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Water quality — Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water

Qualité de l'eau — Isolement et identification des oocystes de Cryptosporidium *et des kystes de Giardia*

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

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Foreword

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 15553 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

Introduction

Cryptosporidium and *Giardia* are protozoan parasites that can cause enteric illness in humans. Both organisms are characterized by an ability to survive in the aquatic environment. *Cryptosporidium* in particular is resistant to chlorine at the concentrations used in the treatment of drinking and swimming pool waters. Consequently the absence of vegetative bacteria as indicators of faecal contamination does not necessarily indicate the absence of *Cryptosporidium* oocysts or *Giardia* cysts. The methods described in this document may be used to determine whether *Cryptosporidium* and/or *Giardia* are present in water supplies. The techniques have been selected on the basis of method development and peer review publication of the data thus obtained. They are further selected to give comparable recoveries of the methods or reagents used in the isolation of the organisms.

Water quality — Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water

1 Scope

This International Standard specifies a method that is applicable for the detection and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts in water. It is applicable for the examination of surface and ground waters, treated waters, mineral waters, swimming pool and recreational waters.

This method does not allow identification to species level, the host species of origin or the determination of viability or infectivity of any *Cryptosporidium* oocyst or *Giardia* cyst which may be present. These procedures are for use by experienced analysts who have successfully completed competency tests prior to commencing analysis. In addition, such analysts should continue to demonstrate competency by examining seeded samples at regular intervals and taking part in external quality assurance schemes.

NOTE Bodies resembling *Cryptosporidium* or *Giardia* in morphology can be present and these may be mistaken for oocysts or cysts. Results should be interpreted with care. Where there is doubt about the identity of oocysts or cysts or where an unusually high result is obtained, it is advisable to have the slides examined by experts from other laboratories to confirm or refute the findings.

2 Terms and definitions

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

2.1
Cryptosporidium
protozoan parasite, concentrated and selected from water samples with the methods described, which reacts with specific anti-*Cryptosporidium* antibodies and exhibits the typical morphological characteristics described in 7.4 of this International Standard

NOTE A more complete definition of the parasite and the different genotypes and species is given in Annex G.

2.2
Giardia
protozoan parasite, concentrated and selected from water samples with the methods described, which reacts with specific anti-*Giardia* antibodies and exhibits the typical morphological characteristics described in 7.4 of this International Standard

NOTE A more complete definition of the parasite and the different species is given in Annex G.

3 Principle

3.1 Concentration from water

The isolation of *Cryptosporidium* and *Giardia* from water requires the use of a procedure which allows the volume of the sample to be reduced whilst retaining any oocysts and cysts. The concentration procedure used however, is dependent upon the water type which is to be analysed, the volume of sample and the amount of particulate material in the sample. This document describes the use of two concentration techniques for varying volumes of water using cartridge filtration and elution followed by low speed centrifugation (7.1). Additional methods for the recovery of oocysts and cysts from small volumes of water or very turbid waters are given in Annex B. Some examples of recovery data for these techniques are given in Annex E.

Table 1 — Membrane filters/filtration systems used for the concentration of parasites from water samples

Membrane filter/filtration system	Application
Pall Envirochek™ STD [a]	Concentration of 10-litre to 200-litre (or more) samples of water
Pall Envirochek™ HV	Concentration of 10-litre to 1 000-litre samples of water
IDEXX Filta-Max®	Concentration of 10-litre to 1 000-litre samples of water

[a] It has been shown by some laboratories that this technique may be used successfully for larger volumes of water although the manufacturers' instructions may only include volumes up to 200 litres.

3.2 Purification and further concentration

After concentration of particulate material from filter eluates, oocysts and cysts are isolated using immunomagnetic separation (IMS) (7.2). Oocysts and cysts are attached to para-magnetic beads coated with specific antibody, the beads are separated from the unwanted particulate material using a magnet and then the oocysts and cysts are dissociated from the beads using acid and neutralized using alkali before immunostaining.

3.3 Detection of *Cryptosporidium* and *Giardia*

After IMS, organisms are labelled with monoclonal antibody (mAb) conjugated to a fluorochrome, usually fluoroscein isothiocyanate (FITC). In addition, any nuclear material is labelled with a nucleic acid stain to aid identification (7.3). Each sample is then examined for the presence of labelled *Cryptosporidium* oocysts and *Giardia* cysts using epifluorescence and differential interference contrast (DIC) microscopy (7.4).

4 Reagents

4.1 Reagents required for eluting Pall Envirochek™ STD capsule filters [1)]

4.1.1 Deionized water, 0,2 µm filtered at the point of use.

4.1.2 Laureth 12 detergent.

4.1.3 Tris buffer, pH 7,4 (A.1.1).

4.1.4 EDTA solution, 0,5 mol/l, pH 8,0 (A.1.2).

4.1.5 Antifoam A.

4.1.6 Elution buffer (A.1.3).

4.2 Reagents required for eluting Pall Envirochek™ HV capsule filters [1)]

4.2.1 Deionized water, 0,2 µm filtered at point of use.

4.2.2 Pre-treatment buffer (A.1.4).

4.2.3 Laureth 12 detergent

1) All products and reagents are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

4.2.4 Tris buffer, pH 7,4 (A.1.1).

4.2.5 EDTA solution, 0,5 mol/l, pH 8,0 (A.1.2).

4.2.6 Antifoam A.

4.2.7 Elution buffer (A.1.3).

4.3 Reagents required for eluting IDEXX Filta-Max® filters [1)]

4.3.1 Phosphate buffered saline (PBS) (A.2.1).

4.3.2 Polyoxyethylene(20)sorbitan monolaurate (Tween 20).

Store at room temperature (20 ± 5) °C. Expiry date one year.

4.3.3 Elution buffer (A.2.2).

4.4 Concentration and detection reagents

4.4.1 Methanol, analytical grade.

4.4.2 Magnetic beads, for the detection of *Cryptosporidium* and *Giardia.*

Expiry date printed by the manufacturer.

NOTE See Annex H for a list of suitable suppliers.

4.4.3 Fluorescently labelled monoclonal antibodies (mAbs) against *Cryptosporidium* and *Giardia*.

Store at (5 ± 3) °C. Expiry date as stated by the manufacturer. When stains are prepared from concentrated material using a diluent supplied by the manufacturer, the prepared solution is stored at (5 ± 3) °C for no longer than 6 months.

NOTE See Annex H for a list of suitable suppliers.

4.4.4 Immunofluorescence mounting medium (A.3.1).

NOTE See Annex H for a list of suitable suppliers.

4.4.5 4′,6′-Diamidino-2-phenylindole dihydrochloride dihydrate (DAPI) freeze dried reagent.

Store according to the manufacturer's instructions.

Expiry date printed by the manufacturer on each vial.

4.4.6 DAPI stock solution (A.3.2).

4.4.7 DAPI working solution (A.3.3).

4.4.8 Phosphate buffered saline (PBS) (A.2.1).

4.4.9 Non-fluorescing immersion oil.

Store at room temperature (20 ± 5) °C.

4.4.10 Stock suspensions of *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts.

Store at (5 ± 3) °C, never allow the suspension to freeze and check quality regularly. Ideally, suspensions of oocysts and cysts should be no more than 3 months old. Stock suspensions should be checked microscopically to confirm that they are monodispersed and discarded if clumps or aggregates are detected. In addition, if mAb and DAPI staining become weak and oocysts become deformed, they should also be discarded.

4.4.11 Parasite storage medium (A.3.4).

5 Apparatus

Use usual laboratory equipment and, in particular, the following.

5.1 Scientific apparatus, required for concentration using Pall Envirochek™ STD or HV. [2)]

5.1.1 Sampling capsule, Envirochek™ STD or HV (Pall).

5.1.2 Peristaltic pump, capable of a flow rate of 2 l/min.

5.1.3 Silicon tubing, for use with the peristaltic pump.

5.1.4 Seeding container, 10 l, if seeding filters is required.

5.1.5 Wrist-action shaker, with arms for the agitation of the Envirochek™ STD or HV sample capsules.

5.1.6 Centrifuge, capable of a minimum of 1 100 g.

5.1.7 Centrifuge tubes, conical, plastic, screwtop, 250 ml capacity.

5.1.8 Centrifuge tubes, conical, plastic, screwtop, 50 ml capacity.

NOTE A flow meter and flow restrictor are required for taking water samples with the filter.

5.2 Specific apparatus, required for concentration using IDEXX Filta-Max®. [2)]

5.2.1 Sampling housing, Idexx Filta-Max®.

5.2.2 Sampling module, Idexx Filta-Max®.

5.2.3 Filter membranes, Idexx Filta-Max®.

5.2.4 Laboratory pump, capable of supplying 500 kPa (5 bar) pressure.

5.2.5 Peristaltic pump, capable of flow rate of 4 l/min.

5.2.6 Silicon tubing, for use with peristaltic pump.

5.2.7 Seeding container, 10 l, if seeding filters is required.

5.2.8 Wash station, automatic or manual, and wash station clamp set, Idexx Filta-Max®.

5.2.9 Vacuum set, includes plastic hand pump, waste bottle, tubing and magnetic stirring bar. Idexx Filta-Max®.

2) All apparatus are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

5.2.10 Tubing set, includes elution tube, and middle section, concentrator tube and base, with line tap and steel rod Idexx Filta-Max®.

5.2.11 Membrane, for tubing set.

5.2.12 Plastic bag, for washing membrane.

5.2.13 Centrifuge, capable of 1 100 *g*.

5.2.14 Centrifuge tubes, conical, plastic, 50 ml capacity.

5.2.15 Forceps.

NOTE A flow meter and flow restrictor are required for taking water samples with the filter.

5.3 General apparatus [2].

5.3.1 Incubator, at (36 ± 2) °C.

5.3.2 Refrigerator, at (5 ± 3) °C.

5.3.3 Magnetic stirrer, and magnetic stirring bars.

5.3.4 Vortex mixer.

5.3.5 Wash bottles, polypropylene, 250 ml.

5.3.6 Calibrated micropipettes, adjustable: 1 µl to 10 µl with 1 µl to 10 µl tips; 20 µl to 200 µl with 10 µl to 200 µl tips; 200 µl to 1 000 µl with 100 µl to 1 000 µl tips.

5.3.7 pH meter.

5.3.8 Magnetic particle concentrators, with suitable tubes.

5.3.9 Well microscope slides, with special hydrophobic coating and coverslips.

5.3.10 Epifluorescence microscope, with a UV filter (350 nm excitation, 450 nm emission), FITC filter (480 nm excitation, 520 nm emission) filters,™ differential interference contrast (DIC) optics and an eye piece graticule. Total magnification 1 000 ×.

5.3.11 Microscope stage micrometer, 1 mm, ruled in 100 units.

5.3.12 Eyepiece graticule, ruled in 100 units.

5.3.13 Humidity chamber, e.g. consisting of a tightly sealed plastic container containing damp paper towels on which the slides are placed.

5.3.14 10 l containers, graduated in 1 l.

5.3.15 Neubauer haemocytometer slide.

6 Sampling and transport

The size of the sample is dependent on the type of water being sampled, the purpose of the analysis, the sensitivity to be achieved and the speed with which the result is required. Small volume samples (10 l to 100 l) can be collected in the field and transported and analysed quickly whereas large volume samples (1 000 l) require on-site filtration. Filtration may take up to 24 h because of a restricted flow rate through the filter. Small volume samples (10 l) will give an indication of water quality at the time of sampling whereas large volumes (1 000 l) will give an indication of water quality over an extended period. Since the concentration of *Cryptosporidium* and *Giardia* is usually very low, large test volumes (10 l to 1 000 l) are required. The volume to be examined may be dictated by regulatory limits.

For large volume filtration, connect the device in-line with the water supply, making sure that the flow through the filter is in the direction indicated on the housing by the manufacturer. A flow meter should be included with the filter and this should be read before and after sampling. If the filter is to be transported to the laboratory, it should be sealed, after sampling, with end caps provided by the manufacturer. Due care shall be taken to ensure that the flow rates do not exceed those recommended by the manufacturers of the filtration devices.

Take small volume grab samples and transport them to the laboratory in the dark at ambient temperature. Once at the laboratory, samples should be stored at (5 ± 3) °C unless they are to be analysed immediately. Samples should be analysed preferably within 24 h of collection and no longer than 4 d.

If the samples are filtered in the field, transport the filters in the dark at ambient temperature. Once at the laboratory, samples should be stored at (5 ± 3) °C unless they are to be analysed immediately. Samples should be analysed preferably within 24 h of collection and no longer than 4 d. If filters are stored at (5 ± 3) °C, they shall be allowed to warm to room temperature before elution starts.

No information is available to date on the behaviour of *Cryptosporidium* and *Giardia* during sample or filter storage. It is therefore advisable to examine the samples as soon after sampling as possible.

A pre-treatment step using sodium polyphosphate before the elution buffer was introduced to improve the removal of particulate material bound to the filter.

NOTE 1 The Envirochek™ STD filter consists of a pleated polyether sulfone membrane sealed in a polycarbonate shell. The filter is supported on a loose polypropylene support. It is supplied packaged with two end caps which can be used to seal the filter. The filter can be connected to a water supply by connecting to a ribbed inlet and the direction of flow through the filter is clearly marked. The flow through the filter should not exceed 2,3 l/min and the differential pressure across the filter should not exceed 210 kPa (2,1 bar).

NOTE 2 The Envirochek™ HV capsule is comprised of 1 µm pore size polyester track-etched membrane permanently enclosed in a polycarbonate housing. The polyester membrane is directly laminated to a polypropylene support which offers a significant strength improvement over the standard Envirochek™ STD. The capsule housing burst strength exceeds 1 000 kPa (10 bar) and the differential pressure across the filter membrane is rated to 410 kPa (4,1 bar). Each Envirochek™ HV capsule is 100 % integrity tested after assembly to ensure product performance. The effective filtration area of the Envirochek™ HV is 1 300 cm^2. The filter is supplied with two end caps which can be used to seal the filter for transport to the laboratory. The filter can be supplied with a tamper evident label containing a unique identification number. The flow through the Envirochek™ HV should not exceed 3,4 l/min.

NOTE 3 The Filta-Max® filter consists of a foam filter module comprising 60 open cell reticulated foam discs with an external diameter of 55 mm and an internal diameter of 15 mm. The discs are sandwiched between two retaining plates and compressed by tightening a retaining bolt to give a nominal porosity of 1 µm. The filter module fits into a filter housing which has a screw top and seal. The filter housing has stainless steel barbed inlet and outlet ports. The sample enters through the lid of the housing and exits through the base. Water flows into the housing, through the compressed foam rings into the centre of the module and through the outlet port. Removal of the retaining bolt during the elution stage allows the filter to expand during washing. Filter housings are supplied with two tools for the removal of the top and two rubber bungs to seal water in the sample. After sampling, Filta-Max® should be kept wet during storage and transportation. If stored or transported in the filter housing, the inlet and outlet should be securely plugged with the rubber stoppers provided. During transportation or storage, the filter module may be removed from the housing and aseptically placed in an airtight container along with several milliliters of additional deionized water.

7 Procedure

7.1 Concentration

7.1.1 Pall Envirochek™ STD Filtration

Support the filter vertically with the white bleed valve uppermost. Remove the two end caps and allow any water in the sample to drain out through the filter. Replace the bottom end cap, fill the cartridge with elution buffer (4.1.6) through the inlet fitting until it covers the filter by approximately 1 cm. Replace the upper end cap and secure the cartridge horizontally into the wrist shaker (5.1.5) with the white bleed valve in the 12 o'clock position. Shake at 600 cycles per minute (cpm) ± 25 cpm for 5 min ± 30 s.

Remove the upper end cap and pour the washings into a 250 ml conical centrifuge tube (5.1.7). Add a further aliquot of elution buffer into the capsule, replace the upper end cap and shake for a further 5 min ± 30 s. Ensure that the white bleed valve is in the 3 o'clock or 9 o'clock position.

After 5 min of shaking, remove the upper end cap and decant the washings into the 250 ml centrifuge tube and centrifuge at 1 100 × g for 15 min without braking during the deceleration phase. Record the pellet volume (volume of solids) immediately after centrifugation.

A second centrifugation step may be required in a 50 ml centrifuge tube in order to measure the volume. Alternatively, 50 ml centrifuge tubes may be used to concentrate the particulate material eluted from the filter.

Using a pipette and a vacuum source of less than 20 kPa (0,2 bar), carefully aspirate off the supernatant leaving 2 ml to 5 ml above the pellet. If no pellet is visible, extra care shall taken to avoid aspirating oocysts and cysts during this step.

Add deionized water to the centrifuge tube to bring the total volume to 9 ml. Vortex the tube for 10 s to 15 s to resuspend the pellet and either store the sample at (5 ± 3) °C for future IMS or proceed directly to 7.2.

If the pellet volume exceeds that recommended by the manufacturer of the IMS test kit, centrifuge the sample a second time in a tube that permits the pellet volume to be measured accurately. Subdivide the sample into aliquots for IMS such that each aliquot represents the maximum pellet volume recommended by the manufacturer and make up each aliquot to 9 ml with deionized water.

7.1.2 Pall Envirochek™ HV Filtration

Support the filter vertically with the white bleed valve uppermost. Remove the two end caps and allow any water in the sample to drain out through the filter. Replace the bottom end cap, fill the cartridge with pre-treatment buffer (4.2.2) through the inlet fitting until it covers the filter by approximately 1 cm. Replace the upper end cap and secure the cartridge horizontally into the wrist shaker (5.1.5) with the white bleed valve in the vertical position. Shake at 600 cycles per minute (cpm) ± 25 cpm for 5 min ± 30 s.

Secure the filter vertically with the white bleed valve uppermost, remove the end caps and allow the pre-treatment buffer to drain out through the filter. Replace the bottom end cap and fill the cartridge as above with deionized water (4.2.1). Replace the upper end cap and rinse the membrane by gently rotating the filter for 30 s. Secure the filter vertically, remove the end caps and allow the deionized water to drain out through the filter.

Replace the bottom end cap, fill the cartridge with elution buffer (4.2.7) through the inlet fitting until it covers the filter by approximately 1 cm. Replace the upper end cap and secure the cartridge into the wrist shaker (5.1.5) with the white bleed valve in the 12 o'clock position. Shake at 600 cpm ± 25 cpm for 5 min ± 30 s.

Remove the upper end cap and pour the washings into a 250 ml conical centrifuge tube (5.1.7). Add a further aliquot of elution buffer into the capsule, replace the upper end cap and shake for a further 5 min ± 30 s. Ensure that the white bleed valve is in the 4 o'clock position. After 5 min of shaking, turn the filter such that the white valve is in the 8 o'clock position and shake for a further 5 min.

Remove the upper end cap and decant the washings into the 250 ml centrifuge tube and centrifuge at 1 100 g for 15 min without braking during the deceleration phase. Record the pellet volume (volume of solids) immediately after centrifugation.

A second centrifugation step may be required in a 50 ml centrifuge tube in order to measure the volume. Alternatively, 50 ml centrifuge tubes may be used to concentrate the particulate material eluted from the filter.

Using a pipette and a vacuum source of less than 20 kPa (0,2 bar), carefully aspirate off the supernatant leaving 2 ml to 5 ml above the pellet. If no pellet is visible, extra care shall be taken to avoid aspirating oocysts and cysts during this step.

Add deionized water to the centrifuge tube to bring the total volume to 9 ml. Vortex the tube for 10 s to 15 s to re-suspend the pellet and either store the sample at (5 ± 3) °C for IMS or proceed directly to 7.2.

If the pellet volume exceeds that recommended by the manufacturer of the IMS test kit, centrifuge the sample a second time in a tube that permits the pellet volume to be measured accurately. Subdivide the sample into aliquots for IMS such that each aliquot represents the maximum pellet volume recommended by the manufacturer and make up each aliquot to 9 ml with deionized water.

NOTE Warming all the elution solutions to 37 °C improves the removal of particulate material. Elution is also helped by increasing the shaking speed to 900 cpm.

7.1.3 Idexx Filta-Max® Filtration

The elution apparatus consists of an upper and lower wash tube (5.2.10), a wash station (5.2.8) and a vacuum set (5.2.9) designed to reduce the volume of the eluate to 50 ml through a membrane (5.2.11). The elution procedure is as follows:

Place a membrane filter (rough surface uppermost) in the base of the lower wash tube and put the tube into the base. Make sure that the membrane is held securely and that the tap on the base is closed.

Unscrew the housing top using the tools provided, remove the filter module from its housing and screw it into the plunger head of the wash station. Pour any residual water in the filter housing into the lower wash tube.

Place the upper part of the wash tube into the jaws of the wash station and lower the filter module down through the tube.

Using the key provided, remove the retaining screw from the filter module. The filter should begin to expand. Screw the stainless steel tube into the base of the upper wash tube.

Pour approximately 600 ml of wash buffer into the lower wash tube and run a small volume of buffer through the membrane by opening the tap on the base of the lower wash tube. Attach the lower wash tube to the upper wash tube. Pump the plunger up and down four or five times to help the filter module expand. If the filter does not expand, leave it soaking in elution buffer for 5 min, occasionally pumping the plunger to help filter expansion.

Pump the plunger up and down as far as it will travel 20 times only. Disconnect the lower wash tube, pressing the plunger 5 times to remove any residual elution buffer from the foam rings. Rinse the stainless steel tube with elution buffer and plug the end with the small rubber bung provided. Place the elution tube on a magnetic stirrer. Locate the magnetic stirring bar into the top of the elution tube and set the stirrer such that the liquid in the tube is mixed. Connect the vacuum pump and open the tap on the base of the wash tube.

If the sample has little turbidity and the catch bottle is placed below the wash tube, liquid will flow by gravity through the membrane. For turbid liquids, apply a vacuum of no greater than 40 kPa (30 cm of mercury) to filter the washings through the membrane.

Should the membrane become blocked, decant washings into a clean bottle, remove the membrane to a plastic bag (5.2.12) and place a fresh membrane into the lower wash tube with the smooth surface uppermost.

Pour the liquid back into the wash tube, rinsing the bottle, and continue the filtration process. Each membrane shall be washed in a separate bag.

When the washings fall to approximately half way up the stirring bar (approximately 30 ml), close the tap and disconnect the vacuum pump and water trap. Pour the liquid in the wash tube into a 50 ml centrifuge tube (5.2.14).

Add a further 600 ml of elution buffer to the lower wash tube and attach it to the wash station. Repeat the washing procedure using only 10 strokes of the plunger. Remove the lower wash tube, rinsing the stainless steel tube, place it on the stirrer and attach the stirring bar. Concentrate the filter washing down to approximately one inch above the stirring bar as described above. Add the contents of the first elution to the wash tube and continue reducing the volume of eluate until it is again half way up the magnetic bar. Remove the stirring bar and pour the filter washings (approximately 30 ml) into the centrifuge tube.

Unscrew the wash tube from the base and carefully remove the membrane filter with fine forceps. Add the filter to the bag provided together with 5 ml of elution buffer. Seal the bag and wash the filter by rubbing between fingers and thumb for (60 ± 10) s. Pipette off the washings and add to the 50 ml centrifuge tube. Repeat the wash procedure and add the second washings to the centrifuge tube. Make up the volume in the tube to 50 ml with elution buffer.

Centrifuge the 50 ml tube at 1 100 × g for 15 min without braking during the deceleration phase.

Record the pellet volume (volume of solids) immediately after centrifugation.

Using a Pasteur pipette and a vacuum source of less than 20 kPa (0,2 bar), carefully aspirate off the supernatant leaving 2 ml to 5 ml above the pellet. If no pellet is visible, extra care shall taken to avoid aspirating oocysts and cysts during this step.

Add deionized water to the centrifuge tube to bring the total volume to 9 ml. Vortex the tube for 10 s to 15 s to re-suspend the pellet and either store the sample at (5 ± 3) °C for IMS or proceed directly to 7.2.

If the pellet volume exceeds that recommended by the manufacturer of the IMS test kit, centrifuge the sample a second time in a tube that permits the pellet volume to be measured accurately. Subdivide the sample into aliquots for IMS such that each aliquot represents the maximum pellet volume recommended by the manufacturer and make up each aliquot to 9 ml with deionized water.

NOTE 1 The manufacturers provide a compact disc with the apparatus with full instructions on the assembly and operation of the equipment.

NOTE 2 An automated wash station is now available (see Annex H).

Clean the wash tubes with hot water and detergent followed by thorough rinsing in warm water and filtered deionized water.

NOTE 3 Where a number of samples from different sources are examined routinely, it is advantageous to have a separate wash tube set and plunger dedicated to each site to minimize cross contamination.

7.2 Immunomagnetic separation (IMS)

This technique involves the attachment of oocysts and cysts to magnetic beads coated with antibodies to either *Cryptosporidium* or *Giardia*. The oocyst or cyst-bead complex is separated from interfering particles in the water concentrate by using a magnet. After separation, the oocysts and cysts are dissociated from the beads by acid treatment. Oocysts and cysts are transferred in suspension to a microscope slide and the magnetisable beads are discarded.

No detailed instructions can be given in this International Standard because commercial test kits are the only validated methods available for IMS. The test kits shall be used according to the manufacturer's instructions.

NOTE 1 For details of the manufacturers of IMS test kits, see Annex H.

NOTE 2 Automated capture and concentration equipment is now available, see Annex H.

7.3 Sample staining

Label an appropriate well slide with the sample number and the sample volume analysed (the whole of the sample should be analysed).

After addition of NaOH to the wells of the slide, distribute aliquots of the suspension containing the separated oocysts and cysts (7.2) onto the wells.

NOTE 1 The volume of the NaOH and the sample added to each well will depend on the size of the wells.

Prepare two separate well slides with positive and negative controls. The positive control shall consist of a suspension of *Cryptosporidium* and *Giardia* containing a known number of parasites (Annex D). The negative control shall consist of filtered deionized water or PBS. Further positive and negative controls shall be included with each batch of samples stained.

Place the well slides containing the samples in an incubator at (36 ± 2) °C or no higher than 42 °C and evaporate to dryness.

Apply a drop of methanol (4.4.1) to each well containing the dried sample and allow to air dry at (20 ± 5) °C.

Overlay the sample well with FITC fluorescently labelled monoclonal antibodies (mAb) (4.4.3).

Place the slides in a humidity chamber (5.3.13), if required, and incubate at (36 ± 2) °C for the time specified by the manufacturer of the conjugated antibodies.

NOTE 2 The exact volumes and times depend on the type of antibodies and well slides used.

After incubation, remove the slides from the humidity chamber and gently aspirate excess labelled mAb from the side of each well. When performing this step, ensure that the pipette tip does not touch the well surface.

Apply 1 drop of 4′,6′-diamidino-2-phenylindole (DAPI) solution (4.4.7) to each well. Allow to stand at room temperature (20 ± 5) °C for 2 min.

NOTE 3 This timing applies only to slides that have been methanol fixed and subsequently dried.

Remove excess DAPI solution by aspiration (as described above).

Apply a drop of filtered deionized water to each well and then aspirate the excess deionized water (as described above).

NOTE 4 An additional washing step using 0,01 M PBS, pH 7,2 is sometimes used before washing with deionized water.

Allow slides to dry at room temperature (20 ± 5) °C or in an incubator at (36 ± 2) °C.

Store the slides in the dark at (5 ± 3) °C until ready for examination. The slides should be examined as soon after processing as possible and shall be examined the next day.

NOTE 5 Slides have been kept for up to three months in the dark and retained their fluorescence. No detailed investigations have been carried out, however, concerning the loss of fluorescence or DAPI staining upon storage. Keep the slide dry and mount it before examination.

Before the examination, apply approximately 20 µl of slide mountant (4.4.4) to the edge of the well on the sample slide, taking care not to touch the slide with the pipette tip.

Place a coverslip onto the slide, taking care not to create bubbles in the slide mountant (4.4.4). Seal the edges of the coverslip with clear nail polish.

Alternatively, the mounting medium may be pipetted onto the centre of the coverslip and the slide carefully inverted and placed on the coverslip. The slide can then be carefully turned over with the coverslip uppermost. Take care to avoid trapping air bubbles between the slide and the coverslip.

7.4 Microscopy

7.4.1 General

Use an epifluorescence microscope fitted with DIC (5.3.10) for analysis of all sample preparations. Use objectives and eyepieces to a total magnification of 200 × or 400 × and 1 000 ×. Refer to the manufacturer's instruction manual for details of microscope configuration.

Calibrate the eyepiece graticule (5.3.12) at regular intervals. (See Annex C for calibration of the eyepiece graticule.)

Use a magnification of 800 × to 1 000 × for the confirmation of oocysts and cysts.

Within this procedure, oocyst and cyst detection relies upon the manual examination of sample preparations using epifluorescence/DIC microscopy. Although this technique is widely employed, it is time consuming, can cause operator fatigue and, as a result, is open to human error. Consequently, a reliable automated procedure is of considerable benefit. Presently, several instruments that can automatically scan sample preparations are available (e.g. laser scanning cytometry) or are in development. When properly validated, such equipment may be employed.

7.4.2 Examination of fluorescent sample preparations using epifluorescence microscopy

7.4.2.1 General

The output of ultraviolet (UV) light from mercury vapour lamps may vary and will gradually decline as the bulb is used. Check the intensity of the UV light regularly using a fluorescent control slide.

Using the epifluorescence microscope (5.3.10) and a 200 × or 400 × magnification, examine the stained control slides to ensure that, on the positive control slide, oocysts and cysts have been correctly labelled by the mAb and that the negative control slide is free from oocysts and cysts. Repeat this examination at 1 000 × magnification to confirm the staining, the size and appearance of the oocysts and cysts. Examine the contents using the UV excitation filter to ensure that the nuclear material has been correctly labelled by DAPI.

If the positive control slide is negative, repeat the stain before any samples are processed. If the negative control slide is positive, undertake an investigation to determine the source of the contamination. Prepare fresh reagents and stain the control slides again before any samples are processed.

Providing that these checks are satisfactory, examine the samples by scanning each well systematically using epifluorescent microscopy (FITC). Use a side-to-side or top-to-bottom scanning pattern.

When a horizontal row has been completed, identify a feature situated at the bottom centre of the field of view (i.e. sample debris or the edge of the well slide coating). Move the microscope stage so that this feature appears near the top of the field of view. If the scanning has been carried out using a top-to-bottom pattern (vertical rows), then identify a feature situated at the right hand side, centre of the field of view. Move the microscope stage so that this feature appears near the left hand side of the field of view.

— With side-to-side scanning, move the stage horizontally so that the boundary of the well is completely in view, then scan horizontally back across the well.

— With top-to-bottom scanning, move the stage vertically so that the boundary of the well is completely in view, then scan up or down the well as necessary.

Repeat until the whole well has been scanned. Scan using a magnification of 200 × or 400 × and note the number of objects which are presumptive *Cryptosporidium* or *Giardia*. Where there are only one or two objects, examine each object at 800 × to 1 000 × using water or oil immersion objectives to confirm that they are oocysts or cysts. Where there are more presumptive oocysts or cysts, examine the whole slide at 800 × to 1 000 × and confirm each object. This process is easier than switching from a dry low power objective to a high power objective to examine each suspect body.

All objects with typical characteristics of *Cryptosporidium* or *Giardia* (7.4.2.2.) should be further examined and measured using DAPI (7.4.3) and DIC (7.4.4).

7.4.2.2 Identification of *Cryptosporidium* oocysts and *Giardia* cysts: FITC

When labelled with FITC-mAb and examined using epifluorescence microscopy (FITC, filter block), organisms should exhibit the following characteristics.

Table 2 — Identification of *Cryptosporidium* oocysts and *Giardia* cysts

Cryptosporidium oocysts	*Giardia* cysts
Apple green fluorescence (often with bright edges)	Apple green fluorescence (often with bright edges)
Spherical or slightly ovoid in shape Some oocysts will exhibit creases, splits and suture lines	Ovoid in shape Some cysts will exhibit creases and folds
Diameter of 4 µm to 6 µm	Dimensions of (8 µm to 12 µm) × (7 µm to 10 µm)

Count badly distorted and damaged objects with care, particularly when no typical oocysts or cysts are observed on a slide.

NOTE 1 The majority of *Cryptosporidium* oocysts appear spherical or slightly ovoid with brighter even staining around the entire circumference. Some oocysts can deviate from this description. Those which have been in the environment for some time can be weakly stained or appear fuzzy. They may still have contents and sporozoite nuclei can be identified. Often oocysts are split as if a segment has been removed. Under these circumstances, the oocyst may have ruptured during drying on the slide and sporozoites and sporozoite nuclei may be evident adjacent to the oocyst. In addition, oocysts, especially those without contents, may appear to be distorted or partially folded.

NOTE 2 The majority of *Giardia* cysts appear ovoid (8 µm to 12 µm × 7 µm to 10 µm), however, on occasion, cysts may appear spherical with dimensions of approximately 10 µm × 10 µm. Cysts which have been in the environment for some time may stain weakly and be badly distorted, especially those without contents.

NOTE 3 A number of species of both *Cryptosporidium* and *Giardia* have been classified (Annex G). The given size ranges target primarily *C. parvum* and *G. intestinalis*. However other species may be in that size range which may or may not be pathogenic to humans. Alternatively, other species or single bodies of the target species may not be identified as *Cryptosporidium* or *Giardia* due to their size being larger or smaller than the given size range and pathogenicity to humans cannot be ruled out.

When an apple green fluorescent event is observed which is characteristic of a *Cryptosporidium* oocyst or *Giardia* cyst, examine the object with the UV filter block for DAPI staining and subsequently with DIC (7.4.3.and 7.4.4 respectively).

Other objects (e.g. algae) may mimic the size, structure and staining of *Cryptosporidium* and *Giardia*. It is therefore important to further confirm presumptive cells (bodies) by DAPI and DIC.

7.4.3 Identification of *Cryptosporidium* oocysts and *Giardia* cysts: DAPI

Each presumptive oocyst or cyst should be examined to confirm the presence of DAPI staining nuclei using a 100 × oil or water immersion objective. Switch over to the UV filter block on the microscope for DAPI visualization.

NOTE The nuclei of DAPI stained oocysts and cysts appear sky blue upon examination with the epifluorescence microscopy (DAPI UV filter block).

If the object exhibits one of the following characteristics, consider it to be a *Cryptosporidium* oocyst or *Giardia* cyst:

— two to four distinct, sky blue nuclei within a single body;

— nuclear material that may be slightly diffuse giving it a fuzzy or ragged appearance;

— diffuse blue internal staining where distinct nuclear material cannot be identified. (This should not be included in the count.)

Include the two sub-groups in the total count unless they show atypical morphological characteristics such as greater than four nuclei or where one or two large intensely stained nuclei are visible within the object.

7.4.4 Identification of *Cryptosporidium* oocysts and *Giardia* cysts: DIC

Having examined the object using the FITC and DAPI filter blocks, close the light stop for the UV light and switch on the transmitted light source ensuring that the substage condenser is in place. Ensure that the substage condenser turret plate has the correct ICT condenser prism in place.

IMPORTANT — It is important that the light from the mercury vapour lamp is blocked as UV light can damage the DIC filter.

Slide the DIC filter and prism into position and optimize the image by adjusting the light intensity and/or turning the adjustment screw on the prism.

Using DIC, measure the size and look for external or internal morphological characteristics typical of a *Cryptosporidium* oocyst or *Giardia* cyst. Confirmation of size and internal contents should only be done at a magnification of × 1 000 (using a × 100 oil or water immersion objective).

Cryptosporidium oocysts should exhibit one of the following characteristics.

— Spherical or slightly ovoid with a convex central area, the surface of which is irregular in appearance. In addition, a thickened oocyst wall may be observed. This is indicative of an oocyst which contains sporozoites. It may be possible to see sporozoites inside the oocyst as well as a distinct refractile point which is the residual body.

— Spherical or slightly ovoid objects with a thickened oocyst wall. In addition a refractile residual body may be observed. This can be indicative of an oocyst after excystation.

— Spherical or slightly ovoid object that is flat and indistinct. In addition, a thickened oocyst wall is observed. This can also be indicative of an oocyst after excystation.

Giardia cysts should exhibit one of the following characteristics.

— Ovoid with a thickened cyst wall and a convex central area. This is indicative of a cyst with contents. In addition, the nuclei demonstrated by DAPI staining may be observed together with remnants of flagellar axonemes and the median body.

— Ovoid with a thickened cyst wall, the central area appearing flat and indistinct. This is indicative of an empty cyst.

NOTE 1 The identification of organisms using DIC requires much experience. The characteristics given can only be used as guidelines for identification purposes. Misidentification of objects that mimic oocysts and cysts can occur even at this stage of identification.

NOTE 2 *Cryptosporidium*- and *Giardia*-like bodies can show external or internal morphological characteristics atypical of oocysts or cysts (e.g. spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.). The presence of such features indicate that the object is not an oocyst or cyst. Be aware that oocyst contents stained by Evans Blue (present in many mAb preparations) can also fluoresce red.

Under some circumstances, the examination of samples using DIC may not be possible due to the presence of interfering debris. In such circumstances, this should be reported and a decision on the identity of the event should be based on the characteristics of FITC-mAb and DAPI labelling.

8 Quality control procedures

8.1 General

The laboratory undertaking the test shall have a clearly defined quality control system to ensure that the apparatus, reagents and techniques are suitable for the test. The use of positive and negative controls shall be part of the test.

Perform seeding and recovery tests using the combined concentration and separation method at regular intervals (e.g. 1 in 20 samples but a minimum of 1 per month).

See Annex D for preparation of a positive control suspension and recovery tests.

NOTE Suspensions which have been enumerated by flow cytometry and contain known numbers of oocysts are available commercially. Prepare and use in accordance with the manufacturer's instructions. Details are provided in Annex H.

8.2 Equipment cleaning

Wash all equipment that is reused thoroughly in water containing detergent and then rinse in deionized water (4.1.1) to remove any oocysts and cysts that may be attached to the equipment.

NOTE 1 The use of a hypochlorite solution containing at least 1 000 mg/l free chlorine may assist in removing oocysts and cysts from equipment (not recommended for cleaning IDEXX Filta-Max equipment).

Wash equipment used for positive control procedures separately (if possible in a separate area) from equipment used for the analysis of samples.

NOTE 2 The use of disposable material is ideal to avoid carry-over effects. Practice has shown, however, that the use of suitable re-use material, as well as adequate cleaning and quality control measures, can control carry-over problems.

9 Reporting of results

9.1 Expression of results

The whole of the sample pellet should be examined.

Give the number of *Cryptosporidium* oocysts and/or *Giardia* cysts per volume of sample examined. Then calculate the concentration of *Cryptosporidium* oocysts and/or *Giardia* cysts for the standard volume usually given (e.g. 10 l or 1 000 l). Absence of the organism, i.e. none detected, shall be expressed as "not detected" in the sample volume examined.

NOTE The numbers of oocysts or cysts in different aliquots of the pellet can vary considerably. Calculation of the numbers of oocysts and/or cysts in the total volume from a number found in an aliquot of the pellet can therefore result in an over- or underestimation of oocyst or cyst concentration.

9.2 Test report

The test report shall refer to this International Standard and include the following information:

a) all details necessary for complete identification of the sample including the sample site, the nature of the sample and the sampling point;

b) the date of sample collection;

c) the volume of sample collected;

d) the date of sample receipt by the laboratory;

e) the date examination completed;

f) the volume of the sample analysed;

g) the method used;

h) any particular occurrence(s) observed during the course of analysis which may have influenced the result obtained;

i) the number of Cryptosporidium oocysts and Giardia cysts detected;

j) the results expressed (9.1).

Annex A
(normative)

Preparation of reagents

A.1 Elution buffers for Gelman Envirochek™ STD and HV capsule

A.1.1 Tris buffer

Tris	121,1
Deionized water	1 000 ml

To prepare, dissolve the Tris in 700 ml of deionized water and adjust the pH to 7,4 with 1,0 mol/l HCl or NaOH. Make up to 1 000 ml with deionized water. Filter sterilization is not necessary. Store at room temperature (20 ± 5) °C. Expiry date 3 months.

A.1.2 EDTA solution

EDTA (ethylenediamine tetraacetic acid, disodium salt, dihydrate)	186,1 g
Deionized water	100 ml

To prepare, dissolve the EDTA in 800 ml of hot deionized water. Cool to room temperature (20 ± 5) °C and adjust pH to 8,0 with 6,0 mol/l NaOH for initial adjustment and 1,0 mol/l HCl or NaOH for final adjustment. Make up to 1 000 ml with deionized water. Store at room temperature (20 ± 5) °C. Expiry date 3 months.

A.1.3 Elution buffer

Laureth-12	1 g
Tris buffer (A.1.1)	10 ml
EDTA solution (A.1.2)	2 ml
Antifoam A	150 µl
Deionized water	1 000 ml

To prepare, weigh Laureth-12 in a glass beaker and add 100 ml of filtered deionized water (4.1.1).

Heat the beaker to melt the Laureth-12 (approximately 1 min) and transfer the solution to a 1 000 ml graduated cylinder.

Rinse the beaker several times to ensure the transfer of the detergent to the cylinder.

Add 10 ml of Tris solution, pH 7,4, 2 ml of EDTA solution, pH 8,0 and 150 µl of Antifoam A.

Dilute to 1 000 ml with filtered deionized water.

Store the solution at room temperature (20 ± 5) °C, expiry 2 months.

NOTE For frequent use, volumes of up to 10 l can be prepared.

A.1.4 Pre-treatment buffer

Sodium polyphosphate $(NaPO_3)_n$	5 g
Deionized water	1 000 ml

To prepare, dissolve the sodium polyphosphate in the water.

Store at room temperature (20 ± 5) °C. Expiry date 1 week.

A.2 Buffers for IDEXX Filta-Max® filters, phosphate buffered saline with volume fraction 0,01 % Tween 20

A.2.1 Phosphate buffered saline (PBS)

Sodium chloride	8,0 g
Di-sodium hydrogen phosphate (Na_2HPO_4)	1,15 g
Potassium di-hydrogen phosphate (KH_2PO_4)	0,2 g
Potassium chloride	0,2 g
Deionized water	1 000 ml

Dissolve the ingredients in the water and adjust the pH to 7,3 ± 0,2 with 1,0 mol/l HCl or NaOH.

Store at room temperature (20 ± 5) °C. Expiry date 3 months.

A.2.2 Elution buffer

Polyoxyethylene(20)sorbitan monolaurate (Tween 20)	1 ml
PBS (A.2.1)	10 l

To prepare, add approximately 8 l of PBS (A.2.1) to a 10 l container (with tap). Stir this liquid using a magnetic stirrer and stir bar. Dispense 1 ml of Tween 20 to a 50 ml centrifuge tube and dissolve in approximately 10 ml of warm deionized water (4.1.1).

Carefully pour the contents of the centrifuge tube into the 10 l container.

Rinse the tube twice with 10 ml of deionized water (4.1.1), adding the contents of the tube to the 10 l container each time.

Finally, fill the 10 l container to the 10 l mark using PBS (A.2.1). Store at room temperature (20 ± 5) °C, expiry date 1 month.

A.3 Concentration and detection reagents

A.3.1 Immunofluorescence mounting medium

1,4-Diazabicyclo[2.2.2]octane (DABCO)	2,0 g

Glycerol	60 ml
PBS (A.2.1)	40 ml

To prepare, dissolve the DABCO in the glycerol and the PBS. Adjust the pH to 7,1 ± 0,2 with 0,1 mol/l HCl or NaOH.

Store vials in use at room temperature (20 ± 5) °C. Store additional vials at (5 ± 3) °C.

NOTE See Annex H for a list of suitable suppliers. Expiry date printed by the manufacturer on each vial.

A.3.2 DAPI stock solution

DAPI	1 mg
Methanol	0,5 ml

To prepare, add 0,5 ml of methanol to a vial containing 1 mg of DAPI (4.4.5).

Store at (5 ± 3) °C. Expiry date 1 month.

A.3.3 DAPI working solution

DAPI stock solution (A.3.2)	10 µl
PBS	50 ml

Prepare by diluting 10 µl of DAPI stock solution in the PBS.

Store at (5 ± 3) °C. Expiry date one day.

A.3.4 Parasite storage medium — stock solution

Sodium azide (NaN_3)	100 mg
Deionized water	5 ml

To prepare, dissolve the sodium azide in the water.

Store at (5 ± 3) °C. Expiry date 1 month.

A.3.5 Parasite storage medium — working solution

Parasite storage medium — stock solution (A.3.4)	1 ml
Deionized water	100 ml

To prepare, add 1 ml of the parasite storage medium stock solution to 100 ml of deionized water.

Store at (5 ± 3) °C. Expiry date 1 month.

Annex B
(informative)

Concentration of oocysts and cysts from small (10 l) volumes of water

B.1 General

Cartridge filtration can be used for small volumes of water (typically 10 l) but, based on the cost of these filters, this may be an expensive approach in some circumstances. Alternative methods for the concentration of oocysts and cysts from small volumes of water are described in this section. Some of these techniques do not require the purchase of expensive equipment and materials and rely on chemical flocculation. Three different concentration techniques are described here. These are calcium carbonate flocculation, ferrous (II) sulfate flocculation and membrane filtration. These techniques allow the analysis of small volumes of water which can be collected and delivered to the laboratory in a relatively short period of time. Where results are needed urgently, they can be obtained within a few hours. The principles of IMS (7.2) and staining and detection (7.3 and 7.4) described in this International Standard can be followed once oocysts and cysts have been concentrated from water samples.

NOTE Flocculation is not suitable for particle-free water. Water containing a substantial amount of particulate material can be difficult to process by membrane filtration.

B.2 Reagents required for use with calcium carbonate flocculation

B.2.1 Deionized water, 0,2 µm filtered at point of use.

B.2.2 Calcium chloride dihydrate, 1 mol/l.

Add 1 470 g of $CaCl_2 \cdot 2\ H_2O$ to 10 l of water. Store at room temperature (20 ± 5) °C. Expiry date 3 months.

B.2.3 Sodium hydrogen carbonate, 1 mol/l.

Add 840 g of $NaHCO_3$ to 10 l of water. Store at room temperature (20 ± 5) °C. Expiry date 3 months.

B.2.4 Sodium hydroxide, 1 mol/l, 400 g of NaOH per 10 l of water.

Store at room temperature (20 ± 5) °C. Expiry date 3 months.

B.2.5 Sulfamic acid, 10 %.

Add 1 000 g of NH_2SO_3H to10 l of water. Store at room temperature (20 ± 5) °C. Expiry date 3 months.

B.2.6 Polyoxyethylene(20)sorbitan monolaurate (Tween 20), volume fraction 0,01 %.

B.3 Preparation of reagents

For each reagent, add the required amount of the respective chemical to approximately 9 l ± 200 ml of filtered, deionized water (B.2.1) and place on a magnetic stirrer until the chemical has fully dissolved.

Finally, add sufficient filtered deionized water (B.2.1) to bring the volume to 10 l ± 100 ml and mix well.

B.4 Specific apparatus required for concentration using calcium carbonate flocculation

B.4.1 Measuring cylinders, 10 ml, 100 ml, 1 000 ml.

B.4.2 Aspiration tubes.

B.4.3 Vacuum source, with vacuum gauges and vacuum catch bottles/reservoirs.

B.4.4 Centrifuge, capable of 7 200 g and 1 100 g.

B.4.5 Centrifuge bottles, plastic, screwtop, 1 000 ml capacity.

B.4.6 Centrifuge tubes, conical, plastic, 50 ml capacity.

B.4.7 Flocculation container, 10 l carboy.

B.5 Procedure for concentration using calcium carbonate flocculation

To a 10 l water sample, add 100 ml of 1 mol/l $CaCl_2$ (B.2.2) and 100 ml of 1 mol/l $NaHCO_3$ (B.2.3) and then shake the container (B.4.7) to mix.

Add 100 ml of 1 mol/l NaOH (B.2.4) and then shake the container to mix.

Allow the contents to stand at room temperature for a minimum of 4 h. Samples shall be left for no longer than 24 h.

After the floc has settled, aspirate the supernatant to just above the floc precipitate using a suction vacuum pressure of no greater than 20 kPa (0,2 bar) (B.4.3). Take care not to disturb the precipitate.

Add sufficient (100 ml to 200 ml) 10 % sulfamic acid (B.2.5) to dissolve the floc completely.

Swirl the contents of the container to mix and pour into a 1 l centrifuge bottle (B.4.5) labelled with the sample number.

NOTE 1 Larger deposits may require the use of two centrifuge bottles.

Add (200 ± 20) ml of 0,01 % volume fraction Tween 20 (B.2.6) to the container, shake vigorously to rinse and add to the 1 l centrifuge bottle.

Add a further (200 ± 20) ml of 0,01 % volume fraction Tween 20 to the container, rotate the container slowly to pick up froth from around the edges and add to the 1 l centrifuge bottle.

Carefully adjust the contents of the 1 l centrifuge bottle to pH 6 to 6,5 by the addition of 1 mol/l NaOH (B.2.4). Ensure that the pH does not exceed this level as this will result in re-formation of the floc.

Balance 1 l centrifuge bottles in pairs to within 1 g using filtered deionized water. Centrifuge at 7 200 g maximum for 12 min without braking during the deceleration phase.

Immediately after centrifugation, remove the bottles from the centrifuge and carefully aspirate the supernatant liquid to just above the pellet using a vacuum pressure of no greater then 20 kPa (0,2 bar) (B.4.3).

Shake the remaining liquid vigorously to re-suspend the deposited material and transfer to a 50 ml centrifuge tube (B.4.6).

Using a wash bottle add (20 ± 2) ml of 0,01 % volume fraction Tween 20 to the 1 l centrifuge bottle to suspend any remaining sample debris and transfer to the 50 ml centrifuge tube. Make up the volume in each tube to approximately 50 ml with filtered deionized water.

Centrifuge the 50 ml tubes at 1 100 × g for 15 min without braking during the deceleration phase. Record the pellet volume (volume of solids) immediately after centrifugation.

NOTE 2 IMS test kits usually have a defined pellet volume to be used for the test, e.g. between 0,5 ml and 2 ml.

Using a Pasteur pipette and a vacuum source of less than 20 kPa (0,2 bar), carefully aspirate off the supernatant leaving 2 ml to 5 ml above the pellet. If no pellet is visible, take extra care to avoid aspirating oocysts and cysts during this step.

Add deionized water to the centrifuge tube to bring the total volume to 10 ml. Vortex the tube for 10 s to 15 s to re-suspend the pellet and either store the sample at (5 ± 3) °C for IMS or proceed to 7.2.

If the pellet volume exceeds that recommended by the manufacturers of the IMS test kit, centrifuge the sample a second time in a tube which permits the pellet volume to be measured accurately. Sub-divide the sample into aliquots for IMS such that each aliquot represents no more than the maximum pellet volume recommended by the manufacturer and make each aliquot up to 10 ml with filtered deionized water.

B.6 Reagents for use with iron (II) sulfate flocculation

B.6.1 Deionized water, 0,2 µm, filtered at point of use.

B.6.2 Sodium hydroxide, 1 mol/l.

Add 400 g of NaOH to 10 l of water. Store at room temperature (20 ± 5) °C. Expiry date 3 months.

B.6.3 Iron(II) sulfate, 1 mol/l.

Add 2 780 g $FeSO_4 \cdot 7\ H_2O$ to 10 l of water. Store at room temperature (20 ± 5) °C. Expiry date 3 months.

B.6.4 Oxalic acid, 10 % (mass/volume).

B.6.5 Polyoxyethylene(20)sorbitan mono-oleate (Tween 80), 0,1 % volume fraction.

B.6.6 PBS-Tween, 10 mmol/l PBS (pH 7,4) and 0,1 % Tween 80.

B.6.7 Polyoxyethylene(20)sorbitan monolaurate (Tween 20), 0,01 % volume fraction.

B.7 Preparation of reagents

See B.3.

B.8 Special apparatus required for iron(II) sulfate flocculation

See B.4.

B.9 Procedure for concentration using iron(II) sulfate flocculation

Fill a flocculation container (B.4.7) with 10 l of surface water. Adjust the pH to 9,0 ± 0,2 with 1 mol/l NaOH (B.6.2).

NOTE 1 Usually, around 2 ml of NaOH is needed.

Add 20 ml of the ferrous sulfate solution (B.6.3) while stirring and mix for 5 min at 280 rev/min.

After overnight sedimentation (18 h) at room temperature (20 ± 5) °C, remove the supernatant carefully until 4 cm to 5 cm above the pellet (sediment).

Place a beaker under the flocculation tank and collect the sediment (600 ml to 1 200 ml according to the surface water used).

Rinse the flocculation tank with a sufficient volume (100 ml to 150 ml) of 10 % oxalic acid (B.6.4) in order to allow a complete dissolution of the ferric sulfate precipitate.

Rinse the tank with 150 ml of the 0,1 % volume fraction Tween 80 solution (B.6.5) and add this solution to the sediment.

Centrifuge washings at 1 500 g for 10 min in 250 ml or 500 ml conical centrifuge tubes (without braking). Carefully aspirate the supernatant until 1 cm above the pellet. Do not disturb the pellet as the supernatant is discarded.

Measure the pellet volume and re-suspend the pellet with 5 volumes of 0,1 % Tween 80 (5 volumes of Tween 80 for 1 volume of pellet).

Repeat the last washing-centrifugation step.

Centrifuge at 1 500 g for 10 min and resuspend the pellet with 1 volume of PBS-Tween (B.6.6).

NOTE 2 The volume of PBS-Tween added is equivalent to the volume of the pellet, total volume 60 ml to 80 ml usually.

Control the pH of the water concentrate and adjust it to pH 7,2 to 7,4 with PBS (10 mmol/l pH 7,4) if necessary.

Transfer the suspension to 50 ml centrifugation tubes (B.4.6). Add (20 ± 2) ml of 0,01 % volume fraction Tween 20 (B.6.7) to the 1 l centrifuge bottle to suspend any remaining sample debris and transfer to the 50 ml centrifuge tubes. Make up the volume in each tube to approximately 50 ml with filtered deionized water.

Centrifuge the 50 ml tubes at 1 100 g for 15 min without braking during the deceleration phase. Record the pellet volume (volume of solids) immediately after centrifugation.

Using a Pasteur pipette and a vacuum source of less than 20 kPa (0,2 bar), carefully aspirate off the supernatant leaving 2 ml to 5 ml above the pellet. If no pellet is visible, extra care shall be taken to avoid aspirating oocysts and cysts during this step.

Add deionized water to the centrifuge tube to bring the total volume to 10 ml. Vortex the tube for 10 s to 15 s to re-suspend the pellet and either store the sample at (5 ± 3) °C for IMS or proceed to 7.2.

If the pellet volume exceeds that recommended by the manufacturer of the IMS test kit, centrifuge the sample a second time in a tube that permits the pellet volume to be measured accurately. Subdivide the sample into aliquots for IMS such that each aliquot represents the maximum pellet volume recommended by the manufacturer and make up each aliquot to 10 ml with deionized water.

B.10 Reagents required for eluting 142 mm membrane filters

B.10.1 Tween 80 in deionized water, 0,1 % volume fraction.

B.10.2 Deionized water, 0,2 µm filtered at point of use.

B.11 Preparation of reagents

See B.3.

B.12 Scientific apparatus required for concentration using 142 mm membrane filtration

B.12.1 142 mm stainless steel filter housing.

B.12.2 142 mm filter membranes, cellulose acetate, no greater than 2,0 µm pore size.

B.12.3 Peristaltic pump, capable of a flow rate of 1 l/min.

B.12.4 Silicon tubing, for use with the peristaltic pump.

B.12.5 Seeding container, 10 l, if seeding filters is required.

B.12.6 Suitable polythene bag, for washing the filter, e.g. Stomacher bag.

B.12.7 Centrifuge, capable of 1 100 g.

B.12.8 Centrifuge tubes, conical, plastic, capacity 50 ml.

B.13 Procedure for concentration using membrane filtration

Place the membrane filter (B.12.2) into the housing (B.12.1) and clamp on the upper part. Pump the water sample through the filter at a rate of no greater than 1,5 l/min. Rinse the container with 2 l of filtered deionized water (B.10.2) and pump the washings through the filter.

Remove the filter from the filter housing and place into a suitable clean polythene bag (e.g. a stomacher bag) (B.12.6). Add 25 ml of 0,1 % volume fraction Tween 80 (B.10.1) and gently rub the surface of the filter for 1 min through the bag to remove particulate material.

Decant the washings into a 50 ml centrifuge tube (B.12.8). Repeat the wash procedure with a further 25 ml of 0,1 % volume fraction Tween 80 and add this to the centrifuge tube.

Centrifuge the tube at 1 100 × g for 15 min.

Using a Pasteur pipette and a vacuum source of less than 20 kPa (0,2 bar), carefully aspirate off the supernatant leaving 2 ml to 5 ml above the pellet. If no pellet is visible, extra care shall be taken to avoid aspirating oocysts and cysts during this step.

Add deionized water to the centrifuge tube to bring the total volume to 10 ml. Vortex the tube for 10 s to 15 s to re-suspend the pellet and either store the sample at (5 ± 3) °C for IMS or proceed to 7.2.

If the pellet volume exceeds that recommended by the manufacturer of the IMS test kit, centrifuge the sample a second time in a tube that permits the pellet volume to be measured accurately. Subdivide the sample into aliquots for IMS such each aliquot represents the maximum pellet volume recommended by the manufacturer and make up each aliquot to 9 ml with deionized water.

Annex C
(informative)

Calibration of eyepiece graticule

Place the stage micrometer on the microscope stage, turn on the transmitted light and focus the micrometer image. The measuring ruler is usually 1 mm in length, subdivided into 100 units, making each unit 10 µm in length.

Adjust the microscope stage and the eyepiece graticule so that the ‘zero’ line on the eyepiece graticule is exactly superimposed on the ‘zero’ line on the stage micrometer.

Without changing the stage adjustment, find a point as distant as possible from the two ‘zero’ lines where a line on the eyepiece graticule is again superimposed exactly on a line on the stage micrometer.

Determine the number of divisions on the eyepiece graticule and the number of µm on the stage micrometer between the two points of superimposition.

Divide the number of micrometres (µm) by the number of graticule divisions to calculate the number of micrometres (µm) per graticule division.

EXAMPLE If 40 divisions on the eyepiece graticule measure exactly 100 µm on the stage micrometer, then one division on the graticule measures 2,5 µm.

Follow the procedure for each objective. Record the information and keep with the microscope. The microscope should be calibrated at frequent intervals.

Annex D
(informative)

Preparation of positive controls and recovery tests

D.1 General

Positive control suspensions are prepared for monoclonal antibody and DAPI stains used in microscopy. They are also prepared for seeding filters or water samples to determine the recovery efficiency of test procedures and the ability of analysts to operate those procedures. Seeding deionized water samples does not provide an adequate challenge to assess the recovery of *Cryptosporidium* or *Giardia* from normal water samples.

D.2 Determination of the concentration of oocysts or cysts in a stock suspension

Use an improved Neubauer haemocytometer to determine the concentration of *Cryptosporidium* oocysts or *Giardia* cysts in stock suspensions received from suppliers. Use this data to prepare an accurate dilution of stock suspensions for the preparation of seed doses.

If the stock suspension requires dilution to enable enumeration with a haemocytometer slide, use the assumed concentration of organisms stated by the supplier to prepare a 1 ml suspension of oocysts or cysts in a 1,5 ml microcentrifuge tube. Do this by diluting an appropriate volume of the stock suspension in filtered (0,2 µm) 0,01 % volume fraction Tween 20, to obtain a final oocyst or cyst concentration of approximately 1×10^6 per ml. Record the volume used (in microlitres, µl) as volume A, V_A, to be used later.

Use the following Equation (D.1)

$$\frac{c_{req}}{c_{stock}} \times V_{tot} = V_{req} \qquad \text{(D.1)}$$

where

c_{req} is the required concentration of oocysts or cysts per millilitre;

c_{stock} is the concentration of (oo)cysts per millilitre of stock solution;

V_{tot} is the total volume required, in millilitres;

V_{stock} is the volume of stock (oo)cysts required in total volume, in millilitres;

and converting the volume from millilitres to microlitres

$$V_A = V_{stock} \times 1\,000$$

where V_A is the volume of stock (oo)cysts required in total volume, in microlitres.

EXAMPLE If the stock suspension of oocysts is supplied at a concentration of approximately 1×10^8 oocysts per ml, add 10 µl of the stock oocyst suspension to 990 µl of filtered Tween 20 solution. Vortex the stock suspension for a minimum of 30 s prior to the removal of an aliquot.

Prepare the haemocytometer slide and coverslip by rinsing both with 90 % ethanol and drying with lint-free microscope lens tissue.

Wet a piece of lint-free microscope lens tissue with filtered deionized water (4.1.1) and apply a thin layer of water to the surface of the haemocytometer slide where the coverslip will make contact with the haemocytometer slide. Press the coverslip firmly down onto the haemocytometer slide until stable Newton's rings are observed.

Vortex the diluted oocyst or cyst suspension for 0,5 min and load the prepared haemocytometer with a volume of the suspension sufficient to fill it using a micro-pipette.

NOTE Extending vortexing might damage the oocysts and/or cysts. Stock suspensions can be homogenized by tilting the tube at least 20 times.

After a resting period of 1 min (which is required to allow oocysts and cysts to sediment onto the surface of the slide), enumerate the oocysts and/or cysts using differential interference contrast microscopy (7.4.4).

When viewed using DIC, the surface of the haemocytometer slide is subdivided into five main squares which are further divided into either 25 or 16 squares for ease of counting. Initially the number of oocysts or cysts contained within the boundaries of the central main square should be determined. This involves counting the number of oocysts or cysts contained within each of the smaller dividing squares sequentially in a sweeping motion left to right down the main square. To avoid double-counting of organisms, those touching the boundary lines of the small dividing squares should only be counted if they touch either the left or top line. A minimum count of 100 oocysts or cysts is required. If the number is less than this, further main squares need to be enumerated until a count of at least 100 is obtained. The total number of organisms counted and then the number of main squares counted needs to be recorded.

The area of the counting chambers in the main square is 1 mm^2. In order to determine the number of oocysts per millilitre, the count in the main square or the mean count for a number of squares is multiplied by 100 to convert it into square centimetres. The depth of the counting chamber is 0,1 mm so the value is multiplied by a further 100 to give the count per millimetre.

The following Equation (D.2) should be used to determine the number of oocysts or cysts in the diluted stock suspension.

$$\frac{n_{\text{(oo)cysts}}}{n_{\text{square}}} \times 10\,000 = c_{\text{(oo)cyst}} \qquad \text{(D.2)}$$

where

$n_{\text{(oo)cysts}}$ is the total number of oocysts or cysts counted;

n_{square} is the number of main squares counted;

$c_{\text{(oo)cyst}}$ is the number of oocysts or cysts per millilitre.

Perform three replicate counts on the diluted stock suspension and calculate the mean number of organisms.

Use the following formula to determine an accurate number of oocysts or cysts per millilitre in the original concentrated stock suspension. Use this value to calculate subsequent dilution factors, Equation (D.3).

$$\frac{1000}{V_{\text{A}}} \times \bar{c}_{\text{(oo)cyst}} = c_{\text{(oo)cyst}} \qquad \text{(D.3)}$$

where

V_{A} is Volume A, in microlitres;

$\bar{c}_{\text{(oo)cyst}}$ is the mean number of (oo)cysts per millilitre (diluted suspension);

$c_{\text{(oo)cyst}}$ is the number of oocysts or cysts per millilitre.

D.3 Performance data for the positive control suspensions

Prepare a 10 ml suspension of *Cryptosporidium* oocysts and *Giardia* cysts in a 25 ml plastic universal vial, by diluting an appropriate volume of both concentrated stock suspensions in parasite storage media (4.6.11) to obtain final concentrations of approximately 1×10^3 per millilitre per organism, i.e. approximately 100 oocysts and cysts of each organism per 100 µl.

Vortex the suspension of *Cryptosporidium* and *Giardia* for 30 s and then dispense 100 µl aliquots of the suspension onto 10 microscope well slides. Dry, fix and stain the slides (7.3). Determine the number of oocysts and cysts upon the slides using epifluorescence microscopy (7.4.2). Calculate the mean number of each organism per slide, the standard deviation and the coefficient of variation. Record the results.

Ensure that the coefficient of variation for each batch of spike doses prepared using manual procedures is less than 20. If greater variation is observed, prepare a fresh batch. Ensure that the mean number of organisms within each dose is between 80 to 120.

NOTE The coefficient of variation of suspensions prepared using flow cytometry is much smaller (1 % to 2 %).

Commercially prepared suspensions containing a known number of *Cryptosporidium* oocysts are now available. These oocysts have been sorted by flow cytometry, making it possible to add accurate numbers of organisms to any recovery procedure.

D.4 Recovery tests

For recovery exercises, prepare and count a fresh test suspension each week.

D.4.1 Small volumes

Vortex the test suspension for 30 s. Add approximately 8 l of water sample to a suitable 10 l container. Pipette 100 µl of test suspension into the container and make the volume up to approximately 10 l. Process the sample according to the test procedures in Annex B or filter the samples according to 7.1.1 to 7.1.3. Care should be taken to ensure that the flow rate does not exceed that specified by the manufacturer. Where filters are used, once the water has been pumped through the filter, add a further 2 l of filtered deionized water to the container. Rinse the container and process this rinse water through the filter. Process the filters according to 7.1.1 to 7.1.3 depending on the type of filter used and determine the percentage recovery.

D.4.2 Large volumes

Seed the filters according to D.4.1. Connect the filters to the water source under test. If filters are being transported into the field, they should be sealed to prevent water loss and transported as described in Clause 6. The maximum flow should be restricted to that recommended by the manufacturer. A water meter should also be fitted to the filter to determine the volume of water filtered. The filter should be allowed to run until a minimum volume equivalent to that normally analysed in the laboratory has been processed. The filter should then be removed and transported back to the laboratory for analysis.

It is impracticable to give guidance on the percentage recovery that should be achieved. Each water type may give different recovery values. The values obtained in the published literature and provided in Annex E should be used as a guide. Recoveries on the methods described should be an on-going process using water types that the laboratory routinely analyses.

Annex E
(informative)

Examples of indicative performance data

E.1 Calcium carbonate flocculation

Vesey *et al*.[9], describe calcium carbonate flocculation for recovering *Cryptosporidium* from water. A seed of 608 oocysts was inoculated into 10 l of deionized, tap and river water respectively. After flocculation, samples were centrifuged, the pellets stained and counted by flow cytometry. Recoveries for deionized water were 70,8 % to 79,7 % ($n = 3$), tap water 69,0 % to 76,9 % ($n = 3$) and river water 69,0 % to 79,0 % ($n = 3$).

Stanfield *et al.,* 2000[6] also evaluated flocculation with calcium carbonate, ferrous sulphate and aluminium sulphate. Mean recoveries for *Cryptosporidium* in 2 laboratories ranged from 75,0 % to 95,4 % for aluminium sulphate and 60,6 % to 64,1 % for calcium carbonate. Mean recoveries for *Giardia* ranged from 62,2 % to 93,0 % for aluminium sulphate and 50,0 % to 61,8 % for calcium carbonate.

E.2 Iron(II) sulfate flocculation

Stanfield *et al.,* 2000[6] also evaluated flocculation with Iron (II) sulphate. Mean recoveries for *Cryptosporidium* using ferrous sulfate ranged from 74,1 % to 77,1 % and for *Giardia* ranged from 67,6 % to 81,3 %.

E.3 Membrane filtration

E.3.1 General

Dawson *et al*., 1993[5] used membrane filtration with 142 mm, 1,2 µm acrylic copolymer membranes (Sartorius) to recover oocysts from seeded 10 l tap water samples containing particulate material. Samples were cleaned with potassium citrate. The mean seed was 187,5 oocysts and the mean recovery was 25,5 % ($n = 19$).

Several types of membrane filters were examined by Stanfield *et al*., 2000 for both the recovery of oocysts and cysts. Oocysts and cysts at a concentration of 10^5 were seeded into 100 l of water and processed through the filters. No cleaning process was used. One laboratory achieved much better results than a second. The good results were polycarbonate membranes 42,3 % to 83,5 % for *Cryptosporidium* and 20,0 % to 70,2 % for *Giardia*, cellulose acetate membranes 52,5 % to 80,8 % for *Cryptosporidium* and 101,6- 33,1 for *Giardia* and for acrylic copolymer 27,0 % to 96,1 % for *Cryptosporidium* and 31,6 % to 75,6 % for *Giardia*. No details are given of membrane size, porosity or manufacturer.

Francis *et al*., 2001[8] reported recoveries of *Cryptosporidium* from 10 l seeded tap water samples of 72,8 % with a range of 24,0 % to 103,9 % ($n = 45$) over a 12 month period. The recovery range for *Giardia* was 64,3 % ranging from a minimum of 15 % to a maximum of 103,1 %. The data is based on weekly recoveries and includes IMS using Dynal beads.

E.3.2 Idexx Filta-Max®

Sartory *et al*., (1998)[7] evaluated the Idexx Filta-Max® for recovery of oocysts from seeded tap (100 l to 2 000 l) water and river (10 l to 20 l) water. The seed concentration was between 1 040 and 3 600 oocysts. The recoveries for tap water were between 76,9 % and 106,7 % ($n = 16$) and for river water between 79,0 % and 97,2 % ($n = 8$). These samples were counted without further processing.

E.3.3 Aureon IMS — Cryptosporidium

Rock *et al.*, 2001 [3], compared the recovery of *Cryptosporidium* oocysts using the Aureon and Dynal IMS kits. Recoveries using packed pellet volumes of 0,5 ml, 1,0 ml and 2,5 ml demonstrated that the Aureon IMS performed better at the higher pellet volumes. Water concentrates used were from the Rio Grande River and two drinking water concentrates obtained from regulatory monitoring in the United Kingdom. In addition, the pH of the IMS test using the Dynal buffers was found to vary with packed pellet volume and time. Adjusting the pH to 7,00 using the Dynal beads improved recoveries. The Aureon buffers did not require pH adjustment.

The Aureon IMS performed better than the Dynal system with a variety of seeded water concentrates split between the two systems (80,87 % against 55,7 % $n = 7$) and (77,23 % against 27,77 % $n = 12$).

E.3.4 Dynal IMS — Cryptosporidium

Campbell *et al.*, (1997) [4] compared the Dynal IMS beads with recoveries in clean and turbid waters using standard UK and USA methods. In reverse osmosis water using 'aged' oocysts, recoveries of 97,4 % were obtained with a high seed ($2{,}1 \times 10^4$) and in turbid waters 65,8 % ($n = 5$). Using a seed of only 8 aged oocysts recoveries of 7 % in clean water and 6,8 % in turbid waters were recorded ($n = 2$). Using viable (6 months old) oocysts, recoveries in clean water were 90,4 % and in turbid waters were 64,3 % ($n = 4$). This work was done by seeding concentrates.

Watkins *et al.*, (2001) [10] compared the performance of Dynal IMS beads with that of Isolate IMS beads using 1 000 l water samples taken over a 24 h period and Idexx Filta-Max® filters seeded with between 80 and 120 oocysts. Recovery ranged from 45,2 % to 58,1 % with a mean value of 52,0 % ($n = 11$). In an inter-laboratory trial using the same protocols, recoveries ranged from 13,3 % to 71,7 % with a mean value of 45,4 % ($n = 30$). Low recoveries observed in one laboratory were thought to be due to the water chemistry.

E.3.5 Isolate IMS — Cryptosporidium

Watkins *et al.,* (2001) [10] compared the performance of Isolate IMS beads with that of Dynal IMS beads using 1 000 l water samples taken over a 24 h period and filters seeded with between 80 and 120 oocysts. Recovery ranged from 34,7 % to 79,8 % with a mean of 58,8 % ($n = 11$). In an inter-laboratory trial using the same protocols, the recoveries ranged from 2,7 % to 90,3 % with a mean of 42,5 % ($n = 30$). Low recoveries observed in one laboratory were thought to be due to the water chemistry.

E.3.6 CelLabs mAb — Cryptosporidium

Watkins *et al.,* (2001) [10] compared the performance of Isolate IMS beads with that of Dynal IMS beads using 1 000 l water samples taken over a 24 h period and filters seeded with between 80 and 120 oocysts. Recoveries ranged from 51,7 % to 74,1 % with a mean value of 60,5 % ($n = 10$). In an inter-laboratory trial using the same protocols, the recoveries ranged from 4,6 % to 84 % with a mean value of 41,1 % ($n = 30$).

E.3.7 Microgen mAb — *Cryptosporidium*

Watkins *et al.,* (2001) [10] compared the performance of Isolate IMS beads with that of Dynal IMS beads using 1 000 l water samples taken over a 24 h period and filters seeded with between 80 and 120 oocysts. The recoveries ranged from 34,2 % to 72,5 % with a mean value of 54,2 % ($n = 10$). In an inter-laboratory trial using the same protocols, the recoveries ranged from 8,3 % to 68 % with a mean value of 41,3 % ($n = 30$).