for *Cryptosporidium* or *Giardia* can be added to each aliquot, so that individual and separate examinations can be carried out for either organism. Alternatively, a combination of beads for both *Cryptosporidium* and *Giardia*, may be added to a single aliquot.

The Leighton tube is placed on a rotating mixer and mixed at ambient temperature, typically at a speed of approximately 20 rpm for a minimum of 60 minutes.

D5.3 *Immuno-bead concentration*

Once the (oo)cysts are complexed to the beads, the beads should be separated from the remaining suspended particulate material. This is achieved using a magnet, housed in a particle concentrator. The Leighton tube is held in the concentrator in such a way that the flat side of the tube is held against the magnet. The particle concentrator is positioned so that the Leighton tube lies horizontal, with its flat side positioned downwards. The concentrator is then gently rocked in a vertical-horizontal motion through 90 ° (at 45 ° each way from the horizontal position) at an approximate speed of one complete movement per second. The tube is gently rocked for a specified period of time, typically 2 - 5 minutes. Alternatively, the rocking process can be carried out using a mechanical rocker, whereby the Leighton tube and magnet are fixed to a rotating mixer. During this process, the IMS beads and any complexed (oo)cysts are "trapped" by the magnet onto the flat side of the Leighton tube. It is important during this stage that rocking is continuous, to prevent

unwanted particulate material adhering to the beads. If the rocking process is interrupted, for more than 10 seconds, the tube should be removed, the beads gently re-suspended, the tube replaced, and the rocking procedure repeated for the full period. Once this process is complete, the rocking motion is stopped, and the cap of the tube removed and any particulate material (not binding or adhering to the beads) is transferred to another tube. This particulate material should be retained in case further processing is needed, or may be discarded. In instances where excessive amounts of fibrous or particulate material are present, beads (with complexed (oo)cysts) may fail to be retained or attracted to the magnet. Diluting the sample and repeating the process may help trap the beads. The Leighton tube should remain in position in the particle concentrator during this process to ensure the beads are not disturbed or discarded.

D5.4 *Dissociation of oocysts and cysts from beads*

Once the beads have been separated from the unwanted particulate matter, the beads should be re-suspended. This is achieved usually by adding 0.7 ml or 0.8 ml (depending on the IMS kit manufacturer's instructions) of a 1 in 10 dilution of IMS reagent solution A (detergent buffer) re-suspending the beads and transferring them from the Leighton tube to a smaller tube, for example an Eppendorf tube. The Leighton tube is then rinsed with a further 0.3 ml or 0.2 ml of diluted IMS reagent solution A to ensure complete transfer of beads to the Eppendorf tube. The volume of final suspension contained in the Eppendorf tube should be about 1 ml. The Eppendorf tube is then placed in a second particle concentrator appropriately designed, and gently rocked through 180 ° (in a vertical-horizontal-vertical motion) at an approximate speed of one complete movement per second for a specified period of time, typically 1 - 2 minutes. Alternatively, the rocking process can be carried out using a mechanical rocker, whereby the Eppendorf tube and magnet are fixed to a rotating mixer. During this time the beads should be magnetically "attracted" to a very small area within the Eppendorf tube in the proximity of the magnet. It is equally important that the rocking process is continuous during this stage. Once this process is complete, the rocking motion is stopped, the cap removed and the liquid in the cap and in the Eppendorf tube is discarded. Care should be taken not to disturb the beads during this process and the Eppendorf tube should remain motionless in the particle concentrator. It is important to ensure that all of the diluted IMS reagent solution A is removed.

Occasionally, particularly with samples containing large amounts of particulate material, the particulate material may be carried over from the first stage of the IMS concentration to the second stage (i.e. from the Leighton tube to the Eppendorf tube). Thus, the beads may be rinsed at this stage to remove any un-wanted material that may be present. The Eppendorf tube is removed from the particle concentrator and an aliquot of diluted IMS reagent solution A, typically 1 ml, is added. The beads are re-suspended by gentle mixing and processed, as described in the preceding paragraph, to re-concentrate the beads. The beads should not be vortex mixed during any part of this operation. Where there is little or no particulate material in the original sample, this washing step may not be necessary.

The Eppendorf tube containing the washed beads is removed from the particle concentrator and an aliquot, typically 50 µl, of 0.1M hydrochloric acid solution (D4.1.3) added. The Eppendorf tube is now vortex mixed, typically between 10 - 15 seconds and allowed to stand for a period of time, typically 5 - 10 minutes at room temperature. During this time, the (oo)cysts should be dissociated from the beads, and form a suspension in the acid. To ensure this process is complete, the Eppendorf tube is vortex mixed for a further 10 seconds and returned to the particle concentrator using positions and settings

recommended by the IMS kit manufacturer, for any remaining beads to be separated from the acid suspension. During this period, the beads are collected in a very small area of the Eppendorf tube and the acid containing the dissociated (oo)cysts can then be carefully removed.

D5.5 *Loading of microscope slides*

A suitable microscope slide (D3.1) should be labelled, and 1M sodium hydroxide solution (D4.1.4) typically 5 µl, added to the centre of the well. Sufficient alkali should be added to exactly neutralise the volume of acid used to dissociate the (oo)cysts from the beads. Taking care not to disturb the beads, the hydrochloric acid containing the (oo)cysts is removed, and quantitatively transferred to the well containing the sodium hydroxide solution. The resulting solution on the slide is gently mixed. A second separation stage may need to be incorporated. Under these circumstances, a further aliquot of hydrochloric acid solution should be added to the beads, the tube vortex mixed and the dissociation procedure repeated. The suspension from the second dissociation stage may then be added to a second microscope slide containing a further quantity of sodium hydroxide solution (D4.1.4) or it may be added to the original slide along with a further aliquot of sodium hydroxide solution (D4.1.4).

All microscope slides should then be dried by incubating the slide at a temperature not exceeding 42 °C until all the solution has evaporated. Methanol (D4.1.5) typically 25 µl, should then be added to each slide and allowed to air-dry until the methanol evaporates. This “fixes” the (oo)cysts present onto the slide. Slides can now be stained as appropriate, see section D6. Where there is a delay in staining the (oo)cysts, the slides may be stored in the dark at ambient temperature until they are required for staining.

D6 Staining and mounting of slides

For particulate material obtained from samples, the mounting of *Cryptosporidium* oocysts or *Giardia* cysts directly on slides rather than membrane filters has become common practice in the UK. This technique enables the examination of slides to be carried out using differential interference contrast (DIC) microscopy.

Once the (oo)cysts are fixed, they should be stained with a fluorescently conjugated antibody. The stain binds with specific epitopes on the (oo)cyst wall, rendering the wall visible by fluorescence microscopy. Additionally, a counter stain, for example Evans Blue, may be included in the stain formulation to quench background fluorescence, thus increasing the contrast. A DAPI stain is used to highlight the DNA of the nuclei of the sporozoites within the *Cryptosporidium* oocyst, or the nuclei within *Giardia* cysts. The stained preparation should then be mounted under a cover slip using a suitable mounting medium which contains anti-fading agents (to help minimise fading of the fluorescence on exposure to ultraviolet light). Finally, the cover slip should be sealed around the edges with a suitable sealant, for example nail varnish, to facilitate long term storage and reduce evaporation of the mounting fluid.

Current fluorescent monoclonal antibody stains are not wholly specific either for *Cryptosporidium* oocysts or *Giardia* cysts, and cross reactions may occur with other environmental particulate material that may be present in environmental waters, for example spores, algae etc. This material may resemble the appearance and morphology of *Cryptosporidium* oocysts or *Giardia* cysts, and it can be difficult to distinguish other

environmental particulate material from (oo)cysts unless DIC microscopy is used for confirmation.

If, during the microscopic examination, problems are experienced with residual highly fluorescent particles causing background fluorescence (which may be highly distracting for the microscopist) it may be necessary to filter the stain before further use. The reagent should be filtered through a suitable filter, with a pore size nominally of 0.2 µm or 0.45 µm. The reagent bottle should then be washed with (oo)cyst-free water and drained. The filtered stain should then be transferred to the washed bottle. Alternatively, fresh reagent may be obtained.

Different mounting fluids can vary in their performance with respect of their anti-fading properties, and should therefore be checked for their effectiveness. DAPI stains used for staining DNA are especially vulnerable to fading.

A comparison of the fluorescence can be carried out by preparing two slides, each slide containing stained (oo)cysts. One slide should be prepared using new mounting fluid and the other slide using the old or previous batch of mounting fluid. A comparison of the fluorescence observed after 24 hours (or a longer time period as deemed necessary) should then be undertaken. The new batch of mounting fluid should be rejected if its anti-fading properties are significantly poorer than those observed with the previous batch of mounting fluid.

D6.1 *Precautions needed to be taken in the preparation of slides*[5]

Unless great care is taken, this stage of the analysis is particularly vulnerable to losses of (oo)cysts from the slide. These losses may occur at any stage during the process, particularly the addition of reagents and their subsequent removal.

When selecting microscope slides, care should be taken to ensure that the glass surface in the well area is not hydrophobic. Hydrophobicity can be observed when a drop of liquid "stands up" more than normal from a glass surface. Thin polyethylene sheets, for example "Cling film™", are often used as packaging materials, and can make glass surfaces more hydrophobic. Where the well surface of a slide has been in direct contact with such packaging materials, the slide should not be used if it shows any sign of being hydrophobic. A clean non-hydrophobic slide enables (oo)cysts to more readily adhere, or become 'fixed' to the slide. This will significantly reduce the risk of (oo)cysts being lost or dislodged during the staining and mounting process.

During all procedures where reagents are added to the well of a microscope slide, the pipette tip should be held in such a position and at such a distance above the slide to prevent "bridging" between the slide and the reagent, i.e. contact being made. Normally, a distance of 10 mm between pipette tip and slide is sufficient to prevent this, but this distance would need to be determined beforehand. At the same time, the impact of placing the reagent on the slide should be sufficiently gentle to ensure (oo)cysts are not lost or dislodged from the slide. In addition, the reagent should be added to the slide in a drop-wise motion. If reagent is added as a jet or stream of liquid, this may cause (oo)cysts to be dislodged, and subsequently to be lost during aspiration or evaporation of reagents. This risk is much greater when suspensions of (oo)cysts are used as quality control samples, than when real samples are examined. This is probably due to other environmental particulate material being present in the sample, which often facilitates the "fixing" of

(oo)cysts to the slide. In quality control samples, other environmental particulate material is usually absent.

Because (oo)cysts are easily lost or dislodged during reagent addition, extra care should be taken during this process and the subsequent aspiration of the reagents from the well. Aspiration of reagents from slides should be carried out from the edge of the well in such a way that the speed of removal of the reagent is as slow as possible, irrespective of the technique used, i.e. whether a pipette or venturi type vacuum source is used. Pipettes with micro-capillary tips have been found useful for this purpose. To prevent cross contamination, pipette tips should be used only for one operation, and changed every time a new operation is carried out. Figure D1 shows details of the appropriate angles and pipette positions used during the aspiration of reagents from slides. The speed of removal of the liquid from the slide should be as slow as possible in order to minimise the risk of dislodging (oo)cysts as the liquid is removed.

Figure D1 Technique for gentle aspiration of reagents from slide wells

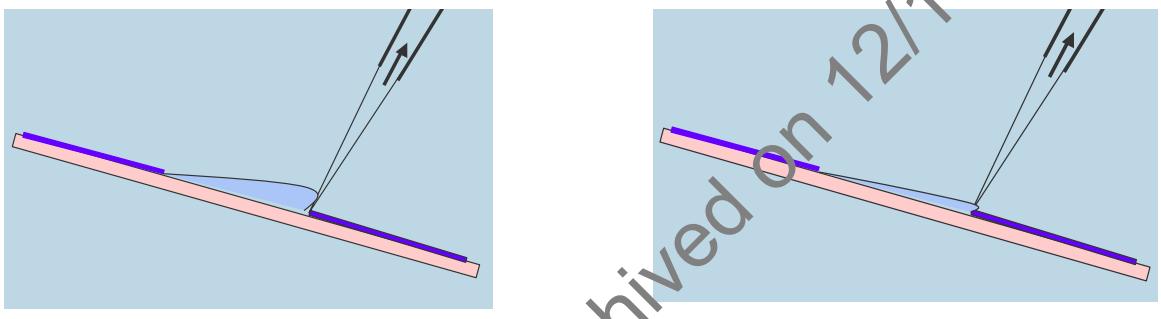
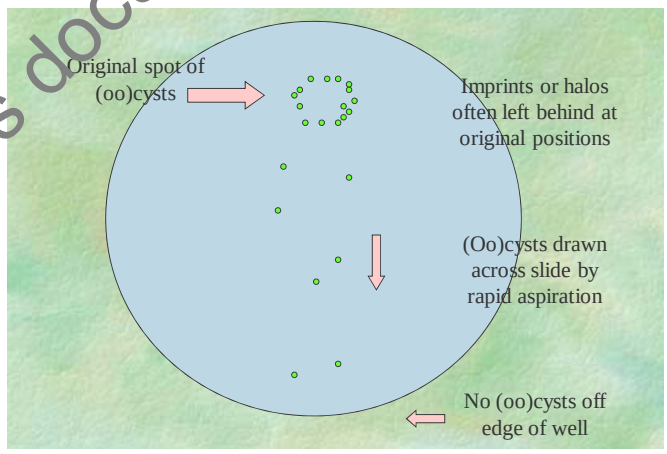


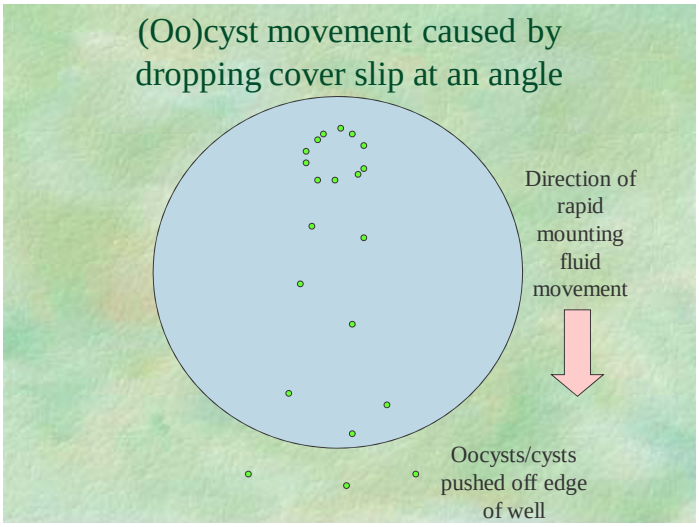
Figure D2 shows some of the effects observed when excessive aspiration speeds are used when (oo)cysts are loosely attached onto a slide. Aspirating the liquid at too high a speed can cause some (oo)cysts to become detached and to migrate down the slide. As a consequence, some (oo)cysts may move from their original position, and some may be lost in the liquid removed from the slide. Unlike (oo)cyst movement caused by poor mounting technique (see Figure D3), this technique does not normally cause (oo)cysts to move beyond the edge of the well area.

Figure D2 Observed symptoms of excessive aspiration speeds



If an inappropriate technique is employed when adding the cover slip to the well of the slide, for example too much pressure is applied or the angle is inappropriate, then the following effects may be observed, see Figure D3.

Figure D3 Effects observed due to incorrect mounting



If the cover slip is added incorrectly or too much pressure is applied or the angle is inappropriate, (oo)cysts that are loosely attached to the slide may move and be repositioned in the well of the slide as a result of lowering the edge of the cover slip over the well, and then allowing the opposite edge of the cover slip to be "dropped" rather than gently "placed". The resulting rapid movement of mounting fluid down the well (in the direction of the arrow) may dislodge (oo)cysts from the well and displace them onto the area surrounding the well. Subsequent microscopic examination of (oo)cysts outside of the well area is difficult and prone to mis-interpretation. This poor technique may also lead to the loss of (oo)cysts as excess mounting fluid is withdrawn from the slide prior to sealant being applied. An appropriate technique to be used is described in the following section.

D6.2 *Staining and mounting procedure*

Fluorescent stains are light sensitive and colours may fade upon exposure to light. Hence, staining procedures should not be carried out in direct sunlight or under bright light conditions.

Following fixing of the (oo)cysts onto the slide (see section D5.5) manufacturer's instructions, specific for individual FITC-labelled monoclonal antibody stains should be followed whether it applies to either *Cryptosporidium* oocysts (D4.2.1) *Giardia* cysts (D4.2.2) or a combination of *Cryptosporidium* oocysts and *Giardia* cysts (D4.2.3).

Incubate the slides for a time and temperature, typically 37 ± 2 °C for 15 minutes (specified by the stain manufacturer). Incubation should be carried out in a humidified chamber. A plastic tray containing a layer of damp paper towel, and fitted with a clip-on sealing lid has been found suitable.

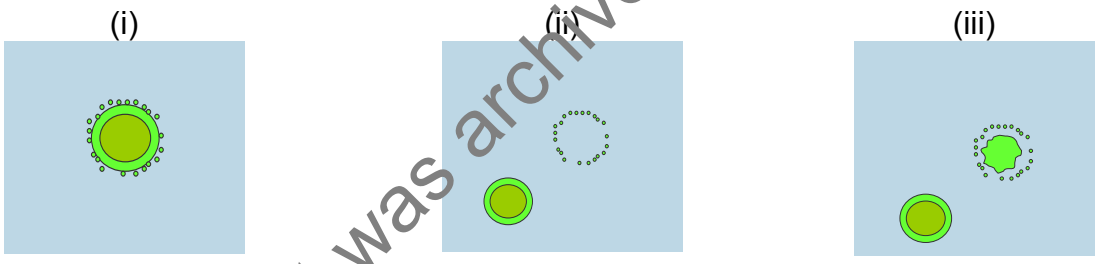
After incubation, aspirate the antibody reagent from the slide using, for example a hand-held micro-pipette or a disposable micro-pipette tip attached to a gentle source of vacuum. Tilt the slide gently to move the stain carefully to one side and aspirate the stain from the lower edge of the well (see Figure D1) taking all necessary precautions to minimise the risk of (oo)cysts being lost or dislodged from the slide.

Unless otherwise directed by the manufacturer, add a drop (typically 30 - 50 µl) of the DAPI-PBS stain (D4.2.5) to the well of the slide. Allow the mixture to stand at room temperature, typically for 2 minutes and then aspirate the staining reagent from the slide.

Add one drop (typically, 30 - 50 µl) of water to the well and allow the mixture to stand at room temperature for approximately 1 minute. Aspirate the water from the slide, then allow any remaining water to evaporate. This is to remove any excess DAPI to minimise interferences in the subsequent microscopic examination.

Add a small drop (typically 10 - 30 µl) of mounting fluid (D4.2.6) to the centre of the well. Carefully, lower a cover slip onto the well, taking precautions to avoid trapping any air bubbles under the cover slip. If too much mounting fluid is used the cover slip may exhibit the tendency to move across the slide, potentially dislodging (oo)cysts. Care should be taken when adding the cover slip to the well to minimise the rate at which mounting fluid is transferred from the centre of the well towards the periphery of the well, as excess fluid is squeezed out between the cover slip and the well of the slide. This is to prevent (oo)cysts being lost, i.e. being pushed outside of the area of the well, or being pushed from under the cover slip. Pressure should not be applied to the cover slip. In addition, the cover slip should not be allowed to drop onto the slide since rapid movement of the mounting fluid across the slide may dislodge (oo)cysts that are loosely attached to the slide, and cause them to be lost (see Figures D3 and D4).

Figure D4 Effects observed of (oo)cyst movement during staining and mounting



In Figure D4(i) a stained (oo)cyst is shown in its original position in the well of the slide. Often present is a “halo” of deposited stain around the (oo)cyst.

In Figure D4(ii) a stained (oo)cyst is shown to have moved from its original position in the well of the slide, leaving the “halo” in the original position.

In Figure D4(iii) a stained (oo)cyst is shown to have moved from its original position in the well of the slide, leaving the “halo” in the original position, but also showing an “imprint” of associated material. This material consists of part of the outer layer of the epitope of the stained (oo)cyst. The material remains within the “halo” and, when observed under low magnification, may lead to an incorrect interpretation leading to the reporting of two (oo)cysts, not one. The use of DIC microscopy should rectify this mis-interpretation.

When these actions are completed and the cover slip is correctly placed, excess mounting fluid should be carefully withdrawn, for example using tissue paper. The edge of the cover slip should then be sealed with a suitable sealant, for example clear nail varnish. Care should be taken to ensure sufficient sealant is effectively applied all around the edge of the cover slip to prevent leakage of mounting fluid from the well or ingress of air into the well. The use of coloured sealant facilitates the observation of the sealing process and enables

potential flaws in the seal to be visibly enhanced. However, the use of fluorescent sealant should be avoided.

Following staining and mounting, slides should be examined as soon as practicable. After examination, slides may be stored in the dark in the range 5 ± 3 °C. Any subsequent examination of stored slides should be interpreted with caution, as the fluorescence may fade over time, if not stored correctly.

D6.3 *Quality control for staining and mounting*

Slides known to contain (oo)cysts (i.e. positive quality control slides) and slides known to be free of (oo)cysts (i.e. negative quality control slides) should be prepared, stained, mounted and examined with each batch of sample slides. This should demonstrate that staining and mounting procedures have been carried out correctly and that stains, where applicable, produce levels of fluorescence that are acceptable.

Positive quality control slides should be prepared by adding an aqueous suspension of (oo)cysts to the well of a slide and allowing the water to evaporate. This suspension should not be prepared in phosphate buffered saline solution as the salt content of these buffer solutions may cause some of the (oo)cysts to become trapped within clusters of salt crystals following evaporation. These trapped (oo)cysts may then not be able to be "fixed" to the slide correctly. Consequently, following fixing, staining, mounting and aspiration procedures, these (oo)cysts will be lost.

Negative quality control slides (blanks) may be prepared by transferring a volume, typically, 50 µl of water into the well of a slide and allowing the water to evaporate, before fixing, staining and mounting.

D7 Microscopic examination

Incident light epifluorescence microscopy is used for the initial examination of prepared slides. Firstly, using a low power magnification, to indicate whether (oo)cysts may be present (as apple-green fluorescing bodies). Secondly, any particles on the slide which fluoresce are then examined under high power magnification to observe morphological features such as shape, size, structure and fluorescence (to indicate characteristic features of *Cryptosporidium* oocysts and *Giardia* cysts). Additionally, all positive identifications should be examined using DIC microscopy. Microscopy should be carried out under subdued lighting, for example in a room or area where the light can be dimmed to enhance or maximise the detection of fluorescent objects.

Microscopy can be applied to any material that has been processed and then mounted and stained on a microscope slide. A range of *Cryptosporidium* and *Giardia* species can be detected in this way. Other OLBs can be found in samples and these should be differentiated from (oo)cysts during microscopic examination.

A high pressure mercury vapour lamp in a special housing is used to generate a broad spectrum of light including ultra-violet and blue light. This light is filtered by band-pass filters to produce a specific wavelength (490 nm excitation, 520 nm emission) which will give maximum excitation for the fluorochromes being used to stain the (oo)cysts. Fluorescein isothiocyanate (FITC) bound to specific antibodies is used to stain the walls of (oo)cysts, whilst 4' 6-diamidino-2-phenylindole (DAPI) is used to stain nuclear material.

The stained material is examined using a light wavelength suitable for FITC and a low power magnification, for example the 20x objective. The whole of the material on each slide should be examined for characteristic (oo)cyst features. Where these are observed, the slide should be re-examined using a high power magnification, for example 100x water or oil immersion objective. Each fluorescing body should be carefully assessed for its size, shape, stain and morphological characteristics. It is more advantageous to use the microscope in the 20x and 100x objective, both in fluid immersion mode, to enable switching from lower magnification to higher magnification during microscopic examination to be carried out more easily. Each fluorescing body should then be examined, firstly, using light of a suitable wavelength (350 nm excitation, 450 nm emission) for DAPI to indicate the presence of characteristic nuclear material, and secondly, by Nomarski differential interference contrast (DIC) microscopy to identify any internal structures within the fluorescent body. Only when the whole slide has been examined can the number of confirmed bodies be reported as a final count.

Prior to use, it is important that the microscope is optimised for Köhler illumination and Nomarski DIC microscopy. The procedure for this technique will vary depending upon each manufacturer and model of the microscope, and it is therefore essential to follow specific manufacturer's instructions.

The transfer of particulate material or beads to a slide during the dissociation stage of the IMS procedure can partially or totally obscure (oo)cysts, and can interfere with the confirmation of internal structures using DIC microscopy. The inclusion of air bubbles during the mounting of the slide can also affect DIC microscopy. The presence of fluorescent particulate material in the fluorescent stain can make correct microscopic examination of (oo)cysts difficult. This is because large amounts of non-specific fluorescing material may be transferred to the slide during the staining procedure.

D7.1 *Calibration of the microscope*

Fluorescing bodies observed on a microscope slide should be measured to determine their size. Such measurements should be undertaken using a graticule inserted into one of the eye-pieces. This graticule comprises a measuring scale which is usually sub-divided into 100 units. The graticule should then be calibrated using a calibrated slide micrometer enabling the microscopist to calibrate the measuring scale units by comparison of these units with a scale of known length on the calibrated slide micrometer. For example, the scale on the slide micrometer is normally I mm (equivalent to 100 units) with each unit representing 10 µm.

Place the calibrated slide micrometer on the microscope stage, turn on the transmitted light and then focus the micrometer image. Ensure that the eye-piece is correctly and appropriately adjusted to focus the graticule for each individual microscopist. Using the 10x objective, adjust the microscope slide and the eye-piece so that the zero line on the graticule scale is exactly superimposed on the zero line of the slide micrometer.

Without changing the stage adjustment, locate a point (as far removed as is possible from the two zero lines) where a line on the graticule scale is again superimposed exactly on a line on the slide micrometer. Determine the number of units on the graticule scale and the length (expressed in µm) on the stage micrometer between the two points of superimposition.

Divide the length (expressed in µm) by the number of graticule units and calculate the number of µm per graticule unit. For example, if 100 units on the graticule scale is equivalent to 100 divisions on the slide micrometer, then one unit on the graticule scale equates to 10 µm. This is usually the case for the 10x objective combined with 10x eye-pieces.

This procedure should be carried out for each objective. This information should be recorded and made available when required. The microscope should be calibrated at frequent intervals, and following any maintenance, relocation, or change to any microscope component. The microscope calibration should not change significantly over time and if significant changes are noted, an investigation into the causes should be instigated and appropriate corrective action taken.

It is prudent to re-calibrate the graticule on occasions when significant numbers of oo(cysts) are detected. This should ensure accurate measurements, and therefore identifications, are made.

D7.2 *Technique for observing and scanning slides*

It is important that all the material on the prepared slide is observed and scanned systematically. It is equally important that the same material should not be counted and recorded more than once, resulting in the subsequent counting and recording of incorrect high numbers of (oo)cysts. In addition, areas on the slide that are not observed or scanned will result in a reduced count and the subsequent recording of low numbers of (oo)cysts. The slide may be scanned by moving the microscope stage in a top to bottom motion (i.e. vertically) and then repeating the step in a reverse motion (i.e. bottom to top) or from a side to side motion (i.e. horizontally). In this way the well of the slide should be thoroughly observed and scanned. Once a single motional (i.e. top to bottom or side to side) scan is completed, the well of the slide should be moved a distance equivalent to a single field of view, and the scanning process repeated. The extent of scanning process may be assessed using a feature on the slide, for example using the hydrophobic coating on the edge of the well, or sample debris fixed onto the slide. In this way, the whole process is repeated until the scan is complete. Where an uneven plane of focus is encountered, the microscope should be correctly focused within each field. A hand-held or electronic counter should prove useful for recording the number of (oo)cysts that are observed during the whole scan.

To carry out a microscopic examination correctly requires extensive training and practical experience, and the examination and counting of known numbers of stained (oo)cysts should be encouraged in order to identify and record characteristic features. For training purposes, it is useful for a collection of prepared slides to be readily available for use that contain *Cryptosporidium* oocysts, *Giardia* cysts and OLBs that resemble (oo)cysts, so that appropriate features can be distinguished (see Appendix 5). Whilst the use of visual aids, such as two-dimensional pictures and diagrams, are helpful for distinguishing characteristic morphological features, they are not a substitute for practical experience, where three-dimensional structures are observed.

In addition, carrying out a microscopic examination correctly can be extremely tiring for individuals. Thus, before microscopists become too tired and the recording of results become affected, sufficient time should be allowed for individuals to terminate the microscopic examination and to refresh themselves. Furthermore, it may be necessary to

consider eye tests for individuals to assess whether colour blindness affects an individual's ability to examine *Cryptosporidium* oocysts, *Giardia* cysts and OLBs correctly.

D7.3 *Examination of slides*

The correct interpretation of a microscopic examination and morphological study of (oo)cysts may be assisted by referring to the images shown in Appendix 5.

Before use, the microscope should be checked, for example for cleanliness and correct adjustment, and the fluorescence intensity of the ultra-violet light checked and recorded. This should be followed by a microscopic examination of a prepared slide containing (oo)cysts in order to demonstrate that the staining reagents are suitable and that the microscope can be operated correctly. The slide should be examined under high magnification to ascertain that (oo)cysts and their nuclear contents have been correctly stained and their morphological features correctly observed. Information relating to this examination should be recorded. Where dried immersion oil distorts the appearance of any material on a slide, the objective should be cleaned before examining samples. The use of this prepared slide can also act as an "aide memoir" for the subsequent examination of samples.

Stained samples should be examined for FITC fluorescence using a low power magnification, for example a total magnification of 200x. During the observation and scan, any small, fluorescent bodies should be noted and counted. If only a small number of fluorescent bodies are present, for example 2 or 3, their position should be noted and the bodies examined individually, using oil- or water-immersion techniques. Where there are larger numbers of fluorescent bodies, the whole slide should be scanned under a high power (100x) oil- or water-immersion lens, giving a total magnification of 1000x. Alternatively, if a 20x fluid immersion lens is used, the repeat scan may be carried out on low power, switching from the 20x to the 100x lens when needed to confirm a fluorescent body. If the latter technique is used, it will be necessary to accurately note the position of the fluorescent body in the scanning field before centralising it for examination at 100x, then return the fluorescent body to that position when switching to the 20x lens to continue the scan. A double scale graticule (vertical and horizontal crossed scales) can facilitate the accurate positional measurement of this.

D7.3.1 *Appearance of Cryptosporidium oocysts with FITC staining*

Depending on the species present *Cryptosporidium* oocysts appear as bright apple-green fluorescent bodies that are round or slightly ovoid in shape. The intensity of the fluorescence decreases, the longer it is viewed. The circumference of the oocyst, which is usually intact and even, stains more intensely than the centre. Appendix 1 highlights the mean size of a range of different *Cryptosporidium* species. Most species are within the size range of 4 - 6 µm.

Occasionally, an oocyst may rupture, giving the appearance of excystation. This causes a break or gap in the oocyst wall and it may appear "pac-man-like" in shape (a circle with a small segment removed, i.e. ). Part or all of the contents of the oocyst may be seen on the outside of the missing segment. If the FITC stain contains a counter-stain such as Evans Blue, the contents of the oocyst may fluoresce pink or red.

Commercially available oocysts often exhibit a typical appearance, but oocysts that have been exposed to the environment may appear different. Environmentally-aged oocysts

may exhibit a weakly stained or diffuse appearance. In addition, environmental exposure and/or sample processing may cause an oocyst to collapse or become distorted, making its size difficult to measure accurately. Under such circumstances, the sporozoites may be lost, giving rise to empty shells which may or may not exhibit an obvious “pac-man-like” appearance. In these cases, they may not be confirmed as oocysts if sporozoites are not present.

D7.3.2 *Appearance of Cryptosporidium oocysts with DAPI staining*

An intact oocyst contains four sporozoites, and each sporozoite contains a single nucleus. These nuclei are stained a sky-blue colour with DAPI. Each nucleus is ovoid, measures approximately 1 µm but not more than 1.5 µm, and not all nuclei may be visible at any one time. In some circumstances, only two or three nuclei may be visible and where the oocyst has ruptured, the nuclei may be visible outside of the oocyst. Fluorescing bodies which contain more than four nuclei are not *Cryptosporidium* oocysts. Care should be taken as normal sporozoite nuclei may sometimes appear “comma-shaped”. This is due to the presence of a DAPI-stained mitochondrion, forming the “tail” of the comma. This may lead to the false impression of more than 4 nuclei being present. Care should be taken to scan the full depth of focus (see Appendix 5). Empty oocysts do not exhibit any characteristic DAPI fluorescence. Other (oo)cyst-like bodies (OLBs), for example algae which possess structured internal contents, do not exhibit characteristic DAPI fluorescence.

Micro-organisms such as bacteria situated either above or below the fluorescing body may stain with DAPI. In these cases, it may be difficult to distinguish these from sporozoite nuclei. Careful focusing and using DIC optics should resolve this difficulty.

Occasionally, the sporozoite nuclear envelope may be damaged in environmentally-aged oocysts, thus the genetic material may not be contained in a localised area. This results in the DAPI-stained bodies appearing more diffuse around their edges, rather than appearing as clear, well defined sky-blue images. This can make interpretation more difficult.

D7.3.3 *Confirmation of Cryptosporidium oocysts with DIC microscopy*

Once the body has been viewed by FITC and DAPI fluorescence, characteristic features should be confirmed by differential interference contrast (DIC) microscopy. The advantage of DIC microscopy is that it enables a more intense scrutiny of the body to be carried out, without quenching the fluorescence. The size of the oocyst and the presence and number of sporozoites can be confirmed by making numerous observations. A disadvantage of DIC microscopy is that particulate material on the slide may obscure the oocyst. The ultra-violet light and far blue used in fluorescence microscopy can damage DIC filters. For this reason these filters are only used to make bright field observations. Engaging the DIC optics and increasing bright field illumination whilst still using the far blue light helps to keep the body in view. Once the body has been located under bright field optics, the far blue light should be blocked off. Fluorescence, and particularly DAPI fluorescence, is subject to quenching on exposure to excitation, and may be lost completely on prolonged exposure. The bodies, however, remain visible with DIC microscopy.

Other organisms, such as algal cells and fungal spores, can exhibit some of the typical features of oocysts. This is because these organisms fall within the same size ranges as oocysts, and exhibit FITC- and DAPI-fluorescence. For this reason, it is essential that the DIC optics and illumination are set up correctly in order to ensure confirmation of internal structures can be made.

The size of the oocyst can be confirmed by taking measurements across two axes. In addition, the number, size and position of sporozoites and their nuclei can be confirmed. Sporozoites are often clustered together and may lie in a range of positions (see series of DIC images of individual oocysts in Appendix 5 - taken at different points in the focal plane). The larger “head” at one end of the sporozoite contains the nucleus and this can be confirmed by opening the epifluorescence shutter with the DAPI filter selected, and observing the fluorescent nucleus position superimposed on the DIC image (see Appendix 5).

A protoplasmic “residual body” visible by DIC microscopy may be observed within an oocyst when it actively excysts, but is often absent when an oocyst ruptures and loses its contents. The absence of identifiable contents in apparently empty oocysts can therefore be confirmed by DIC microscopy.

Bodies having only protoplasmic “residual bodies” may be described as having amorphous contents, i.e. having no discernable shape or morphological feature.

Each fluorescent body on the slide should be examined to confirm whether or not it is an oocyst. An environmental sample may contain a mixture of oocysts and other fluorescing bodies which resemble oocysts and therefore each body should be checked. Bodies which exhibit typical morphological features, or have ruptured, such that 1 - 4 sporozoites can be identified should be counted as oocysts.

Other fluorescing bodies, which resemble oocysts but are not, or which exhibit internal contents but which lack sporozoites should not be counted as oocysts. In addition, bodies with more than four nuclei or nuclei that are larger than 1.5 µm should also not be counted as oocysts . Environmental bodies that are poorly stained, badly mis-shapen and have no internal contents should not be counted as oocysts. The presence of empty bodies having amorphous contents may be recorded but should not be regarded as oocysts, as confirmation cannot be undertaken. Care should be exercised during identification as environmental oocysts can often be significantly mis-shapen, and the contents may be found outside the outer wall of the oocyst. However, empty bodies, bodies of anomalous shape or which are so mis-shapen that they are unlikely to be oocysts or which have amorphous contents which cannot be identified as sporozoites by DIC should not be counted as oocysts.

D7.3.4 *Seeking a second opinion*

Where significant numbers of oocysts are counted for a sample, it is advisable to confirm these findings by arranging for a second microscopist to examine the slides. Where doubt still remains, additional independent microscopists should be used to confirm the findings and consideration given to repeating the sampling and analysis.

D7.3.5 *Appearance of Giardia cysts with FITC staining*

Where the presence of both *Cryptosporidium* oocysts and *Giardia* cysts are to be investigated in a sample, (oo)cysts may be stained on the same slide. *Giardia* cysts exhibit a similar characteristic apple-green fluorescence shown by *Cryptosporidium* oocysts. For cysts, the fluorescence is brighter around the perimeter of the body. Cysts are generally ovoid in shape, although some cysts may be almost circular, depending on the alignment on the slide. Some cysts exhibit creases or folds. *Giardia* cysts vary greatly in size and

have been reported to measure between 8 - 18 µm long by 5 - 15 µm wide, although those involved in human infection are generally 11 - 14 µm long by 7 - 10 µm wide. Cysts which have been in the environment even for small periods of time may become badly distorted and mis-shapen. In cysts where the wall is no longer intact, the contents may appear red with Evans Blue, if this reagent is present.

D7.3.6 *Appearance of Giardia cysts with DAPI staining*

Fresh *Giardia* cysts contain between 2 - 4 distinct nuclei, usually positioned at one end of the cyst, although not all may be visible. The nuclear material in cysts which have undergone environmental exposure may deteriorate and be difficult to observe, even though the contents of the cyst remain.

D7.3.7 *Confirmation of Giardia cysts with DIC microscopy*

In fresh cysts, internal contents can be readily observed. In addition, the nuclei as shown by DAPI staining may be observed together with organelles including flagellar axonemes. Cysts which have been in the environment may lose these characteristic features in addition to their internal contents.

All bodies showing characteristic features should be counted as *Giardia* cysts. Where bodies which are empty are observed, these should be noted and recorded but should not be counted as *Giardia* cysts, as confirmation cannot be assured between *Giardia* cysts and other cyst-like bodies. Since environmental cysts tend to be empty, it may be appropriate to record these bodies as presumptive cysts.

Typical and atypical images of *Cryptosporidium* oocysts, *Giardia* cysts and other fluorescing bodies that resemble (oo)cysts are presented in Appendix 5.

D8 Calculations

The number of (oo)cysts should, for example be reported as (oo)cysts per litre, (oo)cysts per 10 litres or (oo)cysts in a larger volume of sample analysed. The following formula can be used to calculate the number of (oo)cysts per 10 litres:-

$$C = (N / V) \times 10$$

where

C is the number of (oo)cysts per 10 litres of sample;
N is the sum of the number of (oo)cysts observed and counted on all slide preparations for that sample; and
V is the volume (litres) of sample filtered.

D9 Expression of results

The counts of (oo)cysts from all the slides associated with a single sample should be reported. The count should include those bodies which satisfy the criteria as confirmed (oo)cysts. Bodies which do not satisfy the criteria may be counted and recorded but should not be included in the confirmed count. The confirmed count may be expressed as (oo)cysts per litre, (oo)cysts per 10 litres or (oo)cysts in a larger volume. Where no characteristic bodies are found, the report should state that no (oo)cysts were detected in

the original volume of water sampled. *Cryptosporidium* should be reported as *Cryptosporidium* species since individual species cannot be accurately determined by microscopy alone. Similarly, *Giardia* should be reported as *Giardia* species.

D10 Quality control

Laboratories determining the enumeration of *Cryptosporidium* oocysts and *Giardia* cysts in drinking and environmental waters should be able to demonstrate the adequacy of their procedures. This may be affected by the:

- presence of particulate material in the sample;
- equipment used to process the sample, for example, centrifuges;
- the time between filtration and elution and concentration;
- use of inappropriate or poor quality reagents;
- interferences in the IMS process;
- inadequate training of staff.

Particulate material obtained during the sampling process may include organic and inorganic material, including fibrous material, for example cellulose, and algae. This material may prevent (oo)cysts from being eluted from filters or complexing to beads, or inhibit the beads from being magnetically "attracted" by the magnet during the IMS process. In addition, an excess of particulate material transferred to the microscope slide following dissociation may make (oo)cysts difficult to study during the microscopic examination. This is particularly the case during DIC microscopy.

Centrifuges will vary in their ability to separate (oo)cysts from suspensions. It is important that the efficiency of the centrifugation process is assessed before the separation procedure is undertaken routinely. The centrifuge speed and time should be established for the optimum separation of (oo)cysts from centrifuged suspensions.

Materials and reagents used in the recovery of (oo)cysts should be verified before being used routinely. Also, materials or reagents which are past their shelf life or expiry date should be discarded. New batches of materials or reagents should be checked to ensure they give similar performance to the materials or reagents they replace. For example, (oo)cysts can be processed through the IMS stage with new batches of beads to determine whether recovery is consistent.

Care and attention to detail during sample processing is important in obtaining good recoveries. Initially, this is achieved by good analytical training and careful supervision. Ultimately, diligence throughout the technique is important, as is the precision of replicated actions, if recoveries are to be consistent. Staff should be assessed, for example on the recovery of (oo)cysts from replicate seeded samples, and the mean recovery and the standard deviation of these replicated determinations should be assessed against pre-established criteria. In general terms, the larger the standard deviation, the poorer the technique, even if the mean recovery value is considered adequate. Care in the preparation and storage of reagents together with careful processing of samples should lead to consistent recoveries.

In the UK, for samples requiring analysis for regulatory purposes, under the “competent analyst” scheme, analysts are required to be assessed for their individual performance in each analytical method. In addition, the laboratory is required to demonstrate its ability to obtain fit for purpose results. Recovery tests are related to the nature and type of sample being processed by the laboratory, for example where 1000 litres of raw water are routinely processed, it would be appropriate to perform recovery tests on 1000 litres of water, and not 10 litres of water. Because of the effect of sample matrix on recoveries, it is prudent for a laboratory to establish recoveries for the full range of sample matrices examined routinely.

Oocysts and cysts are available commercially either as viable suspensions, formalin-fixed suspensions or flow cytometry sorted suspensions. Viable suspensions of *Cryptosporidium* should be replaced at regular intervals. Formalin-fixed suspensions of *Giardia* should be replaced less frequently, depending on how frequently the analysis is carried out. Flow cytometry sorted suspensions should be kept and used in accordance with manufacturer’s instructions. When suspensions for recovery tests are prepared from commercial suspensions, dilutions should be prepared in water to provide a suspension containing approximately 100 (oo)cysts in 100 µl of water. These suspensions may be stored in the range 5 ± 3 °C but should be used within 7 days and a representative portion counted before they are used.

D10.1 *Seeded samples*

Laboratories undertaking daily *Cryptosporidium* or *Giardia* analysis as part of a routine or information-gathering programme should include a daily quality control seeded sample prepared in a similar manner as for real samples as part of their routine quality assurance programme. The data generated from these seeded samples can be used to demonstrate laboratory performance and that materials and reagents are appropriate and correctly used. In this respect, the use of quality control charts may prove useful in determining trends in analytical performance.

D10.1.1 Treated and ground waters

The quality control of treated and ground waters can be achieved by preparing seeded filters in a manner which, as closely as reasonably practical, emulates real samples. This can be carried out, for example by passing sufficient (oo)cyst-free water through a filter to thoroughly wet it, then adding a known number of oocysts onto the filter, maintaining the optimum flow of water recommended by the manufacturer until the required volume of water has passed through the filter. The filter should then be processed according to the procedures described in this booklet. Recoveries may then be plotted on a quality control chart. Action and warning limits can then be established and trends in the analytical performance followed.

D10.1.2 Raw waters

The recovery from raw waters can similarly be assessed by seeding a suitable volume of raw water sample.

D10.1.3 Filter seeding

Assuming the water to be processed contains no (oo)cysts or the numbers present are accounted for, a number of (oo)cysts can be seeded directly into a suitable volume of treated water. This seeded sample is then passed through the filter system at a flow rate recommended by the filter manufacturer whilst the filter is connected to a validation rig or other suitable apparatus. Once the seeded sample has been filtered, the container should be rinsed with water to ensure complete transfer of all the (oo)cysts. The validation rig can then be connected to the supply of treated water and a suitable volume of treated water passed through the filter. The filter can then be removed and processed.

In the case of raw water, a number of (oo)cysts can be seeded directly into a raw water sample and filtered. Once filtration is complete, the filter can be processed.

A suitable validation rig can be prepared by using standard plumbing fittings, water meter and a flow restrictor to ensure that the flow does not exceed that recommended by the filter manufacturer. Alternatively, a rig can be prepared by using commercially available pipe and fittings and this can be connected directly to a tap, or a proprietary rig can be purchased.

D10.1.4 Spiking suspension

Spiking suspensions of (oo)cysts may consist of:

- 'In-house' flow cytometry sorted (oo)cysts providing that suitable performance can be demonstrated.

- Commercially available suspensions of flow cytometry sorted (oo)cysts.

The results of quality control tests should be plotted on control charts. Control conditions can then be identified and remedial action taken in the event these conditions are breached.

If neither of the above options is available, laboratories may prepare their own suspensions by dilution and enumeration of commercially available suspensions of (oo)cysts. However, these suspensions may exhibit a performance (in terms of standard deviation) that is not as good as that achieved using flow cytometry sorted (oo)cysts.

Commercial suspensions should be stored and used according to the manufacturer's instructions. The suspensions should be diluted and stored in water and mounted, stained and counted using an appropriate number of aliquots (for example 10) of a suitable aliquot volume (for example 100 µl). Fresh suspensions should be prepared and counted before they are used to establish recovery values.

D10.1.5 External quality control

Laboratories undertaking regular analysis of drinking and raw waters should participate in a suitable external quality assurance scheme, i.e. proficiency testing (PT) scheme. The PT scheme organiser should provide prepared filters or suitable samples for analysis, as well as appropriate (oo)cyst suspensions and/or prepared slides for counting. Laboratories should keep details of such schemes and implement appropriate investigations if adverse results are obtained in the scheme. Filters, suspensions and slides should, over time, contain interfering material and blanks and (oo)cysts of various species and genotypes. These species and genotypes may or may not comply with criteria for inclusion in the count or national reporting requirements. Experience covering a range of organisms and interfering material on slides is especially important for demonstrating, maintaining and developing the competence of microscopists.

Appendix 1 Biology, transmission and public health significance of *Cryptosporidium* and *Giardia*

Cryptosporidium and *Giardia* are both genera of protozoan parasites that infect vertebrates. These parasites cause diarrhoeal disease in humans and share some common features that contribute to their water-borne transmission. These include:

(i) multiple hosts and large numbers of (oo)cysts excreted in faeces increase potential for environmental contamination;
(ii) prolonged survival of (oo)cysts in moist environments;
(iii) common occurrence of (oo)cysts in some source water;
(iv) incomplete removal of (oo)cysts by inadequately designed or operated conventional water treatment;
(v) low dose of (oo)cysts causes infection in susceptible host.

Whilst *Cryptosporidium* oocysts and *Giardia* cysts are more resistant to chlorine disinfection than most bacteria, *Cryptosporidium* oocysts are much more resistant than *Giardia* cysts.

Cryptosporidium

Cryptosporidium is a coccidian protozoan, found world-wide in a wide variety of vertebrate hosts, including humans. Some species of this parasite cause disease (namely, cryptosporidiosis) which is usually manifested as diarrhoea, although other symptoms are often present. In the last 25 years, *Cryptosporidium* has been identified as a commonly reported infectious cause of acute self-limiting diarrhoeal illness in immuno-competent individuals. The incubation period (the time from ingestion to the onset of illness) is between 2 - 14 days, and the illness commonly lasts up to three weeks. About 5000 cases of cryptosporidiosis are reported in the UK each year. In developed countries, most cases of cryptosporidiosis are reported in children aged five years or younger, although adults are also susceptible. A rise in reported adult cases may be an indicator of an outbreak situation. In developing countries, most cases of illness occur in children under the age of one year, and adult cases rarely occur. Prior exposure and acquired immunity provide some protection against the disease, particularly with repeated exposure over time. To date, there is no reliable treatment for cryptosporidiosis.

In profoundly immuno-compromised individuals with T-cell deficiencies, cryptosporidiosis can be a life-threatening condition, causing profuse intractable diarrhoea with severe dehydration, mal-absorption and wasting. An insidious form of disease may develop, affecting the biliary tract and predisposing the patient to the development of sclerosing cholangitis, cirrhosis and cholangiocarcinoma. People susceptible to particular risks should be given specific advice on the prevention of acquisition of the disease[1].

Cryptosporidium is transmitted between hosts by the faecal-oral route, either by direct contact with faeces from an infected person or animal, or via contaminated water, food or contact with a contaminated object. The transmissive stage of the disease occurs via the oocyst which acts as a protective shell for the four infective sporozoites contained within. When oocysts are ingested, sporozoites (triggered by exposure to heat, acid, trypsin and/or bile salts within the intestinal tract) subsequently emerge. This process is called excystation. The motile sporozoites invade the epithethial cells of the lining of the gastro-intestinal tract. Asexual reproduction then occurs, followed by a sexual reproductive cycle.

Infection can result in physiological and transport defects in the gut epithelium, leading to profuse watery diarrhoea, which may contain mucus. Other symptoms include abdominal pain, nausea, vomiting, fever, and anorexia resulting in dehydration and weight loss, although infection can also occur without resulting illness ("asymptomatic" carriage). The *Cryptosporidium* life cycle is "direct", requiring no intermediate host, and culminates in the shedding of large numbers of oocysts from infected hosts.

The oocyst is important in facilitating transmission of the parasite. It is also the main target for the recovery and detection in environmental samples and the diagnostic target in clinical laboratories. The sensitivity of these tests relies on efficient recovery and detection of oocysts. These tests only provide identification to the genus level. The taxonomy of *Cryptosporidium* is continually under review, and there are currently 20 species, of which 18 are supported by genetic data (see Table 1). To identify species and subtypes, genetic loci are targeted using molecular techniques (see Appendix 2).

Two species of *Cryptosporidium* predominate in human disease, *Cryptosporidium parvum* and *Cryptosporidium hominis.* Neonatal disease in livestock is caused by *Cryptosporidium parvum*. These two species present the biggest public health risk (see Table 2) and other species (to date) have not been identified in the causes of outbreaks of cryptosporidiosis. Other species have been identified in humans and animals (Table 1) and may be present in water or environmental samples. These species vary in their human infectivity and pathogenicity. Since traditional diagnostic tests and immunologically-based methods for oocyst recovery and detection, which are widely used in water testing laboratories, are genus-specific, it is likely that any of these species may be detected but not differentiated. Multiple species may be present in environmental samples that are influenced by faecal contamination from numerous animal hosts.

Infectivity studies using *Cryptosporidium parvum* and *Cryptosporidium hominis* isolates in humans have been undertaken and show that, while differences occur between isolates, the infectious dose is low. The lowest ID_{50} (i.e. the number of oocysts required to cause infection in 50 % of those people exposed) for *Cryptosporidium parvum* was 9 oocysts and for *Cryptosporidium hominis*, 18 oocysts. However, the ID_{50} is not a measure of the minimum infectious dose and it is widely accepted that, based on extensive animal studies and mathematical modelling of experimental infections, a single oocyst might infect and cause disease in some susceptible people.

Giardia

Giardia duodenalis (synonymous with *Giardia intestinalis* and *Giardia lamblia*) is the only species of the genus *Giardia* known to infect humans. This flagellated protozoan parasite was discovered by van Leeuwenhoek about 300 years ago, and is now recognised as one of the most common world-wide protozoan parasitic causes of diarrhoea (giardiasis) in humans. Similar numbers of giardiasis and cryptosporidiosis cases are reported in the UK each year. Giardia infects the small intestine of humans, causing a spectrum of diseases ranging from asymptomatic to severe diarrhoea. Other symptoms are abdominal pain, bloating, flatulence, malaise, sulphurous belching, nausea and vomiting. The incubation period is between 5 - 25 days. The acute phase is usually self-limiting in immune-competent individuals but lasts for at least 10 days, and may persist for several months. Symptoms during the chronic phase can relapse in short recurrent bouts during which other symptoms appear. Immune-deficient patients often suffer chronic disease, and, if untreated, mal-absorption and debilitation may occur. Asymptomatic infection (carriage of the parasite) also occurs.

Table 1 Named species of *Cryptosporidium* with mean size range

Cryptosporidium species	Mean oocyst size (range)* (µm)	Host from which originally isolated	Natural host range	Confirmed in humans (using molecular techniques)
Intestinal species				
Cryptosporidium parvum	5.0 x 4.5 (4.5-5.4 x 4.2-5.0)	mice	mammal	Yes
Cryptosporidium hominis	5.2 x 4.9 (4.4-5.9 x 4.4-5.4)	human	human	Yes
Cryptosporidium felis	4.6 x 4.0 (3.2-5.1 x 3.0-4.0)	cat	mammal	Yes
Cryptosporidium canis	5.0 x 4.7 (3.7-5.9 x 3.7-5.9)	dog	mammal	Yes
Cryptosporidium wrairi	5.4 x 4.6 (4.8-5.6 x 4.0-5.0)	Guinea pig	Guinea pigs	No
Cryptosporidium suis	4.6 x 4.2 (4.4-4.9 x 4.0-4.3)	pig	pig, human	Yes
Cryptosporidium bovis	4.9 x 4.6 (4.8-5.4 x 4.2-4.8)	cattle	cattle, sheep, pig	No
Cryptosporidium scophthalmi **	4.4 x 3.9 (3.7-5.0 x 3.0-4.7)	fish	fish	No
Cryptosporidium fayeri	4.9 x 4.3 (4.5-5.1 x 3.8-5.0)	Red kangaroo	marsupial, sheep	No
Cryptosporidium macropodum	5.4 x 4.9 (5.0-6.0 x 4.5-6.0)	Eastern grey kangaroo	marsupial	No
Cryptosporidium ryanae	3.7 x 3.2 (2.9-4.4 x 2.9-3.7)	cattle	not yet known	No
Gastric species				
Cryptosporidium andersoni	7.4 x 5.5 (6.0-8.1 x 5.0-6.5)	cattle	cattle	Yes
Cryptosporidium muris	7.0 x 5.0 (6.5-8.0 x 5.0-6.5)	mice	mammal	Yes
Cryptosporidium serpentis	6.2 x 5.3 (5.6-6.6 x 4.8-5.6)	snake	snake	No
Cryptosporidium galli (synonymous with *Cryptosporidium blagburni*)	8.3 x 6.3 (8.0-8.5 x 6.2-6.4)	bird	bird	No
Cryptosporidium molnari **	4.7 x 4.5 (3.2-5.5 x 3.0-5.0)	fish	fish	No
Cryptosporidium fragile	6.2 x 5.5 (5.5-7.0 x 5.0-6.5)	toad	not yet known	No
Multi-site species				
Cryptosporidium meleagridis	5.2 x 4.6 (4.5-6.0 x 4.2-5.3)	turkey	mammal	Yes
Cryptosporidium baileyi	6.2 x 4.6 (5.6-6.3 x 4.5-4.8)	chicken	chicken	No
Cryptosporidium varanii (synonymous with *Cryptosporidium saurophilum*)	4.8 x 4.7 (4.8-5.1 x 4.4-4.8)	lizard	lizard	No

* Length x width, per original description where possible (usually on the measurements of at least 50 oocysts).
** No genetic data available.

Table 2 Risk to UK public health posed by different *Cryptosporidium* species and genotypes[1]

High risk [2]	**Uncertain risk [3]**	**No known risk [4]**
Cryptosporidium parvum	*Cryptosporidium meleagridis*	*Cryptosporidium bovis*
Cryptosporidium hominis	*Cryptosporidium felis*	*Cryptosporidium baileyi*
Rabbit genotype	*Cryptosporidium canis*	*Cryptosporidium wrairi*
	Cryptosporidium muris	*Cryptosporidium varani*
	Cryptosporidium andersoni	*Cryptosporidium scophthalmi*
	Cryptosporidium suis	*Cryptosporidium serptentis*
	Cervine genotype	*Cryptosporidium molnari*
	Skunk genotype	*Cryptosporidium galli*
	Cryptosporidium hominis monkey genotype	*Cryptosporidium fayeri*
	Horse genotype	*Cryptosporidium macropodum*
	Pig genotype II	*Cryptosporidium ryanae*
	Chipmonk genotype II	*Cryptosporidium fragile*
	Cryptosporidium parvum mouse genotype	Numerous genotypes found in animals or raw waters

[1] *Cryptosporidium* species are named based on multiple criteria, but genetic data only are often available and many genotypes are described without designation of species.
[2] Known human pathogens causing a high burden of sporadic illness or causative agent in an outbreak.
[3] Known human pathogens causing a low burden of sporadic illness or isolated from humans but pathogenicity uncertain.
[4] No reported isolates from humans.

Transmission of the disease is often direct from person-to-person, and within families is common. Water-borne disease occurs as the cysts exhibit some resistance to chlorine. The *Giardia* life cycle is direct, requiring no intermediate host, and the parasite exists in two distinct morphological forms, namely, the reproductive trophozoite (which attaches to the enterocytes of the upper small intestine) and the environmentally-resistant cyst, excreted in faeces, which is the main transmissive stage. The cyst is the usual target for detection and diagnosis, although trophozoites are sometimes seen in faeces and they share common antigens.

Only *Giardia duodenalis* has been associated with human disease, but other species occur in other animal hosts (Table 3). *Giardia* genotypes are commonly grouped in assemblages. Only *Giardia duodenalis* assemblages A and B have so far been found in humans (Table 3), and the prevalence varies geographically.

Transmission of *Cryptosporidium* and *Giardia*

Transmission can occur by any route where infective *Cryptosporidium* oocysts or *Giardia* cysts are ingested by a susceptible host. Both *Cryptosporidium* oocysts and *Giardia* cysts, can survive for prolonged periods in cool, moist environments. Person-to-person transmission is the most commonly documented route for *Cryptosporidium hominis* and *Giardia duodenalis* and probably *Cryptosporidium parvum*. Onward spread to, and by, secondary cases can be significant, as can transmission from asymptomatic carriers. Food-borne transmission has been reported for *Cryptosporidium parvum* and *Giardia duodenalis.*

Water can be a major route of transmission of *Cryptosporidium* and *Giardia*, especially in untreated supplies, facilitated by the robust nature of (oo)cysts and varying resistance to chemical disinfection. *Cryptosporidium* oocysts are resistant to chlorine at concentrations used to treat drinking and swimming pool waters and *Giardia* cysts show some resistance to chlorine disinfection. Both drinking waters and recreational waters have been linked to outbreaks of cryptosporidiosis and giardiasis, although outbreaks caused by *Cryptosporidium* occur more frequently than those caused by *Giardia* in the UK, where most outbreaks are linked to farm visits, travelling abroad and use of swimming pools.

Detection of *Cryptosporidium* and *Giardia* and assessment of risk

The methods for the detection of *Cryptosporidium* and *Giardia* described in this booklet are limited in the information they provide for the assessment of risk to public health since they provide no information about the species or potential infectivity for humans, neither in terms of the species specificity nor the viability of the organisms detected.

Methods for determining species, genotypes or subgenotypes are essential for distinguishing human from non-human pathogens and are useful for investigating the source of contamination or infection, causative links in outbreaks and tracking the spread of disease. These methods are based on amplification and analysis of genetic loci, and it is important that the methods used are validated for the sample matrix under investigation. Only methods for typing *Cryptosporidium* from water samples have been validated, and are described in outline in Appendix 2.

Table 3 *Giardia* species and *Giardia duodenalis* genotypes*

Species	Genotype (proposed species)	Subgroup	Host range
*Giardia duodenalis***	Assemblage A	AI	humans, livestock, dogs, cats, beavers, Guinea pigs, slow loris
		AII	humans
	Assemblage B (*Giardia enterica*)		humans, slow loris, chinchillas, dogs, beavers, rats, siamang
	Assemblage C (*Giardia canis*)		dogs
	Assemblage E (*Giardia bovis*)		cattle, goats, pigs, sheep, alpaca
	Assemblage F (*Giardia cati*)		cats
	Assemblage G (*Giardia simondi*)		domestic rats
Giardia agilis			amphibians
Giardia microti			muskrats, voles
Giardia muris			rodents
Giardia psittaci			birds
Giardia ardae			birds

* Adapted from reference 2.
** synonymous with *Giardia lamblia and Giardia intestinalis*

Methods for the determination of *Cryptosporidium* oocyst viability in water or environmental samples are not widely available and are not extensively evaluated or standardised. These methods are mainly based on the use of cell culture and quantification of cell invasion following *in vitro* excystation, by reverse transcriptase PCR (which amplifies mRNA transcripts, present only in viable cells) or by staining and microscopic observation of infectious foci in host cells. Ribosomal RNA-directed probes have also been used in fluorescence *in situ* hybridisation assays.

References

1. Chief Medical Officer 1999. CMO's update 23. *Cryptosporidium* in water: clarification of the advice to immunocompromised.

2. Unravelling *Cryptosporidium* and *Giardia* epidemiology. *Trends in Parasitology*, S M Caccio, R C A Thompson, J McLauchlin and H V Smith, 2005, **21**, pp430-437.

3. General Biology in Cryptosporidium and Cryptosporidiosis, R Fayer, Eds R Fayer and L Xiao, 2007 CRC Press Boca Raton pp1-42.

4. The species of *Cryptosporidium* (Apicomplexa: Cryptosporididae) infecting mammals, *J Parasitol*, S J Upton and W L Current, 1985, **71**, 625.

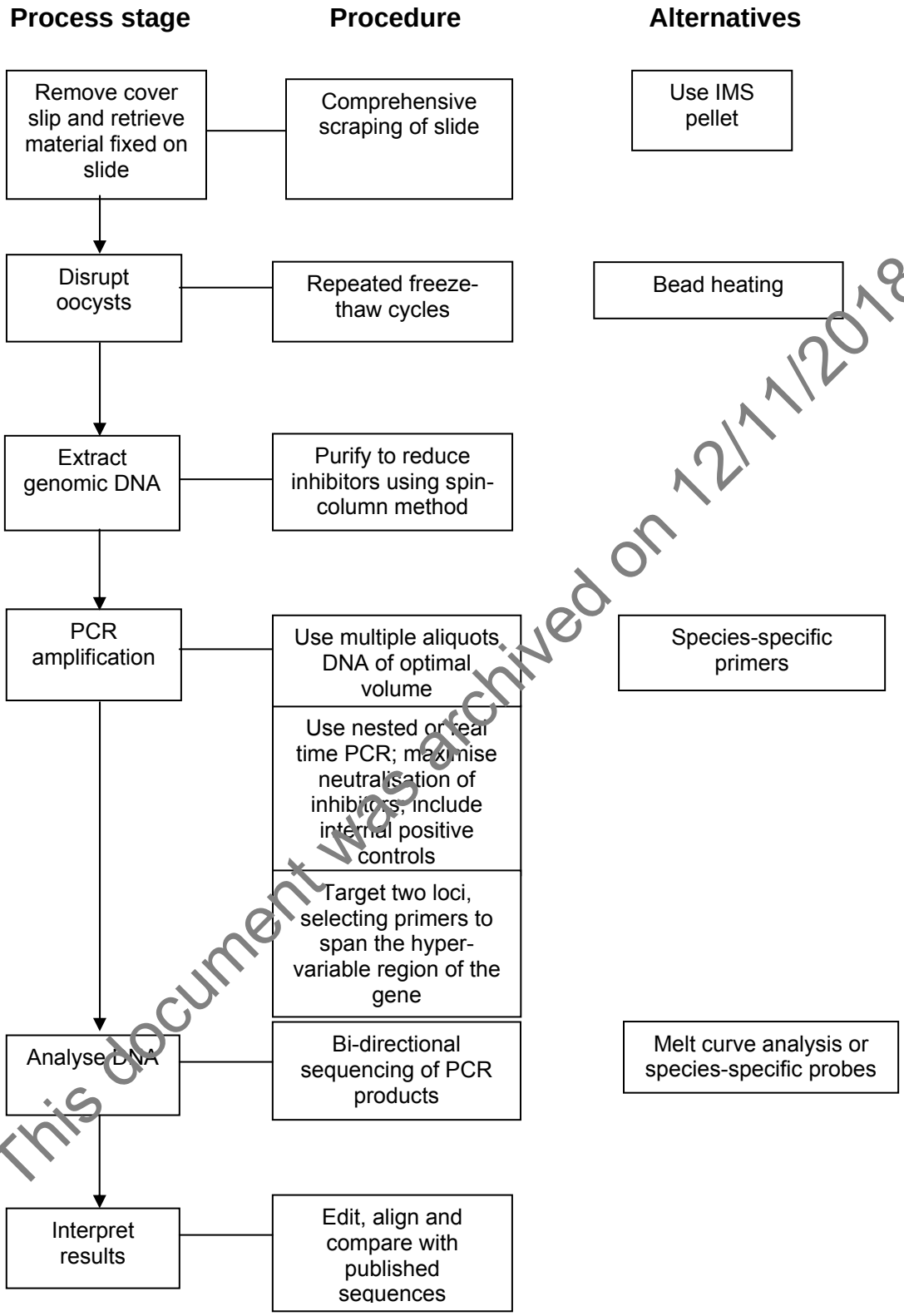
Appendix 2 Overview of the rationale and principles of using molecular methods for the identification of *Cryptosporidium* species

The methods described in this booklet for the isolation and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts in waters and associated matrices use antibody-based procedures to isolate and identify the (oo)cysts by microscopy. The antibodies are genus specific, and do not currently enable the identification the species of *Cryptosporidium* to be ascertained. Of the 20 currently recognised *Cryptosporidium* species, 8 have been identified in humans, and two, *Cryptosporidium parvum* and *Cryptosporidium hominis*, cause the majority of human cryptosporidiosis, particularly in developed countries. Not all *Cryptosporidium* oocysts detected are infectious or pathogenic to humans (see Appendix 1, Table 2). Differentiation of species is also helpful in catchment management and risk assessment and in epidemiologic (for example, outbreak) investigations since it may help identify the source of contamination and infection, and link cases of illness to outbreaks.

A variety of oocysts have been found in the aquatic environment, often in the same sample[1, 2, 3] and the detection of low numbers of oocysts in waters when investigating the species present is analytically challenging. Genetic analysis is central to species identification, and involves optimised oocyst and sporozoite disruption, DNA extraction, DNA amplification by polymerase chain reaction (PCR) and finally, analysis and comparison of the PCR products. Methods have been developed and evaluated for testing oocyst-positive microscope slides from drinking water samples[3], IMS pellets from storm water samples[4] and from IMS pellets from raw surface and waste waters[1, 5] with optimisation of DNA extraction and relief from PCR inhibitors[6]. The process is outlined in Figure 1. As with all microbiological tests applied to water, information from a single sample may not be sufficiently informative and a structured sampling programme should be established to enable interpretation of the results. Likewise, the number of analytic replicates at optimal DNA concentration is important for improving sensitivity and increasing the chance of detecting co-contaminating species or genotypes in the same sample[4]. The use of appropriate internal positive control DNA increases the confidence, where negative results are reported.

Prior to DNA extraction, oocysts are disrupted to expose the sporozoites contained within. A freeze-thaw technique has been shown suitable for waters and environmental samples[3, 7]. A method for identifying *Cryptosporidium* species and/or genotypes present in water samples involves sequence analysis of the small subunit ribosomal DNA (ssu rDNA), amplified by PCR. There are five copies of the ssu rRNA gene in each sporozoite and PCR primers are available that amplify all known species and genotypes. However, careful primer selection is required to avoid non-specific amplification. The use of restriction fragment length polymorphism (RFLP) profiles to identify the species and/or genotypes is limited by the overlap between the profiles of those species that may be present. Although DNA sequence analysis provides unequivocal identification, it is not without difficulty, particularly since water and environmental samples frequently contain more than one *Cryptosporidium* species and/or genotype. Sequencing in these circumstances can be facilitated by optimally diluting the DNA and undertaking multiple PCR amplifications[4, 8]. Up to date knowledge is required to avoid mis-interpretation of RFLP and sequence data in this evolving field. Validation trials have shown that a proportion of samples do not amplify at particular loci. This does not seem to be related to the number of oocysts present, and may be overcome by targeting an alternative locus[3]. If this includes the ssu rRNA gene, the results from one or the other can be used with

Figure 1 Optimal methods for identification of *Cryptosporidium* species from environmental samples



confidence. The use of a positive control known isolate is valuable but should be an appropriate rarely isolated species or genotype.

This method is cumbersome, expensive and time-consuming to perform and results are not usually available quickly (i.e. the process may take 3 - 5 days). In some circumstances, it may be sufficient to identify whether the oocysts present are those that present the greatest risk to public health, i.e. *Cryptosporidium parvum* and *Cryptosporidium hominis*. In this case, species-specific primers or probes may be used. Other approaches for comprehensive identification may include the development of micro-arrays and reverse line blotting. An alternative approach to the molecular analysis of extracted DNA is the use of fluorescence *in situ* hybridisation (FISH). This can be combined with routine microscopy by the use of fluorescently-labelled target-specific probes (for example for *Cryptosporidium parvum* and/or *Cryptosporidium hominis*). However, full scale evaluation has not been undertaken and the staining protocol would need to be revised.

References

1 Molecular characterisation of *Cryptosporidium* oocysts in samples of raw surface water and wastewater, L Xiao, A Singh, J Limor, T K Graczyk, S Gradus and A Lal, *Applied and Environmental Microbiology*, 2001, **67**, pp1097-1101.

2 Distribution of *Cryptosporidium* genotypes in storm event water samples from three watersheds in New York, J Jiang, K A Alderisio, L Xiao, *Applied and Environmental Microbiology*, 2005, **71**, pp4446-4454.

3 Molecular fingerprinting of *Cryptosporidium* oocysts isolated during water monitoring, R A B Nichols, B M Campbell, H V Smith, *Applied and Environmental Microbiology*, 2006, **72**, pp5428-5435.

4 Detection of *Cryptosporidium* oocysts in water: effect of the number of samples and analytic replicates on test results. L Xiao, K A Alderisio, J Jiang, *Applied and Environmental Microbiology*, 2006, **72,** pp5942-5947.

5 Identification of species and sources of *Cryptosporidium* oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool, L Xiao, K Alderisio, J Limor, M Royer, A A Lal, *Applied and Environmental Microbiology*, 2000, **60**, pp5492-5498.

6 Development of procedures for direct extraction of *Cryptosporidium* DNA from water concentrates and for relief of PCR inhibitors, J Jiang, K A Alderisio, A Singh, L Xiao, *Applied and Environmental Microbiology*, 2005, **71**,1135-1141.

7 Investigating the public health significance of *Cryptosporidium* in the environment, G Robinson, PhD thesis, 2005, Cardiff University.

8 Molecular forensic profiling of *Cryptosporidium* species and genotypes in raw water, N J Ruecker, N Bounsombath, P Wallis, C S Ong, J L Isaac-Renton, N F Neimann, *Applied and Environmental Microbiology*, 2005, **71**, pp8991-8994.

Appendix 3 Typical recovery efficiencies for *Cryptosporidium* oocysts and *Giardia* cysts

Cryptosporidium oocysts

Water type	Concentration method	Sample clean-up	Recoveries (%)	Reference
Reagent (50 litres)	Filta-Max	IMS	42 - 62	1
Surface (50 litres)	Filta-Max	IMS	20 - 55	1
Surface (50 litres)	Filta-Max	IMS	11 - 41 *	7
Final (>1000 litres)	Filta-Max	IMS	16 - 60	3
Tap (1000 litres)	Filta-Max	IMS	8 - 57	8
Treated (>1000 litres)	Envirochek	IMS	34 - 73 (n=10)	9
Treated (>1000 litres)	Filta-Max	IMS	19 - 53 (n=10)	9
Treated (>1000 litres)	Filta-Max	IMS	33 - 57 (n=10)	9
Treated (>1000 litres)	Envirochek	IMS	72 - 84 (n=5)	9
Treated (>1000 litres)	Envirochek	IMS	43 - 61 (n=5)	9
Treated (>1000 litres)	Envirochek	IMS	45 - 97 (n=5)	9
Treated (>1000 litres)	Envirochek	IMS	31 - 50 (n=5)	9
Reagent (10 litres)	Envirochek	IMS	31 - 59	2
Surface (10 litres)	Envirochek	IMS	17 - 61	2
Surface (10 litres)**	293 mm polycarbonate	IMS	36 *	4
Surface (10 - 20 litres)**	293 mm polycarbonate	IMS	10 - 88	6
Final (100 litres)	293 mm polycarbonate	IMS	10 - 78	6
Tap (10 litres)	Calcium carbonate flocculation	IMS	6 - 89	5
Surface and ground (10 litres)	Filta-Max *xpress*	IMS	7 - 59 (n=72)	7
Treated (1000 litres)	Filta-Max *xpress*	IMS	5 - 72 (n=66)	7

Giardia cysts

Water type	Concentration method	Sample clean-up	Recovery (%)	Reference
Reagent (50 litres)	Filta-Max	IMS	41 - 70	1
Surface (50 litres)	Filta-Max	IMS	54 - 69	1
Surface (50 litres)	Filta-Max	IMS	5 - 46 *	7
Surface (10 litres)**	293 mm polycarbonate	IMS	48 *	4
Surface (10 - 20 litres)**	293 mm polycarbonate	IMS	12 - 89	6
Final (100 litres)	293 mm polycarbonate	IMS	6 - 91	6
Tap (10 litres)	Calcium carbonate flocculation	IMS	3 - 97	5

* range of mean recoveries from several sources
** membrane 2 µm pore

References

1. USEPA method 1623, "*Cryptosporidium* and *Giardia* in water by filtration/IMS/FA", Validation of Genera Technologies Filta-Max™ foam filters and 50 litres sample volumes under the USEPA performance based measurements system, Z Bukhari, Report 7 March 2000, Clancy Environmental Consultants, Inc.

2. USEPA method 1622, "*Cryptosporidium* in water by filtration/IMS/FA", J L Clancy, Z Bukhari, R M McCuin, Z Matheson and C R Fricker, *Journal of the American Water Works Association*, 1999, **91**, pp60-68.

3. Continuous sampling for *Cryptosporidium* in treated water supplies. Drinking Water Inspectorate Report, August 1999.

4. Comparison of methods for the concentration of *Cryptosporidium* oocysts and *Giardia* cysts from raw waters, C Ferguson, C Kaucner, M Krogh, D Deere and M Warnecke, *Canadian Journal of Microbiology*, 2004, **50**, pp675-682.

5. Data submitted to the Standing Committee of Analysts, A Jonas.

6. Data submitted to the Standing Committee of Analysts, G Ault.

7. Data submitted to the Standing Committee of Analysts, IDEXX Laboratories Inc.

8. Data submitted to the Standing Committee of Analysts, H Shapland.

9. Data submitted to the Standing Committee of Analysts, J Watkins.

Appendix 4 Validation procedures

1 Introduction

Improvements to collection devices, reagents and equipment used to test water for *Cryptosporidium* and *Giardia* may occur at any time. Such improvements might increase the recovery of the parasites whilst at the same time reduce the amount of staff time and analytical expense incurred in sampling and analysis. This part of the booklet provides guidance on the validation procedures to be used for sampling equipment and methods or parts of methods used for analysis. In addition, once a new method or part method has been evaluated, a laboratory may wish to incorporate it as part of its routine procedures. This part of the booklet also gives guidance on the test procedures that a laboratory should use 'in-house' before they use a new or modified method.

The performance of the trial method should be evaluated using a reference material comprising a known number of (oo)cysts in suspension. The target number of (oo)cysts in each aliquot used to compare the trial and reference methods should be 100. In addition, the evaluation should use a variety of different water types to ensure that recovery is not significantly affected by specific types of waters and that the trial method can process the appropriate volume of water, for example as shown in Table A (section 2). The variety of treated waters should include 'hard', 'soft', 'upland surface', 'lowland surface', and 'ground' waters. Similar criteria should be applied to other sample matrices, and to the effects of turbidity on untreated surface waters and similar matrices containing suspended material. Details of the chemical composition of each water type used should also be included. Following validation studies, the procedure for the new method should be fully documented (including statistical analysis) and may then be considered for publication in this series of booklets. The new method should include data derived from the validation studies with appropriate quality control requirements, and guidance on the correct expression of results obtained using the method.

2 The approach to be adopted

For new filtration devices and new or modified analytical methods, the validation is divided into two phases.

(i) Phase 1: a single laboratory validation to determine whether any method used within a laboratory produces results that are statistically superior to or not significantly different from the performance of any one of the methods AA, AB or AD described in section A.

(ii) Phase 2: a multi-laboratory exercise to determine whether, in a representative number of laboratories, the method produces results that are statistically superior to or not significantly different from the performance of any one of the methods AA, AB or AD described in section A.

The outcome of these studies should be that the trial method should be at least better than or equivalent to the reference method at a stated level of confidence. If the trial method does not find significantly larger numbers of (oo)cysts, then the average difference between results from the two methods should be comparable with the null hypothesis of zero difference (with 95 % probability).

3 Performance parameters

The following criteria for method performance are considered relevant and it is expected that these should have been studied in preliminary validation studies before the trial method is used in this validation process:

- Correct identification of (oo)cysts.
- Selectivity, where other objects and particulate material are removed without excessive loss of (oo)cysts.
- Recovery of (oo)cysts at the target number. Un-seeded blanks should be included with each trial.
- Recovery rate at 50 % and 200 % of the target number.

The outcome of phase 1 and phase 2 studies should also assess the ease of use of the trial method.

3.1 *Phase 1 within laboratory study*

The within laboratory study may involve the examination of new filtration devices, new reagents or new equipment for the recovery of (oo)cysts from treated or untreated water or related materials. New filtration devices should be capable of filtering the appropriate volume of water required for the test. All new methods should separate (oo)cysts from water samples compatible with any of the methods described in this booklet. Failure to meet these criteria may require modification of the test protocol or materials and re-testing before phase 2 of the study can be undertaken.

The laboratory undertaking phase 1 of the study should select three sites appropriate to the type of water for which the modification is intended. The sites should include a variety of waters of different characteristics. In the case of treated waters, distribution sites may be selected, and in the case of raw waters, samples of appropriate volume may be transported to the laboratory. For ground waters where only disinfection is carried out, the treated water may be used. The test should be repeated on five separate days although these need not be consecutive.

3.1.1 Filter seeding

Two filters are seeded with the target number of (oo)cysts. This can be carried out by seeding two separate 10 litre quantities of tap water (in a container) with (oo)cysts and passing the seeded water through separate filters whilst connected to a validation rig or other suitable apparatus. The two filters used may be of different construction to enable a comparison to be carried out of the performance of one kind of filter against another, or they may be the same kind and one filter is processed by the test method and the other filter is processed by the reference method. The flow of water through the filter should be that recommended by the manufacturer. Once empty, the containers should be rinsed out with water to ensure complete transfer of (oo)cysts to the filters. For both filters, for treated waters, a suitable quantity of the water is then passed through the filters at a flow rate recommended by the manufacturer and the filters are then processed. In the case of raw waters, the (oo)cysts can be seeded directly into raw water samples and the filters processed once filtration is complete. For each test an un-seeded filter should also be

included, up stream of treated water to act as a negative control.

Oocysts and cysts used for the seeding experiments may be:

- Purchased as commercially available flow cytometry sorted (oo)cysts where numbers are known. The (oo)cysts should be suspended and transferred according to the manufacturer's instructions.

- Laboratories may prepare their own suspensions using flow cytometry.

- Laboratories may prepare their own suspensions using commercially available suspensions of (oo)cysts.

Where laboratories prepare their own suspensions using commercially available (oo)cysts, the concentration of (oo)cysts should be enumerated by performing a number of counts (typically ten) on an aliquot of suspension (typically 100 µl) on each day that a test is carried out using the mounting and staining techniques described in this booklet. In addition, any commercial suspensions used in these studies should be used within the manufacturer's shelf life, expiry or use-by date.

3.1.2 Filter processing

Each filter is processed according to any one the appropriate methods described in this booklet incorporating suitable controls where required. Where there is a modification in the analytical method, for example a change to the IMS stage, the control filter eluate may be divided into two equal portions and one portion processed by each method. The negative control filter used in these studies should not contain (oo)cysts. Should a positive result be obtained, for example with a raw water, and providing that the result is no greater than 10% of the target number, the data may be accepted provided that a correction is made for the level of (oo)cysts present in the control. Where the control level is greater than 10 % of the target number, consideration should be given to using an alternative site. In addition, it may be necessary to use (oo)cysts which have been pre-stained to differentiate them from background organisms.

It is important that, where possible, one site should contain a significant amount of particulate material, for example equivalent to a pellet volume of between 0.3 - 0.5 ml. If none of the sites contains significant particulate material, such material can be prepared (see section 3.1.5). In the case of a treated water, the material can be added to the filter during seeding and for raw waters, the material can be added directly to the raw water sample.

3.1.3 Target number

Checks should also be made on five occasions at one site using half (i.e. 0.5x) the target number and twice (i.e. 2x) the target number of (oo)cysts respectively. Paired samples plus controls should be used on each occasion. These multilevel checks do not need to be carried out on the same day. If recovery with the modified procedures appears to be concentration-dependent, the study should be repeated using further sets of samples.

3.1.4 Extended filtration

For new filtration devices, two extended exercises should be undertaken at each treated

water site. This should cover a minimum period of 48 hours. The laboratory should ensure that the filter continues to operate over the 48 hour period by measuring the flow rate through the filter after 48 hours using a flow meter or a suitable measuring device (for example a measuring cylinder).

3.1.5 Preparation of particulate material

Particulate material may be obtained from surface water using wound polypropylene or pleated membrane cartridge filters with a nominal pore size of 1 µm to separate and collect the material. Water can be pumped through the filter in its housing without the need for flow measurement or flow restriction. Where necessary a 12 volt leisure battery and submersible pump can be used. Alternatively, a filter can be attached to the raw water tap. Water is filtered until a suitable amount of particulate material has been collected.

The particulate material is eluted from the filter by cutting the filter from its core, teasing out the fibres and washing them in 0.1 % polyoxyethylene sorbitan monooleate (for example Tween 80) in water. Washing can be carried out manually or in an appropriate machine (for example a Stomacher device). The particulate material is separated from the detergent by centrifugation at an appropriate speed (for example 1500 rcf for 30 minutes) and discarding the supernatant liquid. The final pellet should be washed in water and centrifuged (for example 1500 rcf for 30 minutes). The pellet volume is measured and the material suspended in water such that a suitable aliquot (for example 1.0 ml) of the suspension provides a pellet volume of 0.5 ml. The material may be stored in the range 5 ± 3 °C for up to 3 months.

3.1.6 Statistical analysis

Once the phase 1 study is complete, a statistical analysis should be conducted to ascertain whether the new or modified method produces results that are superior to or not significantly different from the results produced by the reference method. In addition, adequate documentation of the new method should be produced to ensure that the laboratories participating in phase 2 of the study can perform the procedure clearly and unambiguously without mis-interpretation (see section 6).

4 Phase 2 inter-laboratory trials

Phase two of the study should consist of an inter-laboratory trial involving at least five laboratories, one of which can be the laboratory undertaking phase 1 of the study. This applies to new filtration devices, modifications to the analytical technique or the microscopic examination. The test procedure should be identical to that used in phase 1 of the study. Each laboratory should, where practicable, identify two water sites to be tested and these should be from different sources, i.e. comprise water of different characteristics. If a laboratory is unable to test two types of waters, then the waters from all of the participating laboratories should cover a range of water types likely to be encountered.

Each site is tested for three days using two seeded filters at the target number of (oo)cysts and one un-seeded filter and the recovery of (oo)cysts from each filter is determined. The three day trial does not need to be carried out on consecutive days, but different laboratories should not use the same water source. If a laboratory is unable to test two water types, then the waters analysed by all of the participating laboratories should cover a range of water types likely to be encountered.

The collective data from the phase 2 of the study should be subjected to statistical analysis and, providing that phase 1 and phase 2 of the studies demonstrate statistical equivalence, all the data should be submitted to the Standing Committee of Analysts (SCA) for review. Once reviewed, the new method can be considered for publication into a revised booklet within this series.

5 Adoption by a laboratory

Once published, laboratories can decide whether to use the new method. However, it is important that each laboratory ensures, as appropriate, that it is able to use the new method appropriately and that the new method gives recoveries which are at least equivalent to those produced by the laboratory's existing method. This can be carried out using a single water site and seeding two filters to be tested in parallel either with a treated water or a raw water. Filters are seeded as described in section 3.1.1. A negative control filter is not required. Paired trials should be carried out over 12 days for treated, raw and surface waters although these need not be consecutive, and the filters should be processed according to the procedures defined in this booklet.

The data generated should demonstrate that recoveries with the trial product or method are superior to or not significantly different from the recoveries produced by the reference product or method, at an acceptable confidence level, for example 95 %.

6 Statistical analysis

Statistical analysis of the data derived from phase 1 and phase 2 studies is used to ascertain whether the results produced by the trial or modified method of analysis or equipment is superior to or not significantly different from the results produced by the reference method of analysis or equipment. The number of samples tested and the quality of the data will determine the significance and the confidence in the conclusions made. The number of sample tests suggested are expected to be the minimum required for demonstrating that the trial method is probably fit for purpose. If no satisfactory conclusions can be made from these results then more evidence may be required.

For a statistical analysis, numbers should not be rounded up or down. Any rounding off of decimal places should only be undertaken on the result at the end of the statistical analysis. Any rounding of numbers during the statistical analysis could render the results invalid.

In the following paragraphs, guidance is given on the statistical tests to be used and suitable statistical approaches for use with these tests. The correct statistical tests and the most appropriate statistical methods/approach should therefore be determined. All the raw data should be included in reports on both phase 1 and phase 2 studies, including any data not used in the statistical analysis (i.e. outliers). Data summaries should appear in tables and the key comparisons made between the trial and reference methods.

Statistical results should be presented in an informative way rather than as raw output from a statistical package. For example, statistic values such as averages and test parameters should be given with the number of digits which are significant, rather than as meaningless strings of decimal places. (Decimal places are needed during calculations to avoid cumulating inaccuracies but rounding up or down is carried out only for the final answer which is then presented in the report.)

The main analysis should test the null hypothesis that there is no significant difference in the number of (oo)cysts which the two methods detect. Therefore the numerical difference between paired results should be the key observation analysed. A clear distinction should be made between counts and recovery estimates, and both may have their place. If exact and identical numbers of (oo)cysts were presented to the two methods then the analyses of counts and of recoveries should be equivalent. If there is an unknown variation in numbers of (oo)cysts in the two portions examined by the methods then the recovery 'rates' become ratios of two variables. This introduces complication in the theoretical application of parametric statistical methods. Therefore initial analyses should focus on counts.

The null hypothesis of zero average difference in paired counts can be tested by parametric methods (usually the paired t-test) or by a non-parametric equivalent (for example, the sign test). Parametric analysis can be used if the differences are approximately distributed in a Gaussian manner, i.e. normally distributed. If the data are not normally distributed, then non-parametric statistics provide a more valid approach which is more efficient at detecting true differences between methods.

If, overall, the test method shows a significantly better or significantly worse case, then the trial is conclusive, with the proviso discussed below concerning consistency. If there is 'no significant difference' between the two methods then the 95 % confidence interval of the estimated average difference should be presented and discussed. If the lower value of this interval implies that the trial method could be very much worse than the reference method then this will affect the conclusions. In this case, more samples may need to be examined to increase the power of the study and therefore reduce the confidence interval and clarify the worst-case scenario.

Consistency of the method comparison between sites (in the phase 1 study) or between laboratories (in the phase 2 study) should be apparent from scrutinising the data, for example plotted as graphs. Concerns about inconsistencies should be commented upon. Statistical tests (such as one-way analysis of variance of the differences) can be used to test the null hypothesis of consistency, but the planned study sizes are not large enough to detect small differences. Significant inconsistencies should be discussed as they may affect the prudence of the new method for general use. Preliminary data and data complementary to the main comparison, for example data generated by the manufacturer prior to the phase 1 study, may be useful assessing any inconsistencies.

Tabulations should give details of the data collected and characteristics of the waters from the different sites, giving dates, numbers of (oo)cysts seeded, volumes, controls etc. The numbers of (oo)cysts found by both methods should be given and differences shown. The counts from the two methods should be plotted, with differences highlighted between results from different sites and, where appropriate, levels of contamination. A second plot of recoveries (or estimated recovery ratios) should be drawn. The plots should show the line of equality. Comments on the graphs should be presented.

Summaries of the counts and the differences should be presented (for example means, medians, ranges).

A statistical test of the null hypothesis of zero average difference should be carried out - a paired t-test should be used if the differences are approximately normally distributed or a non-parametric equivalent (for example the sign test) should be used if they are not.

The 95 % confidence interval for the estimated average difference should be calculated and reported.

A one-way analysis of variance of the differences should be carried out to test the null hypothesis of 'same average difference for each site', or a non-parametric equivalent should be undertaken. If appropriate, for the site with 3 contamination levels, the average difference in results from the two methods can be compared between the levels.

In order to clarify the statistical approach outlined above, three examples are given, see Appendix 4A. The first describes a fictitious example, but uses data adapted from real trials. The example illustrates a straight-forward study where no significant difference between two products is demonstrated. The second example describes a previously undertaken study comparing two different para-magnetic (IMS) bead approaches, and illustrates more complex outcomes. Despite much lower recoveries being obtained by both methods at the beginning of the trial, the data have been included in the calculations. The third example presents ‘in-house’ results for the evaluation of a new IMS bead which has passed phase 1 and phase 2 testing and which a laboratory would like to use but which did not participate in either of the phase 1 or phase 2 studies.

In the worked examples the graphics were produced in Excel, making a dot-plot with the results from the reference method plotted on the x-axis and the paired results by the trial method on the y-axis. The ranges on both axes are the same and the plot area is made approximately square with the diagonal line of equality added. Groups of samples (for example from different sites in the phase 1 study and from different laboratories in the phase 2 study) are identified using a 'data series' option.

The statistical analyses were carried out using Minitab. Differences between paired results were calculated and the TTEST and TINT functions were applied to the difference column to provide a paired t-test and 95 % confidence interval. Non-parametric equivalents approaches were obtained with the STEST and WINT functions. Minitab allows one way analysis of variance to compare differences between sites or laboratories when the data are normally distributed, and the Kruskal-Wallis analysis when a nonparametric method is more appropriate.

Appendix 4A Typical examples

Example 1 Fictitious trial method versus reference method

Phase 1 study - Data from the comparison of a trial method and reference method for recovering and enumerating *Cryptosporidium* oocysts - single laboratory study.

Three separate treated water sources were used in the trial. In the study report, the characteristics of these waters should be described, as should the methodology of the study. This worked example concentrates on the presentation and analysis of results.

Five samples were used from two sites, prepared on five different days, and each seeded with 100 oocysts counted using flow cytometry. Nine samples were used from a third site: three samples were seeded with 50 oocysts, three samples with 100 oocysts and three samples with 150 oocysts. The results are summarised in Tables 1.1 and 1.2.

Table 1.1 Summary of counts from the comparison of a trial method with a reference method

Site	Number of seeded oocysts	Trial method count	Reference method count	Difference	Recovery (%)	
					Trial method	Reference method
1	100	44	49	-5	44	49
1	100	52	50	2	52	50
1	100	66	60	6	66	60
1	100	60	49	11	60	49
1	100	49	54	-5	49	54
2	100	38	36	2	38	36
2	100	30	43	-13	30	43
2	100	37	33	4	37	33
2	100	53	30	23	53	30
2	100	44	35	9	44	35
3	50	24	11	13	48	22
3	50	12	22	-10	24	44
3	50	30	30	0	60	60
3	100	65	53	12	65	53
3	100	55	47	8	55	47
3	100	43	52	-9	43	52
3	150	77	83	-6	51	55
3	150	80	90	-10	53	60
3	150	95	70	25	63	47
		12 - 95	11 - 90	-13 to +25		

Samples from 3 sites. All 19 samples were seeded with known numbers of oocysts

Table 1.2 Summary of the statistical analysis of all the data from phase 1 of the study

	Trial method	Reference method	Difference
Number	19	19	19
Mean	50.2	47.2	3.0
Median	49	49	2
Standard deviation	20.58	19.64	10.95
Range	12 - 95	11 - 90	-13 to +25

No oocysts were found in any control tests.

Figure 1.1 shows the plot of oocyst counts by the two methods and includes the line of equality. Eleven points lie above the line where the trial method gave the higher count, 7 points lie below where the reference method gave the higher count and one point lies on the line where the difference was zero. The differences are widely distributed but there is no obvious non-symmetry which would invalidate use of the t-test analysis, which is sufficiently robust for small departures from Gaussian distributed counts i.e. normality. (For 'non-normally distributed' data, the non-parametric analysis will be more efficient, but for approximately 'normally distributed' data the analyses should be equivalent. For illustrative approaches, both approaches will be used in this example. The statistical package Minitab has been used.)

Figure 1.1 Comparison of trial and reference methods using oocyst counts
(Based on 3 sites and 3 levels of seeding at site 3)

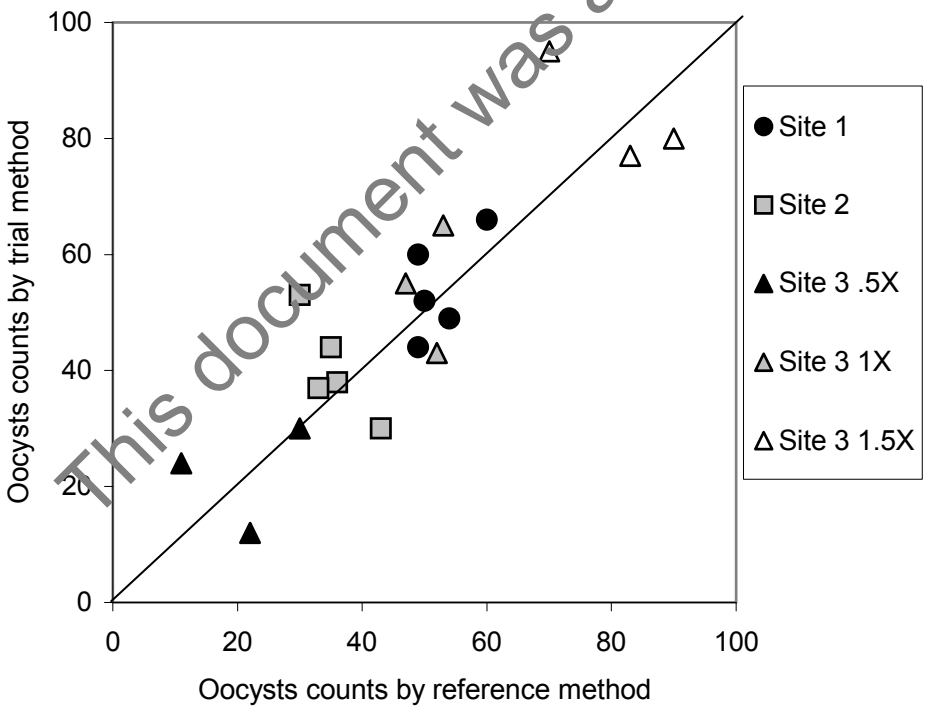
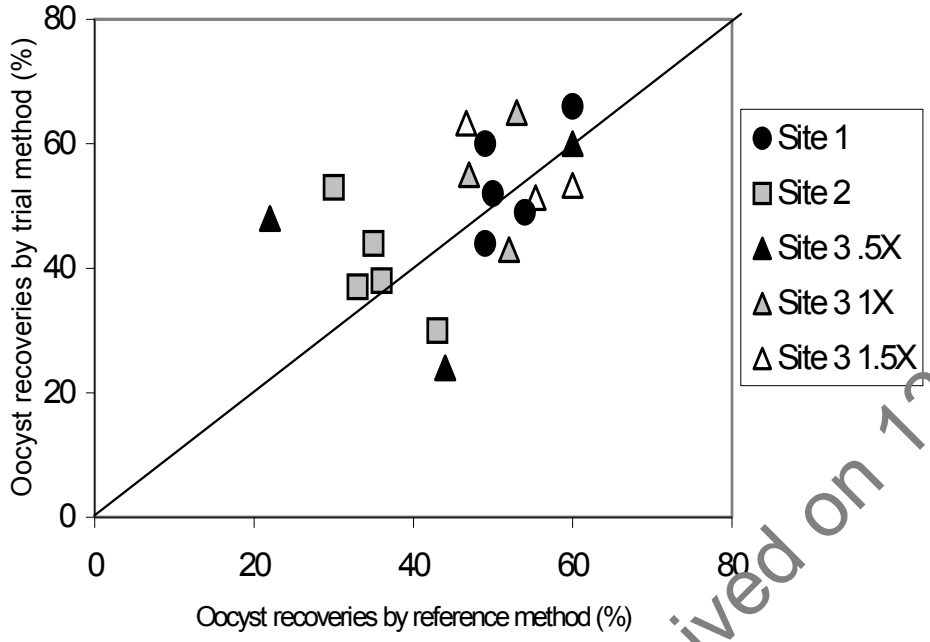


Figure 1.2 shows the plot of recovery percentages which shows similar evidence, except that the scatter of differences appear slightly larger for the three samples seeded with 50 oocysts. This may be an effect of using lower numbers of oocysts to seed some of the samples.

Figure 1.2 Comparison of trial and reference methods using percentage recoveries (Based on 3 sites and 3 levels of seeding at site 3)



Testing the null hypothesis that there is no difference in the average number of counts between the two methods for all the sites

paired t-test (n = 19):
mean difference = +3, t = 1.19, p = 0.25

sign test of the median:
median = +2, where 11 results were positive and four results were negative, p = 0.48, which is the probability of tossing a coin 18 times resulting in 7 or fewer 'heads' or 7 or fewer 'tails'.

95 % confidence for the average difference

From the t-test, the confidence interval, CI, is given by:

$$CI = \text{mean} \pm t_{95} (s/\sqrt{n})$$

where
t_{95} is the t-table entry for 95 % confidence level
(n-1) is the number of degrees of freedom and
s is the standard deviation.

This gives a confidence interval of -2.3 to +8.3 (i.e. 3 ± 5.3)

The Wilcoxon confidence interval for the median difference is -2.5 to +8.5

This study has shown no overall significant difference between the two methods.

Comparison of the sites

The mean differences for the three sites are:

Site 1 mean = 1.8 (from 5 paired samples)
Site 2 mean = 5.0 (from 5 paired samples)
Site 3 mean = 2.6 (from 9 paired samples)

Table 1.3 shows a summary of the one-way ANOVA.

Table 1.3 One way analysis of variance between the three sites

Source	Number of degrees of freedom	Sum of squares	Mean square	F-value	p-value
Between sites	2	29	14	0.11	0.90
Residual	16	2129	133		
Total	18	2158			

There is no significant difference between sites. This is also the finding from carrying out the non-parametric Kruskal-Wallis test (p = 0.95).

Conclusion

The statistical comparison of the methods shows no significant difference.

The 95 % confidence interval for the mean difference give the lower end of the range as -2.3 and upper end of the range as 8.3, estimated from 19 samples where the reference method gave a mean of 47.2 oocysts. This suggests an estimated worse-case scenario of the trial method, on average, finding 95 % as many oocysts as the reference method (*i.e.* 5 % fewer) and the best-case scenario of 118 % as many oocyst (*i.e. 18* % more) (calculated by 100(47.2-2.3) / 47.2 and 100(47.2+8.3) / 47.2, expressed as percentages).

These findings are very similar to non-parametric analyses as, in this example, the differences are approximately normally distributed. Both methods of statistical analysis are given for illustrative purposes. The 95 % confidence interval for the median difference is -2.5 to 8.5 from 19 samples giving a median of 49 oocysts by the reference method. This suggests that the trial method would find between 95 % and 118 % as many oocysts from similar waters.

There is no evidence that the method comparison was affected by the water sites.

As this phase was satisfactory, phase 2 of the study went ahead.

Phase 2 study - Data from the comparison of a trial method and a reference method for recovering and enumerating *Cryptosporidium* oocysts - inter-laboratory study

Five laboratories took part, each examining three samples from each of two different sources, totalling six samples per laboratory and 30 samples overall. Each sample was seeded with 100 oocysts. The resulting counts are shown in Table 1.4. Recovery percentages are the same as the counts, because of the original concentration of 100 oocysts. The statistical summary is shown in Table 1.5.

Table 1.4 Data from the inter-laboratory phase 2 study

Laboratory (Site)	Reference method	Trial method	Difference
1(1)	44	52	8
1(1)	37	39	2
1(1)	55	51	-4
1(2)	55	60	5
1(2)	40	49	9
1(2)	60	50	-10
2(1)	37	68	31
2(1)	38	55	17
2(1)	43	54	11
2(2)	45	47	2
2(2)	50	60	10
2(2)	50	50	0
3(1)	33	38	5
3(1)	35	35	0
3(1)	31	40	9
3(2)	57	49	-8
3(2)	41	57	16
3(2)	63	56	-7
4(1)	50	49	-1
4(1)	41	55	14
4(1)	54	65	11
4(2)	65	44	-21
4(2)	46	66	20
4(2)	45	59	14
5(1)	66	70	4
5(1)	61	65	4
5(1)	59	64	5
5(2)	70	76	6
5(2)	49	59	10
5(2)	68	55	-13
	31 - 70	35 - 76	-21 to +31

Counts from the trial method and the reference methods from 30 samples each seeded with 100 oocysts.

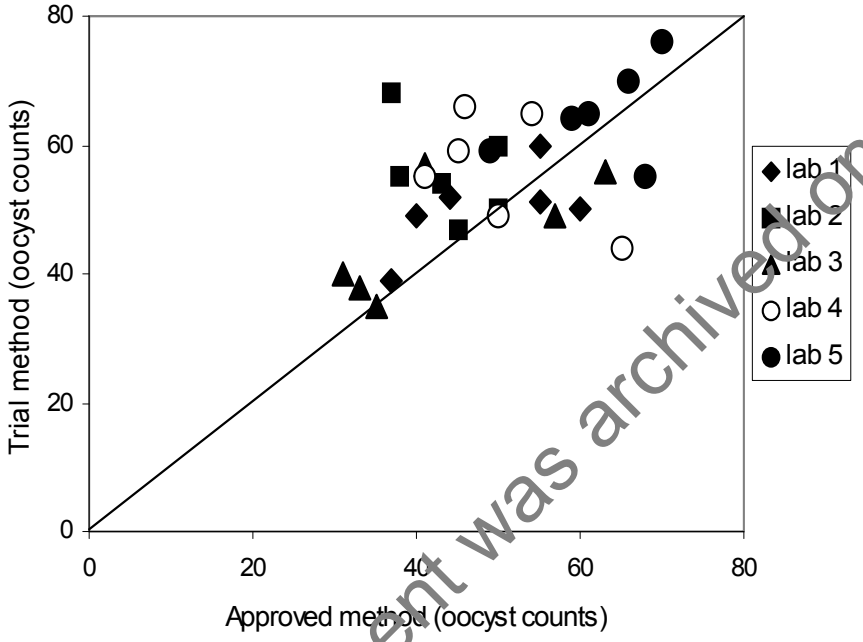
Table 1.5 Summary of the statistical analysis of all the data from phase 2 of the study

	Reference method	Trial method	Difference
Mean	49.6	54.6	5.0
Median	50	55	5
Standard deviation	11.98	9.93	10.53
Range	31 to 70	35 to 76	-21 to +31

No oocysts were found in any control tests.

The data are plotted in Figure 1.3.

Figure 1.3 Oocysts recovered during the phase 2 inter-laboratory study



This figure shows the plot of counts of oocyst recoveries by the two methods and includes the line of equality. Twenty-one points lie above the line, where the trial method gave the higher count and 7 points lie below the line where the reference method gave the higher count. Two points lie on the line, where the difference in counts was zero.

The differences are widely distributed but there is no obvious non-symmetry which would invalidate use of parametric statistics.

Testing the null hypothesis that there is no difference in average counts between the two methods for all sites:

Paired t-test (n = 30)
Mean difference = +5.0, t = 2.58, p = 0.015

Thus, overall, there were significantly higher counts on average from the trial method as compared with the reference method.

95% confidence interval for the average difference

From the t-test, the confidence interval, CI, is given by:

$$CI = \text{mean} \pm t_{95} (s/\sqrt{n})$$

where
t_{95} is the t-table entry for 95 % confidence level
(n-1) is the number of degrees of freedom and
s is the standard deviation.

This gives a confidence interval of +1.03 to +8.90, (i.e. 5 ± 3.97) which lies entirely above zero. A study of the individual laboratories should reveal whether there is agreement.

Figure 1.3 shows that four of the five laboratories indicate a mixture of points above and below the line (with the majority of samples above, in all cases). Laboratory 2 showed no point lying below the line - with five samples giving the higher count by the trial method and the sixth sample showing zero difference.

Individual t-tests of each set of six results show:
Laboratory 1: mean difference = 1.7, t = 0.5, p = 0.6
Laboratory 2: mean difference = 11.8, t = 2.6, p = 0.05
Laboratory 3: mean difference = 2.5, t = 0.7, p = 0.5
Laboratory 4: mean difference = 6.2, t = 1.0, p = 0.4
Laboratory 5: mean difference = 2.7, t = 0.8, p = 0.5

All the laboratories except one showed a positive average difference which was not significantly different from zero. Laboratory 2 showed that the probability of the null hypothesis was exactly 5 %.

Table 1.6 shows a summary of the one-way ANOVA.

Table 1.6 One way analysis of variance between the five laboratories

Source	Number of degrees of freedom	Sum of squares	Mean square	F-value	p-value
Between laboratories	4	425	106	0.95	0.45
Residual	25	2792	112		
Total	29	3217			

There is no significant difference between laboratories. It can be seen from Figure 1.3 that there was variation in the numbers of oocysts recovered from some sites. In particular, for laboratory 3, the first site gave low recoveries for all three samples but this did not affect the comparison between the two methods.

Conclusion

The phase 2 study showed an overall significantly higher recovery of oocysts from the trial method as compared with the reference method. None of the participating laboratories showed significant disagreement.

Example 2 Study of trial method versus reference IMS bead method

Phase 1 study - data from trial IMS-1 method and a reference IMS-2 method for the recovery of *Cryptosporidium* oocysts - single laboratory trial

Three treated waters were used for the phase 1 study. These were an upland river source, a lowland river source and a borehole. Treated water was sampled in distribution as opposed to at water treatment works using paired Idexx Filta-Max^{R} filters. Four paired samples were used at the first site where two paired filters were seeded with 78 oocysts and the other two paired filters were seeded with 124 oocysts. Five paired filters were used at the second site, each seeded with 124 oocysts and four filters were used at the third site each seeded with 124 oocysts. A single additional filter was used as a negative control. All the negative controls were zero. Oocyst concentrations were determined by staining 10 x 20 μl of the seed suspension in accordance with procedures described in this booklet. The studies were conducted in 2000.

The results for the phase 1 study are summarised in Tables 2.1 and 2.2.

Table 2.1 Summary of counts from the comparison of the trial IMS-1 method with the reference IMS-2 method

Site	Number of seeded oocysts	Recovery IMS-1	Recovery IMS-2	Difference	Recovery (%) IMS-1	Recovery (%) IMS-2
1	78	15	15	0	19	19
1	78	18	24	-6	23	31
1	124	71	67	4	57	54
1	124	71	56	15	57	45
2	124	89	59	30	72	48
2	124	79	62	17	64	50
2	124	71	67	4	57	54
2	124	77	62	10	62	54
2	124	99	62	37	80	50
3	124	64	69	-5	52	56
3	124	75	72	3	60	58
3	124	43	62	-19	35	50
3	124	63	67	-4	51	54
		15 - 99	15 - 72	-19 to +37		

Samples from 3 sites with filters seeded with known numbers of oocysts.

Table 2.2 Summary of the statistical analysis of all the data from the phase 1 study

	IMS-1	IMS-2	Difference
Mean	64.2	57.6	6.6
Median	71	62	4
Standard deviation	25.0	17.6	15.3
Range	15 to 99	15 to 72	-19 to +37

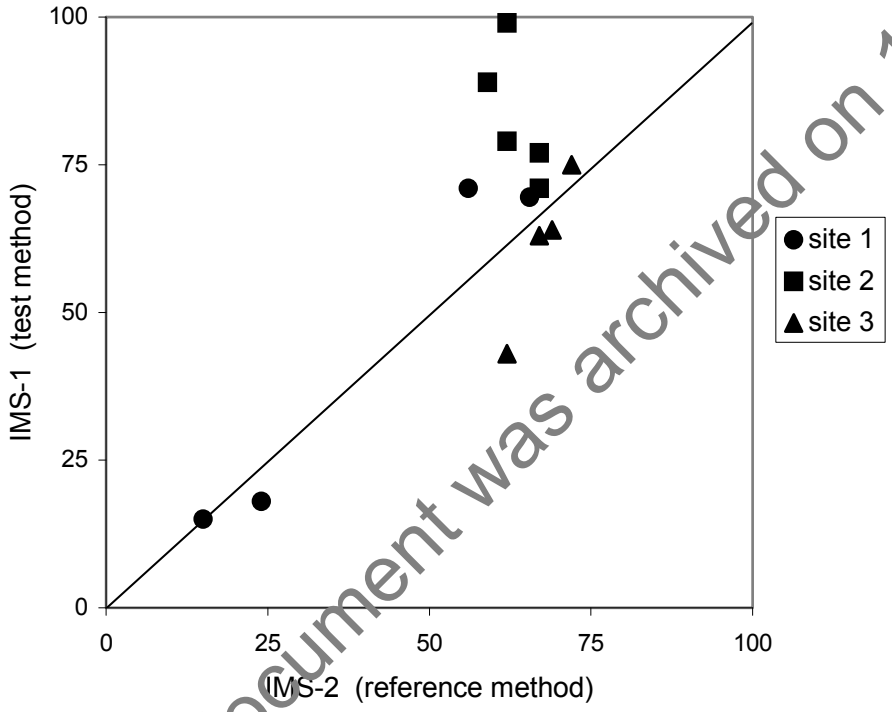
The data are also plotted in Figure 2.1. This shows the plot of the oocyst counts by the two methods and includes the line of equality. Eight points lie above the line where the trial IMS 1 method gave higher counts and four points lie below the line where the

reference IMS 2 method gave higher counts. One point is on the line where the difference in counts was zero.

The differences for the results between the two methods are widely distributed but there is no obvious non-symmetry which would invalidate the use of the t-test, which is robust for small departures from normally distributed data. (For non-normal data the non-parametric analysis will be more efficient but for approximately normally distributed data both statistical analyses should be equivalent). For illustrative purposes both approaches have been used for this example. The statistical package Minitab has been used in this example.

The data plotted in Figure 2.1 comprise the individual counts. Plots of the percentage recoveries show a similar picture.

Figure 2.1 Oocysts recovered during the phase 1 study



Testing the null hypothesis that there is no difference in the average counts between the methods for all the sites

paired t-test (n = 13):
mean difference = +6.6, t = 1.56, p = 0.14

sign test of the median:
median = +4, where 8 results were positive and four results were negative, p = 0.38.

95% confidence for the average difference

From the t-test, the confidence interval, CI, is given by

$$CI = \text{mean} \pm t_{95} (s/\sqrt{n})$$

where
t_{95} is the t-table entry for 95 % confidence level
(n-1) is the number of degrees of freedom and
s is the standard deviation.

This gives a confidence interval of -2.6 to +15.8 (i.e. 6.6 ± 9.2)

The Wilcoxon confidence interval for the median difference is -2 to +16.5

Comparison of the sites

Figure 2.1 appears to show inconsistency between the sites in that site 2 has all five samples above the line, *i.e.* there was a higher recovery of oocysts with the trial IMS-1 method. The other two sites had a mixture of positive and negative differences, with site 3 having three of the four points below the line.

The average difference (IMS-1 - IMS-2) in counts for the three sites are:

Site 1 mean = 3.3, median = 2 (from four paired samples)
Site 2 mean = 19.6, median = 17 (from five paired samples)
Site 3 mean = -6.3, median = -4.5 (from four paired samples)

Table 2.3 shows a summary of the one-way ANOVA.

Table 2.3 One way analysis of variance between the three sites

Source	Number of degrees of freedom	Sum of squares	Mean square	F-value	p-value
Between sites	2	1550	775	6.25	0.017
Residual	10	1243	124		
Total	12	2793			

There is therefore significant difference between the sites. This is also the finding from the non-parametric Kruskal-Wallis test. If the three sites are looked at separately, site 2 shows significantly higher counts by the trial IMS-1 method than by the reference IMS-2 method (paired t-test, t = 3.19, p = 0.03). Sites 1 and 3 show no significant difference from zero average difference (site 1, p = 0.5, 95 % confidence interval for the mean difference is between -11 to +17; site 3, p = 0.3, 95 % confidence interval for the mean difference is between -21 to +8).

Conclusion from the phase 1 study

Figure 2.1 shows more samples where the trial method gave the higher count. The statistical comparison overall of the methods shows no significant difference with a 95 % confidence interval for the mean difference of -2.6 to +15.8. This lower value implies a worst case of the trial IMS-1 method giving mean counts of (2.6/57.6) x 100 percent, 4.5 % worse than the reference IMS-2 method and at best (15.8/57.6) x 100 per cent, i.e. 27.4 % better i.e. a range of -4.5 % to +27 %.

However, there is statistically significant variation between the three sites from which the samples were taken. This might not affect the overall conclusion that the trial IMS-1 method was as good or better than the reference IMS-2 method if there can be confidence that it was not significantly worse at any particular site. In fact, with only four samples from two of the sites it is unlikely that significant differences, even if they were large, would be detected.

At this stage of the study, the evidence for the trial IMS-1 method looked promising and phase 2 of the study went ahead. There may be some issues about how the reference IMS-2 method behaves with the sample types derived from site 3. In this scenario, more samples may need to be taken from site 3 to establish whether there is a significant difference at this site.

Phase 2 study - data from the trial IMS-1 method and the reference IMS-2 method for the recovery of *Cryptosporidium* oocysts - inter-laboratory study

Five laboratories, each using two sites for source water participated in the study. Each laboratory examined six paired samples, three from each of their sites. The results are summarised in Table 2.4.

Table 2.4 Data from the inter-laboratory study

Laboratory (Site)	Oocyst concentration	Recovery IMS-1	Recovery IMS-2	Difference	Recovery (%) IMS-1	Recovery (%) IMS-2
1(1)	94	60	50	10	64	53
1(1)	94	60	48	12	64	51
1(1)	94	46	61	-15	49	65
1(2)	94	52	48	4	55	51
1(2)	94	43	51	-8	46	54
1(2)	94	41	36	5	44	38
2(1)	86	22	21	1	26	24
2(1)	86	45	18	27	52	21
2(1)	86	35	28	7	41	33
2(2)	86	45	55	-10	52	64
2(2)	86	51	30	21	59	35
2(2)	86	32	31	1	37	36
3(1)	113	48	52	-4	42	46
3(1)	113	39	55	-16	35	49
3(1)	113	45	53	08	40	47
3(2)	113	3	15	-12	3	13
3(2)	113	7	17	-10	6	15
3(2)	113	7	20	-13	6	18
4(1)	113	21	96	-75	19	85
4(1)	113	102	60	42	90	53
4(1)	113	48	85	-37	42	75
4(2)	113	62	88	-26	55	78
4(2)	113	68	91	-23	60	81
4(2)	113	42	71	-29	37	63
5(1)	98	34	30	4	35	31
5(1)	98	36	34	2	37	35
5(1)	98	34	32	2	35	33
5(2)	98	38	52	-14	39	53
5(2)	98	44	46	-2	45	47
5(2)	98	44	34	10	45	35
		3 - 102	15 - 96	-75 to +42	3 - 90	13 - 85

Figure 2.2 shows a plot of oocyst counts by the two methods and includes the line of equality. Fourteen points lie above the line where the test IMS-1 method gave the higher count and 16 points lie below the line where the reference IMS-2 method gave the higher count. The scatter of results for laboratory 4 appears larger than for the other laboratories.

Recovery percentages are also shown in Table 2.4 and are plotted in Figure 2.3. Figure 2.3 shows similar findings to Figure 2.2.

Figure 2.2 Oocyst counts during the phase 2 inter-laboratory study

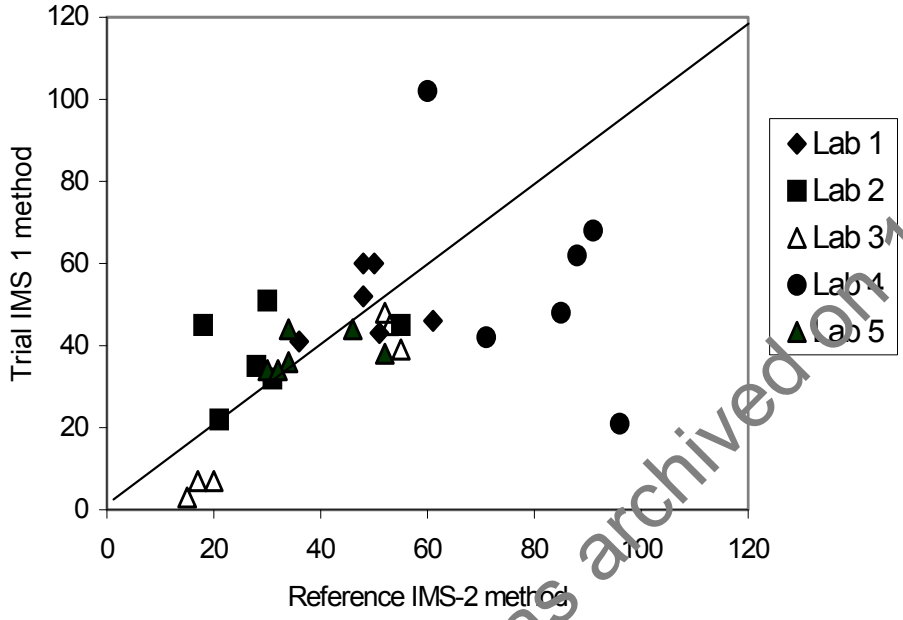
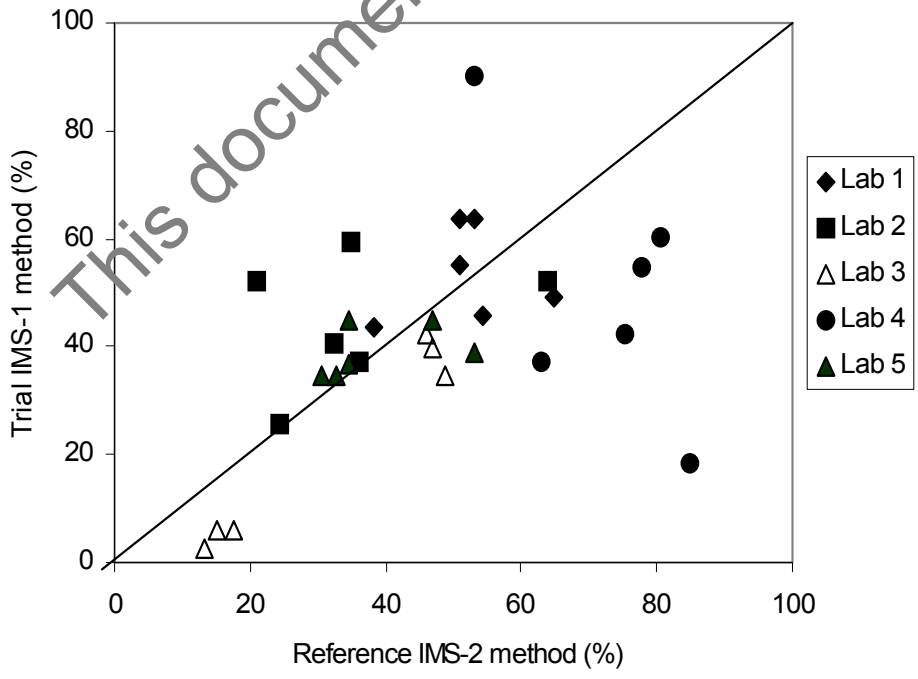


Figure 2.3 Oocyst recovery rates during the phase 2 inter-laboratory study



The statistical summary is shown in Table 2.5

Table 2.5 Summary of the statistical analysis of all the data from the phase 2 study

	IMS-1 counts	IMS-2 counts	Difference	IMS-1 % Recovery	IMS-2 % Recovery
Mean	41.8	46.9	-5.1	42	46
Median	43.5	48	-3	42	7
Standard deviation	19.3	22.5	21.2	18.2	19.4
Range	3 to102	15 to 96	-75 to 42	3 to 90	13 to 85

Testing the null hypothesis that there is no difference in the average counts between the methods

paired t-test (n = 30):
mean difference = -5.1, t = -1.33, p = 0.19.

sign test of the median:
median = +4, where 14 results were positive and 16 were negative, p = 0.8.

95 % confidence interval for the average difference

From the t-test, the confidence interval, CI, is given by

$$CI = \text{mean} \pm t_{95} (s/\sqrt{n})$$

where
t_{95} is the t-table entry for 95% confidence level
(n-1) is the number of degrees of freedom and
s is the standard deviation.

This gives a confidence interval between -13.0 to +2.8 (i.e. -5.1 ± 7.9)

The Wilcoxon confidence interval for the median difference is -11 to +2.5

Comparison of the laboratories

Figure 2.2 appears to show inconsistency between the laboratories. Four of the five laboratories show a mixture of points above and below the line of equality and none of these four laboratories show a significant difference between the methods (either by t-test or by sign test). Laboratory 3 shows all six samples giving higher counts by the reference IMS-2 method and therefore the points are all below the line. This laboratory found significantly higher counts by the reference IMS-2 method than by the test IMS-1 method (* see Table 2.6).

Table 2.6 Average differences (IMS-1 - IMS-2) in counts for the five laboratories

Laboratory	Mean	Median	Paired samples	p-value from t-test	95 % confidence interval
1	1.3	4.5	6	0.77	-10 to +12
2	7.8	4	6	0.22	-7 to +22
3	-10.5	-11	6	0.001	-15 to -6*
4	-24.7	-27.5	6	0.17	-64 to +16
5	0.3	2	6	0.92	-8 to +9

Table 2.7 shows a summary of the one-way ANOVA.

Table 2.7 One way analysis of variance between the five laboratories

Source	Number of degrees of freedom	Sum of squares	Mean square	F-value	p-value
Between sites	4	3901	975	2.69	0.055
Residual	25	9080	363		
Total	29	12981			

This shows that the significant difference between laboratories is not quite significant, with the probability being 0.055, slightly greater than 0.05. The corresponding non-parametric Kruskal-Wallis test does show significant difference between laboratories (p=0.02). Because of the varying scatter of observations from laboratories the non-parametric analysis may be more reliable. The data have already established that laboratory 3 found the reference IMS-2 method to be significantly better whereas the other laboratories found no significant difference between the methods.

Conclusion

Figure 2.2 shows slightly more samples where the trial method gave the lower count. The statistical comparison overall of the two methods shows no significant difference with a 95% confidence interval for the mean difference -13 to +3. The lower level implies a worst case scenario of the trial IMS-1 method giving mean counts of (13.0/46.9) x 100 percent, i.e. 27.7 % worse than the reference IMS-2 method and at best (2.8/46.9) x 100 percent i.e. 6 % better, i.e. a range of -28% to +6%.

However, there is statistically significant variation between laboratories and for one laboratory (laboratory 3) the trial IMS-1 method came out as significantly worse than the reference IMS-2 method. For the other laboratories there was no significant difference. This makes it difficult to draw a conclusion which can be safely extrapolated to other laboratories. Under these circumstances, further samples may need to be analysed by laboratory 3 to determine whether there is a significant difference.

Example 3 Laboratory study of a new reference IMS bead

In this example, a new reference IMS bead method is compared with a previous reference bead method for the recovery of oocysts from treated water, where the laboratory wishes to use the new reference method but did not take part in either of the phase 1 or phase 2 studies. Two filters were seeded with 100 oocysts and 1000 litres of water passed through each filter in the laboratory. Twelve paired samples were run on this occasion. Filters were eluted, the particulate material concentrated by centrifugation and then particulate material from one filter processed using the previous reference IMS method and the second processed using the new reference IMS method. The results of these tests are summarised in Tables 3.1 and 3.2.

Table 3.1 Statistical analysis of a previous reference IMS method with a new reference IMS method

Paired sample	Previous reference IMS method	New reference IMS method	Difference
1	41	50	-9
2	49	53	-4
3	46	52	-6
4	41	39	2
5	41	49	-8
6	45	23	22
7	48	57	-9
8	53	46	7
9	19	23	-4
10	46	48	-2
11	43	42	1
12	34	40	-6
	34 - 53	23 - 57	-16

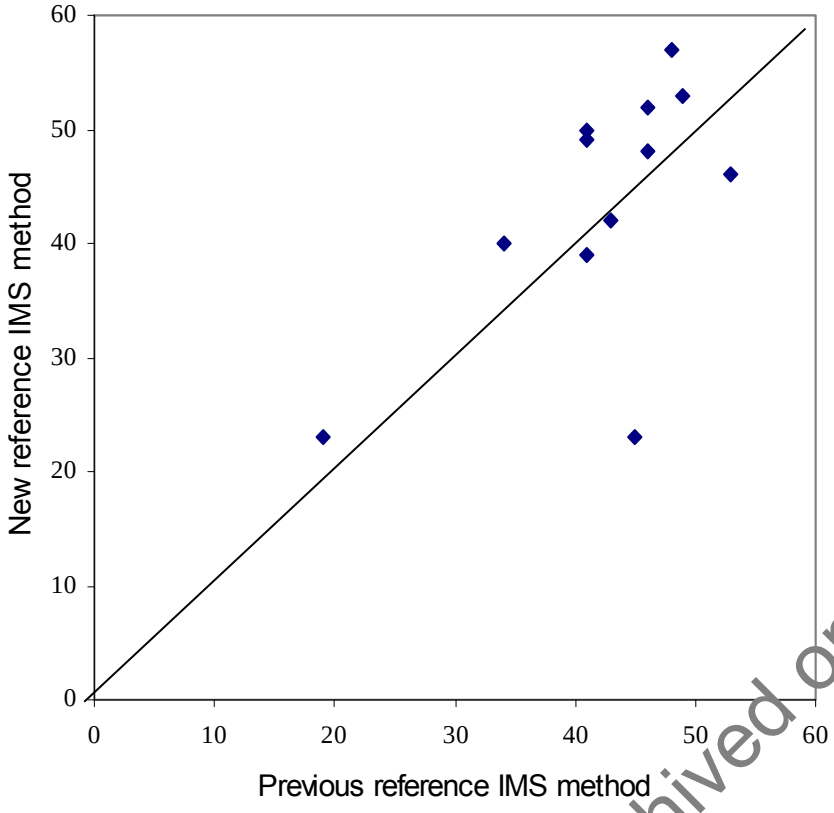
The data are plotted in Figure 3.1.

Table 3.2 Data summary

	Mean	Median	Standard deviation	Range
Previous reference IMS method	42.2	44	8.76	19 to 53
New reference IMS method	43.5	47	10.95	23 to 57
Differences (previous IMS - new IMS)	-1.3	-4	8.79	- 9 to +22

In this set of data recovery with the previous reference IMS method and the new reference IMS method are not significantly different from zero (t = - 0.53; p = 0.61). Both the mean and median recoveries for the new reference IMS method were slightly higher than those for the previous reference IMS method.

Figure 3.1 Comparability plot of the previous reference IMS method against the new reference IMS method



Conclusion

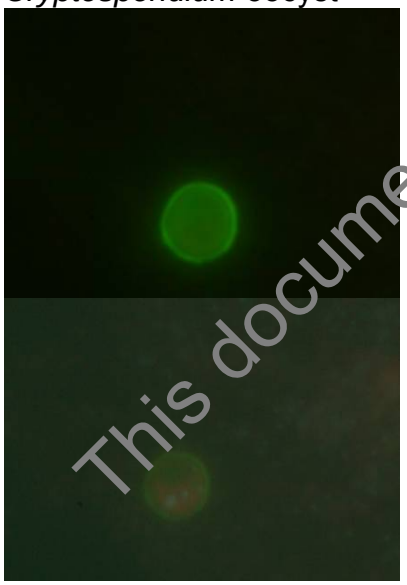
The twelve paired samples demonstrate that there is no significant difference between the two IMS methods.

Appendix 5 Images of *Cryptosporidium* oocysts, *Giardia* cysts and (oo)cyst-like bodies

The Standing Committee of Analysts would welcome the addition of any new images of *Cryptosporidium* oocysts, *Giardia* cysts or (oo)cyst-like bodies which can be added to this collection. Please contact the Secretary of SCA.

Images included here are for illustrative purposes only in order to highlight the difficulties that can arise when attempting to microscopically identify stained (oo)cysts.

Where appropriate, images appear as a series. For example, the same slide is shown as an FITC stain followed by a DAPI stain, followed by observation using DIC microscopy.

Image	Description	Acknowledgement
***Cryptosporidium* oocysts**		
Cryptosporidium oocyst 	FITC stain	R Down Southern Water
Cryptosporidium oocyst 	FITC stain with Evans Blue stain *Cryptosporidium* oocyst looks normal under FITC. DAPI stain with Evans Blue stain Same oocyst showing sporozoite nuclei and red fluorescence due to ingress of Evans Blue stain.	J Watkins CREH *Analytical*

Cryptosporidium parvum

FITC stain

Two oocysts, the contents of the oocyst on the left showing red fluorescence due to ingress of Evans Blue stain.

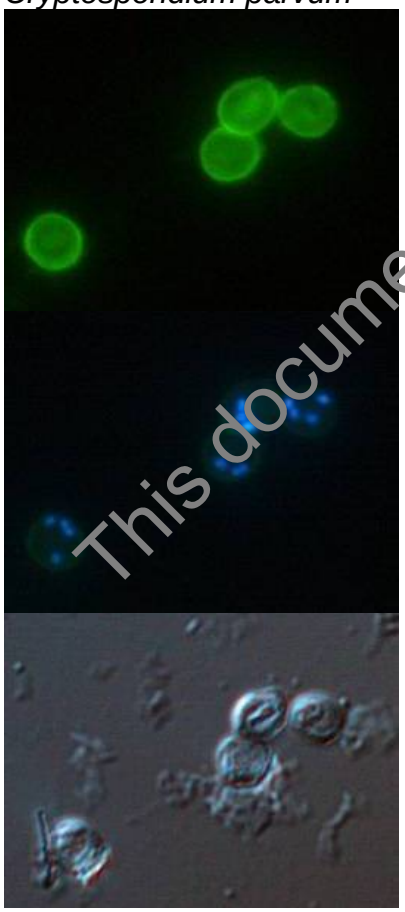
P Walker
United Utilities

DAPI stain

Same oocysts, the left hand oocyst showing sporozoite nuclei with a 'comma'-shaped morphology, sometimes seen in aged oocysts.

DIC microscopy

Cryptosporidium parvum



FITC stain

R Down
Southern Water

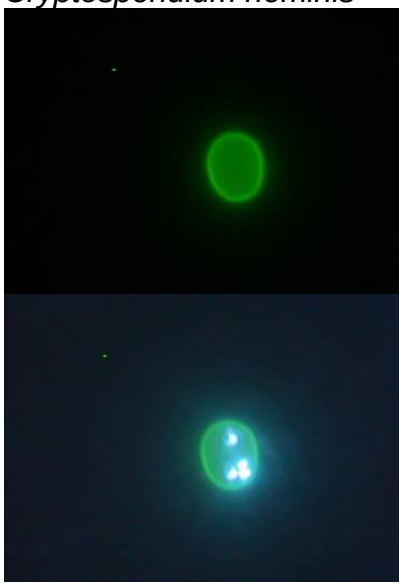
DAPI stain

Two, three or four sporozoite nuclei are visible.

DIC microscopy

The sporozoites are clearly visible although some sporozoites may be obscured by particulate material from the sample that has been deposited on the slide.

Cryptosporidium hominis



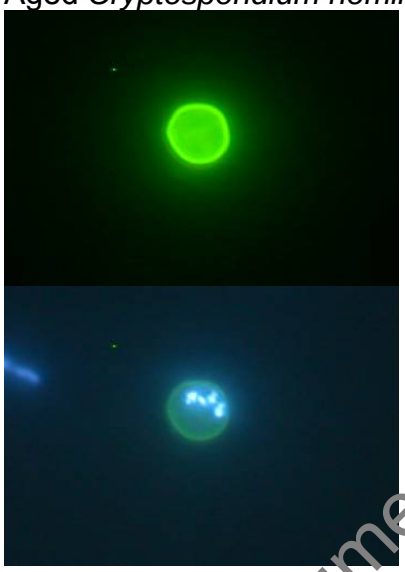
FITC stain
Cryptosporidium hominis exhibits the same round to ovoid shape as *Cryptosporidium parvum*. *Cryptosporidium hominis* cannot be distinguished from other species by shape or morphology.

P Walker
United Utilities

DAPI stain

Showing four sporozoite nuclei.

Aged *Cryptosporidium hominis*



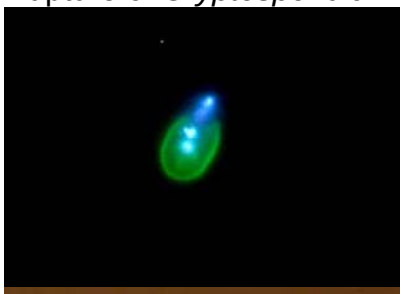
FITC stain

P Walker
United Utilities

DAPI stain

Showing ‘comma’-like nuclei with mitochondrion at end of tail. This may be confusing, falsely suggesting the presence of more than 4 nuclei.

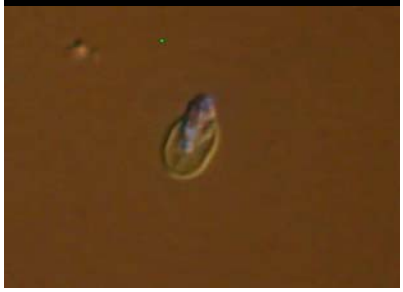
Rupture of *Cryptosporidium* oocysts



DAPI stain with FITC fluorescence visible.

Oocyst in the process of rupturing - giving the appearance of excystation.

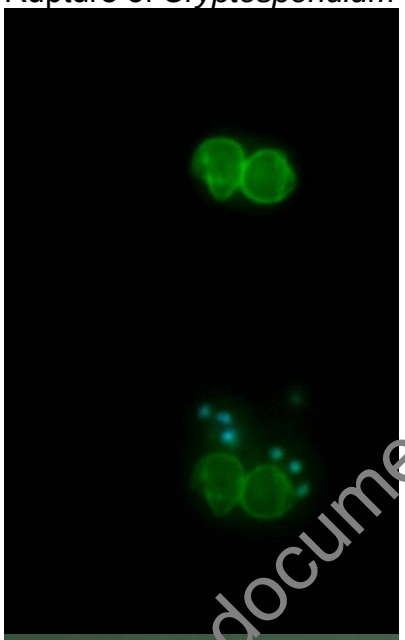
P Walker
United Utilities



Observation using DIC microscopy with superimposed DAPI stain.

This image shows position of sporozoites with DIC and their nuclei revealed by DAPI fluorescence.

Rupture of *Cryptosporidium* oocysts



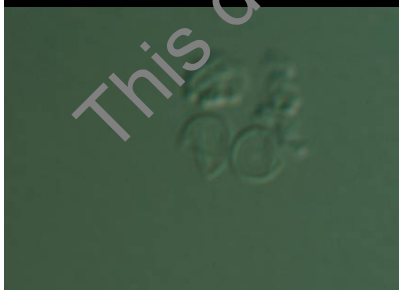
FITC stain

Two ruptured oocysts showing 'pac-man' appearance.

J Watkins
CREH *Analytical*

DAPI stain

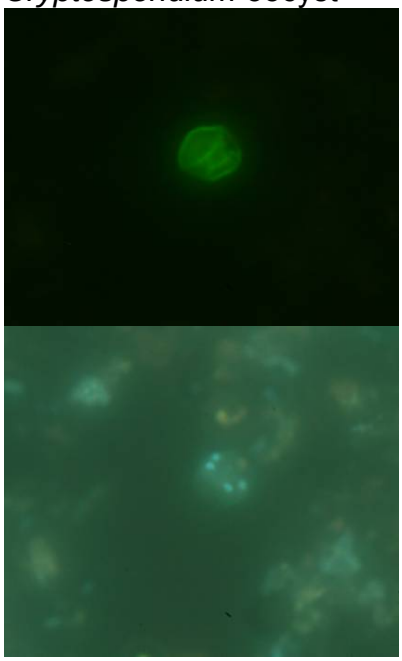
Same oocysts showing the position of the released sporozoites.



DIC microscopy

Same oocysts showing the position of the sporozoites in relation to the empty oocysts.

Environmentally damaged *Cryptosporidium* oocyst



FITC stain

Old and battered environmental oocyst with folded wall. Staining is often poor.

J Watkins
CREH *Analytical*

DAPI stain

Same oocyst showing four sporozoites, the nuclei of which can be clearly observed and counted.

Oocyst in the presence of bacterial interference

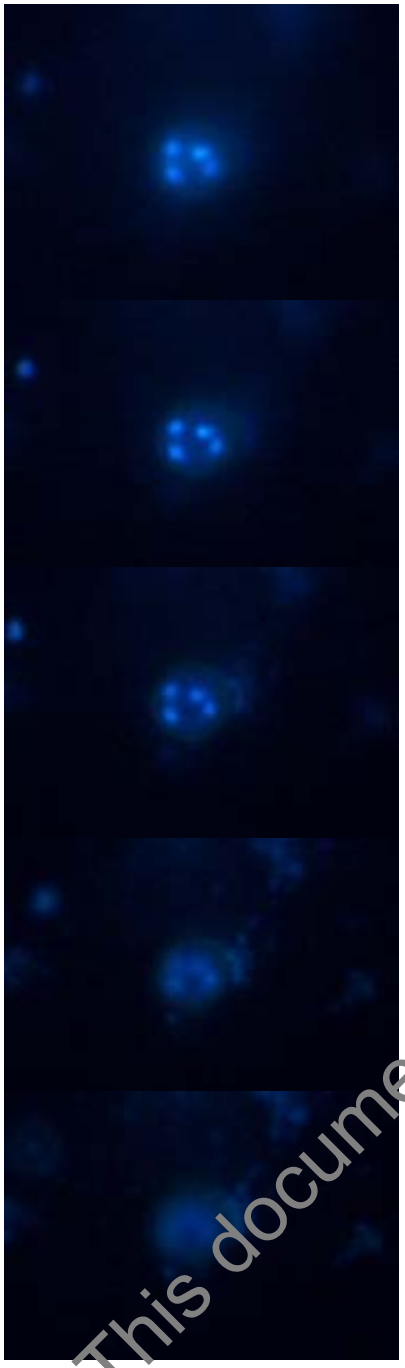
FITC stain

P Walker
United Utilities

DAPI stain

Showing bacterial interference.

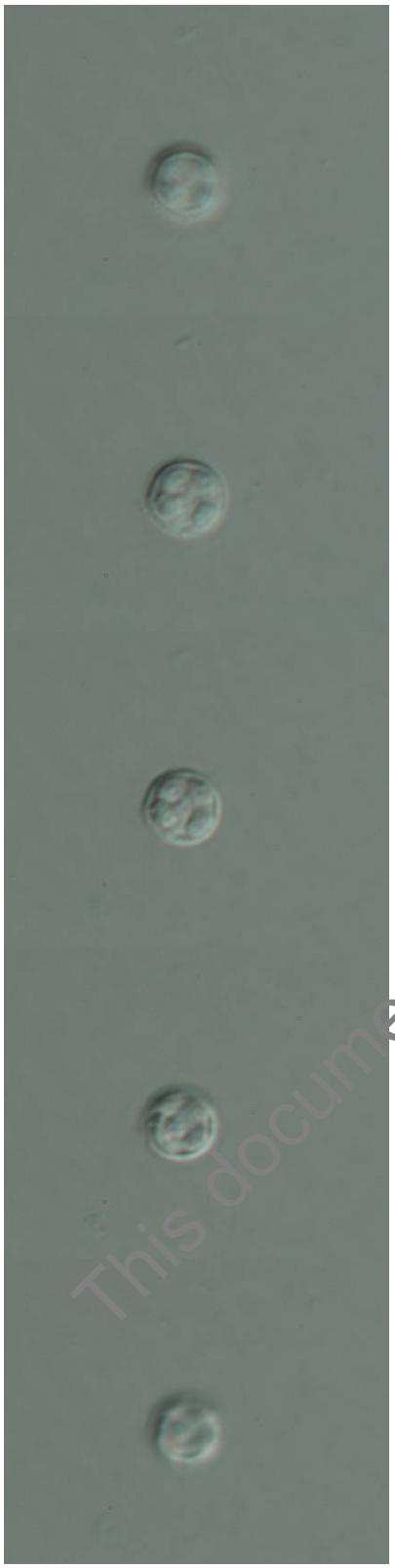
Sequence of *Cryptosporidium* oocyst images through different planes of focus



In this sequence of images, the DAPI focus has been taken through different planes of focus; all 4 sporozoite nuclei are reasonably close to the same plane of focus. In other examples the individual nuclei can appear at different points of focus.

G Robinson
Cryptosporidium Reference Unit UK

Sequence of *Cryptosporidium* oocyst images through different planes of focus

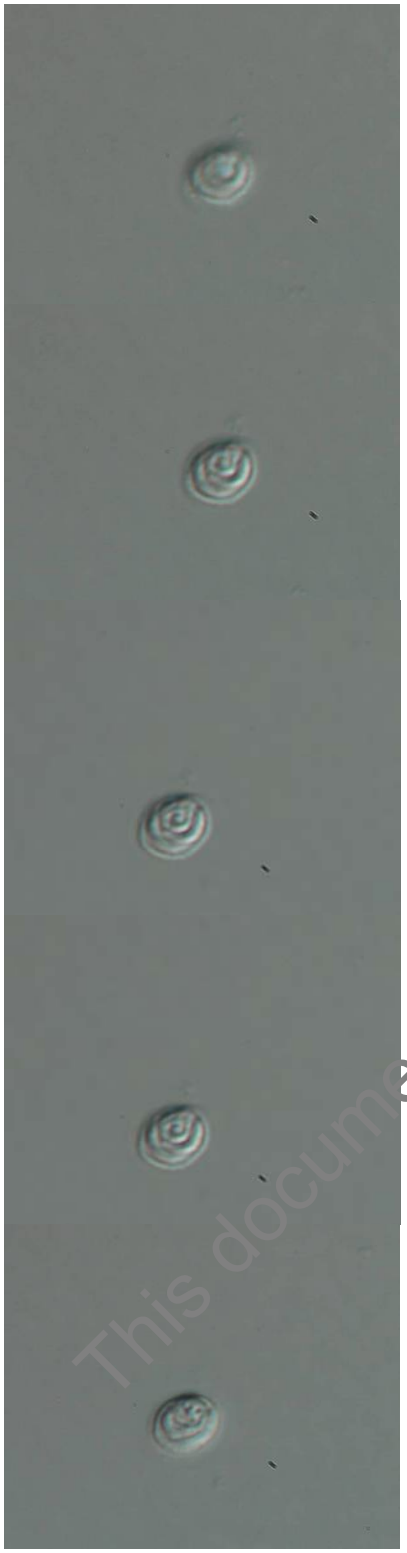


The use of DIC microscopy to focus on an oocyst to determine the position and number of sporozoites.

In this sequence of images the DIC focus has been sequentially taken through the oocyst revealing the packing of the sporozoites within.

J Watkins
CREH *Analytical*

Sequence of *Cryptosporidium* oocyst images through different planes of focus

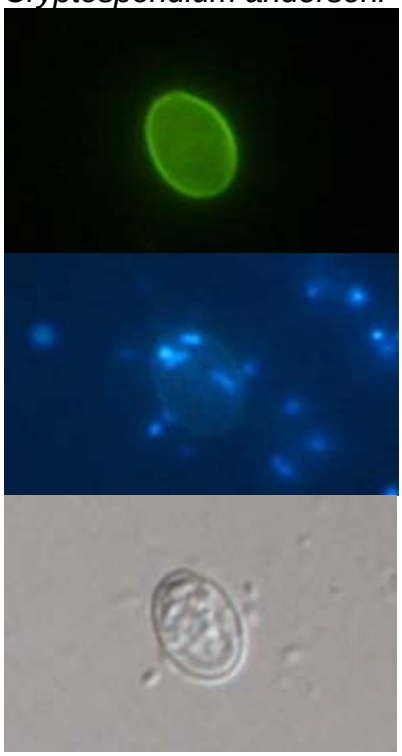


The use of DIC to focus on an oocyst to determine the position and number of sporozoites.

In this second sequence of images, the DIC focus has been sequentially taken through the oocyst revealing the packing of the sporozoites within.

J Watkins
CREH *Analytical*

Cryptosporidium andersoni



FITC stain

This oocyst is larger and more oval than those of *Cryptosporidium parvum* or *Cryptosporidium hominis.*

G Robinson
Cryptosporidium Reference Unit UK

DAPI stain

DIC microscopy

Cryptosporidium andersoni

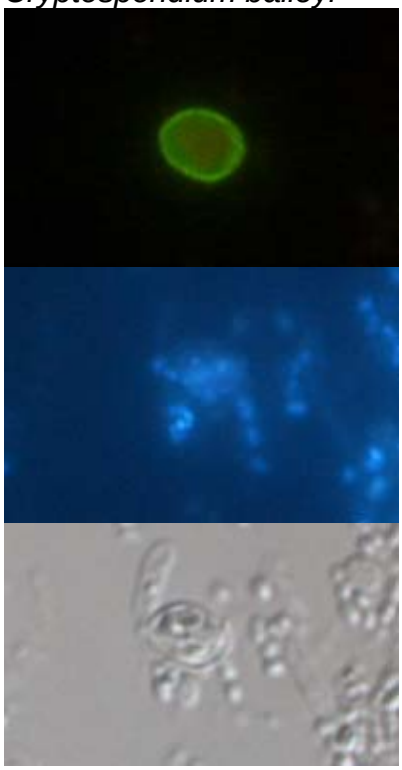
FITC stain

This oocyst is larger and more oval than that of *Cryptosporidium parvum* or *Cryptosporidium hominis*.

P Walker
United Utilities

DAPI stain

Cryptosporidium baileyi



FITC stain

This oocyst is generally slightly larger than that of *Cryptosporidium parvum* and *Cryptosporidium hominis*, but may be in the same size range.

G Robinson
Cryptosporidium Reference Unit UK

DAPI stain

DIC microscopy

Cryptosporidium baileyi

FITC stain

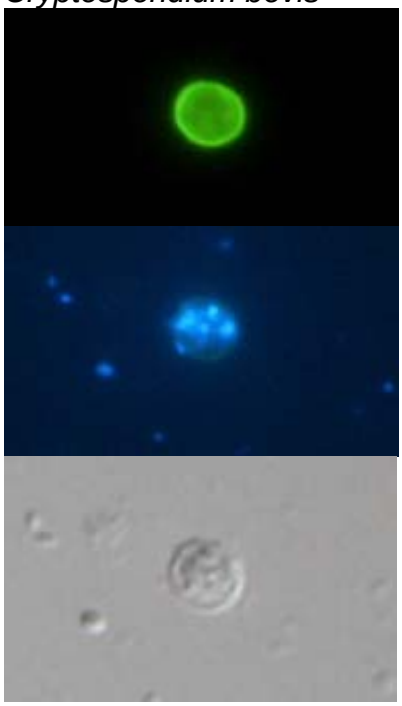
This oocyst is generally slightly larger than that of *Cryptosporidium parvum* and *Cryptosporidium hominis*, but may be in the same size range.

P Walker
United Utilities

DAPI stain

Ingress of Evans Blue stain resulting in slight red fluorescence.

Cryptosporidium bovis



FITC stain

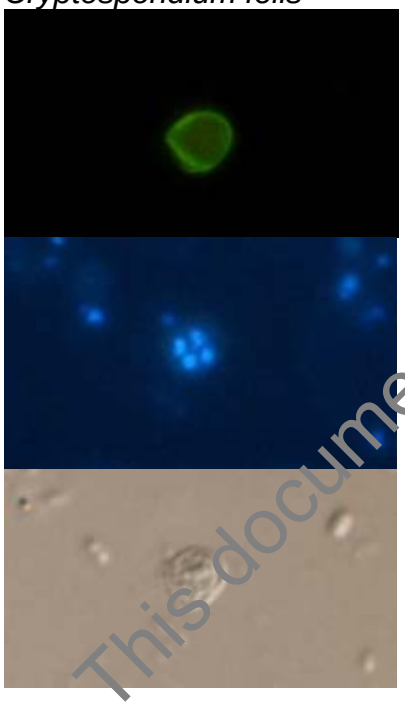
Cryptosporidium bovis is morphologically indistinguishable from *Cryptosporidium parvum* or *Cryptosporidium hominis*.

DAPI stain

DIC microscopy

G Robinson Cryptosporidium Reference Unit UK

Cryptosporidium felis



FITC stain

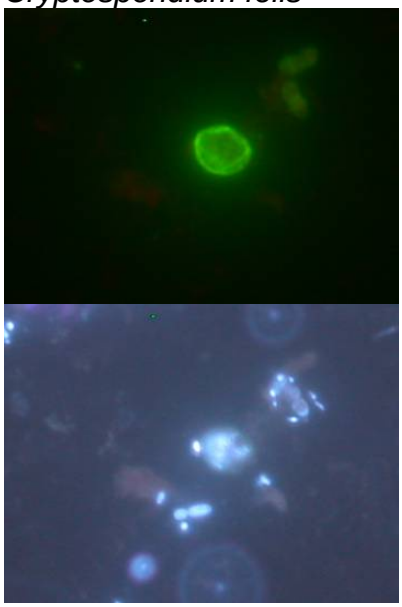
This oocyst may be of a similar or slightly smaller size and morphology than that of either *Cryptosporidium parvum* or *Cryptosporidium hominis*.

DAPI stain

DIC microscopy

G Robinson Cryptosporidium Reference Unit UK

Cryptosporidium felis



FITC stain

This collapsed oocyst is of a similar size to that of *Cryptosporidium parvum* and *Cryptosporidium hominis*.

P Walker
United Utilities

DAPI stain

Cryptosporidium meleagridis

FITC stain

Morphology indistinguishable from other species of same size.

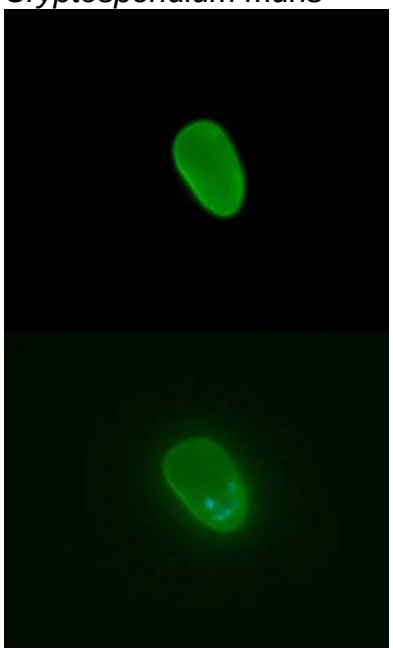
G Robinson
Cryptosporidium Reference Unit UK

DAPI stain

The presence of bacteria (stained with DAPI) can be clearly observed around the oocyst.

DIC microscopy

Cryptosporidium muris



FITC stain

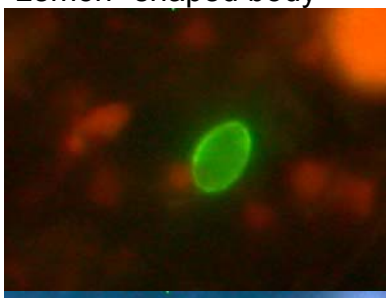
This oocyst is larger and more oval than those of *Cryptosporidium parvum* or *Cryptosporidium hominis*.

DAPI stain

J Watkins
CREH *Analytical*

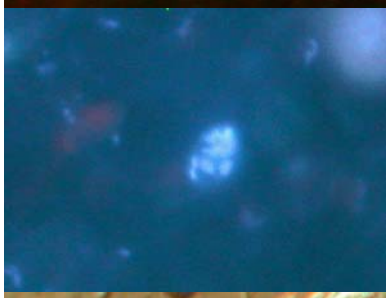
Oocyst-like bodies

"Lemon"-shaped body



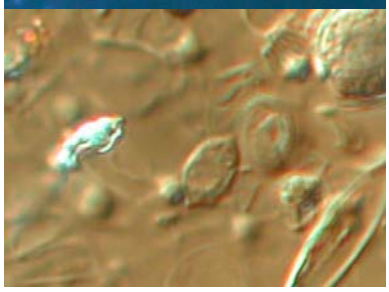
FITC stain

P Walker
United Utilities



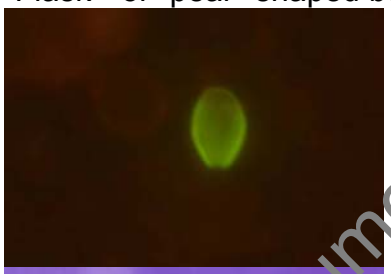
DAPI stain

Showing a number of nuclei.



DIC microscopy

"Flask"- or "pear"-shaped body



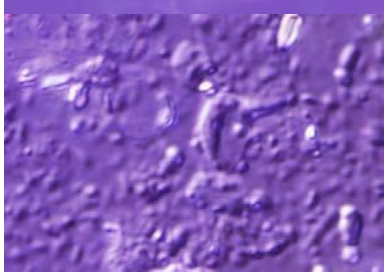
FITC stain

This shows the presence of pore at lower left end.

J Green
Scottish Water

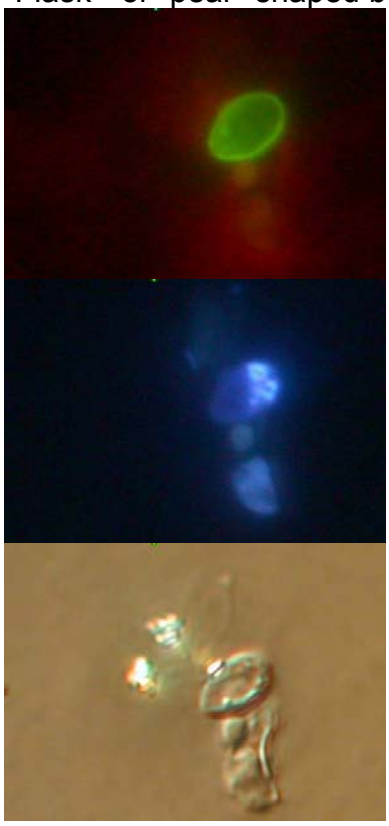
DAPI stain

A cluster of DNA at opposite end to pore.



DIC microscopy

“Flask”- or “pear”-shaped body



FITC stain

This image shows the presence of pore at lower left end.

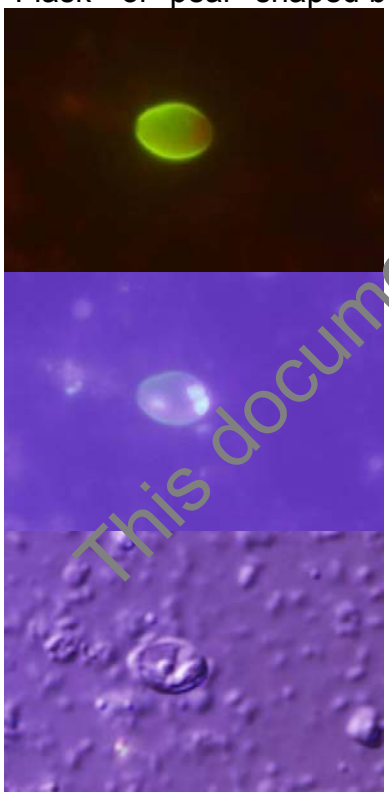
P Walker
United Utilities

DAPI stain

This image shows a cluster of DNA at opposite end to pore.

DIC microscopy

“Flask”- or “pear”-shaped body



FITC stain

This shows the presence of pore at lower left end.

J Green
Scottish Water

DAPI stain

A cluster of DNA at opposite end to pore.

DIC microscopy

Striated body

FITC stain

Tapering “spindle”-shape.

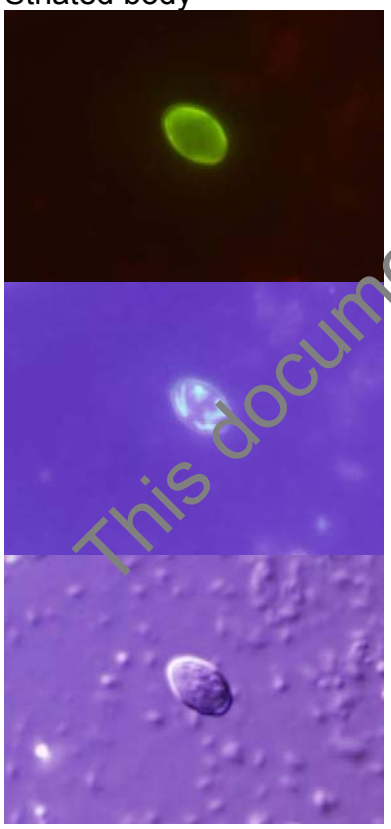
P Walker
United Utilities

DAPI stain

Body containing striated DNA material giving “N”-, “Z”- and “W”-shaped bodies when the DAPI stain is focussed through the body.

DIC microscopy

Striated body



FITC stain

Tapering “spindle”-shape.

J Green
Scottish Water

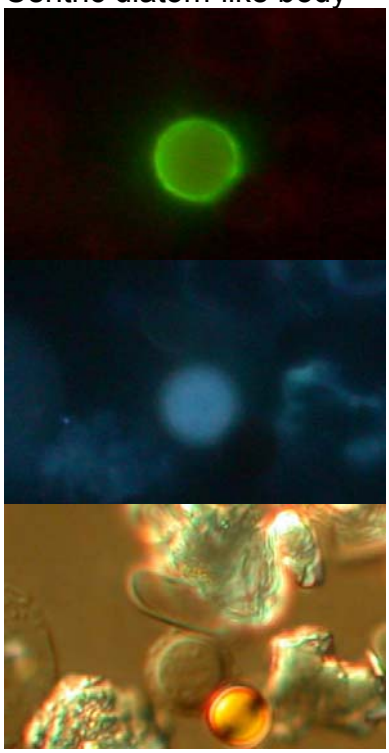
DAPI stain

Body containing striated DNA material giving “N”-, “Z”- and “W”-shaped bodies when the DAPI stain is focussed through the body.

DIC microscopy

This image is more clearly defined than previously seen.

Centric diatom-like body



FITC stain

Possibly a centric diatom which has been stained by the FITC stain.

P Walker
United Utilities

DAPI stain

Showing diffuse, overall fluorescence.

DIC microscopy

This image reveals valve-like structure.

Round diffuse body

FITC stain

J Green
Scottish Water

DAPI stain

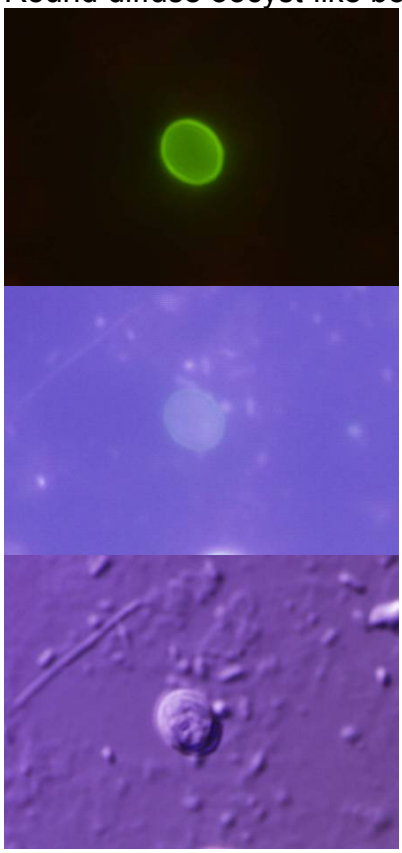
Fluorescence diffused evenly throughout the cell.

DIC microscopy

No discernable structure within the contents.

(These images may be those of an IMS bead)

Round diffuse oocyst-like body



FITC stain

J Green
Scottish Water

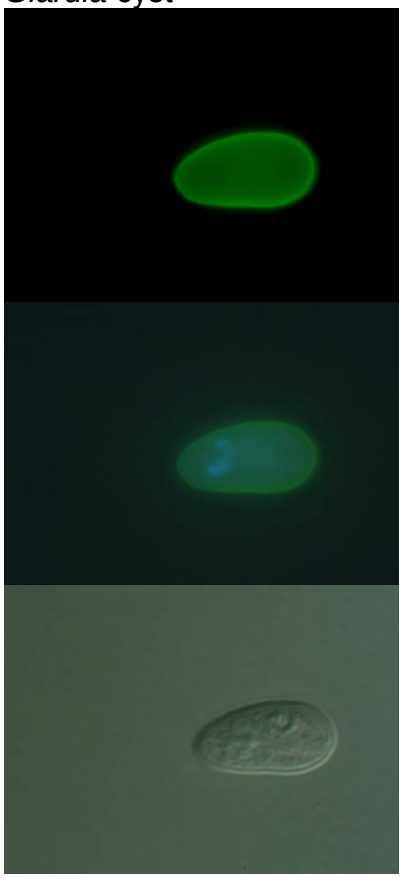
DAPI stain

Fluorescence diffused evenly throughout the cell.

DIC microscopy

Giardia cysts

Giardia cyst



FITC stain

Pear-shape cyst.

J Watkins
CREH *Analytical*

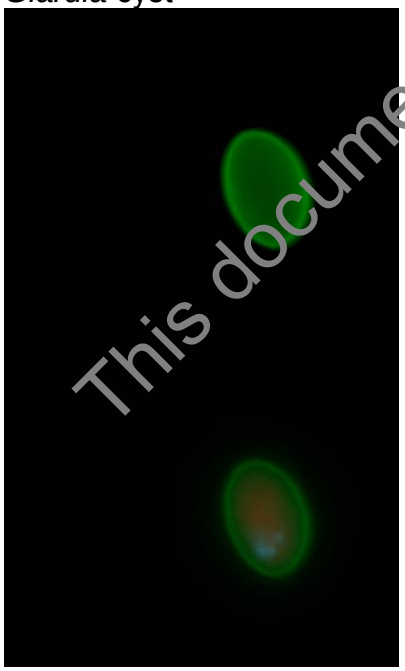
DAPI stain

2 - 4 trophozoite nuclei may be visible.

DIC microscopy

The nuclei, remnants of the flagellar axonemes and the median body should be visible.

Giardia cyst



FITC stain incorporating Evans Blue stain

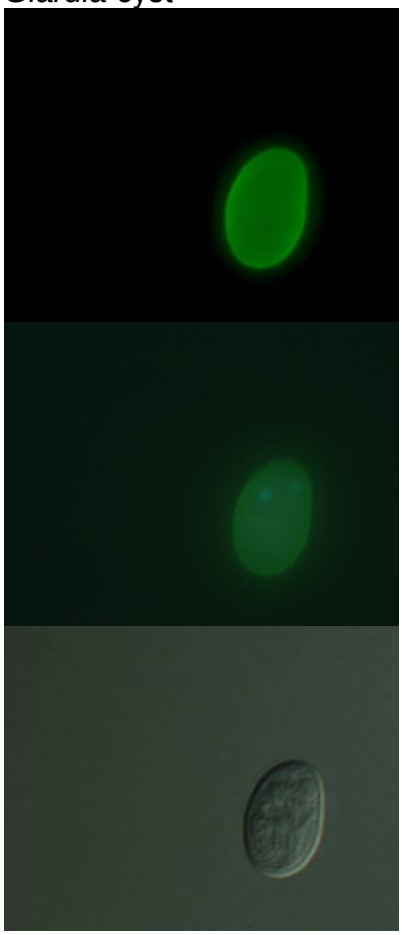
Pear-shaped cyst.

J Watkins
CREH *Analytical*

DAPI stain incorporating Evans Blue stain

Cyst showing stained nuclei and penetration of Evans Blue.

Giardia cyst



FITC stain

Pear-shaped cyst

J Watkins
CREH *Analytical*

DAPI stain

2 - 4 trophozoite nuclei may be visible.

DIC microscopy

The nuclei, remnants of the flagellar axonemes and the median body should be visible.

Giardia cyst - atypical view

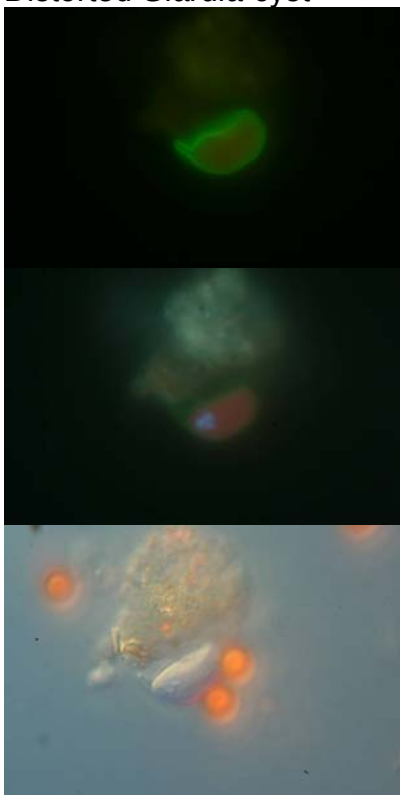
FITC stain

Circular cyst may be seen if the cyst is viewed “end-on”.

J Watkins
CREH *Analytical*

DAPI stain

Distorted *Giardia* cyst



FITC stain

Giardia cyst attached to particulate material. There is some distortion of the cyst.

J Watkins
CREH *Analytical*

DAPI Stain

Giardia cyst attached to particulate material. Careful focusing down through the cyst reveals three nuclei.

DIC microscopy

Giardia cyst attached to particulate material. The presence of three nuclei can be seen by DIC.

Possible *Giardia* cyst

FITC stain

Possible *Giardia* cyst stained with FITC.

J Watkins
CREH *Analytical*

DAPI stain

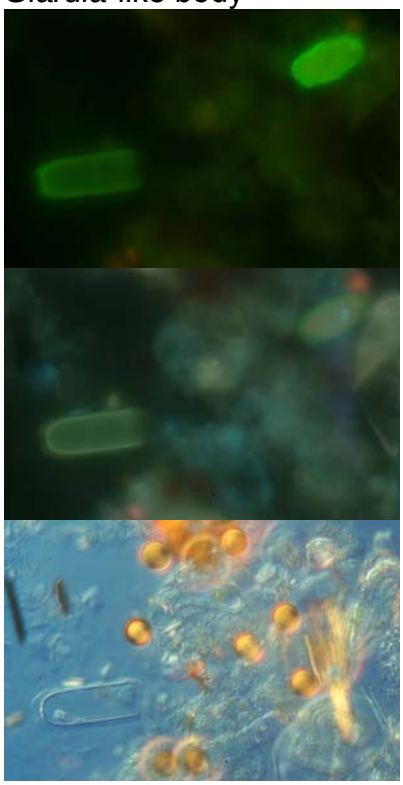
The image suggests three nuclei.

DIC microscopy

Image shows particulate material attached to the outside of the suspected cyst.

Cyst-like bodies

Giardia-like body



FITC stain

Giardia-like body (left) and *Giardia* cyst (right).

J Watkins
CREH *Analytical*

DAPI stain

Three nuclei are visible in the *Giardia* cyst (right) but not in the *Giardia*-like body (left).

DIC microscopy

Giardia-like body (left) appears almost empty and has no end to the right hand side. The *Giardia* cyst (right) is partly obscured by particulate material

Giardia-like body

FITC stain

Giardia-like body both in size and staining.

J Watkins
CREH *Analytical*

DAPI stain

Giardia-like body, no internal nuclei are visible.

DIC microscopy

Giardia-like body appears to be empty.

Giardia-like body

FITC stain

Giardia-like body both in size and staining.

J Watkins
CREH *Analytical*

DAPI stain

Giardia-like body, no internal nuclei are visible.

DIC microscopy

Giardia-like body appears to be empty.

Giardia-like body

FITC stain

Giardia-like body, circular and intensely stained.

J Watkins
CREH *Analytical*

DAPI stain

Weakly stained suggesting internal nuclear material.

DIC microscopy

Image suggestive of a centric diatom.

Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users wish to receive advanced notice of forthcoming publications, please contact the Secretary.

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Environment Agency
Standing Committee of Analysts

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Without the good will and support given by these individuals and their respective organisations SCA would not be able to continue and produce the highly valued and respected blue book methods.

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R Down	Southern Water
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J Green	Scottish Water
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H Hawkins	Veolia Water
S Meikle (nee Houston)	Northumbrian Water Scientific Services
P Johal	Severn Trent Laboratories
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S Jones	Drinking Water Inspectorate
A Mitchell	South West Water
M Morgan	Drinking Water Inspectorate
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