STANDARD OPERATING PROTOCOL

FOR THE MONITORING OF *CRYPTOSPORIDIUM* OOCYSTS

IN TREATED WATER SUPPLIES IN ACCORDANCE WITH THE

CRYPTOSPORIDIUM (SCOTTISH WATER) DIRECTIONS 2003

Part 2 - Laboratory and Analytical Procedures

January 2009

CONTENTS

1. INTRODUCTION TO THE STANDARD OPERATING PROTOCOL

1.1 This Standard Operating Protocol (SOP) provides guidance from the Drinking Water Quality Regulator on behalf of Scottish Ministers on the sampling and analysis requirements associated with the *Cryptosporidium* (Scottish Water) Directions 2003 (The Directions).

1.2 This Standard Operating Protocol is published by DWQR in two parts:

- Part 1 Sampling and Transportation of Samples.
- Part 2 Laboratory and Analytical Procedures.

With Part 3 and Part 4 of the Standard Operating Protocol being published by the DWI

- Part 3 Validation of New Methods or Parts of Methods for Sampling and Analysis.
- Part 4 Requirements for the Inter-laboratory Proficiency Schemes.

1.3 Wherever the terms *Cryptosporidium* or *Cryptosporidium* oocysts, or oocysts are used in this Standard Operating Protocol, they refer to all species (active or inactive) of that genus within the size range 4-6μm, [i.e. *Cryptosporidium* spp.]

1.4 Any proposed deviation from the requirements contained in Parts 1 and 2 of this Standard Operating Protocol will require a full laboratory appraisal to be carried out in accordance with part 3 of the Standard Operating Protocol published by the DWI. Results of such appraisal must be submitted to the DWQR for consideration of approval. This approval must be obtained in writing before any changes are made to the relevant sections of the Standard Operating Protocol.

1.5 This part of the protocol provides guidance on the preparation of the approved collection device, receipt of samples in the laboratory and the approved methods of analysis. It also provides details of analytical quality control and validation tests.

1.6 For any supply requiring monitoring a continuous sample of water, consisting of at least 40 litres per hour shall be taken from each point at which the water leaves the treatment works. An exception can be made if the water is subjected to the same treatment at the same treatment facilities before it leaves the treatment works then a continuous sample need only be taken from one of these points. The DWQR must be contacted to determine how many sampling points are required from a treatment works or if you wish to change or reduce the number of regulatory sampling points at a treatment works.

1.6.1 A sample will be taken from a regulatory sampling point at the frequencies specified by the Directions and determined by the risk assessment process as defined in Annex A of the Directions subject to the following:

(a) an interruption of less than one hour in any one day for changing a collection device and any maintenance that may be required; or:

(b) an interruption in the taking of a continuous sample during a period when the water is not being supplied from the treatment works (ie those works operating on an intermittent basis).

1.6.2 Where less than 365 samples are required from a site per annum, each sample shall be taken for a minimum period of 24 hours and a maximum period of 36 hours. Where 365 samples are required per annum, the collection device must be changed at least once per day. Note that a 'day' means the period of 24 hours commencing immediately after midnight. Thus a collection device must be installed on one 'day' (ie within the 24 hours) and taken off the following 'day' (ie within the 24 hours).

1.6.3 At pumping stations which operate intermittently and less than 200 litres of water passes through the collection device during the 'day' then the collection device does not require changing until the day the total volume passing through the collection device equals or exceeds 200 litres unless a period of 72 hours has elapsed since the collection device was fitted.

2. APPROVED LABORATORIES

2.1 Any analytical laboratory participating in the monitoring of *Cryptosporidium* oocysts in treated water under the Direction must have been previously approved for this work by the DWQR**.**

2.2 The Analytical Laboratory

The analytical laboratory must undertake and maintain external auditing and approval by United Kingdom Accreditation Service, (UKAS) of methodology employed for the receipt, preparation and analysis of samples for *Cryptosporidium* oocysts.

2.3 Competence of authorized staff

2.3.1 All authorized staff designated to work on the preparation of sampling equipment, elution, concentration and enumeration of *Cryptosporidium* in samples taken under the Directions must demonstrate that they are competent of undertaking such work. Such demonstrations will initially consist of undertaking test recoveries of oocysts by means of spiking trials. The data obtained from these tests must be stored in their training records, see Appendix F. Methods for such tests are given in Appendices D and E.

2.3.2 A current summary record must be kept for all designated staff working in the *Cryptosporidium* Analytical Laboratory detailing which part or parts of the analysis they are currently authorized to undertake.

2.4 Analysts undertaking Microscopy

2.4.1 All registered microscopists must be able to calibrate the microscope using an eyepiece graticule and stage micrometer. In addition they must be able to determine the correct alignment of both bright-field and epifluorescence illumination, to use both epifluorescence and DIC microscopy and be capable of identifying internal structures of *Cryptosporidium* oocysts correctly. For the initial examination of slides by epifluorescence microscopy, the microscope is set up and objects viewed with the FITC filter (480nm – excitation, 520nm – emission) in place. This set up is used to determine the size and shape of bodies which may be subsequently identified as oocysts. Fluorescent bodies of the appropriate size (4-6μm diameter) are then viewed under the UV filter (350nm-excitation, 450nm-emission) in order to identify, measure and enumerate sporozoite nuclei. DIC is used to measure the size of suspect bodies and to confirm the presence, specifically the number of sporozoite nuclei and sporozoites, or absence of oocyst contents, particularly when no DAPI stained nuclei can be seen under the UV filter. DIC is also helpful in revealing atypical structures that may help to exclude some objects from the criteria identified for *Cryptosporidium* oocysts. All measurements must be undertaken at a total magnification of not less than x 1000 for them to be meaningful**.**

2.4.2 All registered microscopists must be able to change any of the bulbs in the microscope, especially the mercury vapour lamps (paragraph 6.5.2) and set the correct alignment for the bulb. This should be included as part of the training of the microscopists.

2.5 Eye test and Colour Blindness Test Requirements

2.5.1 Prior to being approved, staff working on the microscopic identification and enumeration of *Cryptosporidium* oocysts must take a colour blindness test and an initial eye test. Thereafter, all approved staff working on the microscopic identification and enumeration of *Cryptosporidium* oocysts must take annual eye tests. Such tests may be carried out using spectacles or contact lenses, if these are normally worn whilst working at a microscope. A signed certificate on headed note paper and signed by the optician to demonstrate that the eyesight is suitable for microscopy is required and should be available for audit. A certificate is also required to demonstrate that the person is not colour blind.

2.6 Time Allowed on Microscopic Examination

2.6.1 Authorised staff working on the microscopic identification and enumeration of *Cryptosporidium* oocysts via an eyepiece must spend no more than 1 hour at the microscope without a break and no more than 4 hours in any working day. A minimum break of 15minutes between each hour of microscopy is required. Staff must keep documentary evidence of all time spent in examination of slides. The counting of a slide must not be interrupted. If counting is undertaken via a VDU, the times given in the Visual Display Regulations must be used.

2.7 Interlaboratory Proficiency Tests

2.7.1 In addition, all such designated staff authorised to work on the microscopic examination of slides will be required to participate in monthly inter-laboratory proficiency tests on samples and slides supplied by the DWI or a DWI approved supplier (details of such tests are given in Part 4 of this SOP). Each analyst carrying out microscopical analysis must participate in 10 tests in any 12 month period. Failure to do so will result in DWQR approval for that analyst being withdrawn until such time as competence can be re-established. Action required in respect of unacceptable results is detailed in Part 4 of this Protocol.

2.7.2 The current 'inter-laboratory proficiency scheme' covers all authorized staff designated to work in an analytical laboratory approved to monitor for *Cryptosporidium* oocysts. The test materials consist of:

i) a microscope slide;

ii) a suspension; and

iii) a filter (Filta-Max® or Envirochek™ HV) for analysis.

2.7.3 Microscope Slides

The microscopic slides are either:

i) stained slides displaying only *Cryptosporidium* oocysts at a level near the regulatory limit; and

ii) stained slides displaying typical interferents or oocyst-like bodies other than *Cryptosporidium,* with or without *Cryptosporidium* present.

2.7.4 Suspensions

These are formulated to give a target concentration and are analysed by authorised staff following the procedure normally used in the laboratory. The analysis of suspensions may be varied from time to time at the discretion of the Scheme Organiser.

2.7.5 Filter Modules

Approved filter modules will be spiked with the target concentration which may range between 50 and 200 *Cryptosporidium* oocysts.

2.7.6 The samples analysed as part of the proficiency scheme must be analysed in accordance with the procedure outlined in this SOP. The analysis or part of analysis is to be undertaken by those authorised staff who are analysing the samples for *Cryptosporidium* oocysts under the Regulations. The analysis of the samples should be rotated around all the authorised staff in the analytical laboratory. The DWQR considers that it is important that all designated staff authorised to analyse *Cryptosporidium* oocysts are shown to be competent in the areas of *Cryptosporidium* analysis for which they have been trained. A permanent record shall be maintained of all the results of the inter laboratory proficiency scheme and these records shall be subject to audit by the DWQR or DWQR approved agent(s).

2.8 Analytical Procedures and Health and Safety Issues

2.8.1 Whilst this part of the SOP has been written as if one analyst would complete the whole analysis it is accepted that the work may be divided between a number of analysts. If this is the case then each analyst's work must be fully documented against each sample handled.

2.8.2 With regard to the analytical procedures, every effort has been made to ensure that the following procedures involve a minimum level of risk. Laboratory Managers should however ensure that a full risk and hazard analysis is done, in compliance with Health and Safety Regulations (e.g. COSHH), and to observe safe working practices at all times.

2.8.3 The laboratory environment where analysis of *Cryptosporidium* samples are undertaken should comply with guidelines[1] for category 2 containment. Guidelines include provision of sealed non-absorbent floor surfaces, work surfaces that are impervious and resistant to chemicals, and separate hand washing facilities that are close to the exit of the laboratory. In addition, all cupboards should be labelled with their contents and lighting for all purposes should be adequate. The laboratory environment should be cleaned frequently and in particular, work surfaces should be cleaned and disinfected frequently.

2.8.4 Whilst laboratory acquired infection is rare, staff should be adequately trained in the prevention of infection, not only to themselves but also to other colleagues. Training should include the understanding of risks from *Cryptosporidium* and the potential for infection.

2.9 Documentation

2.9.1 All entries for records must be made in ink using an indelible ballpoint pen. Any mistake must have a single line through it. The correction is then added, signed, dated. All entries and records shall be legible and remain legible throughout the period of use and storage. All such records must be stored in a secure and fire-resistant cabinet when not in use for a minimum period of twelve calendar months following sampling, unless they were associated with a sample that exceeded the treatment standard or a sample that was being investigated by the DWQR. On the rare occasions it may be necessary to retain the records relating to a specific sample for longer, the DWQR will notify Scottish Water in writing. Records must be stored under secure conditions until written authorization is given by the DWQR for their disposal. The requirement to retain records for one year is in addition to the regulatory requirements to retain information entered on the public record.

2.10 Announced Audits

2.10.1 Each laboratory certified to carry out analysis for *Cryptosporidium* under the terms of the *Cryptosporidium* Directions may be subject to at least one announced audit per year. The audit will be undertaken by the DWQR or DWQR approved agent(s) and will cover those areas of sampling, analysis and records of analysis for the regulatory analysis *Cryptosporidium* oocysts undertaken by the laboratory as the DWQR considers necessary.

2.11 Unannounced Audits

2.11.1 The approved laboratory will be subject to unannounced audits when log books, analysis work books, security, analytical methods, storage of samples, slides, reporting arrangements, and other matters of interest may be audited without prior notice being given.

[1] Categorisation of pathogens according to hazard and categories of containment, 1995. Advisory Committee on Dangerous Pathogens. London. Stationary Office.

3. SAMPLING EQUIPMENT

3.1 Provision of an IDEXX Filta-Max® Filter Module and IDEXX Filta-Max® Filter Housing

3.1.1 Scottish Water Scientific Services will supply the sampling team with a suitable filter module and filter housing for sampling at each of the designated water treatment sites. The filter housing must be supplied with a unique number on both parts (base and top) of the housing and must be fitted with the appropriate connectors on the inlet and outlet of the filter housing. Each filter housing must also have a label attached clearly identifying the monitoring point or group of monitoring points at which it is intended to be used. This label will contain the site identification code(s) which will match that on the outside of the sampling cabinet on site.

3.1.2 Prior to use the IDEXX Filta-Max® housing must be cleaned by normal domestic washing procedures in accordance with the manufacturer's instructions (see Appendix A).

3.1.3 An IDEXX Filta-Max® Filter with unique identification number must be fitted into the IDEXX Filta-Max® housing by the laboratory in accordance with the manufacturer's instructions, either the IDEXX Filta-Max® MK I filter housing (Product No. FMC-10502 now discontinued) or the IDEXX Filta-Max® MKII filter housing (Product No. FMC-10505). For the IDEXX Filta-Max® MK I filter housing secure the lid onto the base leaving a gap of approximately 0.5 mm between the lid and the base[2]. For the IDEXX Filta-Max® MKII filter housing the lid should be screwed into the base until the tag holes line up with the serial number leaving a narrow gap of approximately 0.5mm between the lid and the base**. See Appendix A for full instructions and a diagram.** The IDEXX Filta-Max® MKI or II housing must then be air pressure tested to minimum 500kPa (5 bar) but not exceeding 600kPa (6 bar) (by connecting to a laboratory pump) to ensure that there are no leaks. The air supply hose outlet must be fitted with a stainless steel QC6 Swagelok™ Quick-Connect body and stem. The hose line must be connected to the outlet side of the filter housing. A pressure of between 500kPa (5 bar) and 600 kPa (6 bar) must be maintained for not less than 15 minutes with no drop in pressure greater than 10 kPa (0.1 bar) in order for the test to be deemed satisfactory. The IDEXX Filta-Max® MKI or II housing should then be depressurised before it is removed from the pressure testing rig.

3.2 Details of fitting the filter on site are covered in Part 1 on this Standard Operating Protocol.

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[2] Do not over-tighten the housing top as this may make it difficult to undo, and it is unnecessary as there is only a need to apply sufficient pressure to create a seal between the filter module and the "O" rings in the base and top of the housing. If necessary use a light smear of silicone grease on the "O" rings to effect a seal. Care should be taken to ensure the "O" ring is not over greased.

4. RECEIPT OF SAMPLES

4.1 The transportation of samples under the directions must either be carried out by Scottish Water, or a designated courier or a representative of the approved laboratory. The transportation of the sample from end of sampling to receipt by the approved laboratory must be carried out as soon as reasonably practical to ensure that results of the analysis are available as required by The Directions. (See section 7 of Part 1 'Sampling and Transportation of Samples' of the Standard Operating Procedure for details.)

4.2 The sample should be logged into the laboratory and the relevant information from the sampling log sheet transferred into the Laboratory Information Management System (LIMS.)

4.3 Where fast track analysis is specified, the representative of the approved laboratory must ensure that the sample is assigned for fast tracking for analysis.

4.4 It is recommended that where the recorded head loss is significantly higher than normal and/or greater than 4 bar that sample should be placed on fast track for analysis. However, if Scottish Water can clearly show that the high head loss is solely due phosphate dosing then there is no requirement for express analysis. All results must be reported within the timescales set out in The Direction.

4.5 Unless analysis is to start on the day of receipt, the filter unit must be placed in a designated laboratory refrigerator 5 ± 3°C until examination can commence.

5. SAMPLE PREPARATION

5.1 A list of current DWQR approved equipment and materials required to carry out the analysis for *Cryptosporidium* oocysts is given in Appendix B. Other equipment may subsequently be approved.

5.2 A list of general equipment and materials is given in Appendix C.

5.3 Storage of Samples During Analysis

Where indicated in the following method a sample may be stored under temperature controlled conditions.

5.4 Elution and Primary Concentration for IDEXX Filta-Max® Filter Module

5.4.1 In the case of high risk sites it is recommended that the apparatus used for the elution of samples from a designated sample point be used solely for the elution of samples from said point to minimise the risk of cross-contamination. The apparatus should carry an identification label clearly showing the sample location that it is designated for use with. Alternatively, if the wash station is to be used for samples from a number of high risk sites then a separate plunger head and tubing set (concentrator and elution tubing) must be used for each site and must be labelled clearly to indicate the sample site for which they are to be used.

5.4.2 A IDEXX Filta-Max® membrane is carefully placed and positioned in the concentrator, so that it lies flat. **Ensure that the membrane is rough side up in the wash station assembly** (this step in the procedure has been confirmed by the manufacturer and applies to both existing and new stock). Screw the concentrator tube (the longer of the two tubes) onto the concentrator base, taking care to avoid cross-threading and damaging the membrane. Ensure that a tight seal is created by placing the concentrator base into the jaws of the wash station as the tube is screwed into place. Take the assembled concentrator tube out of the jaws and place on the bench.

5.4.3 If the filter has been stored in the refrigerator, allow the filter to reach room temperature before elution commences.

5.4.4 Pour excess liquid from the filter housing into the assembled concentrator tube, and screw the filter module onto the base of the plunger head. Note the appearance of filter in analysis workbook. E.g. ‘clean’, ‘heavily loaded’ or ‘stained brown’. Pull the plunger down until the filter module is located at the base of the elution tube. The locking pin (located at the top left-hand side of the wash station) should “click” in to lock the plunger in position.

5.4.5 Remove the filter module bolt using an Allen key by turning the key in an anti-clockwise direction (when viewed from below the elution tube base – see manufacturer’s instructions) and attach the steel tube to the elution tube

base. Screw the assembled concentrator tube into the elution tube base. Take care to avoid cross threading. Release the locking pin.

5.4.6 Pour 600ml ± 20ml phosphate buffered saline/tween (PBST) into the assembled concentrator tube. Open the valve on the base of the concentrator, allow a small volume of buffer to pass through and close the valve.

5.4.7 Perform 5 plunges to "wet" the filter and leave to soak with the plunger arm in the up position to permit re-expansion prior to washing. Providing that you can see visual expansion of the filter the washing procedure can be undertaken. Wash the filter module by moving the wash station plunger up and down twenty times. To avoid excess foam generation during this process, gentle movements of the plunger are recommended. **It is important that consistency is maintained and to ensure that full strokes of the plunger are undertaken at the elution stage.**

NB

(i) The plunger has an upper limit restriction during the wash process to avoid the plunger "popping out" of the top of the chamber.

(ii) An investigation by IDEXX has shown that expansion is more important than pre-soaking and all that is required to obtain satisfactory recovery of the oocysts is an expansion of 5 mm of the filter module. It is unlikely that any significant increase in recovery would occur if the pre-soak were greater than five minutes. The requirement to pre-soak the filter module for a maximum of two hours has now been superseded because it has been shown to be unnecessary.

(iii) If automated wash stations are used the wash station counters must be calibrated and checked daily. A check count (of at least 10 cycles) must be performed daily prior to regulatory analysis and a record of the number of cycles must be recorded in the analysis workbook. See Appendix A for instructions on the operation of the automatic wash station.

5.4.8 Compress the filter and detach the concentrator module from the base of the elution module and lower it to the point where the stainless steel tube is above the level of the liquid.. Remove the remaining liquid from the elution module by moving the plunger five times up and down and locking the wash station plunger in place (NB: The filter may sometimes over expand and make locking difficult). Rinse the outside of the stainless steel tube with 2-5 ml PBST from a wash bottle. Cover the end of the stainless steel tube to prevent loss of sample.

5.4.9 Place the assembled concentrator tube on a magnetic stirrer, and attach the lid with stirring magnet attached. Connect the IDEXX Filta-Max® waste bottle trap and hand vacuum pump or electrically pumped vacuum system to the valve at the base of the assembled concentrator tube, begin stirring and

open the valve after the liquid has reached a stable rotational velocity. The waste bottle trap shall collect the sample from only one wash station to prevent loss of sample in case the membrane fails. Only if necessary pump to obtain a vacuum ensuring the gauge reading does not exceed a maximum of 40kPa (11.8 inches, 30cm of mercury). Do not drain away all the liquid and ensure that the membrane is not allowed to go dry. **Use the minimum vacuum required to concentrate the sample when necessary. With clean samples it is not always necessary to use a vacuum.**

5.4.10 Allow the sample level to slowly drain until approximately 20ml remains (level with the middle of the magnetic stirrer bar) and then close the valve. Remove the lid, rinse the stirrer bar into the concentrator and pipette or pour out the contents into a 50ml conical centrifuge tube and retain. (NB samples containing excessive deposits may cause the membrane to clog and the next sub-sample(s) may need to be filtered using separate membrane(s)). If the membrane blocks, further membranes must be used and the membrane must be changed avoiding any losses. **At this point the membrane may be used smooth side up.**

5.4.11 Add another 600ml ± 20ml of PBST to the same assembled concentrator tube, remove the bung from the base of the steel tube and screw the assembled concentrator tube back onto the base of the elution module.

5.4.12 Repeat above wash steps, with the following exceptions:

(i) only 10 wash strokes are required instead of the 20 used in the first wash,

(ii) once the sample level is down to approximately 50ml stop flow and turn off the stirrer lift the stirrer and add the concentrate from the first washing and carry on filtering until the total volume is again down to approximately 20ml;

(iii) before collecting the concentrate, rinse the magnetic stirrer bar with 2-5ml PBST from a wash bottle into the assembled concentrator module,

(iv) collect the concentrate in the same 50ml conical centrifuge tube used for the first run,

(v) for excessively dirty samples it may be necessary to use two or more membrane filters during the concentration step. **At this point the membrane may be used smooth side up** (this step in the procedure has been confirmed by the manufacturer and applies to both existing and new stock)**.** The membranes must be changed avoiding any losses; and

(vi) the magnetic stirrer must be rinsed between filtrations.

5.4.13 Insert the empty assembled concentrator tube in the jaws of the wash station, detach the concentrator tube, remove the membrane with appropriate forceps

and place it in the bag supplied by IDEXX (or equivalent). Add 5ml of PBST, seal the bag and rub the surface of the membrane for approximately 1 minute between thumb and forefinger until the membrane appears to be clean. When a large number of membranes are used for a highly turbid sample they should be washed individually and the washings combined with the rest of the concentrated eluate before centrifugation. Remove the eluate using a pipette and add to the concentrate fraction obtained at the end section 5.4.10. The washing process should be repeated using a further 5ml of PBST. This may result in more than 50ml of eluate and the washings requiring centrifugation. In this case, the combined eluate should be divided equally into two or more 50ml centrifuge tubes and each processed in accordance with paragraph 5.5.

5.4.14 During concentration and separation, the filter eluate is further concentrated by centrifugation, and any oocysts in the sample are then separated from other particulates using Dynal immunomagnetic bead separation (IMS).

NB Samples may be stored at this stage under secure conditions in a refrigerator at a temperature of between $2^{o}C$ and $8^{o}C$ until ready for centrifugation.

5.5 Secondary Concentration (for Dynal IMS and Cellab FITC)

Centrifuge the 50ml centrifuge tube containing the filter eluate at 1100g, for 15 minutes. The relative centrifugal force (RCF) of the centrifuge must be optimised for maximum recovery of oocysts. If an alternative relative centrifugal force (RCF) is used this must be validated. Full details of the method of validation and the data produced must be available for audit. Allow the centrifuge to coast to a stop without braking, this is very important and failure to do so could affect recovery due to their extended path length and resuspension of the pellets due to currents created during deceleration. With a Pasteur pipette or venturi vacuum pump with a disposable micro-pipette tip, and using gentle suction carefully aspirate off the supernatant to just above the pellet so that approximately 2-5ml of liquid remain above the pellet. Care should be taken to ensure that the pellet is not disturbed to minimize the possibility of losing oocysts.

The relationship between rpm and RCF is detailed in Appendix G

5.5.1 If the pellet volume is less than or equal to 0.5ml, record the pellet volume accordingly and the date and time that concentration was completed in the analysis workbook. Add reagent water to the centrifuge tube to bring the total volume to 7.5-9ml. Cap the tube and vortex for 10 to 15 seconds to re-suspend the pellet. Proceed to section 5.6 Dynal IMS Procedure.

NB Samples may be stored at this stage in a refrigerator at a temperature of between 2 and 8°C until ready for stage 5.6. If a number of samples were generated at paragraph 5.4.13 the oocysts found in the whole deposit is the sum of the number of oocysts in all the sub samples.) After the sample is

taken out of the fridge it is brought to room temperature and re-vortexed for 10-15 seconds.

5.5.2 If the pellet volume exceeds 0.5ml then make up to 10ml using oocyst-free reagent water (distilled, de-ionised or reverse osmosis water) in the centrifuge tube. Cap the tube and vortex for 10 to 15 seconds to re-suspend the pellet. Transfer the re-suspended deposit to a 15ml conical centrifuge tube. Rinse out the 50ml centrifuge tube with a further 2ml reagent water and transfer into the 15ml conical centrifuge tube. Repeat this wash step again if necessary. Centrifuge the 15ml conical centrifuge tube at 1100g, or another suitable speed for 15 minutes. Allow the centrifuge to coast to a stop without braking.

5.5.3 When more than one 50ml tube is required for very turbid samples (see paragraph 5.4.13) then full records must be kept for each 50ml tube and their contents subject to separate preparation for the IMS procedure as in paragraph 5.5.2. The sample pellet volume is the sum of the sample pellet volumes for each tube. Each 15ml centrifuge tube then used is treated as a sub-sample of the whole sample.

5.5.4 Record the pellet volume (volume of solids) and the date and time that concentration was completed in the analysis workbook. Without disturbing the pellet, with a Pasteur pipette or venturi vacuum pump with a disposable micro-pipette tip, and using gentle suction, carefully aspirate off the supernatant. Care should be taken to ensure that the pellet is not disturbed to minimize the possibility of losing oocysts.

5.5.5 Add reagent water to the centrifuge tube to bring the total volume to 10ml. Cap and vortex the tube for 10 to 15 seconds to re-suspend the pellet. Then split the sample between a number of 15ml centrifuge tubes to give no more than 0.5ml deposit in any one tube, i.e. if the pellet volume was 0.7ml use two centrifuge tubes and if 1.2ml use three tubes. Make up the volume in each tube to 7.5-9ml and proceed to section 5.6 (Dynal IMS Procedure) treating each centrifuge tube as a sub-sample.

NB Samples may be stored at this stage in a refrigerator at a temperature of between 2 and 8°C Until ready for stage 5.6. If a number of samples were generated at paragraph 5.5.3 and also 5.4.13,, the oocysts found in the whole deposit is the sum of the number of oocysts in all the sub samples.) After the sample is taken out of the fridge it is brought to room temperature and re-vortexed for 10-15 seconds.

5.6 Dynal IMS Procedure

The Dynabeads® anti-*Cryptosporidium* is for the rapid, selective separation of *Cryptosporidium* oocysts from water sample concentrates using IMS. See Appendix B.3.5 for storage details. *Cryptosporidium* spp. oocysts can be separated from samples of not more than 10 ml volume with Dynabeads anti-*Cryptosporidium*. The quantity of particulate matter in each 10ml water

sample concentrate should be such that the packed pellet volume is 0.5ml or less (5%) or less.

5.6.1 Preparation of reagents.

5.6.1.1 For each 10ml of sample, sub sample or control the following quantities of buffers will be required:

1ml of 1 x SL™-buffer-A" (clear, colourless solution)

1ml of 10 x SL™-buffer-A" (clear, colourless solution)

1ml of 10 x SL™-buffer-B" (supplied magenta solution)

5.6.1.2 To prepare a 1 x dilution of SL-buffer-A from the "10 x SL™-buffer-A" (clear, colourless solution) supplied using oocyst-free reagent water as the diluent. For every 1ml of 1 x SL-buffer-A required, take 100µl of "10 x SL™-buffer-A" and make up to 1ml with reagent water. Retain the 1 x SL™-buffer-A in a labelled container for use later in the procedure.

5.6.1.3 To a Dynal L10 tube (125 x 16 mm flat-sided Leighton tube) with 60 x 10mm flat sided area add 1ml of the "10 x SL™-buffer-A".

5.6.1.4 Add 1ml of the "10 x SL™-buffer-B" (supplied magenta solution) to the Dynal L10 tube containing the "10 x SL™-buffer-A".

5.6.1.5 The Dynal L10 tube containing the mixed SL-buffer A and B is then ready for use in oocyst capture in 5.6.2.

NOTE: a crystalline precipitate may form in the 10 x SL™-buffer-A if exposed to prolonged storage at 0 to 4^{o}C. Ensure that the precipitate has dissolved by equilibration to room temperature (15 - 22 ^{o}C) before use.

5.6.2 Oocyst Capture

NB Care must be taken to avoid interruptions to the following procedure, except where indicated.

5.6.2.1 Transfer the water sample concentrate (from section 5.5.1 or section 5.5.5) to the Dynal L10 tube containing the mixed SL-buffer prepared above and use a further 1 to 2.5ml oocyst-free reagent water to rinse out the centrifuge tube. Label the Dynal L10 tube with the sample number and place open tube in a tube rack. If the sample has been stored overnight then it must be vortexed before proceeding with the oocyst capture.

NOTE: As said above the total IMS sample volume (excluding buffers) should not exceed 10ml.

5.6.2.2 Vortex the Dynabeads® anti-*Cryptosporidium* reagent for approximately 10 seconds to resuspend the beads. Ensure that the beads are fully resuspended

by inverting the vial and making sure that there is no residual pellet at the bottom.

5.6.2.3 Add 100µl of the resuspended beads to the Dynal L10 tube (see paragraph 5.6.2.1) containing the water sample concentrate and the mixed SL-buffer. Ensure that the Dynabeads® are well mixed before adding to each sample and revortex the Dynabeads® when necessary. Cap the tube.

5.6.2.4 Affix the Dynal L10 tube to a rotating mixer (eg Dynal-MX1 or Dynal Sample Mixer) and rotate at 15-25rpm for at least 1 hour at room temperature.

5.6.2.5 After rotating for at least 1 hour, remove the Dynal L10 tube from the mixer and place in the Dynal Magnetic Particle Concentrator (Dynal MPC-1) or a Dynal multi-tube Magnetic Particle Concentrator (Dynal MPC-6) with flat side of Dynal L10 tube towards the magnet.

5.6.2.6 Without removing the Dynal L10 tube from the Dynal MPC-1 (or Dynal MPC-6), place the magnetic side of the Dynal MPC-1 (or Dynal MPC-6) downwards, so the Dynal L10 tube is horizontal and the flat side of the tube is facing down and above the magnet.

5.6.2.7 Gently rock the Dynal L10 tube by hand end-to-end through approximately 90^{o}, tilting cap-end and base-end of the tube up and down in turn. Continue the tilting action for two minutes with approximately one tilt per second. To achieve this the user needs to do one tilt per second for the to and another for the fro[3]. Ensure that the tilting action is continued throughout this period to prevent binding of low-mass material that is magnetic or magnetizable. If the sample in the Dynal MPC-1 (or Dynal MPC-6) is allowed to stand motionless for more than 10 seconds then the tube should be removed and the beads re-suspended by gentle shaking. Then the rocking procedure must be repeated for two minutes before continuing.

5.6.2.8 Return the Dynal MPC-1 (or Dynal MPC-6) to the upright position so that the Dynal L10 tube is vertical with the cap at the top. Immediately remove cap and decant all the supernatant from the tube held in the Dynal MPC-1 (or Dynal MPC-6) into a suitable container. Carefully decant the tube such that the flat face and the magnet are uppermost to help retain particles. Providing the particles have not been disturbed during the decanting process the supernatant can be discarded. Do not shake the tube and do not remove the tube from the Dynal MPC-1 (or Dynal MPC-6) during this step. The supernatant from the Dynal tube should be retained as a separate sample until the step has been satisfactorily completed. For guidance see the Dynal *Cryptosporidium* CD.

5.6.2.9 Remove the Dynal L10 tube from the Dynal MPC-1 (or Dynal MPC-6) and resuspend the sample in 0.75ml-0.9ml 1 x SL-buffer-A. Mix very gently to resuspend all material in the tube. **Do not vortex.**

[3] Email from Dynal Biotech Ltd 28/11/02.

5.6.2.10 Transfer all the liquid from the Dynal L10 tube to a labelled, 1.5ml microcentrifuge tube; add 0.1ml-0.25ml of dilute buffer to the L10 tube to rinse then pool in the same microcentrifuge tube ensuring that no beads are left behind.

NOTE: The combined volume from 5.6.2.9 & 5.6.2.10, collected in the microcentrifuge tube, should be approximately 1ml

5.6.2.11 Place the microcentrifuge tube in the second magnetic particle concentrator (Dynal MPC-M or Dynal MPC-S), with the magnetic strip in place. **Important with the Dynal MPC-S magnetic particle concentrator the magnetic strip must be in the vertical position.**

5.6.2.12 Without removing the microcentrifuge tube from Dynal MPC-M or Dynal MPC-S gently rock/roll the tube through 180^{o} by hand. Continue for 1 minute with one gentle 180^{o} roll and rock per second. The magnet is rocked 180 degrees in one second in one direction and then rolled back in another second[4]. At the end of this step, the beads and oocysts should produce a well-formed brown dot on the back of the tube.

5.6.2.13 Uncap and carefully aspirate all the supernatant from the tube and the cap held in the Dynal MCP-M or Dynal MCP-S, and recap the tube. Take care not to disturb the material attached to the wall of the tube adjacent to the magnet. If more than one sample is being processed, conduct three gentle 180^{o} rock and roll actions before removing the supernatant from each tube. Do not shake the tube. Do not remove the tube from the Dynal MPC-M or Dynal MPC-S while conducting these steps.

5.6.3 Dissociation of Beads/Oocysts Complex

5.6.3.1 Remove the magnetic strip from the Dynal MPC-M or Dynal MPC-S.

5.6.3.2 Uncap and add 50µl of 0.1M hydrochloric acid (HCl), recap and then vortex thoroughly for 10 seconds.

5.6.3.3 Place the tube in the Dynal MPC-M or Dynal MPC-S and allow to stand in a vertical position for 5 minutes at room temperature.

5.6.3.4 Whilst the tube is standing remove the required number of 9mm diameter well slides from the box stored at room temperature and ensure that they are clean and grease free. Then at the end of the slide label slide with sample ID. Use slides which are approved for use with the appropriate IMS kit (normally those from the same manufacturer as the kit used, see Appendix B.2.6.

5.6.3.5 After the tube has stood for five minutes, vortex it thoroughly for 10 seconds. After vortexing ensure that all the sample is at the base of the tube.

[4] Email from Dynal Biotech Ltd 28/11/02.

5.6.3.6 Either:

(i) Replace the magnetic strip in Dynal MPC-M and replace the tube in Dynal MPC-M. Allow the tube to rest horizontally for a minimum of 10 seconds so that the magnetic beads attach to Dynal MPC-M magnet; or

(ii) Replace the magnetic strip in Dynal MPC-S **in the tilted position** and allow the tube to stand undisturbed for a minimum of 10 seconds so that the magnetic beads attach to Dynal MPC-S magnet.

5.6.3.7 Add 5µl of 1.0M sodium hydroxide (NaOH) to the slide sample well. See paragraph 6.2.1 of 'Guidelines for Calibration in Laboratories' February 2001, Water Services Unit, Scottish Executive Information Letter 5/2001.

5.6.3.8 Return the microcentrifuge tube in the Dynal MPC-M or Dynal MPC-S to the vertical position and transfer the entire sample from the tube to the slide sample well containing the NaOH. Do not disturb the beads at the back wall of the tube. Gently mix sample with the NaOH using the transfer pipette.

NB Samples may be stored at this stage at room temperature until dry and then in a secured refrigerator at 5 ± 3°C in a dry box until ready for Section 6, or incubated at a temperature not exceeding 42°C until the evaporation step is complete. Samples are stained and examined according to the Standard Operating Protocol Part 2, 'Laboratory and Analytical Procedures' Section 6.

6. EXAMINATION (Staining, Detection and Enumeration)

6.1 Daily Positive and Negative Control Slides

6.1.1 A daily positive and negative control slide must be prepared at the beginning of each day prior to the examination of any batch of slides to determine that:

(i) the reagents are satisfactory;

(ii) there has been no cross-contamination with *Cryptosporidium* oocysts;

(iii) the fluorescence is satisfactory; and

(iv) the oocyst suspension is still satisfactory.

In addition, the laboratory should check that the 0.1M hydrochloric acid used to dissociate the oocysts from the beads and the 1.0M sodium hydroxide to neutralize the acid, are still satisfactory. This is easily accomplished by spotting on an appropriate pH paper to determine that the acid and alkali solutions are satisfactory. Providing both the acid and alkali as well as the initial positive and the negative control slide(s) are satisfactory then the batches of regulatory slides can be stained. More than one initial positive control slide may be prepared by the laboratory. The positive control is prepared from an approved source of oocysts. Exact counts of oocysts are not

required as the test is a daily check on the reagents and oocysts and not a quantitative check.

6.1.2 The positive control slide(s) must be prepared in a spatially separate designated area to minimize any risk of any cross-contamination. In addition, the positive control slide(s) should placed on a separate tray for staining and when pipetting the stain onto the slides ensure that a clean pipette tip is used to avoid cross contamination.

6.1.3 Each positive control slide is prepared in accordance with the Standard Operating Protocol used for the analysis of regulatory samples.

6.1.4 The negative controls should be handled with the samples to detect any possible cross contamination on their journey through the procedure.

6.1.5 A fresh negative control slide must be stained with every additional batch of regulatory slides.

6.2 Checks on the Initial Staining of the Positive Control Slide

6.2.1 Examine the initial positive control slide(s) at a minimum magnification of X200. The positive control slides should contain typically FITC stained oocysts.

6.2.2 Engage the X100 oil immersion lens and examine at least 5 oocysts using the FITC filter, DAPI filter and Nomarski differential interference contrast (DIC) microscopy to determine that the oocysts have been stained satisfactorily.

6.2.3 Ensure that the oocysts demonstrate typical FITC staining characteristics in addition to clearly identifiable DAPI stained nuclei (no greater than 4 sporozoite nuclei per oocyst).

6.2.4 Using DIC ensure that all five oocysts demonstrate typical characteristics and have diameters between 4 – 6µm.

6.2.5 Record all staining and DIC is satisfactory after examination of this control slide.

6.2.6 For each negative control, pipette 25 – 50µl (equivalent to the volume used to prepare the positive control slides)) of oocyst free water into the centre of a slide well and allow to spread over the well area. Ensure that the sample does not spread beyond the non-coated area of the well.

6.2.7 A minimum of one negative control slide should be examined to determine that it does not contain oocysts under the FITC filter at a total magnification of not less than X200. If any of the negative control slides contain oocysts, an immediate investigation must be undertaken and a written report prepared on how the slides became contaminated with oocysts. The anomaly must be reported to the DWQR.

6.2.8 A record should be made of the results of the examination of the initial positive and negative control slide.

6.2.9 If the conditions described in section 6.1 are not met, new positive and negative control slide(s) must then be prepared according to section 6.1 and stained using a fresh batch / bottle of FITC labelled monoclonal antibody and a freshly prepared DAPI solution. No further samples should be stained until it has been established that the new reagents are working optimally. Note the background fluorescence, if any.

6.3 Checks on Microscopy

6.3.1 In addition to staining controls it is essential to ensure correct microscope performance prior to each microscope session (maximum period of 1 hour).

6.3.2 The suggested sequence of examination prior to each microscope session is detailed below.

(i) Check microscope performance using the fluorescence control slide and record the result. If correct performance is not confirmed the microscope should not be used until microscope performance has been optimised.

(ii) View a positive slide at a screening magnification of not less than x200 to ensure that typical characteristics are observed (oocyst size, shape and fluorescence). Enumeration of oocysts is not required. The microscope shall not be employed unless typical characteristics are observed.

(iii) View a positive slide under an immersion objective at a magnification of not less than x1000 to observe five oocysts. Typical characteristics should be observed (FITC/ DAPI fluorescence and DIC). The microscope shall not be employed unless typical characteristics are observed.

(iv) Examine the regulatory slides.

(v) If necessary during the examination of regulatory slides, where difficulty in identifying an object is encountered, then positive control slides should be used as a reference.

(vi) Finally, after examining regulatory slides check the performance of the microscope using the fluorescence control slide and record the result. If correct performance of the microscope is not confirmed all regulatory slides examined within the session shall be re-examined using an alternative microscope.

6.3.3 Microscopy of the Validation Sample

6.3.3.1 The daily validation sample for quality control has replaced analyst, site and weekly validations which are now defunct. For training purposes analysts will still be required to undertake initial analyst validations as a demonstration of competence.

6.3.3.2 As a minimum the analyst will count the oocysts on the slide at a magnification of X200 and will check to ensure that the oocysts are typical of the genus *Cryptosporidium* and are approximately 5µm in size and have a maximum of 4 nuclei no larger than 1µm in diameter. (See section 6.6)

6.4 Sample Staining

NB In the following procedures it is essential to hold reagent droppers sufficiently far above the slide to prevent "bridging" between the slide and the dropper by reagent. Note that strong aspiration from slides during the staining procedure may significantly reduce oocyst recovery efficiencies. A vacuum aspirator should be used with caution.

6.4.1 Incubate all slides at a temperature not exceeding 42°C until the evaporation step is complete (see section 5.6.3.8), keep the positive control slides separate until the evaporation stage has been completed (see section 6.1.2).

6.4.2 After evaporating to dryness, remove the slides from the incubator and apply 25-50µl of absolute methanol (standard reagent grade) to each slide well and allow to air dry for 3 to 5 minutes. Do not allow the methanol to spill outside the well.

NB Slides may be stored at this stage at 5 ± 3°C in a refrigerator. Before proceeding with the analysis allow slides to equilibrate to room temperature (usually 20 to 30 minutes).

6.4.3 Follow the Immuno-Fluorescent Antibody Test (IFAT) reagent manufacturer's instructions in preparing anti-*Cryptosporidium* sp. fluorescein-labelled monoclonal antibody (mAb). Overlay the sample slide well, the negative-control slide and the positive-control slide well, with 50µl of fluorescein-labelled mAb. Place the slides in a humid chamber and incubate at 37°C for 15 to 45 minutes in the dark. [A suitable humid chamber consists of a sealable plastic container containing damp paper towels on which the slides are placed on supports (e.g. swab sticks), or a suitable alternative.] The humid chamber is warmed to 37°C to ensure rapid equilibration of contents.

NB Some stain preparations may deposit an excess of fluorescent particulate material onto the slide during incubation. Although the deposit does not resemble oocysts, it does make the counting of slides more difficult. If there is a problem with fluorescent particulate material as a background to the slide this can be overcome by filtering each new batch of stain through a suitable 0.2µm syringe filter or centrifuge filter before it is used for regulatory samples. The stain bottle(s) should be rinsed out with reagent water before the filtered stain is returned to them.

6.4.4 After staining, remove the slides and using either (a) a hand held disposable micro-pipette or (b) a disposable micro-pipette tip attached to a gentle vacuum source (e.g. Venturi), tilt the slide and carefully aspirate fluorescein-labelled mAb from the side of each slide well. When performing this step, ensure that the vacuum source is set at minimum suction (<5 cm Hg vacuum) and ensure that the pipette tip does not touch the well surface, tipping the slide to permit the stain to drain towards the pipette tip.

6.4.5 Apply 50µl of 4',6-diamidino-2-phenylindole (DAPI) working solution (1/5000 dilution in phosphate buffered saline (PBS), prepared daily by adding 10µl of 2mg/ml DAPI stock solution to 50ml of PBS) to each slide well. Allow to stand at room temperature for approximately 2 minutes.

6.4.6 Carefully remove the excess DAPI solution by aspiration as described above in section 6.4.4.

6.4.7 Apply 50µl of oocyst-free reagent water to each slide well and allow to stand for approximately 1 minute, then aspirate the excess reagent water. Leave to dry for 2-3 minutes. When removing the excess water ensure that the pipette tip does not touch the well surface.

6.4.8 Apply a drop of mounting medium containing an anti-fadant (2% DABCO-PBS, or equivalent) to the centre of each slide well, avoiding bridging.

6.4.9 Some commercial mountants contain antifadants that will stabilise fluorescence. However, some may, for reasons that have not yet been identified, cause leaching of stain and loss of fluorescence. It is therefore important to ensure that before the introduction of any new mountant is made, the new mountant is fully checked to ensure that its performance at least matches that of the mountant specified in B.4.1.

6.4.10 Allow mountant to spread on each microscope slide and then place a suitable coverslip on the slide. Do not apply pressure to the coverslip. Use a tissue to remove excess mounting fluid from the edges of the coverslip and then seal the edges of the coverslip onto the slide using a suitable sealant. Record the date and time that staining was completed in the analysis workbook.

NB: Slides may be stored in a dry box in a refrigerator at this stage at 5 ± 3°C until ready for examination. (Allow the slide to equilibrate to room temperature before proceeding, about 20 to 30 minutes). Slides may be stored unmounted if it is so wished.

6.5 Microscopic Examination - General Points

6.5.1 Scanning technique: ensure that the slide is dry underneath to avoid drag on the microscope stage, and that it is properly located in the stage slide holder. Scan each well in a systematic fashion. A top-to-bottom or side-to-side scanning pattern may be used during counting. For example, starting at the top of a well, count each field and move horizontally across the well ensuring

that the lower field of view covers the full width of the well at that point. When a horizontal or vertical row has been completed, identify a feature of the sample debris, or of the edge of the PTFE coating, at the bottom centre of the field and move this point to the top centre; move the centre point of the field of view horizontally to the edge of the well; count the field and then move horizontally back across the well; repeat to the bottom of the well. Problems may arise because of the lack of a counting grid and the uneven focal plane of oocysts resulting from the nature of the samples. It is therefore helpful to use the eyepiece graticule to ensure a methodical examination of each quadrant/sector. Where an uneven focal plane is encountered this should be addressed by focusing up-and-down within each field. Use a hand-held or electronic tally counter to record the count; attempt to identify visual features of oocysts and of adjacent debris to help avoid duplicate counting of oocysts in the absence of a grid line.

6.5.2 The microscopic examination should be carried out in a room with subdued lighting to maximise detection of fluorescence. Motorised stages and motorised focusing devices may be used. If a motorised microscope stage is used a positional control slide must be used at the start and end of the analytical run. The positional control slide must be treated as if it was a sample. The number of fields covered must be recorded. Mercury vapour lamps generally have a useful, safe, working life of 300 hours depending on specification but significant loss of output or bulb failure may occur at less than this period of time. Fluorescent control slides must be used daily as a check for loss of output and results recorded daily. Vigilance is required as performance loss may be gradual or may occur suddenly. Use immersion (oil or water) objective lenses where required; screen using relevant objective lenses. A X100 objective must be used for checking equivocal objects.

6.6 Daily Quality Control Sample from the Laboratory Validation Rig

6.6.1 Each day the laboratory is in operation a quality control sample will be taken and analysed and the results plotted on a quality control chart. (See Appendix E for the procedure) The oocysts recovered from the daily quality control sample from the validation rig are counted and the result recorded in a numerical and graphical format. In addition to counting the numbers a positive identification of the oocysts will be undertaken to confirm that they are *Cryptosporidium* spp. according the definition detailed at the front of this SOP.

6.6.2 Examine the daily quality control slide at a minimum magnification of X200 to determine that oocysts are present and that they exhibit typical FITC characteristics of *Cryptosporidium*.

6.6.3 Engage the X100 oil immersion lens and sequentially examine 5 oocysts using the FITC filter, DAPI filter and Nomarski differential interference contrast (DIC) microscopy.

6.6.4 Ensure that the oocysts demonstrate typical FITC staining characteristics in addition to clearly identifiable DAPI stained nuclei (no greater than 4

sporozoite nuclei per oocyst). Measure the size of the sky blue nuclei, each of which should be approximately 1μm diameter.

6.6.5 Using DIC ensure that all five oocysts demonstrate typical characteristics and have diameters between 4 – 6μm.

6.6.6 Record all staining and DIC observations after examination of this quality control slide.

6.7 Microscopic Examination of Recovered Deposits by Epifluorescence and Nomarski Differential Interference Contrast (DIC) Microscopy

6.7.1 Identification of oocysts cannot be adequately described in text as it is necessarily somewhat subjective and dependent on experience. It is essential to understand that the microscopic examination of recovered deposits is a comparative exercise. The regulatory (SI) protocol describes the use of a daily quality sample for checking the identification and enumeration of oocysts. The following notes identify the points to look for when comparing putative oocysts with oocysts on the quality/positive control slide.

NB It is important to remember that all microscopic techniques need to be employed to determine putative *Cryptosporidium* oocysts accurately.

6.7.2 With the FITC filter in position, proceed to examine the entire well of each sample slide at no less than X200 total magnification. Cover the whole area of the well with either vertical or horizontal sweeps. Ensure that the trailing edge of each sweep slightly overlaps the leading edge of the previous sweep. Search for apple green fluorescing objects (similar to the size of *C. parvum* oocysts on the positive control slide). Where screening suggests that there are only a small number of objects on a slide (20 or less), these should be re-located once the scan is complete and examined using the X100 immersion objective lens. Where screening suggests that there is a substantial number of objects the scan may be repeated using either the X40 or X50 immersion objective lenses.

NB When numerous organisms resembling oocysts are present it is far easier both to scan and to identify using immersion liquid, rather than switching from a low magnification dry to a high magnification immersion lens and vice versa, continuously.

6.7.3 In cases where large numbers of objects are present then a tally count of all objects would be made. 20 randomly distributed objects should then be recorded and relocated for confirmation at x100 magnification. If 75% or more of these objects confirm as *Cryptosporidium* then the calculation for the whole slide may be extrapolated. If less than 75% of objects confirm as *Cryptosporidium* then a further 20 (or the remaining objects) should be recorded and confirmed. The result may then be extrapolated from this count of up to 40 randomly distributed objects. Care must be taken when using immersion liquid that the oocysts do not become detached and move when the oil is cleaned from the cover slip.

In order to calculate the confirmed count for a slide with large numbers of objects use the following equation:

$$N = \frac{X}{Y} \times Z$$

Where
N = *Cryptosporidium* Count, or CLB count, or non-*Cryptosporidium* count
X = Number of objects confirmed as *Cryptosporidium*, CLB or non-Crypto
Y = Number of objects recorded and investigated
Z = Total count of objects on the slide.

6.7.4 In instances where a sample slide contains slightly ovoid or spherical objects with an apple green fluorescence and a size similar to the *C. parvum* oocysts observed on the positive control slide, then such objects must be measured accurately under the x 100 objective lens to determine size and whether they contain identifiable internal contents. Objects which fulfil the definition of an oocyst but which are less than 4 x 4µm or greater than 6 x 6µm may be noted and included with the final report, but should not be included in the Regulatory count.

6.7.5 Engage the x100 immersion objective lens and place the fluorescing object in the centre of the field of view. With the FITC filter in place ensure that the object is within the appropriate size range. Switch to the UV filter and determine whether the object contains any DAPI-stained (sky blue) inclusions/nuclei (see Section 6.9). The measurement of such inclusions/nuclei must be performed quickly and efficiently to avoid quenching of the fluorescence.

6.7.6 Block off the UV light source and engage the Nomarski optics. Fine focus on the putative oocyst and confirm the size measurements. Determine whether sporozoite nuclei and / or sporozoites can be seen under DIC.

6.7.7 Weigh up all the features seen under both epifluorescence and DIC microscopy in order to determine whether the object seen is an oocyst of the genus *Cryptosporidium*. *Cryptosporidium* oocysts have the characteristics described in the following paragraphs:

6.8 Epifluorescence Microscopy Under The X100 Immersion Objective Lens Using The FITC Filter

6.8.1 The advice and procedures contained in sections 6.8 to 6.11 should all be used when deciding whether a particular object meets the criteria for classification as an oocyst.

6.8.2 A summary of the identification process is included in Appendix I. This is provided as an aid to microscopists and is not a substitute for the regulatory guidance given in the following sections. As a further aid a summary of the

species and genotypes of *Cryptosporidium* and the current state of *Cryptosporidium* taxonomy are included in Appendix J.

6.8.3 The putative oocyst must conform to the following fluorescence criteria:

Bright apple green fluorescing, round or slightly ovoid objects which measure between 4 and 6μm (measured length x breadth). Often the fluorescence has an increased intensity around the entire circumference of the oocyst, with no visible breaks in oocyst wall staining. Occasionally, oocysts can exhibit a granular, fuzzy or dull perimeter to the fluorescence especially when they have been exposed to the environment for some time. Environmental ageing can induce changes such as the disruption or dissolution of the sporozoites and/or organelles within an oocyst. Environmental exposure and/or sample processing can cause oocysts to collapse or distort, causing their morphology and morphometry to alter.

6.8.4 Environmental exposure and/or sample processing can cause oocysts to gape, which alters their morphology and morphometry (as if a segment has been removed resembling a 'Pacman'®). Often the fluorescence has an increased intensity around the circumference of the oocyst, however, oocysts can exhibit a granular, fuzzy or dull perimeter to the fluorescence especially when they have been exposed to the environment for some time.

6.9 Epifluorescence Microscopy Under The X100 Immersion Objective Lens Using The UV Filter

6.9.1 Intact oocysts contain nuclei of sporozoites which are highlighted with DAPI under the UV filter. Up to four distinct, round to ovoid, sky-blue nuclei each measuring approximately 1 μm will be seen. The nuclei are normally located within the apple green fluorescent object, but on occasion may be displaced around the fluorescent object if the oocyst has aged or dried unevenly. Sporozoite nuclei of intact but environmentally damaged oocysts can still be demonstrated by DAPI staining.

DAPI stained sporozoite nuclei can sometimes be seen outside oocysts, in proximity to the gape, particularly in oocysts which have ruptured. On occasion, the orientation of the oocyst can prevent the gape from being seen.

6.9.2 Empty oocysts do not exhibit any characteristic DAPI fluorescence. Sporozoites may have been lysed or discharged in the environment or during sample processing.

6.10 Confirmation Of Fluorescent Criteria Under The X 100 Immersion Objective Lens Using Nomarski Differential Interference Contrast (DIC) Microscopy

6.10.1 Unlike fluorescent emissions, features seen by DIC neither quench nor disappear following numerous observations and, where present, can be used in conjunction with residual fluorescence criteria by external approved analysts to confirm findings. Size measurements taken under the FITC filter

and DAPI positive nuclei can be confirmed using DIC as long as contaminating material from the sample does not occlude these structures.

6.10.2 Where difficulties arise in confirming either size or presence of nuclei by DIC, re-engage the UV light beam into the light path and locate the putative oocyst. The fluorescence may appear duller because of the introduction of the DIC optics into the light path. Slowly increase the bright field illumination and as the DAPI fluorescence appears to fade with increasing bright field illumination, the oocyst wall and sporozoite nuclei will come into sharp relief under DIC. Block off the UV light source and measure the size of the object under DIC. Where present, confirm the number, size and position of sporozoite nuclei by DIC. Determine by DIC whether all DAPI positive nuclei are within the putative oocyst. Attempt to locate the numbers and positions of the crescentic sporozoites.

6.10.3 Empty oocysts do not contain sporozoites; hence they do not exhibit DAPI fluorescence. The absence of contents MUST be confirmed by DIC.

6.11 Further Guidance Notes for the Identification of Environmentally Aged Oocysts:

(i) Difficulties in identification can arise when oocysts have been present in the environment for a protracted period of time. Collapsed or distorted empty oocysts are amongst the most difficult to identify. One of the dimensions of a misshapen oocyst can lie outside the Regulatory size range. In such instances, determine whether the putative oocyst contains any DAPI positive nuclei AND whether sporozoite nuclei and / or sporozoites can be seen by DIC.

(ii) Interfering debris can occlude oocyst images and / or organelles, making identification by FITC and UV fluorescence microscopy and DIC microscopy more difficult. DAPI-stained sporozoite nuclei can often be seen under the UV filter when occluding debris masks the presence of nuclei under DIC microscopy.

(iii) Do not confuse the presence of DAPI stained micro organisms, such as bacteria, with sporozoite nuclei. Fine focussing using the X100 immersion objective lens can assist in determining whether a DAPI stained object is surface adherent within a putative oocyst or in a free sporozoite. Size and shape criteria can be used to exclude many micro organisms.

(iv) Objects with typical FITC fluorescence around their entire circumference and amorphous[5] internal contents demonstrable by DIC,

[5] In this context amorphous has the strict scientific meaning of 'without a specified form, shape or structure ie morph', rather than it's meaning in common usage of nebulous. In the context of the above paragraph an amorphous body is one which does not have the morphology of a *Cryptosporidium* oocyst, which is described elsewhere within the SOP Part II and which can be seen under DIC. An empty oocyst does not have internal contents and therefore is amorphous, as are bodies which either

but which do not possess sporozoite nuclei that can be demonstrated by DAPI and / or DIC are not counted as oocysts.

(vi) Objects with typical FITC fluorescence around their entire circumference, with DAPI-stained nuclei larger than 1.5µm under the X100 immersion objective lens, or with more than four DAPI-stained nuclei are not counted as oocysts.

(vii) Environmental objects which stain poorly with the monoclonal antibody, are badly misshapen and whose internal contents cannot be demonstrated by DAPI or DIC are not counted as oocysts as it is impossible to confirm that such objects are *Cryptosporidium* oocysts.

NB If a number of sub-samples were generated at paragraphs 5.7.2 and 5.5.13 the number of oocysts found in the whole sample is the sum of the oocysts found in all the sub-samples.

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6.12 Calculation

$$\frac{\text{(number of oocysts found within total deposit)} \times 10}{\text{number of litres (initial sample volume)}} = \text{oocysts / 10 litres}$$

6.13 Internal Confirmation of Results

6.13.1 Where analysis of a sample gives a preliminary result of 0.5 oocysts or greater in 10 litres, the slide must be examined by another approved person within the laboratory to confirm the findings. Where the result of the internal confirmation does not agree with the initial result, the results will be reviewed by both analysts where agreement will be met. If agreement is not reached the result from the most experienced analyst will be reported to the water company under the Regulations.

6.13.2 On a monthly basis, microscopists are also required to undertake an internal inter-analyst slide check. Each microscopist must randomly select a positive slide examined by them and have it checked by another approved microscopist. Where the original slide count is 30 or less, the check count must lie within ±3 of the original count. For counts above 30, the inter-analyst count must lie within ±10% of the original count. All anomalous counts must be investigated but special consideration must be given to counts out with the stated tolerances. Consideration must also be given to low counts that influence whether a sample is positive or negative or where the count significantly changes the final count per 10 litres. Where the result of the second analyst check does not agree with the initial result, the results will be reviewed by both analysts where agreement will be met. If agreement is not reached the result from the most experienced analyst will be reported. The frequency of the inter-analyst check may be increased, depending on the experience and performance of the microscopist. In

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lack other key morphological features or exhibit atypical morphological features. FITC and DAPI staining are not morphological features. They are artificially produced features to aid identification.

extreme cases it may be necessary to stop an analyst carrying out microscopy, invalidate their work and fully re-train.

6.14 External Confirmation of Results

6.14.1 Where analysis of a sample gives a preliminary result of 0.7 oocysts or greater in 10 litres or where the actual identification of organisms is in doubt confirmation must be obtained from an external approved analyst in an independent laboratory, as well as the slide being examined by another approved person within the laboratory. Until such confirmed identification has been made, all such organisms must be included in the total oocyst count.

NB Where consecutive samples from the same site fall above 0.7 oocysts/10L, it is only necessary to externally check the first sample fitting this criteria. In the latter stages of such incidents, where the site appears to be coming back under control, the first sample that falls below 0.7 oocycsts/10L is also sent for external checking

6.14.2 A list of such approved laboratories is available on the DWI Website: www.dwi.gov.uk. The external approval analyst is an approved analyst from a laboratory which is not under the same control/ownership as the laboratory undertaking the analysis. The purpose of this second opinion is to confirm the identification of the organism. Non-confirmed organisms are excluded from the regulatory count under Appendix I. Where there is disagreement between the internal and external analysts, DWQR must be informed.

6.14.3 The external confirmation must be made either:

(i) via a computer with image grabbing facilities and ISDN telephone link allowing the external independent approved analyst to examine the sample slide at a remote site without undue delay, or

(ii) by inviting the external independent approved analyst to the laboratory to examine the slide in situ, or

(iii) by sending the slide to the external independent approved analyst in an appropriate container by courier or member of laboratory staff.

The external independent approved analyst must ensure that he/she examines the whole slide to assess the number of oocysts on the slide. This examination must be carried out and the result reported back to the licensed laboratory so that the reporting requirements in the Regulations are achieved.

6.15 Storage of Slides after Analysis

6.15.1 All slides must be stored in a refrigerator at 5 ± 3^{o}C, which is designated solely for the purpose of storing *Cryptosporidium* slides, for at least three calendar months following completion of analysis.

6.15.2 All slides containing confirmed Cryptosporidium Oocysts must be stored under conditions defined in 6.15.1 for 1 full rolling year.

6.15.3 The requirement of 6.15.2 shall be superseded when slides are genotyped as requested by Scottish Waters Public Health Team for either internal or Incident Management Team purposes.

7. REPORTING ARRANGEMENTS

7.1 All results of analysis under the Directions must be reported to the relevant customer such that the requirements of the Directions are achieved. Results must include details of:

(i) the water meter reading at end of sample run
(ii) volume of water filtered
(iii) the date and time of both the start and finish of the sample run
(iv) elapsed sample time
(v) headloss over filter
(vi) deposit volume
(vii) concentration of oocysts found for each sample
(viii) the Scottish Water LIMS sample number
(ix) the sample point location or identity number
(x) analytical laboratory (and where appropriate laboratory identity reference)
(xi) the date when the result was reported to Scottish Water.
(xii) The total number of oocysts observed in the sample;

NOTE: Information that must be included in the customers formal report must include: ii, vii, viii, ix, x and xii above as a minimum.

7.2 A monthly summary of all results must be sent to DWQR. The summary may be sent in a standardised electronic form agreed with the DWQR.

7.3 The concentration of oocysts shall be reported as the number of oocysts per 10 litres. Reporting of results shall be to three decimal places. Where the concentration of oocysts is less than 0.01 per 10 litre, this shall be reported as <0.01 oocysts / 10 litres, regardless of sample volume.

7.4 Any results of analysis which confirm the positive detection of *Cryptosporidium* oocysts in a final water sample must be reported by a designated person within Scottish Water to the DWQR in accordance with the Directions.

7.5 The presence of bodies meeting the size requirements for *Cryptosporidium* oocysts, but with amorphous[5] contents may be of significance for the treatment of water and could under some circumstances be of health significance. A prudent water company may wish to have the numbers of such objects reported to it with the results to allow their significance to be assessed and appropriate action initiated.

APPENDIX A. Maintenance Guidelines

A.1 For IDEXX Filta-Max® Automatic and Manual Wash Stations

A.1.1 For specific details follow the manufacturers instructions, the following information is for guidance.

A.1.2 Cleaning

A.1.2.1 The plunger head can be cleaned with soapy water, and wiped clean with a damp cloth.

A.1.2.2 The paintwork can be cleaned with soapy water and/or 70% ethanol. Abrasive washing is not recommended and therefore must not be used.

A.1.3 Maintenance

A.1.3.1 It is recommended that the "O" ring on the plunger is lightly lubricated with silicone grease before each use.

A.1.3.2 Lubrication of the "rack and pinion" mechanism on the manual wash station and the 'robo-cylinder' on the automatic wash station **is not** required, as they are self-lubricating.

A.2 For IDEXX Filta-Max® Tubing/Vacuum Sets

A.2.1 Cleaning

A.2.1.1 It is recommended that all components are disassembled for washing and that they are washed with soapy water, followed by thorough rinsing with oocyst-free de-ionised water. NB: Laboratory washing machines could be used but may cause the plastic to become opaque with repeated washing. If washing machines are used the "O" rings in the base of the concentrator should be removed and washed separately. Any tubing/concentrator sets used for positive control samples must be washed separately from any other tubing/concentrator sets.

NB Tubing/Concentrator Sets must not be autoclaved.

A.2.2 Maintenance

A.2.2.1 Check the "O" rings in the concentrator at least once per week for effectiveness of seal, and replace when necessary.

A.3 IDEXX Filta-Max® MKI and MKII Filter Housings

A.3.1 Cleaning

A.3.1.1 The MKI and MKII filter housings can be cleaned by washing in soapy water, which should be followed by a rinse with oocyst-free de-ionised water.

A.3.1.2 The MKII filter housing may be cleaned in a dishwasher at temperatures not exceeding 40ºC. Note: Rinse aids should NOT be used. Clean the inner and outer surfaces of the filter housing with a non-abrasive sponge or cloth and warm soapy water. Rinse with oocyst free water and dry.

A.3.2 Maintenance

A.3.2.1 Before each use, clean debris and grease from around the housing threads, re-grease with silcone grease before use.

A.4 IDEXX Filta-Max® MKI Filter Housings

A.4.1 Ensure all O-rings are located correctly and lightly lubricated with silicone grease before use.

A. 4.2 Tighten the IDEXX Filta-Max® MKI Filter Housing until the lid is approximately 0.5mm form the base. Take care not to over tighten as this will cause difficulties when trying to remove the filter module.

A.4.3 The IDEXX Filta-Max® MKI Filter Housing may on occasion be used up to a pressure of 500 kPa (5 bar) under normal circumstances, however, 800 kPa (8 bar) is the maximum operating pressure.

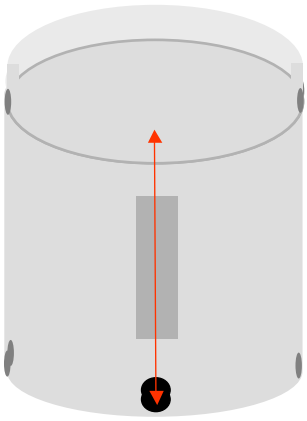
A.4.4 To open the MKI Filter Housing use the tools supplied by IDEXX.

A.5 IDEXX Filta-Max® MKII Filter Housings

A.5.1 Ensure all O-rings are located correctly and lightly lubricated with silicone grease before use.

A.5.2 Use the tools provided by IDEXX to close the MKII housing. Align the lid onto the base and tighten until the two tag holes and serial numbers align. Tag holes are identified by the presence of horizontal and vertical drill holes.

IDEXX Mark II Filter Housing Alignment of the Tag Holes



TAG HOLE ALIGNMENT

A.5.3 When tightened adequately there should be a gap of approximately 0.50mm between base and lid of the housing. The filter module should not move within the housing. **Avoid tightening beyond this point.**

A.5.4 The IDEXX Filta-Max® MKII Filter Housing may on occasion be used up to a pressure of 500 kPa (5 bar) under normal circumstances, however, 800 kPa (8 bar) is the maximum operating pressure.

A.5.5 To open the MKII Filter Housing use the tools supplied by IDEXX.

A.6 Procedure for changing Swageloks from IDEXX Filta-Max® MKI to MKII filter housings

A.6.1 Record both the new and old housing lid and base serial number so that an audit trail of each unit can be kept.

A.6.2 Remove the Swagelok and discard the old washers and housing.

A.6.3 Thoroughly clean the Swagelok using warm soapy water and rinse using oocyst-free, de-ionised water.

A.6.4 Attach the Swagelok using the following directions:

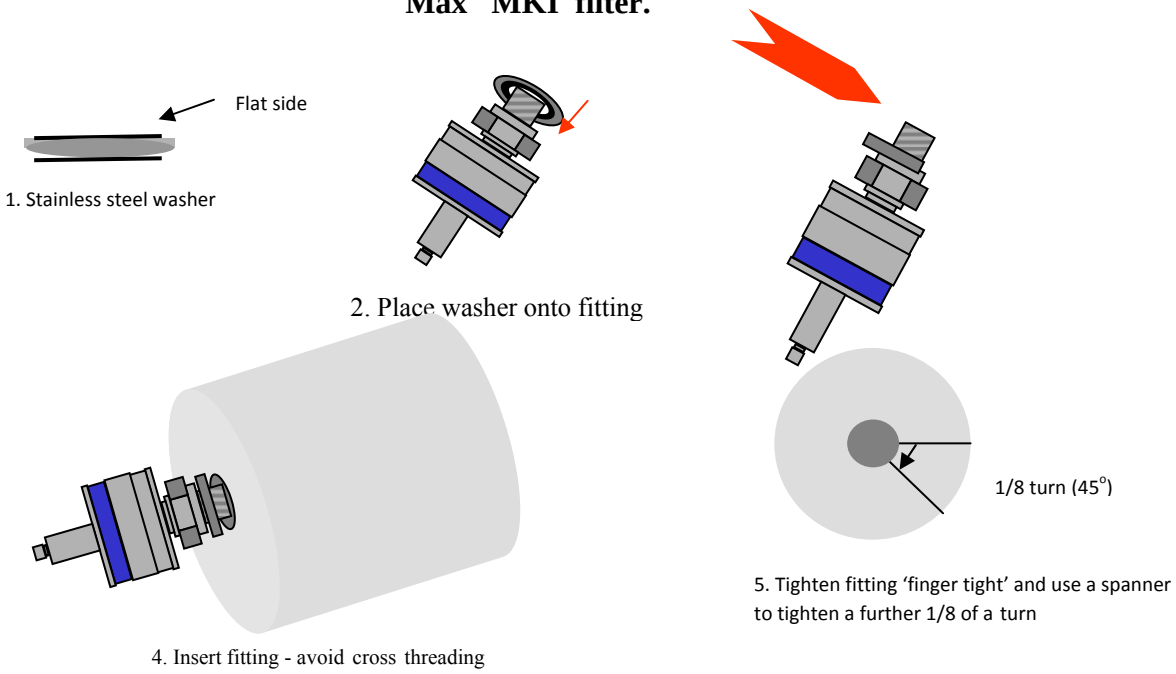
A.6.5 Look carefully at both sides of the stainless steel washers provided. Note that one side is flat and the other is slightly convex. Orientate the stainless steel washer so that the flat side of the washer will make contact with the plastic of the housing. Place the washer onto the Swagelok in this orientation.

A.6.6 Place a small drop of adhesive (supplied by IDEXX) to the thread of the Swagelok fitting.

A.6.7 Screw the Swagelok by hand into the housing, taking care not to cross-thread or over tighten. Stop when a small amount of resistance is felt.

A.6.8 With a spanner tighten the Swagelok 1/8 of a turn (45o) (equivalent to 6 Newton Metres).

Diagram Showing Method of Connecting the Swageloks to the IDEXX Filta-Max® MKI filter.



APPENDIX B. Specific Equipment, Materials & Reagents

B.1 IDEXX Filta-Max® Equipment[6]

B.1.1 The IDEXX Filta-Max® MKI and MKII filter housings are specified sampling devices. Each filter housing base and top must be supplied with a unique number for identification. It is advisable to have at least three of these housings specifically dedicated to each site or group of sites. Each housing so dedicated must have a label firmly fixed to the housing showing the site (or group) location code at which the housing is intended to be used. The use of the housing at only a small group of low or medium risk sites or at one high risk site will minimise the risk of cross-contamination (MKI filter housing Product No. FMC-10501, now discontinued, and MKII filter housing FMC10505) or equivalent device validated in house to ensure equivalent performance.

B.1.2 Filter module must be the IDEXX Filta-Max® filter Module. Each module must be supplied with a unique number for identification purposes (Product No. FMC-10602).

B.1.3 IDEXX Filta-Max® filter membranes (Product No. FMC-10800 for 100 pack). An additional supply of spare membranes is essential for samples containing high deposits.

B.1.4 IDEXX Filta-Max® manual wash station and wash station clamp set (Product No. FMC-10101)

B.1.5 IDEXX Filta-Max® automatic wash station and wash station clamp set (Product No. FMC-10103)

B.1.6 IDEXX Filta-Max® tubing set, vacuum set and magnetic stirrers (Product Nos. FMC-10301, FMC-10401, and FMC-10901).

B.2 Invitrogen Dynal Biotech Equipment

B.2.1 Dynal Sample Mixer (MX-1, Product No. 159.07 - takes 12 samples; or MX-2, Product No. 159.08 - takes 8 samples; or MX-3, Product No. 159.09 - takes 20 samples).

B.2.2 Dynal Primary Magnetic Particle Concentrator DYNAL MPC-1 and DYNAL MPC-6, Dynal, Secondary Magnetic Particle Concentrator Dynal MPC-M or Dynal MPC-S.

B.2.3 Dynal Spot-On, 9mm single well microscope slides with special coating, 100/box (Product No. 740.04) or equivalent slides. Equivalent slides must be validated in house to demonstrate equivalent performance.

[6] For a full list of product codes contact IDEXX Technical Support 01638-723011

B.2.4 Dynal L10 Tubes, Re-useable flat sided glass tubes with Sure-Cap, 5/box (Product No. 740.03) or equivalent tubes. Equivalent tubes must be validated in house to demonstrate equivalent performance.

B.2.5 Dynal Dynabeads, Anti-*Cryptosporidium* kit for 10 tests, contains 1ml Dynabeads plus "10 x SL™-buffer-A" plus "10 x SL™-buffer-B" (Product No. 730.01). Dynabeads GC Combo kit (Product No: 730.02) may also be used. These should be stored at 5 ± 3°C. Ensure that they are equilibrated to room temperature (15-22^{o}C).

B.2.6 Hendley (Essex) Ltd 9mm single well blue-coated microscope slides (Product No. PH246 Hendley).

B.3 Cellab FITC stain

B.3.1 Monoclonal antibody/FITC reagent Cellabs Cryptocel IF antibody (5ml, Product Code Z1RR1).

B.4 Mounting Medium

B.4.1 IFA mounting medium (e.g. TCS DABCO IF mounting fluid, 10ml, Product Code Z1MM10) or equivalent medium validated in house to demonstrate equivalent performance.

B.5 DAPI reagent

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B.5.1 4',6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI) freeze dried reagent (e.g. Sigma Aldrich, Product No. D9542). When the solid was stored at -20^{O}C protected from the light, samples showed no change in purity (TLC) after three years.

B.5.2 DAPI (stock) solution, 2mg/ml DAPI per millilitre of methanol. Prepared by weighing out 1 mg of freeze-dried DAPI reagent and adding 0.5ml methanol. This stock solution is stable for 1 month if stored in a refrigerator at 4^{O}C.

B.5.3 DAPI (working) solution, 10µl DAPI stock solution in 50ml of PBS, prepared daily.

B.6 Phosphate Buffered Saline/Tween (PBST)

B.6.1 Phosphate buffered saline (PBS) (e.g. Oxoid, Product No. BR0014G [was BR0014a] also referred to as Dulbecco A), this is a balanced salt solution without calcium or magnesium. To make up PBS from separate Analar ingredients use the Oxoid formula given in the current catalogue for Product No. BR0014G.

B.6.2 Phosphate buffered saline (PBS) pH 7.4 at 25ºC. Dissolve 1 tablet in 200ml ultra pure or distilled water to obtain (0.01M PBS, 0.137M NaCl, 0.0027M KCl, pH7.4 at 25 degrees centigrade). Supplied by SIGMA product code = P4417 in tablets. B.9.1 Phosphate buffered saline (PBS) pH 7.4 at 25ºC.

Each PBS sachet makes one litre, use ultra pure or distilled water to make 0.01M PBS, 0.138M NaCl, 0.0027M KCl, pH 7.4 at 25 degrees centigrade. Supplied by SIGMA (product Code = P-3813) in sachets.

B.6.3 Tween 20,Polyoxyethylenesorbitan Monolaurate (TWEEN 20) Sigma Ultra, supplied by SIGMA (Product code = P-7949).

Polyoxyethylenesorbitan Monolaurate (TWEEN 20) Merck.

Information supplied by IDEXX Laboratories Inc.

B.6.4 Ready for use elution buffer (PBS/Tween), each carboy labelled with a use by date (Vickers).

B.6.5 To make up PBS from separate Analar ingredients use the Oxoid formula given in the current catalogue for Product No. BR0014G.

Formula	**g/l**
Sodium chloride	8.0
Potassium chloride	0.2
Disodium hydrogen phosphate	1.15
Potassium dihydrogen phosphate	0.2

B.6.6 To make Phosphate buffered saline/Tween to each litre of phosphate buffered saline add 100µl of Tween and stir for 10 minutes.

B.7 Approved sources of oocysts

B.7.1 Supply of *C. parvum* oocysts (Moredun Animal Health).

B.7.2 Supply of *C. parvum* oocysts (***EasySeed***–C100, ***EasySeed***™ from TCS Water Sciences, Product code Z9ES-C100. See below for instructions for use:

Seeding the QC sample

(1) Add 2ml of 0.05% (v/v) Tween 20 (eg Sigma Aldrich fine chemicals) to the Easyseed tube.
(2) Replace cap and shake vigorously.
(3) Remove cap and pour EasySeed into sample.
(4) Add 3ml of reagent grade water to the tube.
(5) Replace cap and shake vigorously.
(6) Remove cap and pour EasySeed into sample.
(7) Repeat steps 4, 5 and 6.

B.7.3 Supply of *C. parvum* oocysts (EasyPC™), using Iowa strain oocysts from TCS Water Sciences. EasyPC™ are test tubes each containing six thousand four hundred oocysts in two millilitres of water, Product Code Z9EPC-C. They have been specifically manufactured for the positive control slides.

B.7.4 Supply of *C. parvum* oocysts, Iowa strain (University of Arizona, Department of Veterinary Science and Microbiology and is known as the Harley Moon isolate). Can also be purchased from TCS Water Sciences.

B7.5 Supply of *C. parvum* oocysts (EasySeed-C100, using Iowa strain oocysts, EasySeed™ from TCS Water Sciences, Product code Z9ES-C100 (University of Arizona Iowa Strain).

APPENDIX C. General Equipment & Materials

C.1 Centrifuge to take up to 50ml conical centrifuge tubes with appropriate bucket cushion inserts capable of running at 2-12 x 10^3 rpm, with timer. If using Pall filters then centrifuge tubes up to 250ml may be used. The appropriate carrier would be fitted for use with the specific size of tube.

C.2 Appropriate conical centrifuge tubes with an accuracy +/- 0.5ml.

C.3 High speed vortex mixer.

C.4 Epifluorescence microscope with UV (340-380nm excitation, 450nm emission) and FITC (450-490nm excitation, 560 nm emission) filters or other validated filters to ensure appropriate sensitivity and calibration traceable to UK national standard, and Nomarski DIC optics and calibrated eye-piece graticule and calibrated stage graticule.

C.5 Appropriate graduated pipettes, straight, polystyrene, disposable eg 10ml.

C.6 Graduated pipettes, straight, polystyrene, disposable, 1.0ml.

C.7 Pasteur pipettes, Sterilin, 1ml graduated, polyethylene, length 155mm, non-sterile. (Bibby Sterilin Ltd).

C.8 Micro-pipettors and tips, variable volume dispensers, Eppendorf or similar, with calibration certification, 5-50µl and 50-200µl ranges.

C.9 Safety pipette fillers.

C.10 Magnetic stirrer and followers.

C.11 Humid chamber (such as a sealable plastic container containing damp paper towels on which the slides are placed on supports (e.g. swab sticks)).

C.12 Incubator, 37-42^{o}C or equivalent device validated in house to ensure satisfactory performance.

C.13 Appropriate forceps, preferably stainless steel, without sharp points.

C.14 Elution buffer (phosphate buffered saline with 0.01% v/v Tween 20), *Cryptosporidium* oocyst free.

C.15 Reagent water, a supply of distilled, de-ionised, or reverse osmosis treated water oocyst-free.

C.16 Hydrochloric acid, 0.1M, oocyst-free.

C.17 Sodium hydroxide, 1.0M, oocyst-free.

C.18 Laboratory pump, capable of supplying 500 kPa (5 bar) pressure.

APPENDIX D. Quality Assurance & Quality Control

D.1 General

D.1.1 It is expected that sampling and analysis for *Cryptosporidium* will be accredited within the DWTS and undertaken in such a way that a chain of evidence can be demonstrated and that the procedures used are scientifically defensible so that Scottish Water can demonstrate due diligence in monitoring water quality. External quality assessment and adequate quality assurance and quality control procedures must therefore be in place, to the standard required by the DWQR.

D.1.2 The laboratory undertaking these analyses must be able to demonstrate its ability to maintain an adequate system of quality assurance and quality control such that the results generated are fit for the purpose for which they are to be used. Such a system must cover the whole process from sampling to reporting for which the laboratory is directly responsible.

D.1.3 The source and age of the oocysts used in the recovery must be documented and be traceable.

D.1.4 All equipment used in the sampling, elution, concentration and analysis of oocysts must be maintained according to the manufacturer's instructions. Such maintenance must be recorded in an appropriate log book, with dates and details of service and replacement parts.

D.1.5 All equipment used for measurements (including microscope eye-piece graticule, all variable volume micro-pipettes, pH meters, thermometers and balances) must be subject to regular calibration checks in accordance with approved calibration regimes. Such calibration checks must be logged with dates on which they were carried out and the name and signature of the person who carried out the check. Calibration checks to be in accordance with "Guidelines for Calibration in Laboratories" February 2001, Water Services Unit, Scottish Executive Information Letter 5/2001. The instrument should be calibrated in the units used by the laboratory where an instrument can record in more than one unit. Eye-piece graticules should be calibrated on a regular basis (monthly) and every time there is a result greater than 0.5 oocysts per 10 litres. In addition, the eyepiece graticule should be re-calibrated following maintenance, repair or relocation of the microscope. A full record must be made of each calibration.

NB After each service or repair the instrument should be checked to ensure that it is fit for purpose and capable of producing valid results.

D.1.6 All new batches of reagents and materials (including monoclonal antibody, DAPI, IMS beads, mounting medium and membranes) used in the analysis of oocysts under the Regulations must be checked against current batches to ensure that they are of suitable quality. Such checks must be logged with

batch numbers, dates on which they were carried out and the name and signature of the person who carried out the check.

D.1.7 For analysis under this SOP purified oocysts must only be obtained from a DWQR approved supplier. Each batch should be checked on receipt against current batches to ensure that it is fit for purpose. It would be prudent to keep previous batches of oocysts (for instance the last two) in the event of:

a) an accident in the laboratory: and

b) no replacement oocysts being available.

D.1.8 All slides produced as a result of analysis of regulatory samples that are currently stored in the laboratory will be subject to periodic audit. In particular, those slides that the laboratory has identified with putative oocyst(s).

D.1.9 Should it so wish, an approved laboratory can submit any slide(s) for confirmation/identification by an external approved analyst. This would be in addition to the requirements of section 6.14 of Part 2 of the Standard Operating Protocol.

APPENDIX E. Laboratory Quality Assurance Tests

E.1 Laboratory Quality Assurance Tests

E.1.1 Each approved laboratory shall have a formal system of internal quality control checks in accordance with the following procedures. Records and relevant charts will be kept and maintained such that not only a daily check can be made but also the results will be trended to determine any long term changes which could affect the analysis.

E.2 Principle

E.2.1 The basic principle of the procedure is that every approved laboratory will carry out every day the laboratory is analysing regulatory samples the analysis of a spiked sample from the validation sampling rig. The spiked sample taken will, as far as is practical, replicate the procedure for a sample taken at a regulatory site. That is, a continuous sample is taken over a period of 24 hours at a minimum flow of 40 litres per hour, on average, this should equate to a flow through the filter of approximately 1000 litres over the 24 hour period. The sample shall be taken in accordance with the Directions and the SOP. A full chain of custody is not required providing that the sampling is undertaken by approved *Cryptosporidium* laboratory staff and the validation sampling rig is in a secure location under the sole control of the *Cryptosporidium* laboratory.

E.2.2 After the spiked sample has been taken from the validation sampling rig the sample will be transferred to the *Cryptosporidium* laboratory and signed in the appropriate analysis book(s). The sample will be treated as 'A N other' sample and analysed alongside the other regulatory samples. If the analysis of the regulatory samples is not completed within the working day then the daily validation sample will be treated in exactly the same way.

E.2.3 The analysis will be undertaken by staff approved to undertake regulatory analysis. Strict rotation of staff is not a specific requirement but over a period of time all approved staff must be involved in the analysis of the daily validation sample. The analysts will process and analyse the daily validation sample using the same procedure for the analysis of regulatory samples. Thus if several analysts are involved in processing and analysing the regulatory samples then the daily validation sample will be treated in the same way. The results of the daily validation sample will be plotted on a chart with upper and lower limits to ensure appropriate action is taken if the result is outside the action limits. Any result outside the action limits must be investigated and the results of the investigation recorded.

E.2.4 Each laboratory shall prepare, run and analyse a daily validation sample on each day the laboratory is analysing regulatory samples. The daily validation sample shall be valid until the next quality control sample result is available. Where a laboratory operates two separate shifts with separate staff operating on each shift then each shift will have to prepare, run and analyse a daily

validation sample on each day that the laboratory shift is analysing regulatory samples.

E.3 The Validation Sampling Rig

E.3.1 The validation sampling rig should conform to the following specification. A suitable sampling rig is available from Hydraulic Modelling Services Limited (HMS). The rig is available in two forms, either in a cabinet or mounted on a board.

E.3.2 Details of a suitable septum, needle and syringe are given below. Whilst these products have been found to be satisfactory for use with the validation rig the SOP does not endorse that they are the only suitable products available. The laboratory may use a product of equivalent specification.

E.4 Specification of the Validation Rig

E.4.1 Technical Specification

Dimensions of 2 line rig (mm)	850 h x 850 w x 250 d (optional enclosure 1000 x 1000 x 320)
Dimensions of 1 line rig (mm)	625 h x 625 w x 250 d (optional enclosure 1000 x 1000 x 320)
Weight (kg) of 2 line rig	approx 15Kg
Weight (kg) of 1 line rig	approx 8Kg
Feed / supply to the rig:	Mains water at around 5 bar pressure
Septa's for Injection Port:	High pressure GC septum part **5183-4757-50** (11mm) 50 or 100 per pack. Or a septum of equivalent specification. (See paragraph 4.3.1).

E.4.1.1 The unit should be wall-mounted for ease of use and to give a rigid fixing. 9mm mounting holes are provided. Standard15mm copper pipe can be used to plumb a mains supply to the rig. A ½ inch BSP male fitting is provided on the inlet. Connect with a ½ inch x 15mm female iron. Fittings are readily available from plumbers merchants or from HMS. The inlet is lower right. The outlets are 8mm nylon tube from the flow restrictors at the top of the board. 2 meters of tube is provided to run to waste. The outlets can run to the left or right. To change direction slacken the upper union on the water meter, slacken the pipe clip. Turn the outlet through 180 degrees. Retighten union and clip.

E.4.1.2 The equipment is specifically designed to provide a means of introducing a known amount of *Cryptosporidium* into a flow of water upstream of a proprietary *Cryptosporidium* filter.

E.4.1.3 The supply water or sample enters the sampling unit through the lower ½ inch BSP fitting on the right side of the unit. The sample, once in the unit, passes a needle valve followed by a pressure gauge. The valve is used to

reduce the line pressure when injecting *Cryptosporidium*. Downstream of the gauge is a tee to split the flow into two lines (line 1, and line 2)

E.4.1.4 Just above the tee on line 2 is another tee into which additional lines can be plumbed. If you have ordered a rig with an extra line a 15mm stainless steel compression fitting will be fitted to the tee and a length of tube supplied to connect to the extra line on a separate board.

E.4.1.5 Each line consists of a check valve followed by a ball valve and then an injection port.

E.4.1.6 The injection port houses a "septa" sandwiched between a stainless steel washer and cone. The nut securing the septa, washer and cone needs to be only hand tight to seal. Septa's are designed to be self sealing when the needle is withdrawn, however, the septa may require renewing regularly.

E.4.1.7 Downstream of the injection port are flexible hoses with swagelok quick connects. Either side of the filter housing are similar quick connect fittings to allow for simple removal of the housing.

NB When disconnected the quick connect fittings automatically shut off. The pressure gauges fitted in the upstream and down stream plumbing allows the head loss across the filter to be measured, and its consequent rate of blocking. The sample proceeds through a water meter, and flow restrictor before leaving the rig. The flow restrictor is set at 1.0 litres/min.(requires 1 bar min' pressure).

E.4.1.8 Once all connections have been made and a filter housing fitted the unit is ready to start. However it is very important to remember to flush the sample line thoroughly before connecting the supply. Fit a septum in to the injection port. Open the needle valve, and the ball valve on the line to be used. Purge the lines of air.

E.4.1.9 When injecting the sample of *Cryptosporidium* through the septa first reduce the line pressure using the needle valve.

NB It is essential that flow is maintained when injecting. If possible only lower the pressure to a minimum of 1 bar. This will allow 1 litre per minute flow rate to be maintained. However, if 1 bar is too high a pressure to inject against, one can reduce the pressure still further but check that there is still a good flow through the water meter.

E.4.2 Changing the Filter Housing

This requires the uncoupling of the quick connect fittings on either side of the housing. Coupling/uncoupling should only be undertaken with the filter housing in its cradle and the flow switched off. Ensure the direction of flow is correct.

NB All the quick connect fittings are self sealing when disconnected.

To couple:
Align body with stem of housing.
Push body onto stem until it 'clicks'.
To uncouple:
Push body sleeve towards stem.

E.4.3 Specification of a Suitable Septum

E.4.3.1 A suitable septum is supplied by:

Crawford Scientific
Holm Street
Strathaven
ML10 6NB

Tel No: 01357 – 522961

High pressure GC septum12mm, 50 or 100 per pack.

Or a septum of equivalent specification.

E.4.3.2 It is recommended that the septum is changed each day of use prior to injection of the oocysts to minimize the possibility of loss of the oocysts.

E.4.4 Injection Syringe and Needle

E.4.4.1 A suitable syringe is supplied by:

BD (Becton, Dickinson and Company)
Plastipak Luer Fitting 10ml
Ref: 302188

3S Healthcare, George House, Unit 6, Delta Park Industrial Estate, Millmarsh Lane, Enfield, EN3 7QJ

Tel: 0870 8734901

Or a syringe with an equivalent specification.

E.4.4.2 A suitable needle:

BD (Becton, Dickinson and Company)
Microlance 3, 19G x 2" (1.1mm x 50mm)
Ref: 301750

Supplied by: 3S Healthcare, George House, Unit 6, Delta Park Industrial Estate, Millmarsh Lane, Enfield, EN3 7QJ

Tel: 0870 8734901

Or a needle with an equivalent specification.

E.4.4.3 It is recommended that each syringe and needle is used once and safely disposed of in accordance with the laboratory's health and safety policy and practice.

E.5 SPIKING SUSPENSION

E.5.1 A spiking suspension may be made either

(a) a flow cytometer may be used to prepare a suspension of 100 oocysts and subject to approved quality control procedures; or

(b) *Easyseed*^{TM}, which are test tubes containing one hundred *Cryptosporidium* oocysts in approximately 1ml of saline solution; or

(c) an approved commercial product containing 100 oocysts with certificated tolerances equivalent to existing approved products.

E.6 PERFORMANCE

E.6.1 Using the method outlined above experience has shown that recoveries of 40% or greater can be achieved. Similarly the recovery should not exceed 100% due to the inherent potential loss of oocysts that could occur during the analysis of the sample.

E.6.2 Experience will determine the recovery each laboratory will make. It is anticipated that there will be a variation in the initial stages. Once the staff have become familiar with the equipment and the analysis it is anticipated that recovery will become more consistent and this will be reflected in the standard deviation. The DWQR will be monitoring the recoveries obtained by the laboratories but each will be looked at on an individual basis noting the percentage recovery, consistency of results and the standard deviation. In addition, the charts will be monitored to determine if they follow the guidance in Section E.9.4.

E.7 PROCEDURE

E.7.1 Procedure for use of Idexx Filta-Max® filter module in the Validation Sampling Rig

E.7.1.1 It is important that the same batch of filters is used for both the validation sample and the regulatory samples. The batch of filter housings used must be detailed as part of the information recorded in the validation log. The filter modules carry a one year shelf life and they may be used beyond the shelf life date in accordance with the following.

Provided that the filter modules from the same batch are being used both as the daily validation sample and for the regulatory samples and there is no discernable deterioration in either recovery or trend. If two consecutive samples breached the warning limits or one sample the action limit then a new batch of in date filter modules must be used. The outdated filter modules may not be used again unless equivalence can be determined according to sections E.9.9 and E.9.10.

E.7.1.2 Insert a filter housing into a filter module and ensure that it is tightened according to the manufacturers instructions to prevent leaking. The filter housing(s) is/are placed in the appropriate holder(s) on the spiking rig. Record the time, date, analyst and meter reading. The water is turned on up to a maximum pressure of 150kPa (1.5 bar) to wet and coat the filters for at least one hour. [The pressure is a guide, it has been found that a pressure of 150 kPa will be sufficient to give a flow rate of 40 litres per hour required in the Directions.] Check to see that the filter is not leaking this should be identified soon after the water has been turned on. After the filter has run a minimum of one hour without leaking then the spike is prepared and injected into the validation rig.

E.7.1.3 Prepare the spike as per instructions. Draw each rinse stage (PBS and RO water) into a 10ml syringe ensuring no liquid remains in the needle. Record the details of batch numbers, date, time and analyst. When more than one line on the validation rig is being used then these must be the same for all the filters. The time between preparing the spike and injecting the spike into the sample line must be kept as short as possible.

E.7.1.4 Reduce the flow so that the pressure reads below 0.5 bar, the actual pressure is not critical, but a flow of water should be maintained through the validation rig. Inject the spike into the sample line ensuring the needle is inserted completely into the septum to inject the oocysts directly into the flow of water to minimize the loss of oocysts. The plunger should be held down for 5-10 seconds to try to ensure that the oocysts are carried by the flow of water onto the filter module. Care should be taken as there may be some back pressure, safety glasses or visor should be worn. As the plunger is released water will be drawn into the syringe. Fill and empty the syringe to rinse twice, without withdrawing the needle from the sample line. Remove the needle and reset the flow to give a minimum flow of 40 litres per hour

filtered over 24hrs. Record the time, date, analyst and meter readings. Check the septum for leakage and replace if necessary.

E.8 RECORDS OF LABORATORY QUALITY ASSURANCE TESTS

E.8.1 Records must be kept of all 'Laboratory Quality Assurance Tests' and these must be available for any audit undertaken by the DWQR. The records can be electronic (such as linked spreadsheets and graphs) or hard copy or both. A graph of the daily validation sample must be maintained and be available for inspection and audit at all times.

E.8.2 A full record of each daily AQC sample shall be kept in a bound and sequentially numbered AQC analysis book. The records will provide information on the water sampled, the spike used (with an auditable trail to the supplier), details of reagents used. All records must be capable of being audited back to either the sample or the analyst.

E.8.3 A full auditable record must be kept of the actions taken in response to any exceedance of the 'triggers' detailed in E.9.4.2. This record must be available for any inspection undertaken at the laboratory.

E.9 THE STATISTICS TO BE USED AND GUIDANCE ON THE CHARTS TO BE USED FOR PLOTTING RESULTS FROM DAILY *CRYPTOSPORIDIUM* QC SAMPLES

E.9.1 Introduction

E.9.1.1 A large daily volume spiked sample is processed through an approved rig after injection of a known number of oocysts. Following analysis of the filter this provides an oocyst count from the filter. The method of analysis of the filter shall be the method used for analysing regulatory samples and the sample shall be treated as another regulatory sample. The spike shall be 100 oocysts within a known tolerance, using a flow cytometer of a standard deviation of less than 2.5.

E.9.1.2 The data from these QC samples will be recorded so that information on process details, names of analysts and any other relevant background facts are readily to hand. The results will be recorded and plotted on charts. See Section 8 above 'Records of Laboratory Assurance Tests'.

E.9.1.3 The aim of these QC charts is to give assurance that oocyst recovery performance is being maintained and to trigger investigations when performance appears to change.

E.9.1.4 Quality control charts were developed for operational control in manufacturing industry and have been used successfully in the water industry for demonstrating control of chemical testing, in particular using suitably modified Shewhart charts. The concept has been extended to

microbiological laboratories to demonstrate consistent microbial enumeration, using reference material.

E.9.1.5 In industry the measurement being controlled might be physical (e.g. life-time of a light bulb) and have a defined tolerance. A sample of the bulbs would be tested and if the average life time was seen to deteriorate and the measurements cross an 'action' line then the production process would be judged to be out of control and appropriate action taken. In water chemistry the ability of the laboratory to make accurate measurements is monitored using reference material. The regular tests would be expected to give consistent and repeatable results. A small amount of variation would be observed and tolerated. If QC results drift or become more erratic, with results on the control chart crossing the 'warning' or 'action' lines, then an investigation would be undertaken to pinpoint any problems in the analytical procedures (or possibly a problem with the reference material).

E.9.1.6 In microbiology there is usually much more variation than in chemistry in the results from sequential samples from reference material - principally because of random variation in the numbers of organisms present in each test portion and partly because of the difficulty in keeping stable reference material. Therefore 'control' charts have been approached in a less judgmental way and are often referred to as 'guidance' charts (*Microbiology of Drinking Water (2002) - Part 3 -Practices and procedures for laboratories, section 8.5.1*). An apparent problem won't necessarily reflect a true deterioration in laboratory performance; it may just be microbial behaviour or problems with the samples. However the chart does act as a screening tool for possible laboratory problems. The charts are designed with 'response' lines which, if crossed, trigger investigation without automatically labelling the laboratory as 'out of control' and thus discrediting all that batch of results.

E.9.1.7 In this *Cryptosporidium* QC scheme laboratories have an advantage over most microbiology QC in that the numbers of organisms spiked into the sample will be known to a much greater accuracy than can be achieved for bacterial spiking suspensions. There may be problems with maintaining consistent reference material for spiking the samples day after day, and with using water with consistent properties.

E.9.2. Aims of Internal QC Daily Samples.

E.9.2.1 The results of the daily QC samples should be used as part of the laboratory's Quality Assurance programme. The results will be plotted on a suitable guidance chart. This will provide a screening tool for possible problems in recovering and enumerating oocysts. Furthermore, the charts and the associated records will provide evidence to DWQR that performance is either being maintained or that observed evidence of possible problems is being promptly investigated, with the results of that investigation properly recorded and any necessary action taken.

E.9.3 Constructing Guidance Charts

E.9.3.1 Plot the numbers of oocysts recovered sequentially. Once the rig is functioning satisfactorily and staff are accustomed to its use then this plot should settle down to show a small scatter around a steady average. It is customary to use a sequence of steady data (a minimum of 20 consecutive results) to calculate the average and standard deviation of the observations. These statistical parameters are then used to construct the warning and response lines against which future QC samples are compared.

E.9.3.2 The guidance chart will show the preliminary sequence of data. Horizontal lines of the mean of these preliminary data and lines at ± 2 x standard deviation (warning lines) and at ± 3 x standard deviation (response lines) are drawn on the chart.

E.9.3.3 The reason these lines are chosen is because, if the data are distributed approximately Normally (*i.e.* with a frequency distribution which would look bell-shaped and have this mean and standard deviation) then the warning lines should not be crossed, by chance, more than about 1 in 20 observations, and the response lines more than 1 in 370 observations. In reality microbiology counts are, at best, only approximately Normally distributed but this approach usually provides a working, objective solution.

E.9.3.4 Part of the management of the QC work will be to re-assess the values of the guidance lines at regular intervals and to check that they are set at suitable levels.

E.9.4 Plotting routine results

E.9.4.1 Once the levels have been set and the guidance chart is functional then the following procedures should be followed and logged:

E.9.4.2 Plot the new QC result on the guidance chart and note whether any of the following has occurred:

(i) this result falls outside a response (action) line;

(ii) two out of three successive results fall outside the same warning line;

(iii) nine consecutive results fall on the same side of the mean line; and

(iv) six consecutive counts show a trend which continuously rises or falls.

These represent four 'triggers' which have been tried and tested in microbiology laboratories and which are very unlikely to occur by chance unless the rate of recovery of oocysts has changed (Microbiological Analysis of Food and Water: Guidance for Quality Assurance. Ed NF Lightfoot & EA Maier, pub Elsevier, 1998. page 170). By having four triggers there is a better chance of detecting problems with gradual as well as sudden onset. These triggers should initiate a pre-planned response. An explanation should

be sought as to whether there is a change in laboratory performance or, for example, a change in the QC sample material. Note, however, that these events can happen, rarely, by chance.

E.9.5 Response to triggers

E.9.5.1 Pre-planned responses to 'trigger' events should be kept on file and be available for audit when required.

E.9.5.2 Responses to any of the four triggers will typically involve an appraisal of the whole analytical process including the preparation of the associated daily samples from which the data have been calculated. Apart from the assessment of the actual analytical and spiking procedures (including observation of the analysts' techniques), it is appropriate to check whether the changes may be associated with the introduction of a new batch of a reagent (e.g. IMS beads, IFA stain *etc.*).

E.9.6 Monthly review

E.9.6.1 Assess the lines on the guidance chart and check that they are fulfilling their required functions.

E.9.6.2 If the warning lines are never being crossed (and they should, by chance, be exceeded occasionally) then the estimate of the standard deviation is probably too high. This can happen when the chart is first set up because 20 observations is quite a small number for obtaining an accurate estimate. Re-calculate the mean and standard deviation using the last 50 results.

E.9.6.3 If the first three of the four triggers have been happening several times without explanation then consider whether the initial standard deviation was too low or the initial mean was too low or high. Re-set them using the last 50 results.

E.9.6.4 Once the QC procedures have settled down it should not be necessary to keep re-calculating the parameters. In theory the 2 x s.d. lines is likely to be crossed approximately one in 20 data points, but this will be a chance and not a regular process. Some months will have no crossings and others will have multiple crossings. Similarly the data points will be expected to be scattered above and below the mean line but some months may have a slight excess on one side and other months on the other side, by chance. Further guidance should be possible in the light of experience - when this scheme has been running in all laboratories for at least a year.

E.9.6.5 Changes in the values of the statistical parameters must be logged. Adjustments in the early months of setting up the guidance charts are justifiable but any changes after the charts have been used successfully for a few months must be highlighted and discussed in the log. They may well indicate a shift in performance.

E.9.7 Summary of procedures

E.9.7.1 A summary of the procedures are detailed below:

(a) daily samples will be processed according to the DWQR standard operating protocol, details recorded and result plotted;

(b) when the routine is established a sequence of at least 20 results will be used to calculate mean and standard deviation and these will be used to plot response lines on the guidance chart;

(c) each subsequent daily result will be plotted and checked against the set of four 'triggers';

(d) the performance of the chart will be reviewed after every month (i.e. initially after 50 data points have accumulated and thereafter monthly) to assess, long-term, the appropriateness of the response lines; and

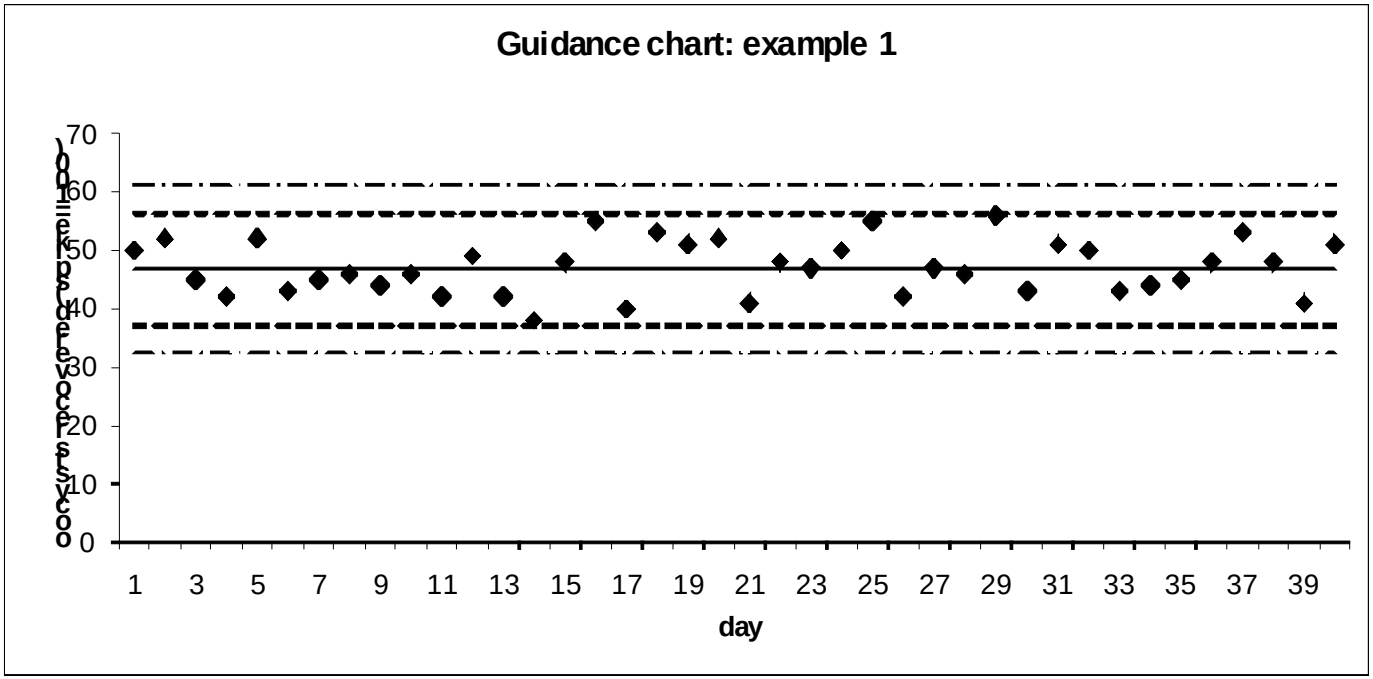
(e) after the scheme has been running for about a year this document will be reviewed. Further worked examples will be provided using real data.

E.9.8 Examples of QC Charts for Information and Guidance

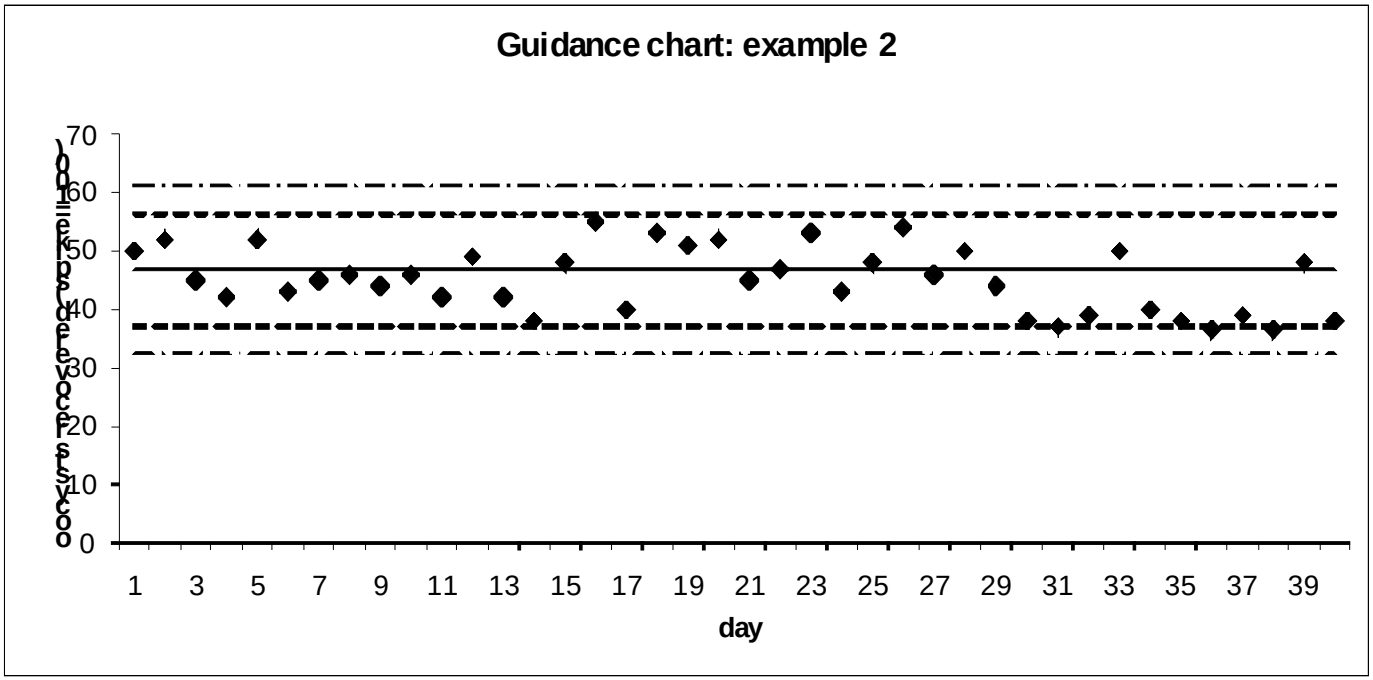
E.9.8.1 Example 1 shows a 40-day series. The first 20 observations were taken from real data and their mean (46.8) and standard deviation (4.83) were used to calculate warning and response lines. Because no further data were available for this example, the next 20 results were computer-generated to have the same parameters (*i.e.* mean and s.d.). Therefore this example illustrates a laboratory performing consistently; no possible problems are detected. Note that the upper warning line is reached once (on day 29) and this was a chance occurrence.

E.9.8.2 In Example 2 the same 20 observations as in example 1 were used to set up the chart. The next 10 results were computer-generated to have the same parameters but then the average recovery was reduced by 10%, from day 31 onwards. Visual inspection would have raised suspicion that the average recovery had in fact fallen. And by day 38 one of the 'trigger' rules had come into effect - two out of three successive results fall below the lower warning line. In real life this would lead to investigations, a record in the log book of this occurrence and a report on the outcome of the investigations and any action taken.

E.9.8.3 Guidance Chart Example 1



E.9.8.4 Guidance Chart Example 2



E.9.9 Introduction of New Approved Products into the Laboratory

E.9.9.1 Before any new approved product can be introduced in an approved laboratory for the sampling and analysis of regulatory samples; it must be shown to be at least as good as or better than recovering oocysts at limit concentration, than the existing approved method of analysis. The method whereby validation is to be achieved is set down below.

E.9.9.2 The revision of the SOP has introduced the concept of 'equivalent' in the testing of new approved products for the analysis of regulatory *Cryptosporidium* samples in approved laboratories. This is defined below:

Equivalent

The required performance of the trial method is that it should be at least equivalent (or better) than the standard method. 'Equivalent' will be interpreted as either 'significantly better' or as 'not significantly different', but the latter must be to a level of confidence acceptable to DWQR. Thus, if the trial method is not finding significantly larger numbers of oocysts then the average difference between results from the two methods should be compatible with the null hypothesis of zero difference (with 95% probability). This statistical analysis will automatically lead to a statement about the likely range of the 'true average difference' between the methods, which is the average you would get from an infinite number of samples of the same types of water. This range is usually expressed as a 95% confidence interval and the lower end of this should rule out unacceptably worse recovery.

So that any new product has to be shown that it is at least 'equivalent' in performance or better than the existing approved product as defined above.

E.9.9.3 The definition of 'equivalent' will be used to introduce new approved products into the laboratory. A new product will be tested, as part of an analysis, against the existing approved method of analysis employed in the laboratory.

E.9.9.4 The laboratory validation sampling rig will be used to produce the samples for analysis in the laboratory to determine 'equivalence'. One line of the validation sampling rig would be the daily QC filter module (the result would be used as the QC sample, as well as, using the result as part of the validation of the new product) and the other line would be used to test the new product. Using the laboratory validation rig, the two filters are tested in parallel using the following procedure for checking the verification:

(i) seed the two filter modules with oocysts, each at the limit concentration. Where a product other than a filter module is being tested then two filter modules of the same type should be seeded;

(ii) connect the filters to the validation sampling rig which is on the laboratory standard water supply. The flow-meter connected

downstream of each filter unit to measure the volume sampled is read prior to turning on the water supply to the validation sampling rig. The water is turned on, so that the pressure gauge reads 100-150 kPa (1-1.5 Bar), for at least one hour. [Providing the filter has run for at least one hour the time the oocysts are injected is not critical.] The pressure to the sampling lines is turned down to 50 kPa (0.5 Bar) to make injection of the oocysts easier. The oocysts are then injected into each filter line. After injection of the oocysts the syringe is flushed at least twice into the flow line. When this has been completed the pressure is increased to 100-150 kPa (1-1.5 Bar) to ensure that at least 1,000 litres of water will pass through the filter over a 24 hour period. The filter is run continuously for a 24 hour period at a flow rate not less than 40 litres per hour. At the end of the 24 hour period the filter is removed and the sample analysed in the laboratory along with the regulatory samples. That is it would be treated in a similar manner to any regulatory sample analysed in the laboratory and not in any special way;

NB The sample taken will as far as is practical replicate the sample taken at a regulatory site. That is, a continuous sample is taken over a period of 24 hours at a minimum flow of 40 litres per hour on average, this should equate to a flow through the filter of approximately 1000 litres over the 24 hour period.

(iii) the validation sampling rig is run on twelve separate days and the filters run for a 24 hour period and the volume filtered is measured, which should be greater than 1,000 litres. Fresh filters are used for each 24 hour period.

(iv) It is not required that a negative control is run with these filters.

(v) The data generated must demonstrate that one approved product is equivalent or (better) than the other approved product. 'Equivalent' will be interpreted as either 'significantly better' or as 'not significantly different', but the latter must be to a level of confidence acceptable to DWQR. Should the data fail to demonstrate equivalence then more replicates will be required for this to be achieved.

E.9.10 Statistics to be used to Validate a New Product in the Laboratory

E.9.10.1 The laboratory checking the *alternative* or modified approved method needs to establish equivalence between the two methods. The statistics for this together with two worked examples are given in Annex B of Part 3 of the DWI SOP ‘Validation Of New Methods Or Parts Of Methods For Sampling And Analysis’. It may also be apparent that there is a difference in recovery of oocysts between the water sources used by different laboratories for the validation. Providing the results are consistent with the QC sample for the supply and the method is ‘in control’ then the result is acceptable.

E.9.11 Documentation

E.9.11.1 Full documentation must be kept of each validation in a separate file. The completed file has to be available for audit purposes to show that any new product has been properly validated. All results, data and calculations (including any rejected results) must be kept and copies put on the file. The written report of any validation should clearly show the setting down of the results, the calculation of the statistics and that the new approved product is 'equivalent' to the existing approved product being used in the laboratory. The format of the report must follow the format set down in Part 3 of the DWI SOP 'Validation Of New Methods Or Parts Of Methods For Sampling And Analysis'.

E.10 INITIAL ANALYST VALIDATION

E.10.1 Each approved analyst must have documented evidence in training records to show that they can meet the level of recovery required by the Protocol. As part of their training each analyst must undertake 10 replicate analyses for method verification/performance assessment using 10 litre spiked samples to show that they are competent to undertake analysis of regulatory samples. (See Appendix F Training and Training Records).

E.10.2 Analysts should be able to reproducibly achieve a recovery not less than 30% of the whole procedure using spiked reagent water. Any recovery less than 30% in any of 10 replicate analyses undertaken by the analysts for method verification/performance assessment may be regarded as **not** demonstrating satisfactory performance with the procedure, unless they can fully explain the causes for the lower recovery. Single recoveries from any set of 10 replicate analyses that are below 30% must be repeated. More frequent recoveries of less than the minimum/maximum of any set of 10 replicate analyses are considered as indicative of poor performance. Analysts failing to achieve these levels of recovery should review their procedures for analysing oocysts and analyst's competencies before revalidating the method. Analysts failing to achieve the required standards must be retrained in those parts of the procedure in which they have under performed before repeating the full set of 10 replicate tests. The results of ALL replicate analyses should be placed in the analysts training records.

E.10.3 The analyst should not undertake more than two analyses per day nor take more than 10 weeks to complete the 10 replicate analyses.

E.10.4 If an analyst has undertaken their validations using the manual wash station then they are automatically approved to use the automatic wash station. However, analysts validated using the automatic wash station are only approved to use the automatic wash station.

E.10.5 Procedure for Initial Analyst Validation

E.10.5.1 Set-Up

E.10.5.1.1 Equipment for these tests consists of a ten litre carboy or aspirator, peristaltic pump capable of delivering 1 litre per minute, and an IDEXX Filta-Max® filter unit. Connections between various units to be made with laboratory plastic tubing. A magnetic stirrer and bar are required to ensure that the contents of the carboy/aspirator are kept stirred during filtration.

E.10.5.1.2 Install the filter module in the filter holder before securing the inlet and outlet ends of the holder.

E.10.5.2 Procedure

E.10.5.2.1 Part fill the carboy/aspirator containing a magnetic stirring bar with approximately 8 litres of reagent water and place on stirrer plate. Start the magnetic stirrer. Start the pump, and pump 1-2 litres of water through the rig to charge the filter unit, switch off the pump. With the stirrer activated, pipette a volume of the oocyst working suspension into the carboy/aspirator to seed the required number of oocysts (80 to 120 oocysts) and bring the volume up to 10 litres of water. Start the pump and filter the sample at a flow rate of approximately 1 litre/minute.

E.10.5.2.2 Turn off the magnetic stirrer when volume in the carboy/aspirator is too low to stir effectively. When the contents of the carboy/aspirator are virtually depleted, turn off the pump and add 1 litre of de-ionised water to the carboy. Swirl to rinse down the sides of the carboy/aspirator. Turn the pump back on and filter the remaining water.

E.10.5.2.3 Switch off the pump. Disconnect the inlet end of the filter housing while maintaining the level of the inlet fitting above the level of the outlet fitting to prevent backwashing and the possible loss of oocysts from the filter.

E.10.5.2.4 Loosen the outlet fitting and allow water to drain as much as possible, then cap the outlet fitting.

E.10.5.2.5 Proceed to elution and concentration, and staining as in sections 5 & 6 of this SOP.

APPENDIX F. Training and Training Records

F.1 TRAINING RECORDS

F.1.1 Prior to undertaking analysis of samples from any Company's regulatory sites each analyst shall undertake a course of training (either internal or external) such that they will be competent and be capable of carrying out their duties without generating any anomalies.

F.1.2 Every person engaged in the regulatory analysis of *Cryptosporidium* oocysts shall have a full and detailed record of all the training undertaken and the dates on which the training was undertaken. The training record shall be available for audit when necessary. Each laboratory shall maintain and revise, when necessary, full details of the course of training given to each person working in the *Cryptosporidium* laboratory, including the significance of chain of custody.

F.1.3 When the laboratory is satisfied that the analyst has satisfactorily completed the initial training course they shall undertake the 10 replicate analyses in accordance with Appendix E. An analyst shall be deemed to be approved to undertake regulatory analysis of samples on completion of the course of training and achieving satisfactory recoveries from the 10 replicate samples as detailed in Appendix E.

F.2 TRAINING

F.2.1 Each person's training record shall include at least the following:

(i) full details of the training undertaken including sample preparation, receipt of samples, examination, including dates commenced and completed;

(ii) full details of the ten replicate analyses, including each individual result;

(iii) record of all further laboratory assurance tests with recoveries;

(iv) results of inter-laboratory proficiency tests on samples and slides provided by DWI and DWQR in a suitable format such that performance of the individual can be easily monitored together with dates and a copy of the work undertaken.

F.3 FURTHER GUIDANCE

F.3.1 If a person is re-employed in a laboratory undertaking regulatory *Cryptosporidium* analysis of samples within a twelve month period the person should undertake 10 replicate analyses to show that the performance is in accordance with Appendix E.

F.3.2 If a person is re-employed in a laboratory undertaking regulatory *Cryptosporidium* analysis of samples longer than a twelve month period the person should undertake retraining, including 10 replicate analyses to show that the performance is in accordance with Appendix E.

F.3.3 If a person moves from one laboratory undertaking regulatory *Cryptosporidium* analysis of samples to another laboratory undertaking regulatory *Cryptosporidium* analysis then the person should undertake 10 replicate analyses to show that their performance is in accordance with Appendix E.

APPENDIX G. Centrifuge

The centrifuge may give a reading in either revolutions per minute (rpm) or relative centrifugal field (RCF or 'g' value). The SOP recommends that the centrifuge is operated at 1100g for 15 minutes.

The relationship between rpm and RCF is detailed below. The acceleration of a centrifuge is usually expressed as a multiple of the acceleration due to gravity (g = 9.8 m s-2 termed relative centrifugal field (RCF or 'g' value). The RCF depends on the speed of the rotor (n, in revolutions per minute, rpm) and the radius of rotation (r, in mm) where:

$$RCF = 1.118r \left(\frac{n}{1000}\right)^2$$

The relationship can be rearranged, to calculate the speed (rpm) for specific values of r and RCF.

$$n = 945.7 \sqrt{\left(\frac{RCF}{r}\right)}$$

However, it should be noted that RCF is not uniform within the centrifuge tube: it is highest near the outside of the rotor (r max) and lowest near the central axis (r min). In practice, it is customary to report the RCF calculated from the average radius of rotation (rav). It is worth noting that RCF varies as a squared function of the speed: thus the RCF will be doubled by an increase in speed of approximately 41%.

EXAMPLES OF CALCULATIONS OF RCF AND rpm FOR A CENTRIFUGE

Suppose you wanted to calculate the RCF of a bench centrifuge with a rotor of rav = 95 mm running at a speed of 3220 rpm.

RCF = 1.118 X 95 X (3.3220)2 = 1100 g

To calculate the speed required to produce a relative centrifugal field (RCF) of 1100 g using a rotor or rav = 85 mm.

945.7 √ (1100/85) = 3402 rpm

APPENDIX H. Glossary

BSP	British Standard Pipe fitting
CLBs	Cryptosporidial oocyst-like bodies
COSHH	Control of Substances Hazardous to Health
DAPI	4',6-diamidino-2-phenylindole
DIC	Differential interference contrast
DWI	Drinking Water Inspectorate
DWQR	Drinking Water Quality Regulator for Scotland
Filta-Max®	Proprietary name for IDEXX filter and wash station assemblies
FITC	Fluorescein isothiocyanate
HCl	Hydrochloric acid
IFAT	Immuno-fluorescent antibody test
IMS	Immunomagnetic bead separation
LIMS	Laboratory Information Management System
M	Molar
Mab	Monoclonal antibody
MPC	Magnetic Particle Concentrator
NaOH	Sodium hydroxide
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline/tween 20
PTFE	Polytetrafluoroethylene
QA	Quality assurance
QC	Quality control
SCADA	Supervisory control and data acquisition
SI	Statutory Instrument

SOP	Standard Operating Protocol
USEPA	United States Environmental Protection Agency
UV	Ultra-violet
WTWs	Water treatment works
Eluent	Solution (ie PBST) used to wash/elute filters
Eluate	Consequent solution generated from washing filters (ie sample)

APPENDIX I.

Epifluorescence microscopy				
	Feature	**Comments**	**Significance**	**Action**
A1	Bright apple green fluorescing round or slightly ovoid objects measuring between 4 and 6µm.	Fluorescence should be bright and even with an increased intensity on the outer perimeter. Occasionally, oocysts can exhibit a granular, fuzzy or dull perimeter to the fluorescence, especially in oocysts exposed to the environment for some time.	Oocyst may be intact. Oocyst was empty prior to sampling or ruptured during air drying.	• Minimum fluorescent criteria for empty oocysts. • Confirm size, shape and presence of nuclei with DAPI, and size, nuclei and sporozoites by DIC.
A2	Bright apple green fluorescing objects with a gape (pac-man) measuring between 4 and 6µm.	Fluorescence should be bright and even with an increased intensity on the outer perimeter. Occasionally, oocysts can exhibit a granular, fuzzy or dull perimeter to the fluorescence, especially in oocysts exposed to the environment for some time.	Sporozoites may be present within oocyst. Oocyst was empty prior to sampling or ruptured during air drying.	• Minimum fluorescent criteria for empty pac-man shaped oocysts. • Search for DAPI stained nuclei within or in proximity to pac-man. Confirm size and presence of nuclei and sporozoites by DIC.
B1	A + four DAPI stained nuclei.	DAPI stained nuclei should be fluorescent foci of approx. 1µm with no diffuseness or thread like projections.	Size, nuclei and sporozoites available for confirmation by DIC.	• Minimum fluorescent confirmatory criteria for intact oocysts or oocysts with gape. • Confirm size and presence of nuclei and sporozoites by DIC.
B2	A + 1 to 3 DAPI stained nuclei.	DAPI stained nuclei should be fluorescent foci of approx. 1µm diameter with no diffuseness or thread like projections.	Some nuclei may be hidden by others. Rack fine focus through plane of object and attempt to confirm the presence of up to 4 nuclei.	• Confirm size and presence of nuclei and sporozoites by DIC.

Nomarski DIC microscopy				
	Feature	**Comments**	**Significance**	**Action**
C	Round or slightly ovoid, smooth, thick walled, colourless and refractile objects measuring between 4 and 6µm.	Measurement using DIC can be more accurate than measurement using fluorescence.	See A1, A2.	• Confirm presence of nuclei and sporozoites by DIC.
D	C + 4 nuclei.	Each nucleus should be approx. 1µm diameter.	Confirmation of B1 by DIC.	• Minimum confirmatory criteria for intact oocysts or oocysts with gape. • Confirm presence of 4 sporozoites by DIC.
E	C + D plus 4 sporozoites.	Only partial images of the 4 sporozoites may be visible.		• Confirmatory criteria for intact oocysts or oocysts with gape.
F	C + 1 to 3 nuclei.	Each nucleus should be approx. 1µm diameter.	Some nuclei may be hidden by others. Rack fine focus through plane of object and attempt to confirm the presence of up to 4 nuclei.	• Confirm presence of sporozoites by DIC.
G	C + F plus 1 to 3 sporozoites.	Only partial images of the sporozoites may be visible.	*Cryptosporidium* spp. oocyst.	Record in Regulatory count.

APPENDIX J.

Table. Species and genotypes of *Cryptosporidium*.

***Cryptosporidium* species**			
Species	**Infectious to humans**	**Major host**	**Oocyst dimensions (µm)**
C. hominis	√	humans	4.5 x 5.5
C. parvum	√	cattle, other livestock, humans	4.5 x 5.5
C. suis	√	pigs	5.05 x 4.41
C. felis	√	cats	4.5 x 5.0
C. canis	√	dogs	4.95 x 4.71
C. meleagridis	√	turkeys	4.5-4.0 x 4.6-5.2
C. muris	√	rodents	5.5 x 7.4
C. andersoni	X	cattle	5.6 x 7.4 (5.0-6.5 x 8.1-6.0)
C. wrairi	X	guinea pigs	4.0-5.0 x 4.8-5.6
C. bovis	X	cattle	4.7-5.3 x 4.2-4.8
C. bailey	X	poultry	4.6 x 6.2
C. galli	X	finches, chicken	8.0–8.5 x 6.2–6.4
C. serpentis	X	reptiles	5.6–6.6 x 4.8–5.6
C. saurophilum	X	lizard	4.2-5.2 x 4.4-5.6
C. molnari	X	fish	4.72 x 4.47
C. scophthalmi	X	fish	3.7-5.0 x 3.0-4.7

***Cryptosporidium* genotypes**					
	Infectious to humans		**Infectious to humans**		**Infectious to humans**
Monkey	√	Deer mice	X	Pig (x2)	X
Cervid (1)	√	Duck	X	Tortoise	X
Cervid (2)	X	Goose (x2)	X	Muskrat (x2)	X
Marsupial (x4)	X	Goose (x2)	X	Muskrat (x2)	X
Mouse	X	Squirrel (x2)	X	Horse	X
Rabbit	X	Bovine	X	Lizard	X
Skunk	X	Bear	X	Fox	X
Ferret	X	Snake	X	Woodcock	X

The current state of *Cryptosporidium* taxonomy

Cryptosporidium is a protozoan parasite that infects a wide range of vertebrates. Species within this genus cause human cryptosporidiosis which probably constitute the most common causes of protozoal diarrhoea, worldwide, and lead to significant morbidity and mortality in both the developing and developed world. Transmission is via the faecal oral route following direct or indirect contact with the transmissive stages (oocysts), including person-to-person, zoonotic, waterborne, foodborne and airborne transmission. Molecular biology has provided powerful new tools for characterising *Cryptosporidium*, and the analysis of previously unrecognised genetic

differences within this genus has revolutionised both its taxonomy and our understanding the epidemiology of human disease. Molecular tools have enabled not only the identification of species / genotypes in the faeces of infected hosts, but also their recognition in environmental samples, including water.
At least seven species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, and *C. muris*) and two genotypes (monkey and cervine) of *Cryptosporidium* are associated with human disease, and molecular approaches have enabled a greater understanding of the contributions of humans and livestock as reservoirs of infection. Using species typing tools, differences in geographical and temporal distribution, disease presentations and risk factors for infection have been identified for *C. parvum* and *C. hominis*, the most commonly reported causes of cryptosporidiosis.