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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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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®.

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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.