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Microbiology of the food chain — Detection and enumeration of *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruits

Microbiologie de la chaîne alimentaire — Recherche et dénombrement de Cryptosporidium *et* Giardia *dans les légumes verts frais à feuilles et les fruits à baies*

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

Introduction

Cryptosporidium spp. and *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) are protozoan parasites that can cause enteric illness in humans. Both organisms are characterized by a robust transmission stage, the *Cryptosporidium* oocyst and the *Giardia* cyst, which can survive in moist environments for prolonged periods. These transmission stages are hereafter referred to collectively as (oo)cysts. *Cryptosporidium* oocysts in particular are highly resistant to chlorine at the concentrations used in the treatment of drinking water, and chemical disinfection of leafy green vegetables and berry fruits, where performed during processing, may also be ineffective. Consequently, the absence of vegetative bacteria on fresh produce as indicators of faecal contamination does not necessarily indicate the absence of (oo)cysts. No practical method exists to culture *Cryptosporidium* spp. and *Giardia duodenalis* for the purpose of detection, and therefore, in order to detect contamination with these parasites, direct removal of the (oo)cysts from the food sample must be performed, followed by visualization of the (oo)cysts by microscopy. The methods described in this International Standard are for determining whether *Cryptosporidium* and/or *Giardia* (oo)cysts are present on the surfaces of fresh produce and for their enumeration. This International Standard is based on published methods that have been tested in a multicentre collaborative trial. Alternative methods can be used following a demonstration of their equivalence with this International Standard following the protocol described in ISO 16140.[1]

Microbiology of the food chain — Detection and enumeration of *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruits

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This International Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

1 Scope

This International Standard specifies a method that is applicable for the detection and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts on or in food products that are described herein as fresh leafy green vegetables and berry fruits. With suitable controls, it may also be applicable for the examination of other fresh produce.

The microscopy descriptions are for *Cryptosporidium* spp. oocysts and *Giardia duodenalis* cysts of size ranges which include those species (*Cryptosporidium*) or assemblages (*Giardia*) known to be pathogenic to humans.

This method does not include any molecular analysis and therefore is not suitable for the determination of the species or genotypes/assemblages of *Cryptosporidium* oocysts and *Giardia* cysts. The method will detect all species and genotypes/assemblages that are known to be pathogenic for humans and also others that are not. For further identification, molecular typing assays are required. However, these cannot be reliably performed if process positive controls have been spiked into the samples, as the result of molecular typing assays will be obfuscated.

This method does not allow the determination of viability or infectivity of any *Cryptosporidium* oocysts and *Giardia* cysts which may be present.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 7218:2007, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

3 Terms and definitions

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

3.1
***Cryptosporidium* oocyst**
transmission stage of *Cryptosporidium* spp.

Note 1 to entry: Its detection is based on reaction with specific anti-*Cryptosporidium* antibodies and morphological characteristics as described in Clause 8.

3.2
***Giardia* cysts**
transmission stage of *Giardia* spp.

Note 1 to entry: Its detection is based on reaction with specific anti-*Giardia* antibodies and typical morphological characteristics as described in Clause 8.

3.3
fresh leafy green vegetable
plant leaves eaten as a vegetable, which have not been subjected to any process, except perhaps cutting and washing

3.4
fresh berry fruit
small, round or oblong, fleshy and juicy fruit, which has not been subjected to any process except perhaps cutting and washing

3.5
internal extraction control
(oo)cysts labelled with specific fluorogenic reporters that may be added in defined numbers to the sample prior to processing to assure that the method is operating properly

3.6
positive control
sample to which (oo)cysts have been added in defined numbers prior to extraction to verify that the method after the elution step is operating efficiently

3.7
negative control
sample having an equivalent quantity of material to the tested sample, which is considered to be free of (oo)cysts and processed in the same manner as the tested sample

4 Principle

The principle of the method is based on removal of the (oo)cysts from the sample by elution procedures, followed by concentration in the eluate by centrifugation and isolation by immunomagnetic separation (IMS). Detection of the (oo)cysts is performed by microscopy after labelling with specific monoclonal antibodies (mAbs) conjugated to a fluorochrome.

5 Reagents

5.1 Reagents required for eluting (oo)cysts from leafy green vegetables and berry fruits

5.1.1 Glycine buffer, pH 5,5 for leafy green vegetables (A.2.1).

5.1.2 Glycine buffer, pH 3,5 for berry fruits (A.2.2).

5.2 Reagents required for concentrating, fixing, staining, detection, and quality control

5.2.1 Methanol, analytical grade.

5.2.2 Paramagnetic beads, coupled with antibodies specific to the walls of *Cryptosporidium* oocysts and/or *Giardia* cysts.

5.2.3 Hydrochloric acid (HCl), 0,1 mol/l (A.3.1).

5.2.4 Sodium hydroxide (NaOH), 1 mol/l (A.3.2).

5.2.5 Fluorescently-labelled monoclonal antibodies (mAbs), against *Cryptosporidium* oocysts and/or *Giardia* cysts.

5.2.6 Immunofluorescence mounting medium (A.3.3).

5.2.7 4',6'-diamidino-2-phenylindole dihydrochloride dihydrate (DAPI), freeze-dried reagent.

5.2.8 DAPI stock solution (A.3.4).

5.2.9 DAPI working solution (A.3.5).

5.2.10 Phosphate buffered saline (PBS), pH 7,3 (A.1.1).

5.2.11 Non-fluorescing immersion oil.

5.2.12 Stock suspensions of *Cryptosporidium* oocysts and *Giardia* cysts (Annex B).

5.2.13 Suspensions of pre-labelled and enumerated non-viable *Cryptosporidium* oocysts and *Giardia* cysts.

The fluorochrome label shall be a different colour than that which is used for the detection of the target organism.

5.2.14 Parasite storage medium (A.3.6; A.3.7).

5.2.15 Demineralized and filtered water (ultrapure water type 1).

5.2.16 Fingernail varnish (for sealing coverslips onto slides as necessary).

6 Apparatus

This method requires common microbiological laboratory equipment (refer to ISO 7218) and, in particular, the following.

6.1 Tweezers, for handling fresh produce as necessary.

6.2 A paddle (peristaltic) blender and compatible filtered bags.

6.3 Swing out centrifuge, to accommodate at least 4 × 50 ml conical centrifuge tubes per run.

6.4 Glass Leighton tubes.

6.5 Rotating mixer, compatible with the Leighton tubes.

6.6 Magnetic clip stand, compatible with the Leighton tubes.

6.7 Magnetic clip stand, for microcentrifuge tubes.

6.8 Welled slides, with hydrophobic coating, wells capable of accommodating the 50 µl volume of processed sample after IMS, and coverslips of appropriate size.

6.9 Slide warmer tray, 37 °C to 42 °C incubator, or equivalent slide-drying apparatus.

6.10 Humidity chamber, lidded box containing damp absorbent material, e.g. a paper towel that can be held at the temperature and humidity appropriate for the immunofluorescent reagent.

6.11 Aspiration device, vacuum source equipped with liquid trap and pipette, or equivalent

6.12 Epifluorescence microscope with 20×, 40× objectives and 100× objective immersion lens and a calibrated eyepiece graticule (reticule), shall include fluorescein isothyocyanate (FITC) filter set (480 nm excitation, 520 nm emission filter) and DAPI filter set (375 nm excitation, >420 nm emission). If internal extraction controls are used, an additional filter set suitable for the fluorochrome will be required. Differential interference contrast (DIC) optics are advantageous. A photographic recording system attached to the microscope may be pertinent for recording positive or presumptive events.

6.13 FITC control slide, for evaluation of fluorescence intensity and verification of proper performance of the optical system of the fluorescence microscope.

7 Sampling and transport

7.1 Sampling

A procedure for sampling is not specified in this International Standard. See the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the relevant stakeholders (e.g. competent authorities, regulators, customers) come to an agreement on this subject.

7.2 Transport

Soft fruit samples and other delicate samples shall be handled carefully during transport to preserve their physical integrity (refer to ISO 7218:2007, 8.2).

7.3 Receipt of samples

Samples should be assessed for acceptance criteria using appropriate guidance as required within the purpose of testing (refer to ISO 7218:2007, 8.3). Fresh leafy green vegetables and berry fruits shall be regarded as perishable and analysis shall commence as soon as possible after acceptance. Criteria for rejection can include the presence of mould, decomposition of sample, or loss of sample integrity in the case of berry fruit.

7.4 Storage

Fresh leafy green vegetables and berry fruits should be stored refrigerated (between 4 °C and 8 °C), to reduce sample deterioration (refer to ISO 7218:2007, 8.4).

7.5 Preparation of test sample

The condition of the fresh produce shall be noted before the analytical procedure is commenced.

Test samples shall be at least 25 g.

It is recommended that no more than 100 g of any individual sample is analysed in a single procedure.

When analysing whole leafy green vegetables, such as lettuce heads, it is recommended that a random selection of intact clean leaves (exclude stems) from different parts of the plant should be examined.

For samples that are in small units, for example, raspberries, the sample shall consist of a random sample of these units.

8 Procedure

8.1 Removal of parasites from leafy green vegetables

a) Place sample in filtered bag. Avoid excessive handling. Use tweezers if necessary.

It is recommended that each sample is spiked with an internal extraction control suspension containing a defined number of pre-labelled and enumerated *Cryptosporidium* oocysts and/or *Giardia* cysts following the manufacturer's instructions. The control suspension should be pipetted on to the surface of the leaves; this should be done after the sample has been placed in the processing container. The fluorescent label used on the internal extraction control should differ from that used to detect the target (oo)cysts in the test samples. If internal extraction controls are not used, a positive process control sample is recommended (see 9.2).

b) Add 200 ml glycine buffer pH 5,5.

c) Process sample in paddle blender for 30 s at 200 rpm to 300 rpm.

d) Collect eluate into 50 ml conical-bottomed centrifuge tubes, dividing the eluate equally between them, ensuring that all the vegetable matter is retained in the filter. Squeeze the bag and the filter tightly in order to ensure that all the eluate is obtained from the sample. The eluate may be collected into a vessel before transferring to the centrifuge tubes.

Alternatively, the eluate can be transferred to a single 250 ml conical-bottomed centrifuge bottle, if an appropriate centrifuge/rotor is available for the subsequent step.

Rinsing the sample following washing could increase recovery efficiencies. After transferring the eluate from the filter bag, and while the tubes are being centrifuged [see point e)], add 10 ml 1 mol/l glycine buffer pH 5,5 to the sample and rinse by manipulating the sample from outside the bag. After removal of the supernatant from the centrifuged tubes, add the rinsate from the sample to the tubes. Add a further 10 ml 1 mol/l glycine buffer pH 5,5 to the bag, again manipulate produce and water from outside the bag, and add rinsate to tubes. Centrifuge as in point e).

e) Centrifuge the eluate at 2 500*g* maximum for 10 min with no braking.

NOTE 1 Centrifugation at 2 500*g* maximum can result in a very compact pellet. A lower speed of 1 100*g* maximum for 10 min with no braking has been reported to give at least equivalent recoveries of *Cryptosporidium* oocysts and *Giardia* cysts.

f) Remove the supernatant, by using a pipette and vacuum source, ensuring the pellet is not disturbed. If no pellet is visible, extra care shall be taken to ensure that parasites are not lost during aspiration. This can be done by leaving a small volume of liquid in the bottom of the tube.

g) Re-suspend the pellet in the residual liquid left in the bottom of each tube and combine the pellets into a single centrifuge tube.

h) Rinse the empty 50 ml conical centrifuge tubes with sterile distilled water and transfer the rinsate into the tube containing the combined pellets. Repeat this process until the volume of suspended pellet in the tube does not exceed the capacity of the tube.

i) Centrifuge the eluate at 2 500*g* maximum for 10 min with no braking.

NOTE 2 Centrifugation at 2 500*g* maximum can result in a very compact pellet. A lower speed of 1 100*g* maximum for 10 min with no braking has been reported to give at least equivalent recoveries of *Cryptosporidium* oocysts and *Giardia* cysts.

j) Remove the supernatant by using a pipette and vacuum source, ensuring the pellet is not disturbed. If no pellet is visible, extra care shall be taken to ensure that parasites are not lost during aspiration. This may be done by leaving a small volume of liquid in the bottom of the tube.

k) Estimate the volume of the final pellet.

l) Re-suspend the pellet in 1 ml water and transfer into a Leighton tube. Rinse the centrifuge tube with 2 ml water and transfer to the Leighton tube. Repeat until the Leighton tube contains 9 ml of sample.

If the pellet volume exceeds that recommended by the manufacturer of the IMS test kit, divide the sample into aliquots that do not. Make up each aliquot to 9 ml with deionized water.

NOTE 3 At this point, the Leighton tube can be capped, refrigerated, and the process halted for up to 24 h.

8.2 Removal of parasites from berry fruits

a) Place sample in 500 ml lidded container. Avoid excessive handling. Use tweezers if necessary.

It is recommended that each sample is spiked with an internal extraction control suspension containing a defined number of pre-labelled and enumerated *Cryptosporidium* oocysts and/or *Giardia* cysts, following the manufacturer's instructions. The suspension should be pipetted onto the surface of the berries; this should be done after the sample has been placed in the processing container. The fluorescent label on the internal extraction control should differ from that used to detect the target (oo)cysts. If internal extraction controls are not used, a positive process control sample is recommended (see 9.2).

b) Add 200 ml 1 mol/l glycine pH 3,5.

c) Agitate sample gently for 1 min (e.g. by rolling or slow-speed shaking) to minimize damage to the sample.

d) Transfer eluate to conical centrifuge tubes. If there is excessive detritus from the sample, pour eluate through a sieve or through a paddle blender bag filter and collect eluate into conical centrifuge tubes allowing drainage through the filter for 5 min.

NOTE 1 The eluate can be transferred to a single 250 ml conical-bottomed centrifuge bottle, if an appropriate centrifuge/rotor is available for the subsequent step.

e) Rinse container (and sieve or filter and paddle blender bag if used in point d) with 50 ml 1 mol/l glycine buffer pH 3,5.

f) Transfer rinsate into the conical centrifuge tubes.

g) Centrifuge the eluate at 2 500*g* maximum for 10 min with no braking.

NOTE 2 Centrifugation at 2 500*g* maximum can result in a very compact pellet. A lower speed of 1 100*g* maximum for 10 min with no braking has been reported to give at least equivalent recoveries of *Cryptosporidium* oocysts and *Giardia* cysts.

h) Carefully remove the supernatant, by using a pipette and vacuum source, ensuring the pellet is not disturbed. If no pellet is visible, extra care shall be taken to ensure that parasites are not lost during aspiration. This may be done by leaving a small volume of liquid in the bottom of the tube.

i) Re-suspend the pellet in the residual liquid left in the bottom of each tube and combine the pellets into a single centrifuge tube.

j) Rinse the empty 50 ml conical centrifuge tubes with sterile distilled water and transfer the rinsate into the tube containing the combined pellets. Repeat this process until the volume of suspended pellet in the tube does not exceed the capacity of the tube.

k) Centrifuge the eluate at 2 500*g* maximum for 10 min with no braking.

NOTE 3 Centrifugation at 2 500*g* maximum can result in a very compact pellet. A lower speed of 1 100*g* maximum for 10 min with no braking has been reported to give at least equivalent recoveries of *Cryptosporidium* oocysts and *Giardia* cysts.

l) Remove the supernatant, by using a pipette and vacuum source, ensuring the pellet is not disturbed. If no pellet is visible, extra care shall be taken to ensure that parasites are not lost during aspiration. This may be done by leaving a small volume of liquid in the bottom of the tube.

m) Estimate the volume of the final pellet. Re-suspend the pellet in 1 ml water and transfer into a Leighton tube. Rinse the centrifuge tube with 2 ml water and transfer to the Leighton tube. Repeat until the Leighton tube contains 9 ml of sample.

If the pellet volume exceeds that recommended by the manufacturer of the IMS test kit, divide the sample into aliquots that do not. Make up each aliquot to 9 ml with deionized water.

NOTE 4 At this point, the Leighton tube can be capped, refrigerated, and the process halted for up to 24 h.

8.3 Immunomagnetic separation (IMS)

This technique involves the mixing of the concentrated sample eluate with paramagnetic beads that are coupled to antibodies specific to the walls of *Cryptosporidium* oocysts or *Giardia* cysts, in a buffered medium that enables maximum binding. The (oo)cyst-bead complexes are then separated from other debris in the sample by using a magnet. After separation, the (oo)cysts are dissociated from the beads by vigorous agitation under acidic conditions. The (oo)cysts are transferred in suspension to a microscope slide, where the acid is neutralized by the addition of NaOH (5.2.4).

A second separation step may be included at the dissociation stage, by adding a further aliquot of hydrochloric acid solution to the beads and repeating the dissociation procedure. Either place the suspension from the second dissociation stage on a second microscope slide containing NaOH or add to the original slide with a further aliquot of NaOH.

NOTE 1 Detailed instructions of the IMS procedure are not provided in this International Standard because these instructions are dependent on the kit that is being used.

NOTE 2 The commercial kits currently on the market are specifically designed for water sample concentrates [treated water or untreated (raw) water which is intended for potable supply or environmental water samples].

NOTE 3 It is possible to reduce the quantities of paramagnetic beads and volumes of buffers recommended by the manufacturers of the IMS kits, with no reduction in separation and concentration efficiency.

8.4 Sample staining

a) Use a FITC control slide to ensure that the intensity of the excitation light generated by the lamp is satisfactory and that the field illumination is uniform.

b) Prepare two separate well slides with positive and negative controls. The positive control shall consist of a suspension of *Cryptosporidium* oocysts and *Giardia* cysts (see Annex B). The negative control shall consist of filtered deionized water or PBS. Positive and negative controls shall be included with each batch of samples stained and shall be stained and examined before the samples [see steps e) to l)]. Only proceed with staining sample slides if control slides have stained satisfactorily.

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

d) Add an appropriate volume of 1 mol/l NaOH to the wells of the slide according to instructions from the manufacturer of the IMS kit used, then distribute aliquots of the suspension containing the separated (oo)cysts onto the wells.

e) Place the well slides containing the samples in a slide warmer tray, 37 °C to 42 °C incubator or equivalent slide-drying apparatus and evaporate to dryness. The slides can dry at ambient temperature overnight, provided that there is no danger of cross-contamination or disturbance.

f) Apply a drop of methanol (5.2.1) to each well containing the dried sample and allow to air dry at ambient temperature.

g) Overlay the sample well with fluorescently labelled monoclonal antibodies (mAbs) specific to *Cryptosporidium* oocysts and/or *Giardia* cysts (5.2.5).

h) Place the slides in a humidity chamber (6.10), if required, and incubate as specified by the manufacturer of the conjugated antibodies.

NOTE 1 The exact volumes and incubation times depend on the manufacturer's instructions and well slides used.

i) Remove the slides from the humidity chamber and gently remove excess labelled mAbs from each well.

j) Apply 1 drop of DAPI working solution (5.2.9) to each well. Allow to stand at ambient temperature for 2 min.

k) Remove excess DAPI working solution gently from each well by tilting the slide. Add a drop of filtered deionized water to each well; after a few seconds, remove excess water gently from each well.

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

l) Apply one drop of immunofluorescence mounting medium to the sample slide well.

m) Place a coverslip onto the slide, taking care not to create bubbles in the mounting medium. If the slide needs to be stored, the sample shall be protected from desiccation by sealing the coverslip with a narrow band of fingernail varnish and protected from bleaching by storage in the dark.

n) Store the slides in the dark until ready for examination. The slides should be examined as soon after processing as possible, preferably within 24 h.

8.5 Microscopy

8.5.1 General comments

An epifluorescence microscope, preferably fitted with DIC, should be used for analysis of all sample preparations.

At least two filter blocks will be needed; three if internal extraction controls are used. In the example given in 8.5.2.1, these are

a) FITC filter set (480 nm excitation, 520 nm emission),

b) DAPI filter set (375 nm excitation, >420 nm emission), and

c) Texas Red filter set (555 nm excitation, 630 nm emission).

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 (reticule) at regular intervals.

8.5.2 Examination of sample preparations using epifluorescence microscopy

8.5.2.1 General

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

Using the epifluorescence microscope and a 200 × or 400 × magnification, examine the labelled control slides to ensure that on the positive control slide, (oo)cysts have been correctly labelled by the mAbs and that the negative control slide is free from (oo)cysts. Examine the contents of the (oo)cysts using the UV excitation filter to ensure that the nuclear material has been correctly labelled by DAPI.

If no fluorescent (oo)cysts are observed in the positive control slide, repeat the stain before any samples are processed. If fluorescent (oo)cysts are observed in the negative control slide, undertake an investigation to determine the source of the contamination. Prepare fresh reagents and stain control slides again before any samples are processed.

Providing that these checks are satisfactory, examine the sample slides and batch or sample process controls as described separately below, scanning each well entirely and systematically using epifluorescence microscopy and a magnification of 200 × or 400 ×. Use a side-to-side or top-to-bottom scanning pattern.

If batch positive controls are used, then slides can be examined using the FITC (blue) filter block. Scan using a magnification of 200 × or 400 × and note the number of objects which are presumptive/putative *Cryptosporidium* oocysts or *Giardia* cysts. Closer examination is required for confirmation; examine presumptive objects at 1 000 × using water or oil immersion objectives. It is easier to examine the whole slide at a lower magnification and then return to presumptive objects and examine them at higher power than to switch from a dry low power objective to a high power objective as each presumptive (oo)cyst is noted.

If sample process positive controls are used, then test slides will need to be examined to identify whether each stained object is a seeded control organism or a natural contaminant and for confirmation of each putative/presumptive natural (oo)cyst. For example, where FITC-labelled mAbs and Texas Red-labelled positive control (oo)cysts are used, use the FITC filter block to scan the slide for fluorescing objects. For each object meeting the characteristics in Table 1, use the Texas Red filter block to identify whether the object is a control organism. If it appears to be, use the DAPI filter block to confirm the DAPI staining pattern, record the count, switch back to the FITC filter block and proceed to the next object.

Objects with characteristics of either *Cryptosporidium* or *Giardia*, that are not seeded control organisms, should be measured using the eyepiece graticule (reticule), measurements recorded, and should be examined further using DAPI filters (see 8.5.2.3) and DIC (see 8.5.3) for confirmation at 1 000 × using water or oil immersion objectives. (Oo)cyst numbers shall be recorded.

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

The common characteristics of *Cryptosporidium* oocysts and *Giardia* cysts when labelled with FITC-mAbs and examined using epifluorescence microscopy are listed in Table 1.

Table 1 — Characteristics used in the identification of *Cryptosporidium* oocysts and *Giardia* cysts labelled with FITC-mAbs

Cryptosporidium oocysts	*Giardia* cysts
Apple green fluorescence around the entire circumference of the oocyst wall – often a double edge is visible	Apple green fluorescence around the entire circumference of the cyst wall
Spherical or slightly ovoid in shape	Usually ellipsoid/ovoid in shape but cysts that have dried to the slide in a perpendicular position may appear spherical or spheroid
Some oocysts will exhibit creases or splits; some oocysts may have a typical "pac-man" appearance (i.e. resemble the eponymous video game character)	Some cysts will exhibit creases and folds
Diameter of 4 µm to 6 µm	Dimensions (for horizontally aligned cysts) of (8 µm to 12 µm) × (7 µm to 10 µm)

Count badly distorted and damaged objects with care, particularly when "typical" oocysts or cysts are not observed.

Although the majority of *Cryptosporidium* oocysts and *Giardia* cysts appear as described in Table 1, deviation from these descriptions can occur, particularly for those parasites that have been in the environment for some time. The most common deviation is weak fluorescence or "fuzzy" appearance. Descriptions should be given for each parasite identified. If it is not possible to confirm that the objects are parasites (by DIC and/or DAPI staining of nuclei, or subsequent molecular techniques), they should be recorded as "presumptive" or "putative".

NOTE 1 Several species of both *Cryptosporidium* and *Giardia* have been recognized. The size ranges described in Table 1 are intended primarily as descriptors of *G. duodenalis* and of the major species of *Cryptosporidium* that infect humans. Some *Cryptosporidium* species have diameters up to 8 µm (e.g. *C. andersoni* and *C. muris*) but these are rarely associated with human infection.

Other objects that may be eluted from fresh produce may have similar size, structure, and staining characteristics as *Cryptosporidium* oocysts or *Giardia* cysts. It is therefore important that all objects with these characteristics are confirmed or refuted by further investigation. If it is not possible to confirm or refute, due to lack of characteristic markers such as nuclei, then the objects should be classified as presumptive/putative. In such instances, it is recommended that the slides are examined by experts from other laboratories to confirm or refute the findings.

NOTE 2 Internal extraction control cysts and oocysts will, in addition, fluoresce according to the fluorochrome with which they are labelled.

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

Each presumptive (oo)cyst should be examined for the presence of DAPI-stained nuclei. Switch to the UV filter block on the microscope for DAPI visualization. Use 40 × objective initially and a 100 × oil or water immersion objective if necessary. The nuclei of DAPI-stained (oo)cysts appear sky blue.

The common characteristics of *Cryptosporidium* oocysts and *Giardia* cysts when stained with DAPI and examined using epifluorescence microscopy are listed in Table 2.

The DAPI-staining characteristics for each presumptive *Cryptosporidium* oocyst and *Giardia* cyst should be recorded.

Table 2 — Characteristic DAPI-staining of *Cryptosporidium* oocysts and *Giardia* cysts

Cryptosporidium oocysts	*Giardia* cysts
Within oocysts, sporozoite nuclei appear as small, distinct dots.	Within cysts, trophozoite nuclei appear as distinct dots, often located at one pole of the cyst.
If the oocyst contains sporozoites (is not just a shell), then the nuclei of all four sporozoites are usually visible, although often not in the same plane of focus. Focus up and down.	If the cyst has contents and distinct nuclei, there are usually two or four, depending on the reproductive stage of the cyst. These may be in different fields of focus, so it is important to focus up and down.
Sometimes, but infrequently, the DAPI staining is diffuse.	Frequently, the DAPI staining is diffuse, as the nuclear material is no longer distinct from the other cyst contents. However, patches of more intense fluorescence located at one pole of the cyst may be observed.

Cryptosporidium oocysts and *Giardia* cysts which are empty (only cyst/oocyst walls) will not have any internal DAPI staining, as they lack nuclei. These shall be reported as presumptive.

Parasites that have ruptured during drying onto the slide (as occasionally may occur, particularly with old or environmentally-stressed parasites) may have internal contents adjacent to them and *Giardia* trophozoite or *Cryptosporidium* sporozoite nuclei may thus be evident adjacent to the cyst or oocyst walls. This should be investigated for those parasites with no apparent internal contents and recorded appropriately.

Objects that resemble *Cryptosporidium* oocysts or *Giardia* cysts but are not actually these parasites may have DAPI-staining characteristics that are atypical of (oo)cysts, including red-fluorescing chloroplasts, crystals, spores, etc. The presence of such features indicates that the object is not an (oo)cyst. However, be aware that Evans Blue (a counter stain that is included in many of the commercially available mAbs preparations) may result in some internal contents fluorescing red. In addition, the red background conferred by Evans Blue is not suitable for examination of slides containing control (oo)cysts stained with Texas Red. DNA from other microflora will also stain with DAPI and care shall be taken to ensure that the DNA is not confused with parasite DNA.

8.5.3 Examination of sample preparations using DIC microscopy

8.5.3.1 General

Having examined the object by epifluorescence using the FITC, Texas Red (or other) and DAPI filter blocks, confirm the identification by DIC microscopy. Block the UV light and switch on the transmitted light source ensuring that the correct objective turret, DIC filter and sub-stage condenser prism are in place.

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.

8.5.3.2 Identification of *Cryptosporidium* oocysts and *Giardia* cysts using DIC microscopy

For inexperienced users particularly, first examine the positive control slide, as a reminder of the external or internal morphological characteristics typical of *Cryptosporidium* oocysts or *Giardia* cysts as visualized by DIC microscopy.

Then examine the presumptive and DAPI-confirmed oocysts and cysts, as identified by epifluorescence. Use 40 × objective initially and then a 100 × oil or water immersion objective.

Measure the size of each parasite or presumptive parasite across two axes and look for external or internal morphological characteristics typical of a *Cryptosporidium* oocyst or *Giardia* cyst.

The characteristics of *Cryptosporidium* oocysts and *Giardia* cysts when examined by DIC microscopy are listed in Table 3.

Table 3 — Characteristic appearance of *Cryptosporidium* oocysts and *Giardia* cysts by DIC-microscopy

Cryptosporidium oocysts		*Giardia* cysts	
Intact oocysts, with contents	**Empty oocysts (excysted, ruptured)**	**Intact cysts, with contents**	**Empty cysts (excysted, ruptured)**
Spherical or slightly ovoid, smooth, colourless and refractile, with convex central area, the surface of which appears irregular. A thickened oocyst wall may be observed. It may be possible to see sporozoites inside the oocyst, as well as a distinct refractile point (the residual body).	a) Spherical or slightly ovoid objects with a thickened oocyst wall. A refractile residual body may be observed. This can be indicative of an oocyst after excystation or rupture. b) Spherical or slightly ovoid object with clear opening ("pac-man" appearance). A thickened oocyst wall may be observed.	Ellipsoid/ovoid with a smooth, thickened cyst wall and a convex central area. If nuclei were seen by DAPI, staining these may be observed at one pole of the cyst. Also remnants of flagellar axonemes diagonally across the long axis of the cyst and the median bodies lying transversally in the mid portion of the cyst.	a) Ovoid with a thickened cyst wall, the central area appearing flat and indistinct. b) Ovoid with a thickened cyst wall, which may show distinct creases, breakages and deformities.

For some samples, examination of presumptive (oo)cysts using DIC may not be possible due to excess interfering debris. In such circumstances, this should be noted and a decision on the identity of the event should be based on the characteristics of FITC-mAbs and DAPI labelling.

NOTE 1 Identification of organisms using DIC requires considerable experience. The characteristics given in Table 3 are only intended as guidelines. Misidentification of objects that resemble (oo)cysts can occur, even at this stage of identification.

NOTE 2 Objects that resemble *Cryptosporidium* oocysts or *Giardia* cysts but are not actually these parasites may have external or internal morphological features atypical of (oo)cysts and that have not been visible using epifluorescence microscopy. Such features include spikes, stalks, appendages, pores, one or two large nuclei filling the cell, chloroplasts, crystals, spores, etc. The presence of such features indicates that the object is not an (oo)cyst.

9 Quality control procedures

9.1 General

The laboratory undertaking the test should have a clearly-defined quality control system to ensure that the apparatus, reagents, and techniques are suitable for conducting this analysis.

9.2 Inclusion and interpretation of controls

Internal extraction controls (see 3.5) may be used to verify that the method after the elution step is operating efficiently. If no labelled (oo)cysts are observed, the analytical procedure has failed.

If internal extraction controls are not used, then a positive process control (see 3.6) sample may be created by spiking an additional sample with approximately 100 *Cryptosporidium* oocysts and/or *Giardia* cysts (see Annex B). The spiking suspension should be pipetted on to the surface of the leaves or berries. The positive process control should be included at regular intervals (e.g. 1 in 20 samples but a minimum of 4 annually is recommended). If no (oo)cysts are observed, the analytical procedure has failed.

A negative control (see 3.7) should be included at regular intervals as required within the purpose of testing. If (oo)cysts are observed in the negative control, cross-contamination has occurred during the analysis and any positive test samples should be regarded as potentially false-positive.

9.3 Equipment cleaning

All equipment to be reused should be thoroughly washed in water containing detergent and then rinsed in deionized water to remove any (oo)cysts that may be attached to the equipment.

Equipment used for positive control procedures should be washed separately (if possible, in a separate area) from equipment used for sample analysis.

Use of disposable material (e.g. paddle blender bags) avoids cross-contamination possibilities but is not feasible for all equipment (e.g. Leighton tubes). Having duplicate equipment sets for positive control procedures is another possible intervention. However, adequate cleaning and quality control measures (such as running a negative control directly after a positive control, using the same, but washed, equipment) can be sufficient.

Analysis of negative control samples should be documented in the same way as positive control samples.

NOTE The use of a hypochlorite solution containing at least 1 000 mg/l free chlorine can be useful on pre-cleaned equipment for avoiding detection of cross-contaminant (oo)cysts, by disrupting the epitopes to which the mAbs binds.

10 Reporting of results

10.1 Expression of results

The result should be given as the number of *Cryptosporidium* oocysts and/or *Giardia* cysts detected per weight of fresh produce sample examined.

If internal extraction controls are used, the number of pre-labelled and enumerated non-viable *Cryptosporidium* oocysts and *Giardia* cysts detected should be compared with the number that was spiked into the sample and the percentage recovery calculated for each sample. If a positive process control is used, the number of *Cryptosporidium* oocysts and *Giardia* cysts detected should be compared with the number that was spiked into the sample and the percentage recovery calculated.

10.2 Test report

The test report shall include the following information:

a) a reference to this International Standard, i.e. ISO 18744;

b) all details necessary for complete identification of the sample, including the sample matrix, the sample source and the chain of production as far as is known, and any lot details or batch identification codes;

c) the date of sample collection;

d) the weight of sample collected;

e) the date of sample receipt by the laboratory;

f) the date analysis commenced;

g) the weight of sample analysed;

h) the method used, particularly including any operating conditions that are not specified in this International Standard, or regarded as optional, together with details of any events which may have influenced the results;

i) the number of *Cryptosporidium* oocysts and *Giardia* cysts detected and any relevant details regarding their identification and verification (e.g. size, DAPI-staining, morphological details);

j) the absence of the organism, i.e. none detected, shall be expressed as “not detected” on the sample weight analysed;

k) whether or not a sample control has been included. Report the percentage recovery, determined for each sample by the internal extraction control, or each batch by the positive process control and the calculated limit of detection;

l) the date analysis completed;

m) the name and signature of the analyst; if more than one analyst has conducted the entire analytical chain, then it shall be specified which analyst conducted which sections.

Annex A
(normative)

Preparation of reagents

A.1 General reagents

A.1.1 Phosphate buffered saline (PBS).

Sodium chloride (NaCl)	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 (KCl)	0,2 g
Deionized water	1 000 ml

Dissolve the ingredients in 800 ml water and adjust the pH to 7,3 ± 0,2 with 1 mol/l HCl or NaOH, then bring to 1 l volume.

Store at room temperature 20 °C ± 5 °C for up to six months.

A.2 Reagents for elution of parasites from fresh produce

A.2.1 1 mol/l glycine buffer, pH 5,5.

Glycine ($C_2H_5NO_2$)	75,07 g

Dissolve the ingredient in 800 ml water and adjust the pH to 5,5 ± 0,2 with 1 mol/l HCl or NaOH, then bring to 1 L volume.

Store at 5 °C ± 3 °C for up to three months.

A.2.2 1 mol/l glycine buffer, pH 3,5.

Glycine ($C_2H_5NO_2$)	75,07 g

Dissolve the ingredient in 800 ml water and adjust the pH to 3,5 ± 0,2 with 1 mol/l HCl or NaOH, then bring to 1 L volume.

Store at 5 °C ± 3 °C for up to three months.

A.3 Reagents used in concentration, fixing, staining, detection and QC

A.3.1 Hydrochloric acid (HCl), 0,1 mol/l.

A.3.2 Sodium hydroxide (NaOH), 1 mol/l.

A.3.3 Immunofluorescence mounting medium.

1,4-Diazabicyclo[2.2.2]octane (DABCO)	2,0 g
Glycerol	60 ml
PBS (A.1.1)	40 ml

To prepare, dissolve the DABCO in the PBS and add the glycerol. Adjust the pH to 7,1 ± 0,2 with 0,1 mol/l HCl or 1 mol/l NaOH. Aliquot into vials and store at 5 °C ± 3 °C until use.

A.3.4 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 (5.2.7).

Store at 5 °C ± 3 °C in the dark for up to 12 months.

A.3.5 DAPI working solution.

DAPI stock solution (A.3.4)	10 µl
PBS	50 ml

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

Store at 20 °C ± 5 °C in the dark for up to one month.

A.3.6 Sodium azide stock solution.

Sodium azide (NaN_3)	100 mg
Deionized water	5 ml

To prepare, dissolve the sodium azide in the water.

Store at 5 °C ± 3 °C for up to one year. Sodium azide is very hazardous by skin contact, eye contact, ingestion, or inhalation. Severe over-exposure can result in death. Follow instructions in the Safety Data Sheet.

A.3.7 Parasite storage medium.

Sodium azide stock solution (A.3.6)	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 °C ± 3 °C for up to one month.