Drinking Water Inspectorate

STANDARD OPERATING PROTOCOL

FOR THE MONITORING OF *CRYPTOSPORIDIUM* OOCYSTS

IN TREATED WATER SUPPLIES

TO SATISFY

THE WATER SUPPLY (WATER QUALITY) REGULATIONS

2000, SI No. 3184 ENGLAND

THE WATER SUPPLY (WATER QUALITY) REGULATIONS

2001, SI No. 3911 (W.323) WALES

Part 2 - Laboratory and Analytical Procedures

16 June 2005

CONTENTS

1 INTRODUCTION TO THE STANDARD OPERATING PROTOCOL

1.1 This Standard Operating Protocol (SOP) provides guidance from the Drinking Water Inspectorate (DWI) on behalf of the Secretary of State and the Welsh Assembly Government on the sampling and analysis requirements associated with the Water Supply (Water Quality) Regulations 2000 (for England) and the Water Supply (Water Quality) Regulations 2001 (for Wales), (The Regulations).

1.2 This SOP is published in four parts:

- Part 1 Sampling and Transportation of Samples.
- Part 2 Laboratory and Analytical Procedures.
- Part 3 Validation of New Methods or Parts of Methods for Sampling and Analysis.
- Part 4 Requirements for the Inter-laboratory Proficiency Scheme.

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

1.4 The conditions of sampling and examination must be such as to permit the use of the analytical results as evidence in a Court of Law. The SOP therefore describes a system that would ensure both the scientific quality of the results, and that a chain of evidence is maintained in compliance with the Police & Criminal Evidence Act 1984. The Protocol covers the minimum requirements to satisfy this requirement.

1.5 Any proposed deviation from the requirements contained in parts 1 and 2 of this SOP will require a full laboratory appraisal to be carried out in accordance with part 3 of this SOP. Results of such appraisal must be submitted to the DWI for consideration of approval. This approval must be obtained in writing before any changes are made to the relevant sections of the SOP. Any approved changes to this SOP will be circulated to all water companies and DWI approved laboratories.

1.6 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.

1.7 Additional guidance is given in the document 'Monitoring Equipment for the Continuous Sampling of Cryptosporidium in Treated Drinking Water' Revision 4 December 1999. This document can be accessed via the DWI web site. A hyperlink is present in the DWI Information Letter 2/2000 paragraph 4 and just click on the title of the document.

1.8 Extracts from 'The Water Supply (Water Quality) Regulations 2000 Relating to the Sampling and Analysis of Cryptosporidium) Oocysts

1.8.1 The treated water entering supply from the treatment works shall contain less than one oocyst in 10 litres of water.

1.8.2 For any supply requiring monitoring a continuous sample of water, consisting of at least 40 litres per hour over the sampling period 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 Inspectorate 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.

1.8.3 A continuous sample will be taken from any regulatory sampling point 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).

1.8.4 The collection device must be changed at least once per day. Note that in respect of 'The Regulations' 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).

1.8.5 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 require changing until the day the total volume passing through the collection device equals or exceeds 200 litres.

1.9 Preferred Units

1.9.1 The preferred units in the 'Standard Operating Protocol' are SI units.

2. APPROVED LABORATORIES

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

2.2 The Analytical Laboratory

2.2.1 The analytical laboratory must designate a room or rooms specifically for the receipt, preparation and analysis of samples for *Cryptosporidium* oocysts. Other analysis may be carried out in the designated room or rooms, but full access and other controls must be in place. Such room or rooms herewith termed the *Cryptosporidium* Analytical Laboratory must be secured with suitable locks on all access doors and windows. Keys to the access door or doors and windows must only be held by authorised designated personnel. Access should be via key, controlled issue, proximity card or other approved methods. A buzzer should sound when the door is opened.

2.2.2 The analytical laboratory must designate authorised staff for working in the *Cryptosporidium* Analytical Laboratory. All authorised staff designated to work in said laboratory must sign and record the date and time when entering and leaving the *Cryptosporidium* Analytical Laboratory, in a purpose held log-book that must be bound and each page sequentially numbered.

2.2.3 The *Cryptosporidium* Analytical Laboratory must not be left unattended without first securing all samples taken under these Regulations or without securing all access doors and windows to prevent unauthorised entry.

2.2.4 Unauthorised staff or visitors (including cleaners, service engineers, etc.) must sign and record the date and time in a visitors' logbook on entering and leaving the *Cryptosporidium* Analytical Laboratory. They must also be accompanied by an authorised member of staff at all times whilst in the *Cryptosporidium* Analytical Laboratory unless all samples and slides are securely stored. The visitors' logbook must be a bound book with all pages sequentially numbered.

2.3 Competence Of Authorised Staff

2.3.1 All authorised staff designated to work on the preparation of sampling equipment, elution, concentration and enumeration of *Cryptosporidium* in samples taken under the Regulations 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 authorised 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 (480 nm excitation, 520 nm 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 (350 nm excitation, 450 nm 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 microscopist.

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 to demonstrate that the person is not colour blind. All certificates should be available for audit.

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. 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 DWI approval for that analyst being withdrawn until such time as competence can be re-established.

2.7.2 The current 'inter laboratory proficiency scheme' covers all authorised 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 (IDEXX Filta-Max® or Envirochek™ HV) for analysis.

2.7.3 Microscope Slides

The microscope slides are either:

i) stained slides displaying only *Cryptosporidium* oocysts; 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 contain any level of *Cryptosporidium* relevant to the assessment of laboratory performance and are analysed by authorised staff following the procedure normally used in the laboratory.

2.7.5 Filter Modules

Approved filter modules will be spiked with the target concentration[1] (100 oocysts) or half or twice the target concentration.

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 Inspectorate considers that it is important that all designated staff authorised to analyse for *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 Inspectorate or DWI 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. If necessary, they should each keep their own analysis workbook logging details of all samples 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 and good working practices at all times.

2.8.3 The laboratory environment where analysis of *Cryptosporidium* samples are undertaken should comply with guidelines[2] 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

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[1] The target concentration is 100 oocysts which equates with 100 oocysts in 1000 litres or 1 oocyst in 10 litres. Continuous sampling over 24 hours at 40 litres per hour equates to approximately 1000 litres.

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

should include the understanding of risks from *Cryptosporidium* and the potential for infection.

2.9 Documentation

2.9.1 All entries for receipts, logbooks, evidence bags, or analysis or other 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 and timed. All entries and records in analysis books, log books or on evidence bags 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 Inspectorate. On the rare occasions it may be necessary to retain the records relating to a specific sample for longer, the Inspectorate will notify a company in writing. Records must be stored under secure conditions until written authorisation is given by the Inspectorate 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 Annual Audits

2.10.1 Each laboratory certified to carry out analysis for *Cryptosporidium* under the terms of the Water Supply (Water Quality) Regulations 2000 (England) 2001 (Wales) shall be subject to at least one announced audit per year. The audit will be undertaken by the Inspectorate or DWI 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 Inspectorate considers necessary.

2.11 Unannounced Audits

2.11.1 If it is considered necessary the approved laboratory may be subject to unannounced audits when log books, analysis work books, security, analytical methods, storage of samples, slides, use of security tags, reporting arrangements, and other matters of interest may be audited without prior notice being given.

2.12 Intermediate Audits

2.12.1 These will be undertaken by the Inspectorate to undertake specific audit tasks or to follow up any significant recommendations or poor working practices identified at the annual audit.

3 SAMPLING EQUIPMENT

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

3.1.1 The *Cryptosporidium* Analytical Laboratory shall supply the IDEXX Filta-Max® Filter and Filta-Max® housing for sampling at each of the designated water treatment sites. The IDEXX Filta-Max® housing must be supplied with a unique reference, clearly and legibly labelled, on both parts (base and top) of the housing and must be fitted with the appropriate connectors on the inlet and outlet of the IDEXX Filta-Max® housing. Each IDEXX Filta-Max® housing must also have a label attached, or be labelled, clearly identifying the monitoring point at which it is intended to be used. This label will contain the site identification code, 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[3]. 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 0.5 mm 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 500 kPa (5 bar) (by connecting to a laboratory pump) to ensure that there are no leaks. The air supply hose outlet must be fitted with a Swagelok™ Quick-Connect body (B-QC6-B-4MS fitted with K5 (blue) key). The hose line must be connected to the outlet side of the filter housing. A pressure of 500 kPa (5 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.

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[3] 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.

3.1.4 After carrying out a successful pressure test, an approved plastic security tag (green) must be threaded through holes in both the base and the top of the IDEXX Filta-Max® MK I or II housing and tightened to ensure that the housing cannot be opened without breaking the tag. All security tags used in the monitoring of *Cryptosporidium* oocysts under the regulations must be of standard design and obtained from DWI or DWI approved suppliers. The records of the delivery and the serial numbers of the tags shall be subject to audit as determined by the Inspectorate.

3.1.5 The identity numbers of the IDEXX Filta-Max® Filter, IDEXX Filta-Max® MKI or II housing base and top, plastic security tag fitted to the housing and the location identity and the laboratory where the sample is to be taken must be recorded in the appropriate boxes on an approved evidence bag.

3.1.6 A log must be kept of the identity numbers of the housing with a record of the date of the pressure test.

3.1.7 The assembled collection device must then be placed in a tamper-evident delivery bag together with the evidence bag to be used for the return journey and the white cabinet tag. The tamper-evident delivery bag as well as the evidence bag should be checked to ensure they are fit for use. The delivery bag must have the "use by" date clearly marked on it together with the site location at which the collection device is intended to be used and the name of the person preparing the device. It is advisable for a second person to check that the preparation of the sampling device and the data on the evidence bag and the sample preparation logbook has been carried out correctly. After the checks have been satisfactorily completed and recorded the tamper-evident delivery bag must then be sealed. The expiry date is to be decided by the preparing laboratory but should be no greater than 6 months from the date of preparation.

3.1.8 Prior to acceptance of the tamper evident delivery bag the sampler or courier should check that there is no evidence of tampering. Details of fitting the filter on site are covered by part 1 of this SOP.

3.2 Provision of a Pall Envirochek™ HV Filter Module And Filter Housing

3.2.1 The C*ryptosporidium* Analytical Laboratory shall supply the Pall Life Sciences Envirochek™ HV filter module and stainless steel filter housing for sampling at each of the designated water treatment sites. The filter module must be supplied with a unique number and the housing 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 at which it is intended to be used. This label will contain the site identification code, which will match that on the outside of the sample cabinet on site.

3.2.2 Prior to use the stainless steel filter case must be cleaned by normal domestic washing procedures in accordance with the manufacturer's instructions (see Appendix A).

3.2.3 A filter module with unique identification number must be fitted into the filter housing by the laboratory in accordance with the manufacturer's instructions. The filter housing must then be air pressure tested to 500 kPa (5 bar) (by connecting to a laboratory pump) to ensure that there are no leaks. The air supply hose outlet must be fitted with a Swagelok^{TM} Quick-Connect body (B-QC6-B-4MS fitted with a K5 (blue) key). The hose line must be connected to the outer side of the filter housing. A pressure of 500 kPa (5 bar) must be maintained with no discernable drop for not less than 15 minutes in order for the test to be deemed satisfactory. The filter housing should then be depressurised before it is removed from the pressure test rig. **Should the pressure test fail, the 'O' rings which seal the filter in the housing should be removed and replaced with new 'O' rings. Also check alignment of capsule, and adjust if necessary.**

NOTE: SILICONE GREASE MUST NOT BE USED ON THE 'O' RINGS IN THE STAINLESS STEEL HOUSING.

3.2.4 After carrying out a successful pressure test, an approved security tag (green) must be threaded through holes in the housing and tightened to ensure that the housing cannot be opened without breaking the tag.

3.2.5 The identity numbers of the filter module, stainless steel filter case base and top, plastic security tag fitted to the housing and the location identity where the sample is to be taken, must be recorded in the appropriate boxes on an approved evidence bag.

3.2.6 A log must be kept of the identity numbers of the case with a record of the pressure test.

3.2.7 The assembled collection device must then be placed in a tamper-evident delivery bag together with the evidence bag to be used for the return journey and the white cabinet tag. The tamper-evident delivery bag as well as the evidence bag should be checked to ensure they are fit for use. The delivery bag must have the "use by" date clearly marked on it together with the site location at which the collection device is intended to be used and the name of the person preparing the device. It is advisable for a second person to check that the preparation of the sampling device and the data on the evidence bag and the sample preparation logbook has been carried out correctly. After the checks have been satisfactorily completed and recorded the tamper-evident delivery bag must then be sealed. The expiry date is to be decided by the preparing laboratory but should be no greater than 6 months from the date of preparation.

3.2.8 Prior to acceptance of the tamper evident delivery bag the sampler or courier should check that there is no evidence of tampering. Details of fitting the Pall Life Sciences Envirochek^{TM} HV filter module on site are covered by part 1 of this SOP.

3.3 Continuity of Chain of Custody

3.3.1 It is a requirement of this SOP that any results should be admissible to a Court of Law in the event of a prosecution by DWI. It is thus imperative that a chain of evidence can be demonstrated and that the procedures used are scientifically defensible such that the result of the analysis would stand up in a Court of Law.

3.3.2 The use of approved filter housing transport bags and evidence bags will ensure chain of custody is maintained. The evidence bags will be subject to audit by the Inspectorate.

3.3.3 Whenever the custody of the evidence bag is transferred on to another person, whether employed by the water company, or by a designated courier company, or by an approved laboratory, the transferee must ensure that the continuity section on the evidence bag is completed by the recipient at the time of transfer.

3.3.4 On delivery to the approved laboratory the person delivering must ensure that the continuity section on the evidence bag is completed by the recipient at the time of transfer. All records made on paper or the evidence bag must be clear and legible in indelible ink using a ball-point pen.

3.3.5 When one or more delivery bags are delivered by courier (whether employed by the water company or analytical contractor) to either, a designated sample site, or secure location in a container secured with padlocks that have a unique identification under secure control of the water company or the approved laboratory, then a record of receipt is only required for the acceptance or delivery of the container irrespective of the number of delivery bags it contains. The record of receipt can be a logbook kept in the secure location where the containers are kept. The laboratory must maintain a record of the number and identification of all the delivery bags in each locked container. The same procedure is required for the return of the sample in the evidence bag from the sample site or collection point to the laboratory in the container (See Section 4: Receipt of Samples in Part 2 of the SOP).

3.3.6 A designated courier service if using transport boxes may have an electronic logging system in place to allow the journey of the transport container to be followed at all times. If this is the case, the requirement for paper receipts at all stages of the transportation of the samples can be waived. However, an electronic logging system does not obviate the requirement for completing the continuity section of the evidence bag at each transfer point during the transportation of the sample if it is not in a sealed tamper evident transport box to the laboratory. However, whilst transported in the locked container all handovers of the locked container must be registered by written record of receipt or by an electronic logging system that allows

a full audit trail to be undertaken. Keys to the locked container must be under the secure control of either the water company, or the approved laboratory.

3.3.7 Where delivery bags are delivered to either a designated sample site or secure location in the approved delivery bags but not in a locked container, then each delivery bag must be recorded in a logbook at the laboratory and a record of receipt obtained. Delivery to a secure store will require a logbook to be maintained and a record of each individual evidence bag recorded. At each stage of onward transmission when the delivery bags are handed over then the continuity section on each evidence bag must be completed at the time of transfer from one person to another to maintain the chain of custody.

3.3.8 At no time must the sample be left unattended whilst not in secure storage. For this purpose a closed and securely locked van may constitute secure storage.

3.4 Logbook For Filter Housings

3.4.1 A log book must be kept of all housings and fittings used by the laboratory recording the allocation of the housing (eg Company and/or site), and any reallocation or disposal. A complete history must be maintained of each sampling device from delivery to disposal.

3.5 Security Tags

3.5.1 All security tags used in the monitoring of *Cryptosporidium* oocysts under the Regulations must be of standard design and obtained from DWI or DWI approved supplier(s).

3.5.2 Security tags used on the sampling line and fittings will be made of plastic, blue in colour and each with a unique number. These security tags will be controlled and fitted only by DWI Inspectors or DWI approved agent(s). If a tag is broken to facilitate repair or modification of the sample line or fittings then a white tag (see Part 1 SOP section 3.2.4) will be fitted after completion of the repair or modification and the numbers of the broken blue tag and the replacement white tag fitted shall be recorded in the relevant log book together with the reason for breaking the tag.

3.5.3 Security tags used on the filter units must be made of plastic, green in colour, each with a unique number prefixed with "FU". These tags will be made available only to DWI approved laboratories preparing the collection devices for use under the Regulations. The approved laboratories will be responsible for the secure storage of these tags. These security tags must be strictly controlled and their use will be subject to audit by DWI Inspectors or DWI approved agent(s).

3.5.4 Security tags used on the sampling cabinet must be made of plastic, white in colour and each with a unique number prefixed with "MC". These tags will be made available to water companies who will be responsible for their secure storage. The

use of these security tags must be strictly controlled and their use will be subject to audit by DWI Inspectors or DWI approved agent(s).

3.5.5 Security tags used by DWI Inspectors or a DWI approved agent to reseal equipment after auditing will be made of plastic, blue in colour, each with a unique number. These security tags will be controlled and fitted only by DWI Inspectors or DWI approved agent(s).

3.6 Spare Delivery Bags

3.6.1 Spare delivery bags must not be issued to a Company. If a delivery bag is found to be damaged the bag and contents should be returned to the laboratory for checking and if necessary cleaning prior to being reissued in a new delivery bag.

3.7 Evidence Bags

3.7.1 The filter unit containing the sample once removed from the sampling cabinet must be enclosed within a plastic evidence bag supplied with a special seal that will show clearly if the seal has been tampered with. The bags must have a continuous design close to the edges to make evident any attempts to cut open a bag edge or base to access the filter unit and then to reseal the bag. The bag will be printed to allow the entry of data required during the sampling and transportation to be entered in clear legible handwriting in indelible ink by a ballpoint pen. These bags must be obtained from the DWI or a DWI approved supplier. Their use must be strictly controlled and unused bags must be stored under secure conditions. Companies are responsible for the control of the evidence bags. The use of these bags may be audited by DWI Inspectors or DWI approved agent(s). A typical layout of an evidence bag is shown in Appendix A. Spare evidence bags must never be issued in order to maintain the chain of custody. Only one evidence bag must be used for each regulatory sample. The reason for this is that the number of the evidence bag is used as an identifier for a sample on the Cryptosporidium database. There must be a clear audit trail from the laboratory, out to site and back to the laboratory and all the information is written in indelible ball point pen.

3.7.2 If the evidence bag is damaged prior to being fitted in the sampling unit the delivery bag and contents are returned to the laboratory and either replaced by a spare delivery bag in the sampling unit or a replacement transport bag is obtained from the intermediate store or laboratory.

3.7.3 If the evidence bag is damaged during fitting of the filter module in the sampling unit the delivery bag and contents are returned to the laboratory and either replaced by a spare delivery bag in the sampling unit or a replacement transport bag is obtained from the intermediate store or laboratory.

3.7.4 If the evidence bag is damaged after the filter module has been changed the reason for the damage is written in the comments section on the evidence bag and signed

dated and timed. The filter should be put inside the evidence bag and delivered to the laboratory as per a regulatory sample. If a sample box is used with the appropriate level of security (see SOP Part 2, section 3.3) then the standard procedure can be used. If there is no secure sample box then at each handover a record must be made in the comments section noting the state of the bag and if the green tag is intact and the filter module numbers agree. The Company may use a sealable bag to enclose the evidence bag and filter module providing the comments section detailing the reason for its use is completed and the sealable bag would be kept with the evidence bag for as long as the regulations require.

4 RECEIPT OF SAMPLES

4.1 The transportation of samples under the Regulations must either be carried out by the water company, or a designated courier or a representative of the approved laboratory. The transportation of the sample from end of sampling to receipt by the DWI approved laboratory must be carried out as soon as reasonably practical to ensure that results of the analysis are available in accordance with the Regulations. (See Section 7 of Part 1 'Sampling and Transportation of Samples' of the Standard Operating Procedure for details). On arrival at the laboratory the transport container that is used must be received by a designated person (one of the authorised laboratory staff) who must check that the secure transport container and/or evidence bag shows no sign of having been tampered with and must sign for receipt of the sample, giving name, date and time in a continuity block on the evidence bag. If the evidence bag shows signs of having been tampered with, the details must be entered in the comments box of the evidence bag, and the matter reported to the designated person within the water company who must inform the DWI designated contact of the matter within the agreed time scale.

4.2 The sample should be logged into the laboratory and the relevant section on the evidence bag completed clearly and legibly in indelible ball-point pen.

4.3 There is no need to open the evidence bag until analysis is about to be started unless there is evidence that the bag has been tampered with or there are adverse comments written on the evidence bag which require immediate action. The designated person must then cut open the base of the evidence bag below the comments, ensuring that the information on the bag is not defaced and remove the collection device from the bag.

4.4 As soon as the bag is opened the designated person must then check that the security tag (green) attached to the filter housing is secure and its identity number corresponds to that written on the evidence bag. The person must then tick the adjacent box on the bag to signify that the security tag details are correct and the tag is intact. If the tag number is incorrect or if the tag was not secure on receipt, the box must not be ticked but the facts must be entered in the comments box on the evidence bag and the matter reported to the designated person within the water company who must then inform the DWI designated contact of the matter within the agreed time scale.

4.5 The designated person must then check that the identity numbers on the filter housing base and top match those given on the evidence bag and if so must tick the appropriate boxes. If the identity numbers do not agree, the boxes must not be ticked but the facts must be entered in the comments box on the evidence bag, and the matter reported to the designated person within the water company who must inform the DWI designated contact of the matter within the agreed time scale.

4.6 If the “express analysis” box on the evidence bag is ticked the designated person must ensure that the sample is put on the fast track for analysis. It is recommended that where the recorded headloss is significantly higher than normal and/or greater than 400 kPa (4 Bar) the sample should be placed on the fast track for analysis. However, if the Company can clearly show that the high headloss is solely due to phosphate dosing then there is no requirement for express analysis. All results must be reported within the timetable set out in the Regulations.

4.7 Unless analysis is to start on the day of receipt, the evidence bag must be placed in a secure designated laboratory refrigerator (+2 ^{o}C to +8 ^{o}C) until examination can commence. If the evidence bag has been opened, the filter unit must then be replaced in the evidence bag which must then be resealed using a security label and placed in a secure designated laboratory refrigerator (+2 ^{o}C to +8 ^{o}C) until examination can commence.

5 SAMPLE PREPARATION

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

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

5.3 Documentation

5.3.1 Record each stage of the process in the bound and sequentially numbered pages of an uniquely identified analysis workbook, including contemporaneous notes as necessary. No lines or pages in this analysis workbook must be left blank. At the completion of analysis a horizontal line must be drawn across the page immediately below the last entry and a diagonal line must be used to cross through any unused portion of a page. Each page used must be signed with date and time. Uniquely numbered whole method or section pro-forma's may be used providing they are in bound form.

5.3.2 Chequerboard Of Permutations Of Different Reagents

CHEQUERBOARD OF PERMUTATIONS						
	Dynal IMS	**Cellab FITC**	**Isolate IMS**	**Microgen FITC**	**Easyseed**	**ChemScan**
Dynal IMS		Y	N	Y	Y	Y
Cellab FITC	Y		Y		Y	Y
Isolate IMS	N	Y		N	Y	N
Microgen FITC	Y		N		Y	N
Easyseed	Y	Y	Y	Y		Y
ChemScan	Y	Y	N	N	Y	

It is important that analysts record which test product they use for each sample for the analysis of the *Cryptosporidium* oocysts in the relevant workbook(s).

5.4 Storage of Samples during Analysis

5.4.1 Where indicated in the following method a sample may be stored under secure conditions. Such conditions must include the placing of security seals over the sample or sample container such that the analyst will be confident that on his/her return to the sample it has not been tampered with. A note of the sealing of a sample and examination of the seal after withdrawal from the secure location together with dates and times must be entered in the analysis workbook. Security seals are not necessary if the analyst has sole access to the secure location. Similar procedures must be undertaken if the sample is left unattended by the analyst responsible for the sample unless the sample has been formally handed over to another analyst.

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

5.5.1 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 designated sample location for which it is to be used. Alternatively, if the wash station is to be used for samples from a number of 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.

5.5.2 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.

5.5.3 Take the sample in its evidence bag from the refrigerator, check the security label used to seal the opened evidence bag, note the state of security label in the analysis workbook (i.e. intact or damaged); if damaged an internal laboratory investigation must be carried out into how the damage occurred. Remove the filter unit from the bag, break the green plastic security tag and undo the filter housing with the appropriate tools. Check that the identity number on the filter agrees with that given on the evidence bag and if so tick the appropriate box on the evidence bag. If the identity number on the filter does not agree with that on the evidence bag, report the discrepancy to the designated person within the water company who must inform the designated DWI contact of the matter within the agreed timescale. If the filter has been stored in the refrigerator, allow the filter to reach

room temperature before elution commences. Tear off one of the attached identity labels from the evidence bag and stick the label in the analysis workbook on the page where the analysis results are to be recorded.

5.5.4 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.

5.5.5 Remove the filter module bolt using the Allen key provided 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.

5.5.6 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.

5.5.7 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.

5.5.8 Compress the filter and detach the assembled concentrator tube from the base of the assembled elution tube and lower it to the point where the steel tube is just above the level of the liquid, taking care to ensure that the seal on the plunger is not misplaced. Remove the remaining liquid from the elution tube by moving the plunger five times up and down and locking the wash station plunger in place (NB: The foam filter may sometimes over expand and make locking difficult). Rinse the outside of the steel tube with 2-5 ml PBST from a wash bottle. Place the bung provided inside the end of the steel tube to prevent loss of sample.

5.5.9 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, 30 cm 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. For requirements of an electrically pumped vacuum system see paragraph 5.5.16.**

5.5.10 Allow the sample level to slowly drain until approximately 20 ml 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 50 ml 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** (this step in the procedure has been confirmed by the manufacturer and applies to both existing and new stock)**.**

5.5.11 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.

5.5.12 Repeat the above wash steps see paragraph 5.5.7, 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 50 ml 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 20 ml;

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

(iv) collect the concentrate in the same 50 ml 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.

5.5.13 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 5 ml 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 of section 5.5.10. The washing process should be repeated using a further 5 ml of PBST. This may result in more than 50 ml of eluate and the washings requiring centrifugation. In this case, the combined eluate should be divided equally into two or more 50 ml centrifuge tubes and each tube processed in accordance with paragraph 5.7.3 page 24.

5.5.14 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 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 to +8 ^{o}C until ready for centrifugation

5.5.15 Centrifuge the 50 ml centrifuge tube containing the filter eluate at 1100g[4], for 15 minutes. The relative centrifugal field (RCF) of the centrifuge must be optimised

[4] 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.

for maximum recovery of oocysts. If an alternative centrifugal field (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 2-5 ml 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 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 (r av). 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%.

See overpage for examples of calculations.

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 r_{av} = 95 mm running at a speed of 3220 rpm.

$$RCF = 1.118 \times 95 \times (3.3220)^2 = 1100 \text{ g}$$

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

$$945.7 \text{ v } (1100/85) = 3402 \text{ rpm}$$

5.5.16 **Requirements of an Electrically Pumped Vacuum System**

5.5.16.1 As an alternative to the manual operated vacuum system specified in paragraph 5.5.9 of the Protocol a laboratory may use an electrically pumped vacuum system provided it meets all of the following requirements:

(a) the system is fitted with a suitable vacuum pump such that it cannot exceed 40 kPa (30 cm of mercury) and master vacuum chamber, and is used solely for *Cryptosporidium* analysis using the approved regulatory method, and is not connected to any other system or equipment;

(b) a dedicated vacuum line for each concentrator, fitted with:

(i) an isolating valve;

(ii) a fine control valve, to allow minimal vacuum to be applied to each sample (as required by the Protocol); and

(iii) a vacuum gauge to ensure excessive vacuum is not applied;

(c) a separate catch pot (or waste pot) is in place for each concentrator, that will retain all the eluate even if the membrane fails and all the liquid is quickly transferred to the pot, to permit recovery of the sample. The pot must be fitted with an effective trap arrangement to prevent liquid being drawn down the vacuum line. When deciding the size of pot and design of trap full allowance must be made for the propensity of the eluate to froth;

(d) an operating procedure needs to be produced in the event of a failure or lifting of the membrane for the vacuum system. This must include a cleaning regime for thoroughly cleaning the catch pot and tubing between samples and a check procedure to determine that no oocysts are left in the catchpot. It must also include the procedure to be followed to achieve maximum recovery of oocysts for re-concentration following a membrane failure; and

(e) provisional approval of the design, specification and operating procedure by the Drinking Water Inspectorate.

5.5.16.2 Details of proposed systems should be sent to the Inspectorate for approval and should not be used for regulatory analysis until written approval has been obtained. The system and its use will be subject to audit, after which the approval may be withdrawn if the system is not considered satisfactory. Alternatively, modifications may be required.

5.6 Elution and Primary Concentration for Pall Envirochek™ HV Filter

5.6.1 Take the sample in its evidence bag from the refrigerator, check the security label used to seal the opened evidence bag, note the state of the security label in the analysis workbook (i.e. intact or damaged); if damaged an internal laboratory investigation must be carried out into how the damage occurred. Tear off one of the attached identity labels from the evidence bag and stick the label in the analysis workbook on the page where the analysis results are to be recorded. Remove the filter unit from the bag, break the green plastic security tag and undo the filter housing.

5.6.2 Remove the sample filter from the filter housing and place the blue end caps on the filter. Check that the identity number on the filter agrees with that given on the evidence bag and if so tick the appropriate box on the evidence bag. If the identity number on the filter does not agree with that on the evidence bag, report the discrepancy to the designated person within the water company who must inform the designated DWI contact of the matter within the agreed timescale. Note the appearance of the filter in the analysis workbook, e.g. 'clean', heavily loaded' or 'stained brown'. If the filter has been stored in the refrigerator, allow the filter to reach room temperature before elution commences.

5.6.3 Heat the pre-treatment buffer, the reagent water for rinsing and the elution buffer to 37 ± 1 °C.

5.6.4 Support the filter vertically with the white pressure release valve uppermost and remove the blue vinyl end caps. Allow the residual water in the filter to drain out. Tilt the filter gently once all the water has drained to remove any excess water. Replace the blue end cap on the filter outlet. Pour warmed pre-treatment buffer into the module to a depth of approximately 13 mm (0.5 inches) above the white pleated filter element. Replace the blue vinyl inlet cap. Place the filter into the wrist shaker with the white pressure release valve in the 12 o'clock position and tighten the clamp securely.

5.6.5 Shake the filter at 900 ± 25 cycles per minute (cpm) for 5 minutes.

5.6.6 Remove the filter module from the shaker and secure vertically with the white pressure release valve uppermost. Remove the end caps and allow the pre-treatment buffer to drain completely from the module.

5.6.7 Replace the lower end cap and pour warmed reagent water into the filter module to approximately 13 mm (0.5 inches) above the white pleated filter element. Replace the upper end cap and gently rotate the filter manually for 30 seconds to rinse. Secure the filter vertically with the white pressure release valve uppermost and remove the end caps. Allow the reagent water to drain completely from the module.

5.6.8 Replace the lower end cap and pour warmed elution buffer into the filter module to a depth of approximately 13 mm (0.5 inches) above the white pleated filter

element. Replace the upper end cap and secure the filter module on the wrist shaker, making sure that the white pressure release valve is facing towards you and in the 12 o'clock vertical position. Shake the filter module at 900 ± 25 cpm for 5 minutes. Remove the filter module from the shaker and decant the elution buffer from the inlet end into three 50 ml centrifuge tubes. Balance the centrifuge tubes if necessary with fresh elution buffer. Centrifuge the 50 ml tubes containing the filter eluate at 1100g or another suitable speed for 15 minutes. Allow the centrifuge to coast to a stop without braking. 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. The speed of the centrifuge must be optimised for maximum recovery of oocysts.

5.6.9 Add a further volume of warm elution buffer to the same filter module and repeat the wash procedure described in 5.6.8. On this occasion, the white pressure release valve must be in the 4 o'clock position. Shake the filter module at 900 ± 25 cpm for 5 minutes. Rotate the filter module until the white pressure release valve is in the 8 o'clock position. Shake the filter module at 900 ± 25 cpm for a further 5 minutes. Decant the elution buffer into the three 50 ml centrifuge and centrifuge as described in 5.6.8. Remove the supernatant after centrifugation.

5.6.10 Vortex the contents of each centrifuge tube to resuspend any particulate material. Combine the contents of the three tubes into a single tube. Wash the two remaining tubes by adding 2 ml of reagent water and vortex for a further 10 seconds. Add the washings to the single tube and make up the volume to 50 ml with reagent water. Centrifuge at 1100 g for 15 minutes[5]. Allow the centrifuge to coast to a stop without braking. Remove the supernatant as described in 5.6.8. 'Vortex the content of each centrifuge tube to resuspend any particulate material. If large volume centrifugation is used, carefully remove the supernatant as described in 5.6.8 leaving approximately 20 ml above the pellet. Re-suspend the pellet by vortexing for 10 seconds and transfer to a 50 ml centrifuge tube. Add 5 - 10 ml of reagent water and vortex for a further 10 seconds. Add all the washings to the single tube and make the volume up to 50 ml with reagent water. Centrifuge at 1,100 x g for 15 minutes. Allow the centrifuge to coast to a stop without braking. Remove the supernatant as described in 5.6.8.

5.6.11. Large volume centrifugation using 250 ml conical centrifuge tubes may be used providing that it can be demonstrated that the recovery of oocysts is not significantly less than that using small volume centrifugation. The washings from 5.6.8 and 5.6.9 are combined into one tube. Centrifuge at 1100 g for 15 minutes and allow the centrifuge to coast to a stop without braking. Remove the supernatant as described in 5.6.8.

[5] See footnote 3 pages 22 and 23 on the relationship of rpm to RCF.

NB: Samples may be stored at this stage under secure conditions in a refrigerator at a temperature of between +2 °C to +8 °C until ready for centrifugation paragraph 5.7

5.7 Secondary Concentration (for Dynal IMS and Cellab FITC) also (for Dynal IMS and Microgen Bio-Products FITC).

NB Please note the following Dynal IMS and Cellab FITC also Dynal IMS and Microgen Bio-Products FITC can be used with the IDEXX filter at present the Envirochek filter is only approved for use with Dynal IMS and Cellab FITC.

5.7.1 If the pellet volume is less than or equal to 0.5 ml, record the pellet volume accordingly and the date and time that concentration was completed in the analysis workbook (see section 5.3.1 above). Add reagent water to the centrifuge tube to bring the total volume to 7.5-9 ml. Cap the tube and vortex for 10 to 15 seconds to re-suspend the pellet. Proceed to section 5.8 Dynal IMS Procedure.

NB: Samples may be stored at this stage under secure conditions in a refrigerator at a temperature of between +2 °C to +8 °C until ready for stage 5.8. If a number of samples were generated at paragraph 5.7.3 (and also 5.5.13) 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.

5.7.2 If the pellet volume exceeds 0.5 ml then make up to 10 ml 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 15 ml conical centrifuge tube. Rinse out the 50 ml centrifuge tube with a further 2 ml reagent water and transfer into the 15 ml conical centrifuge tube. Repeat this wash step again if necessary. Centrifuge the 15 ml conical centrifuge tube at 1100 g, or another suitable speed for 15 minutes. Allow the centrifuge to coast to a stop without braking.

5.7.3 When more than one 50 ml tube is required for very turbid samples (see paragraph 5.5.13) then full records must be kept for each 50 ml tube and their contents subject to separate preparation for the IMS procedure as in paragraph 5.7.2. The sample pellet volume is the sum of the sample pellet volumes for each tube. Each 15 ml centrifuge tube then used is treated as a sub-sample of the whole sample.

5.7.4 Record the pellet volume (volume of solids) and the date and time that concentration was completed in the analysis workbook (see section 5.3.1 above). 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.

5.7.5 Add reagent water to the centrifuge tube to bring the total volume to 10 ml. Cap and vortex the tube for 10 to 15 seconds to re-suspend the pellet. Then split the sample between a number of 15 ml centrifuge tubes to give no more than 0.5 ml deposit in any one tube, i.e. if the pellet volume was 0.7 ml use two centrifuge tubes and if 1.2 ml use three tubes. Make up the volume in each tube to 9 ml and proceed to section 5.8 (Dynal IMS Procedure) treating each centrifuge tube as a sub-sample.

NB: Samples may be stored at this stage under secure conditions in a refrigerator at a temperature of between +2 ^{o}C to +8 ^{o}C until ready for stage 5.8. If a number of samples were generated at paragraph 5.7.3 (and also 5.5.13) 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.

NB: If the laboratory is using ChemScan ®RDI then go to SECTION 5.11

5.8 Dynal IMS Procedure (for Dynal IMS and Cellab FITC) also (for Dynal IMS and Microgen Bio-Products FITC)[6]

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

5.8.1 Preparation of reagents

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

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

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

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

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6 Dynal Biotech Ltd have produced a CD-ROM video of the whole IMS procedure and is available to all users of the kits details in the new revision of the package insert for the Dynabeads® anti-*Cryptosporidium.*

5.8.1.2 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 1 ml of 1 x SL-buffer-A required, take 100 µl of "10 x SL™-buffer-A" and make up to 1 ml with reagent water. Retain the 1 x SL™-buffer-A in a labelled container for use later in the procedure.

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

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

5.8.1.5 The Dynal L10 tube containing the mixed SL-buffer A and B is then ready for use in oocyst capture in 5.8.2.1.

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

5.8.2 Oocyst capture

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

5.8.2.1 Transfer the water sample concentrate (from section 5.7.1 or section 5.7.5) to the Dynal L10 tube containing the mixed SL-buffer prepared above and use a further 1 ml 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.

5.8.2.2 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.

5.8.2.3 Add 100 µl of the resuspended beads to the Dynal L10 tube (see paragraph 5.8.2.1) 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.

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

5.8.2.5 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.

5.8.2.6 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.

5.8.2.7 Gently rock the Dynal L10 tube by hand end-to-end through approximately 90°, 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[7]. 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.

5.8.2.8 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.

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

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

5.8.2.11 Place the microcentrifuge tube in the second magnetic particle concentrator (Dynal 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.**

5.8.2.12 Without removing the microcentrifuge tube from Dynal MPC-M or Dynal MPC-S gently rock/roll the tube through 180° by hand,. Continue for 1 minute with one

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

gentle 180° roll and rock per second. The magnet is rocked 180 degrees in one second in one direction and then rolled back in another second[8]. At the end of this step, the beads and oocysts should produce a well-formed brown dot on the back of the tube.

5.8.2.13 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° 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.

5.8.3 Dissociation of beads/oocysts complex

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

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

5.8.3.3 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.

5.8.3.4 Whilst the tube is standing remove the required number of 9 mm 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 adhere the detachable sample identification label from the evidence bag. 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.3.3 and B.4.3).

5.8.3.5 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.

5.8.3.6 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.

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

5.8.3.7 Add 5 µl of 1.0 M sodium hydroxide (NaOH) to the slide sample well. See paragraph 6.2.1 of 'Guidelines for Calibration in Laboratories.

5.8.3.8 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 +2 ^{o}C to +8 ^{o}C in a dry box until ready for Section 6, or incubated at a temperature not exceeding 42 ^{o}C until the evaporation step is complete. Samples are stained and examined according to the Standing Operating Protocol Part 2, 'Laboratory and Analytical Procedures' Section 6.

5.9 Secondary Concentration (TCS Biosciences Isolate Crypto IMS and Cellabs FITC)

5.9.1 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 Isolate Crypto immunomagnetic bead separation (IMS).

5.9.2 From paragraph 5.6.10 or 5.6.11 (Envirochek filter) or paragraph 5.5.15 (Idexx filter) if the pellet volume is less than or equal to 2.0 ml, record the pellet volume accordingly and the date and time that concentration was completed in the analysis workbook (see section 5.3.1 above). Add reagent water to the centrifuge tube to bring the total volume to 9.0 ml. Cap the tube and vortex for 10 to 15 seconds to re-suspend the pellet. Proceed to section 5.10 (Isolate Crypto IMS Procedure).

5.9.3 If the pellet volume exceeds 2.0 ml then add 10 ml oocyst-free reagent water to the centrifuge tube by pipette. Using the pipette tip re-suspend the pellet. Transfer the re-suspended deposit to a 15 ml conical centrifuge tube. Rinse out the 50 ml centrifuge tube with a further 2 ml reagent water and pipette into the 15 ml conical centrifuge tube. Repeat this wash step again if necessary. Centrifuge the 15 ml conical centrifuge tube at 1100G, or another suitable speed, for 15 minutes. Allow the centrifuge to coast to a stop without breaking.

NB: If a number of sub-samples were generated at paragraph 5.9.3 the oocysts found in the whole deposit is the sum of the number of oocysts in all the sub-samples.

5.9.4 Record the pellet volume (volume of solids) and the date and time that the concentration was completed in the analysis workbook (see section 5.3.1 above). 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. The aspiration of the supernatant is a critical stage which could incur

significant losses of oocysts and great care must be taken not to disturb the pellet. Do not let the pipette tip touch or in any way disturb the pellet.

5.9.5 Add reagent water to the centrifuge tube to bring the total volume to 10 ml. Cap and vortex the tube for 10 to 15 seconds to re-suspend the pellet. Then split the sample between a number of 15 ml centrifuge tubes to give no more than 2 ml deposit in any one tube, i.e. if the pellet volume was 4 ml use two centrifuge tubes and if 5 ml use three tubes. Make up the volume in each tube to 9 ml and proceed to section 5.10 (Isolate Crypto IMS Procedure) treating the contents of each centrifuge tube as a sub-sample.

NB: Samples may be stored at this stage under secure conditions in a refrigerator at a temperature of between +2 °C to +8 °C until ready for stage 5.10.

5.10 Isolate Crypto IMS Procedure

5.10.1 Preparation of reagents

5.10.1.1 For each 10 ml sample, sub-sample or control on which the Isolate Crypto procedure is performed, the following quantities of buffer will be required:

1 ml of 1 x Isolate Crypto Reagent A (clear, colourless solution)
[but see 5.10.2.6/7]

1 ml of 10 x Isolate Crypto Reagent A (clear, colourless solution)

1 ml of 10 x Isolate Crypto Reagent B (magenta coloured solution)

5.10.1.2 Prepare a 1:10 dilution of Reagent A from the '10 x IMS Reagent A' (clear colourless solution) supplied using oocyst-free reagent water as the diluent. For every 1 ml of diluted Reagent A required, take 100 µl of 'IMS Reagent A' and make up to 1 ml with reagent water. Retain the 1 x Reagent A in a labelled container for use later in the procedure.

5.10.1.3 To a Leighton tube (125 mm x 16 mm flat sided tube) add 1 ml of Reagent A and 1 ml of Reagent B.

NB: Reagent A may contain crystals, particularly after removing from the refrigerator. Ensure that the crystals are completely dissolved by gently warming and mixing the reagent before use.

5.10.1.4 The Leighton tube containing the mixed Reagents A and B is now ready for use in the oocyst capture 5.10.2.

5.10.2 Oocyst capture

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

5.10.2.1 Transfer the water sample concentrate (from section 5.9.2 or 5.9.5) to the Leighton tube containing the mixed Reagent buffers prepared above and use a further 1 ml oocyst-free reagent water to rinse out the centrifuge tube. Label the Leighton tube with the sample number.

5.10.2.2 Mix the Isolate Crypto IMS beads by gentle rotation of the bottle. Ensure that they are fully re-suspended by inverting the bottle and checking that there is no residual pellet at the bottom of the bottle. Add 100 µl of the bead suspension to the Leighton tube.

5.10.2.2 Insert the Leighton tube into an Incu-Clip (blue) with the flat side facing down (A). Slide the Incu-Clip and Leighton tube onto the Isolate Rotator disc until it clicks into position, locating the cap of the tube in the Isolate Rotator Hub (B) and rotate at 20 rpm for at least 1 hour at room temperature (C).

NB: As an alternative, the Dynal MX1 rotating mixer can be used to incubate the Leighton tube. After an hour at room temperature, transfer the Leighton tube from the mixer to a Magna-Clip (white) and proceed from 5.10.2.4 (E).

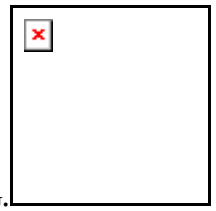
A. B. C.

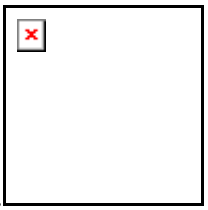
5.10.2.4 Carefully remove the Incu-Clip containing the Leighton tube from the Isolate Rotator. Remove the tube from the Incu-Clip (D) and place it into a Magna-Clip (white) with the flat side facing down against the magnets (E). Slide the Magna-Clip onto the Isolate Rotator disc until it clicks into position, locating the cap of the tube in the Isolate Rotator Hub (F). If the pellet volume was less than 0.5 ml, rotate on the rotating disc at 20 rpm for at least 5 minutes (G). Where the pellet volume was 0.5 ml or greater, rotate for at least 10 minutes (H) to ensure complete capture of the beads.

D. E. F.

5.10.2.5 Remove the Magna-Clip from the rotating disc. If other samples are being processed continue to rotate the disc. Carefully decant the Leighton tube keeping it in the Magna-Clip by pouring off with the flat side downwards. Providing that

the beads have not been disturbed during this process the supernatant can be discarded. Stand the Magna-Clip on the bench so that the Leighton tube in it is upright. With a 1 ml pipette, carefully remove any additional supernatant from the bottom of the tube taking care not to disturb the beads. Process any other samples following the above steps.

G. 

H. 

5.10.2.6 Remove the Leighton tube from the Magna-Clip and add 0.8 ml of Reagent A diluted 1:10 with reagent water. With a 1 ml pipette, rinse the bead pellet from the side of the tube. Pipette the reagent over the beads repeatedly to ensure **ALL** beads are rinsed from the tube.

5.10.2.7 Transfer the beads to a clean labelled microcentrifuge tube. Rinse the Leighton tube with a further 0.2 ml of diluted Reagent A and add this to the microcentrifuge tube.

5.10.2.8 Cap the microcentrifuge tube and place into the Micro-Clip (white). Slot the Micro-Clip onto the Isolate Rotator. If the pellet volume was less than 0.5 ml, rotate at 20 rpm for at least 2 minutes. Where the pellet volume was 0.5 ml or greater, rotate for at least 4 minutes to ensure complete capture of the beads.

5.10.2.9 Remove the Micro-Clip from the rotating disc. If other samples are being processed continue to rotate the disc. With the tube remaining in the Micro-Clip, immediately remove the supernatant from the tube and cap using a suitable pipette with or without a vacuum source. Take care not to disturb the beads attached to the wall of the tube adjacent to the magnet.

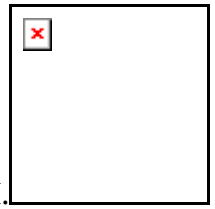
5.10.2.10 With samples containing a large amount of particulate material, it may be advantageous to wash the beads. Remove the tube from the Micro-Clip, add a further 1.0 ml of diluted Reagent A, cap the tube, gently mix the beads and repeat stages 5.10.2.8 and 5.10.2.9. Process any other tubes using the above procedure.

5.10.3 Dissociation of beads/oocyst complex

5.10.3.1 Remove the microfuge tube from the Micro-Clip, add 50 µl of 0.1 M hydrochloric acid (HCl) and vortex the tube thoroughly for 10 seconds.

5.10.3.2 Stand the tube at room temperature for 5 minutes and then vortex the tube again for 10 seconds.

5.10.3.3 Place the tube into the Dissociation Block. Allow the tube to rest horizontally (I) for at least 30 seconds for the beads to attach to the back of the tube.

I. 

5.10.3.4 Remove a 9 mm well slide from the box stored at room temperature and ensure that it is grease free. Then at the end of the slide adhere the detachable sample identification label from the evidence bag. Use slides which are approved from use with the IMS kit (see Appendix B.4.8).

5.10.3.5 Add 5 µl of 1.0 M sodium hydroxide (NaOH) directly to the sample well of the slide. Carefully return the Dissociation Block to the upright position and transfer the supernatant from the tube to the well of the slide without disturbing the beads from the back of the tube. Gently mix the sample with the NaOH using the transfer pipette.

5.10.3.6 Place the slide in an incubator at a temperature not exceeding 42 ^{o}C to begin the drying process or alternatively retain the slide on the bench until the second dissociation is complete and has been added to the slide.

5.10.3.7 Perform a second acid dissociation to ensure complete release of oocysts by repeating steps 5.10.3.1 to 5.10.3.5. Carefully remove the slide from the incubator and add the NaOH and the supernatant to the same well. Alternatively, add the second dissociation to the slide.

5.10.3.8 Place the slide in an incubator not exceeding 42 ^{o} C until dry.

NB: Once the evaporation step is complete and the material on the slide is fixed, the sample may be stored in a secure refrigerator at +2 ^{o}C to +8 ^{o} C in a dry box until ready for section 6. 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.

Providing both 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 up to 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 – 6 µm.

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

6.2.6 For each negative control, pipette 50 µl 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 Inspectorate.

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 may 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 the following validations which are now defunct. Analyst validations, site validations and weekly validations. 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 X 200 and will check to ensure that the oocysts are typical of the genus *Cryptosporidium* and are between 4-6 µm in size and have a maximum of 4 nuclei no larger than 1 µm. (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 ^{o}C until the evaporation step is complete (see section 5.8.3.8 and 5.10.3.6), keep the positive control slides separate until the evaporation stage has been completed (see section 6.1.3).

6.4.2 After evaporating to dryness, remove the slides from the incubator and apply 25-50 µl 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 +2 °C to +8 °C in a secure 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 50 µl of fluorescein-labelled Mab. Place the slides in a humid chamber and incubate at 37 °C for 60 to 90 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 0.2 µm 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 50 µl of 4',6-diamidino-2-phenylindole (DAPI) working solution (1/5000 dilution in phosphate buffered saline (PBS), prepared daily by adding 10 µl of 2 mg/ml DAPI stock solution to 50 ml 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 a drop 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 antifadents 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.7.1.

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 +2 ^{o}C to +8 ^{o}C and under secure conditions 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.

6.4.11 Biotech Frontiers Easystain Instructions for Use

6.4.11.1 The Fixing Buffer has been specially formulated to minimise background and fading. Use Fixing Buffer immediately after antibody incubation. If ice is available, keep the Fixing Buffer on ice whilst using. Alternatively, use directly from the fridge. Allow Fixing Buffer to stay on slide or membrane for 1 minute prior to washing off.

6.4.11.2 The Mounting Medium has been specially formulated for use with IgG1 antibodies. The use of alternative mounting mediums may result in fading of oocysts and cysts.

6.4.11.3 Correct use of the amounts and timings detailed below will produce the best results.

6.4.11.4 The optimized procedure for staining oocysts with both EasyStain™ and DAPI on a microscope well slide is detailed below.

i. Place the sample onto a well slide and allow to dry at a temperature of no greater than 50^{o}C (50^{o}C is optimal).

ii. Add 25 µl methanol to the well and allow to dry at room temperature.

iii. Add 50 µl EasyStain™ (Antibody Solution) to the well and incubate at room temperature for 15 minutes.

iv. Carefully tip the slide, long edge down to aid removal of antibody. Carefully remove the antibody. Ensure that you do not touch the surface of the well slide.

v. Slowly add 200 µl of cold Fixing Buffer to the well and allow to stand at room temperature for 1 minute. The Fixing Buffer should flow over the edges of the well slide. This will help to reduce background staining around the edges of the slide.

vi. Gently aspirate the excess Fixing Buffer from below the well. Whilst aspirating the Fixing Buffer, carefully tip the slide, long edge down to aid removal of the buffer.

vii. Add 50 µl of the working strength DAPI solution and leave for 2 minutes.

viii. Carefully tip the slide, long edge down to aid removal of the DAPI. Carefully remove the DAPI. Ensure that you do not touch the surface of the well slide with the Pasteur pipette.

ix. Add a drop of distilled water to the well and allow to stand for 1 minute.

x. Carefully tip the slide, long edge down to aid removal of the distilled water. Carefully remove the distilled water. Ensure that you do not touch the surface of the well slide.

xi. Add a drop of mounting medium to the well and apply a coverslip.

xii. Record the date and time that staining was completed. Slides may be stored in a refrigerator in the dark at 2^{o}C to 8^{o}C until ready for examination.

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 100-200 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 X 200 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 1 µm diameter.

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

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 (no more than 5), 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. Each object identified should then be examined carefully using the X100 immersion objective lens to determine size and the characteristic features of oocysts. 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.

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 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 4 µm or greater than 6 x 6 µm may be noted and included with the final report, but should <u>not</u> be included in the Regulatory count.

6.7.4 Engage the x 100 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.

6.7.5 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.

6.7.6 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 putative oocyst must conform to the following fluorescence criteria:

Bright apple green fluorescing, round or slightly ovoid objects which measure between 4 and 6 µm (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.

6.8.2 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 'pac-man'). 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.

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 can be confirmed by DIC.

6.11 Further Guidance Notes for the Identification of Environmental 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.

(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 microorganisms, 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 microorganisms.

(iv) It is not sufficient to examine a proportion of the putative oocysts present on a sample slide. A mixture of oocysts and bodies resembling oocysts can be present, therefore each object must be assessed and classified. This principle applies both to the initial examination of the slide and to its analysis by an approved external analyst.

(v) Objects with typical FITC fluorescence around their entire circumference and amorphous internal contents demonstrable by DIC, 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 1.5 µm 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

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

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 results of the internal confirmation do not agree with the initial result, the result of the most experienced analyst shall be the result reported to the water company under the Regulations.

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.

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 6.5. Where there is disagreement between the internal and external analysts, the Drinking Water Inspectorate 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 a sealed tamper-evident bag by courier or member of laboratory staff. The chain of evidence must be maintained during this transfer.

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 requirement in the Regulations are achieved.

6.15 Storage of Slides after Analysis

6.15.1 All slides must be stored in a secure (locked) refrigerator at +2 ^{o}C to +8 ^{o}C, which is designated solely for the purpose of storing *Cryptosporidium* slides, for at least three calendar months following completion of analysis. All slides containing samples that exceed the prescribed concentration for *Cryptosporidium* oocysts under these Regulations must be stored under said secure conditions until written authorisation is given by the Chief Inspector of the Drinking Water Inspectorate for their disposal.

7 ChemScan®RDI: VARIATION OF METHOD OF IMS AND EXAMINATION (staining, detection and enumeration)

7.1 Method Variation for ChemScan®RDI for Detection of Cryptosporidium Oocysts

7.1.1 Principle

7.1.1.1 A modified Immuno Magnetic Separation (IMS) procedure is employed to remove debris prior to oocyst capture on a slide. Each slide is then incubated with a fluorescently labelled monoclonal antibody, specific for Cryptosporidium. Labelled oocysts are detected by a solid phase cytometer, the ChemScanRDI, and subsequently confirmed by microscopic examination of the slide.

7.1.2 Preparation of Water Concentrates

7.1.2.1 **Samples should be prepared according to the SOP up to and including section 5.7.** The samples are then concentrated using a modified IMS. This section of this procedure basically follows the standard SOP, with minor differences, which are highlighted in the text **in red**.

7.1.3 IMS concentration procedure

7.1.3.1 Preparation of reagents

7.1.3.1.1 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 1 ml of 1 x SL-buffer-A required, take 100 µl of "10 x SL™-buffer-A" and make up to 1 ml with reagent water. For each 10 ml sample, sub-sample or control on which the Dynal IMS procedure is performed, the following quantities of buffer will be required: **3 ml** of 1 x SL Buffer A, 1 ml "10 x SL™ Buffer A", and 1 ml "10 x SL™ Buffer B".

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

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

7.1.4 Oocyst Capture

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

7.1.4.1 Transfer the water sample concentrate (section 5.7 of SOP) to the Dynal L10 tube containing the mixed SL-buffer and use a further 1 ml 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.

7.1.4.2 Vortex the Dynabeads® anti-Cryptosporidium reagent for approximately 10 seconds to resuspend the beads. Ensure that the beads are fully re-suspended by inverting the tube and making sure that there is no residual pellet at the bottom.

7.1.4.3 Add 100 µl of the resuspended beads to the Dynal L10 tube containing the water sample concentrate and SL-buffer. Cap the tube.

7.1.4.4 Affix the Dynal L10 tube to the Dynal rotating mixer and rotate at approximately 25 rpm for at least1 hour.

7.1.4.5 After rotating for 1 hour, remove the Dynal L10 tube from the mixer and place in the Dynal magnetic particle concentrator (MPC-1) or a Dynal multi-tube magnetic particle concentrator (MPC-6) with flat side of Dynal L10 tube towards the magnet.

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

7.1.4.7 Gently rock the Dynal L10 tube by hand end-to-end through approximately 90°, tilting cap-end and base-end of the tube up and down in turn. Continue the tilting action for 2 minutes with approximately one tilt per second. 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 MPC-1 (or MPC-6) is allowed to stand motionless for more than 10 seconds, repeat the rocking procedure for the whole 2 minutes before continuing.

7.1.4.8 Return the MPC-1 (or MPC-6) to the upright position so that the Dynal L10 tube is vertical with cap at top. Immediately remove cap and decant all the supernatant from the tube held in the MPC-1 (or MPC-6) into a suitable container. Decant the tube held such that the flat face is on the lower side 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 MPC-1 (or MPC-6) during this step.

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

7.1.4.10 Transfer all the liquid from the Dynal L10 tube to a labelled, 1.5 ml micro-centrifuge tube; add 0.1 ml of diluted buffer to rinse and pool in the same micro-centrifuge tube.

7.1.4.11 Place the micro-centrifuge tube in the second magnetic particle concentrator (MPC-M), with the magnetic strip in place.

7.1.4.12 Without removing the micro-centrifuge tube from MPC-M gently rock/roll the tube through 180° by hand (see Figure 1 in information sheet provided with the IMS kit). Continue for approximately 1 minute with approximately one gentle 180°roll/rock per second. At the end of this step, the beads and oocysts should produce a well-formed brown dot on the back of the tube.

7.1.4.13 Uncap and immediately aspirate the supernatant from the tube and the cap held in the MPC-M, and recap the tube. If more than one sample is being processed, conduct three gentle 180° rock/roll actions before removing the supernatant from each tube. Take care not to disturb the material attached to the wall of the tube adjacent to the magnet. Do not shake the tube. Do not remove the tube from the MPC-M while conducting these steps.

7.1.5 Sample wash and dissociation of beads/oocysts complex (extra wash steps)

7.1.5.1 Remove the magnetic strip from the MPC-M.

7.1.5.2 Add 1ml of 1x SL buffer A to the micro-centrifuge tube.

7.1.5.3 Without removing the micro-centrifuge tube from MPC-M (magnet removed) gently rock/roll the tube through 180° by hand to resuspend the beads. Continue for approximately 10 second with approximately one gentle 180°roll/rock per second.

7.1.5.4 Replace the magnet into the MPC-M.

7.1.5.5 Without removing the microcentrifuge tube from MPC-M gently rock/roll the tube through 180° by hand. Continue for approximately 1 minute with approximately one gentle 180°roll/rock per second. At the end of this step, the beads and oocysts should produce a well-formed brown dot on the back of the tube.

7.1.5.6 Uncap and immediately aspirate the supernatant from the tube and the cap held in the MCP-M, and recap the tube. If more than one sample is being processed, conduct three gentle 180° rock/roll actions before removing the supernatant from each tube. Take care not to disturb the material attached to the wall of the tube adjacent to the magnet. Do not shake the tube. Do not remove the tube from the MPC-M while conducting these steps.

7.1.5.7 Repeat sections 7.1.5.1 - 7.1.5.6 as a further wash step.

Return to the main protocol (volume and slide changes)

N.B See Section 7.4 of this protocol for the controls that must be prepared with each batch of samples and before any analysis may be carried out on the RDI.

7.1.5.8 Uncap and add **100µl** of 0.1M hydrochloric acid (HCl), recap and then vortex for 5 seconds.

7.1.5.9 Stand each tube for not less than 5 minutes not longer than 10 minutes at room temperature in a vertical position.

7.1.5.10 Vortex for 5 seconds.

7.1.5.11 Replace magnetic strip in MPC-M and replace tube in MPC-M. Allow tube to rest horizontally for a minimum of 10 seconds so that the magnetic beads attach to MPC-M.

7.1.5.12 Remove a **Chemunex microscope well slide** from the box (stored at room temperature)

7.1.5.13 Add **10 µl** of 1.0 N sodium hydroxide (NaOH) to the **centre** sample well of the slide.

7.1.5.14 Return the microcentrifuge tube in the MPC-M to the vertical position and transfer the entire sample from the tube to the **centre** sample well of the Chemunex slide 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 at +2°C to +8°C until ready for stage 6 or incubated at a temperature not exceeding 42°C until the evaporation step is complete and then at +2°C to +8°C until ready for stage 6.

7.2 Examination (staining, detection and enumeration) (Volume and slide changes)

7.2.1 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. Also, ensure that the sample does not spread beyond the centre well.)

The batch test positive and negative controls must be stained with the sample slides.

7.2.1.1 After evaporating to dryness, remove slides from the incubator and apply 50µl of absolute methanol (standard reagent grade) to the **slide centre well**. Ensure that the methanol does not spread beyond the **outer well**. Allow to air dry for 3 to 5 minutes.

NB: Slides may be stored at this stage at +2°C to +8°C. Allow to equilibrate to room temperature before proceeding (20 to 30 minutes).

7.2.1.2 Place the slides (samples and/or controls) in a humid chamber and overlay the **centre and outer slide wells** with **150 µl** of fluorescein-labelled monoclonal antibody (mAb) - **provided as ChemId 2 with the Chemunex kit**. Place the slides in a humid chamber and incubate at 37 °C for 60 to 90 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.

7.2.1.3 After staining, remove the slides and use 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 the fluorescein-labelled mAb from the side of each outer 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.

7.2.1.4 Apply **150 µl** of 4',6-diamidino-2-phenylindole (DAPI) working solution (1/5000 dilution in phosphate buffered saline (PBS), prepared daily by adding 10µl of 2 mg/ml DAPI stock solution to 50ml of PBS) to each slide well. Allow to stand at room temperature for approximately 2 minutes.

7.2.1.5 Carefully remove the excess DAPI solution by aspiration as described above in section 7.2.1.3 above.

7.2.1.6 Apply **150 µl** of oocyst-free **B12 buffer provided with the Chemunex kit** to each slide well and allow to stand for approximately 1 minute, then aspirate the excess buffer. Leave to dry for 2-3 minutes. When removing the excess buffer ensure that the pipette tip does not touch the well surface.

7.2.1.7 Apply 20 µl of mounting medium provided with the Chemunex kit to the centre of each slide well, avoiding bridging.

7.2.1.8 Place a **Chemunex coverslip** on each microscope slide and allow the mountant to spread. 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 an approved sealant.

NB: Examine slides immediately or store slides in a dry box, or in a refrigerator at this stage, at +2°C to +8°C and under secure conditions until ready for examination (allow to equilibrate to room temperature before proceeding).

Sections 6.2 and 6.3 of the standard DWI SOP still apply. For convenience a copy has been inserted below

6.2 Checks on the Staining and Numbers of Oocysts

6.2.1 Examine all positive control slides at a minimum magnification of X 200. The positive control slides should contain between 80 and 120 oocysts, if the average result of the three positive control slides fall outside this range on two analytical runs then a fresh working suspension of oocysts must be made up for future testing. If there is a statistically significant change then a fresh working suspension of oocysts must be made up and a set of three positive control slides prepared for counting at the end of the run.

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

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

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 observations after examination of this control slide.

6.2.6 For each negative control, pipette 50 µl 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 The three negative control slides should be examined to determine that they do not contain oocysts under the FITC filter at a total magnification of not less than x 200. A record must be made of the observations. If any of the negative control slides do contain oocysts, an immediate investigation must be undertaken and a written report prepared on how the slides became contaminated with oocysts. The anomaly should be reported to the Inspectorate by telephone under notification code 3.

6.2.8 An identification label must be firmly affixed to the end of each positive and each negative control slide, this label must contain an identification number (with bar code if used).

6.2.9 If the conditions described in section 6.1 are not met, then the whole batch of slides including the positive and negative controls and all sample slides must be considered invalid. New positive and negative control slides 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. The freshly prepared positive and negative control slides can then be used with the sample slides. Note the background fluorescence, if any.

6.3 Checks on Microscopy

6.3.2 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.

(vii) 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.

(viii) View a positive slide at a screening magnification of not less than x 200 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.

(ii) 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.

(iv) 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.

(v) 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.

7.3 Automatic Scanning of Sample Concentrate using the ChemScan®RDI and Microscopic Examination by Epifluorescence and Brightfield Microscopy

7.3.1 Switch on the computer and wait until the Windows NT screen is displayed.

7.3.2 Switch on the ChemScan by turning the key switch located on the left side of the instrument to the ‘I’ position. Switch on the microscope and printer.

7.3.3 Log on to Windows NT by typing in unique operator Username and Password in the space provided for each in the 'Log On' window, then click on the 'OK' button. Run the ChemScan program by double clicking on the ChemScan icon. . Allow the

ChemScan a warm up period of 30 minutes before performing any analysis. Enter onto the data sheet the time of the ChemScan analyser power up.

7.3.4 To create a new session, select 'New Session' in the 'File Menu' of the 'Log Window' and enter a unique session ID.

7.3.5 Before the first scan is commenced ensure that the 30 minute warm-up time for the ChemScan analyser has elapsed. Prior to scanning any slides, the instrument controls must be performed and be within the defined acceptance criteria (Section 7 of this document). If they are acceptable the positive and negative control slides must be examined before proceeding with the sample slides (Section 6.3.2 in DWI SOP).

7.3.6 **Scanning technique:** Open the ChemScan drawer, insert the slide into the ChemScan analyser.

7.3.7 Start a scan by clicking on the 'Scan' button in the 'Log Window'. A window appears with space for Sample ID, Batch ID and comments. Enter details into each of these. Enter the time of the first scan onto the data sheet.

7.3.8 Click on the ‘Start Scan’ button to activate the scanning. On completion of scanning results are displayed in the 'Log Window, ChemScan results' column. Click on the ‘Scan name’ button corresponding to the slide analysed. The 'Scan Map' window will appear, displaying an image of the events detected by the ChemScan.

7.3.9 Each event can now be microscopically validated. Open the ChemScan drawer and remove the slide from the ChemScan analyser and transfer to the microscope stage. Click on the ‘Begin Validation’ button.

7.3.10 The microscope will position automatically at the first event to be validated. Examination should be performed using the guidance criteria specified in the DWI SOP Sections 6.3.2 onwards. Each event for control and sample slides should be validated (See section 6.4.11 below). When brilliant apple-green fluorescing slightly ovoid or spherical objects 4 to 6µm in diameter (using calibrated eye piece graticule) are observed with a brightly highlighted periphery, switch the microscope to the UV filter block for DAPI. If necessary, use differential interference contrast (DIC) microscopy for confirmation, look for typical oocyst internal structures.

7.3.11 **Microscopic validation:** Each fluorescent event must be validated using the microscope keypad. The keypad buttons correspond to the following: ‘M’ = validated Cryptosporidium oocyst; ‘P’ = particle; ‘?’ = unknown. After each validation mark the keypad will give an audible ‘beep’, and the stage will automatically go to the next object to be validated. The keypad will give a longer than usual ‘beep’ to let you know that you have arrived at the last object for validation purposes. Equivocal objects must now be evaluated using a x100 objective and oil immersion.

7.3.12 When the validation is complete, click on ‘Save’ button and then 'OK ' to record and store the results. The scan and validation results must also be saved permanently on a CD. Select 'Export data' from the 'File' menu and ensure that the CD drive is the target. The results must then be saved to a folder on the CD.

7.3.13 Record the date and time that the sample examination and confirmation was completed on the data sheet.

7.3.14 All slides must be stored in a secure (locked) refrigerator designated solely for the purpose of storing Cryptosporidium slides for at least three calendar months following completion of analysis.

7.3.15 When a session is complete the operator must log off. Select "Exit' from the 'File' menu. Select 'Start" from the Windows NT taskbar and then 'Shut down'. From the 'Shut down' window select 'Close all programs and log on as a different user' and then 'OK'. The 'Log On' window will then appear for the next operator.

7.3.16 **Re-validation of sample slides**. Log on to the ChemScan program ensuring that the Username and Password is that of the operator in charge of the re-validation. From the 'Log Window' click on the 'Scan name' button corresponding to the slide requiring re-validation. The 'Scan Map' window will appear, displaying an image of the events detected by the ChemScan. Position the slide on the slide support and insert into the slide support recess on the microscope stage. Click on the 'Begin Validation' button. Validate the slide as described in sections 6.4.10 - 6.4.11

7.3.17 When the re-validation is complete, click 'Save'. A new file name needs to be typed in as the original can not be over written. A suggestion is to use the same name but to add the initials of the person conducting the validation. Once complete press 'OK' and 'OK' again.

7.3.18 **ChemScan shut down**: Turn the instrument key switch to the 'O' position.Select 'Start" from the Windows NT taskbar and then 'Shut down'. From the 'Shut down' window select 'Shut down' and then 'OK'. When the message; "It is now safe to turn off your computer" appears, press the ON/OFF computer button. Switch off the microscope. Switch off the printer.

7.4 Controls and QA/QC

7.4.1 Instrument Controls

7.4.1.1 Scan the Chemunex ScanCheck slide.

7.4.1.2 From the 'Scan Map' window, click on the 'Print' button to generate a hard copy of the scan map.

7.4.1.3 With the scan map print out ensure that:

The date and time are correctly shown:
The complete test circle image is printed, with no gaps or discontinuities:

The complete test cross image is printed, with no gaps or discontinuities:

If all the above checks are correct then write “ChemScan <serial number> scan check OK” on the scan map print out, then date and sign.

7.4.1.4 Click on the 'Save' button and then the 'Close' button to return to the 'Log Window'

A Daily Control Check slide should be read on the ChemScan at the beginning of each day.

7.4.1.5 Homogenise the Chemunex Daily Control solution (containing fluorescent beads) using a vortex mixer for 30 seconds.

7.4.1.6 Apply 50 µl of the Daily Control solution to the centre sample well of a Chemunex slide.

7.4.1.7 Incubate the slide until the evaporation step is complete at a temperature not exceeding 42°C..

7.4.1.8 Apply 20 µl of Chemunex mounting medium

7.4.1.9 Place a Chemunex coverslip on each microscope slide and allow the mountant to spread. 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 an approved sealant

7.4.1.10 Scan the Daily Control slide. From the 'File' menu select 'View' and then 'Scan Histogram'. Click the 'Print' button in the 'Scan Histogram' window to generate a hard copy of the Peak Intensity results

7.4.1.11 Check that results are within the range quoted for the MIR (in the Commissioning Document provided with the Instrument). If the result is within range then sign and date. Photocopy and attach to data sheets. Click on the 'Close' button to return to the 'Scan map' window.

7.4.1.12 If the result is outside this range then repeat using Daily Control solution from a different unopened bottle. If the result is still outside the acceptance range then proceed to section 7.3

7.4.1.13 Using the daily control scan map, select 'Microscope set up' button and then click on the ‘Set Home' button. The following message appears ‘Do you wish to use the centre of the reference membrane to align the microscope?
,

7.4.1.14 Click on 'YES', and use the joystick to align the green mark of the motorised stage with the cross hairs of the ocular directly over the centre of the fluorescent mark.

7.4.1.15 When the alignment is complete, click on 'OK', the following message will appear. ‘Do you wish to set the stage marker’ Click on ‘NO’. The microscope alignment is now complete.

7.5 Positive and negative controls

N.B. All reagents must be QC checked prior to use and records maintained. Most reagents for this procedure overlap with the standard SOP, so routine laboratory QC procedures will suffice. Similar controls to those prepared for the standard method should also be employed as detailed below.

A positive control slide prepared from the Chemunex reagents must be stained and examined on the ChemScan at the start of the day before any samples are stained. This will QC the antibody and DAPI working stocks on a daily basis to ensure efficient performance and ensure that contamination with oocysts has not occurred.

Oocyst-positive and negative control slides must be stained every time a batch of test slides is stained.

7.5.1 Vortex the working solution for a minimum of 15 seconds

7.5.2 Pipette a 50µl aliquot to the centre sample well of each Chemunex slide. This will give 80 to 120 oocysts on the control slide. Label with Chemunex Mab and then DAPI. Analyse this sample on the ChemScan RDI prior to staining and analysing any samples.

7.5.3 Prepare a further three positive and three negative control slides with each batch of samples prepared, as in 7.2.2 above for the positives, and using 50µl of deionised water for the negative controls. These should be analysed before any sample slides are analysed.

A mean count of between 80-120 oocysts should be observed on the positive control slides. When validating the positive controls, check that they contain oocysts with typical bright apple green FITC fluorescence of sufficient intensity that the organisms may easily be observed and identified. In addition a significant number of oocysts should be DAPI positive exhibiting sufficient staining intensity that individual sporozoite nuclei may easily be observed. Check observations with the x100 oil-immersion lens, and with the DIC and DAPI measure the sizes of the oocysts. Ensure that the negative control slides do not contain oocysts.

If any of the criteria are not met, then the whole batch of slides, controls and sample slides must be considered invalid. Fresh control slides must then be prepared and all

sample slides in the batch must be carefully washed with reagent water. The control and washed sample slides must then be stained with freshly prepared fluorescein-labelled monoclonal antibody solution. If any of the negative control slides contain oocysts an immediate investigation must be undertaken and a written report prepared into how the slides were contaminated. The other positive and negative control slides shall be used during the analytical run.

N.B. Each control slide must be analysed on the Chem*Scan* and validated on the motorised stage microscope. Each slide must then be transferred to a standard microscope, examined and enumerated. All results should be included on the data sheets.

7.6 Control failure

7.6.1 If any of the instrument controls are not within the criteria and/or limits specified then this must be documented and the ChemScan cannot be used for sample analysis until the cause has been identified, resolved and documented. If the positive or negative control slides to not produce expected results, the DWI SOP, Sections 6.2.6, 6.2.7 and 6.3.2 should be followed.

8 REPORTING ARRANGEMENTS

8.1 All results of analysis under the Regulations must be reported to the relevant water company such that its requirements of the Regulations are achieved. Results must include the following details:

(i) name of the water company and the company identity number;

(ii) sample point identity reference code and name of sample point;

(iii) name of the laboratory and laboratory identity reference;

(iv) the analysis identity number (this is the number on the evidence bag);

(v) the date and time of both the start and finish of the sample run;

(vi) the date when the result was reported to the company;

(vii) the water meter reading at end of sample run;

(viii) the volume of water filtered;

(ix) the timer reading at end of sample run;

(x) the elapsed sample time;

(xi) the headloss over filter;

(xii) the deposit volume;

(xiii) the appearance of the filter, eg slightly brown, very dirty etc;

(xiv) the concentration of oocysts (in regulatory units) found for each sample; and

any other information that the water company requires.

8.2 The concentration of oocysts shall be reported as the number of oocysts per 10 litres. The limit of detection of this method for any positive results shall be deemed to be 0.01 oocysts per 10 litres. Any result of 0.005 oocysts per 10 litres or less than shall be reported as less than 0.01 oocysts per 10 litres. Any result greater than 0.005 oocysts per 10 litres and less than 0.01 oocysts per 10 litres shall be reported as 0.01 oocysts per 10 litres.

8.3 If no oocysts are detected in the sample then the results will be recorded as less than (<). The actual value that it is 'less than' will be determined by the volume

filtered, but in all cases where more than 667 litres was filtered and no oocysts detected the result is recorded as <0.01 oocysts/10 litres. If the amount filtered is between 401 and 666 litres and no oocysts are detected the result should be reported as <0.02 oocysts per litre. If the amount filtered is between 286 and 400 litres and no oocysts are detected the result should be reported as <0.03 oocysts per litre. If the amount filtered is between 182 and 285 litres and no oocysts are detected the result should be reported as <0.04 oocysts per litre.

8.3 Any results of analysis which contravene the prescribed concentration for *Cryptosporidium* oocysts set under these Regulations must be reported to a designated person within the relevant water company by telephone as soon as practical after completion of analysis, and a copy of a draft certificate of analysis must also be sent to a designated facsimile number or email address within the relevant water company as soon as practicable after completion of the analysis.

8.4 Any results of analysis which contravene the prescribed concentration for *Cryptosporidium* oocysts set under the Regulations must be reported by a designated person within the water company to a designated person within the Drinking Water Inspectorate in accordance with stated procedures.

8.5 A monthly summary of all results from each site must be sent to the DWI in accordance with the procedures set down in DWI Information Letters (IL) 10/1999, 4/2000, 10/2000, 13/200 and 28/2000. Such summaries must include volume of water filtered, headloss over filter, deposit volume, and concentration of oocysts found for each sample. The summary will be sent in a standardised electronic form stipulated by the DWI.

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 40 ºC. 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 0.5 mm form the base. Take care not to overtighten as this will cause difficulties when trying to remove the filter module.

A.4.3 The IDEXX Filta-Max® MKI Filter Housing can be used up to a pressure of 500 kPa (5 bar). 800 kPa (8 bar) is the maximum operating pressure.

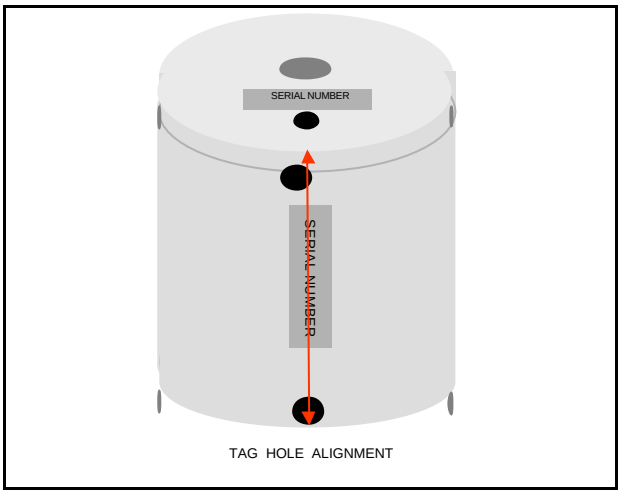
A.4.4 To open the MKI Filter Housing use the tools supplied by IDEXX.

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



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.**

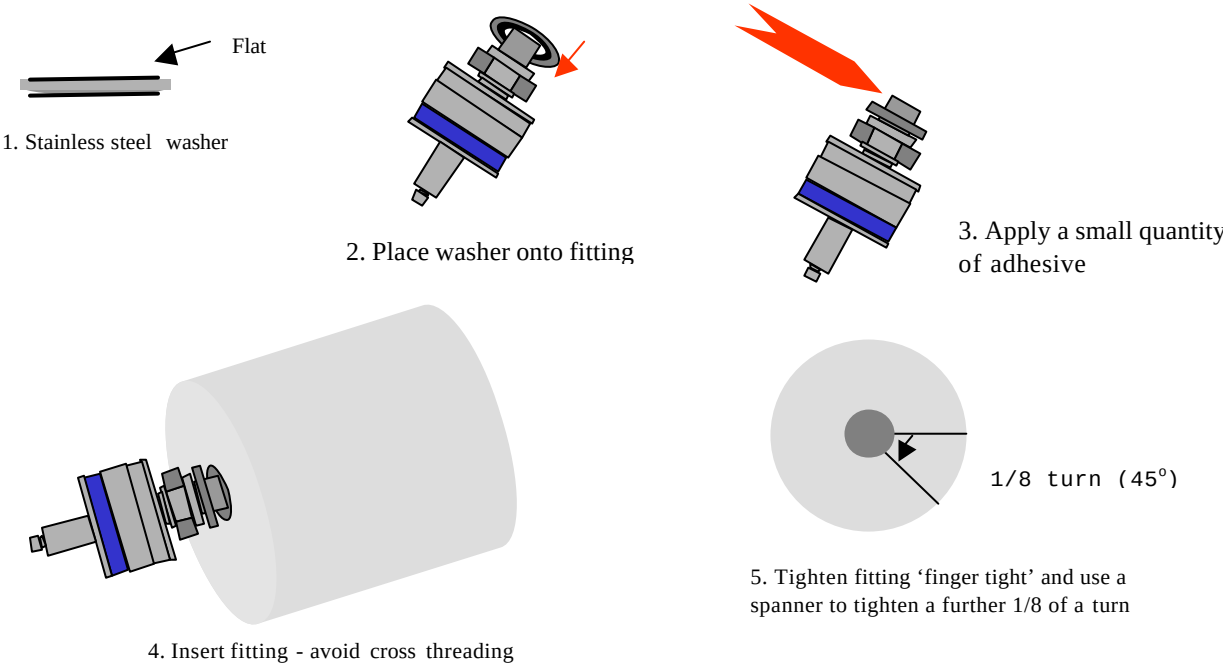
A.5.4 The IDEXX Filta-Max® MKII Filter Housing can be used up to a pressure of 500 kPa (5 bar). 800 kPa (8 bar) is the maximum operating pressure.

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 in accordance with paragraph 3.4.1 of the Part 2 of the SOP.

Diagram showing method of connecting the Swageloks to the IDEXX Filta-Max® MKI filter.



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 overtighten. Stop when a small amount of resistance is felt.

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

A.7 PALL ENVIROCHEK™ HV

A.7.1 Stainless Steel Filter Case

A.7.1.1 Cleaning

A.7.1.1.1. The stainless steel filter case can be cleaned by washing with soapy water, which should be followed by a rinse with oocyst-free water.

A.7.1.2.1 Should the assembled filter case and filter module leak during the pressure test, replace the 'O' rings.

A.7.1.2.2 'O' rings should be replaced every 6 months.

Housing Assembly Schematic

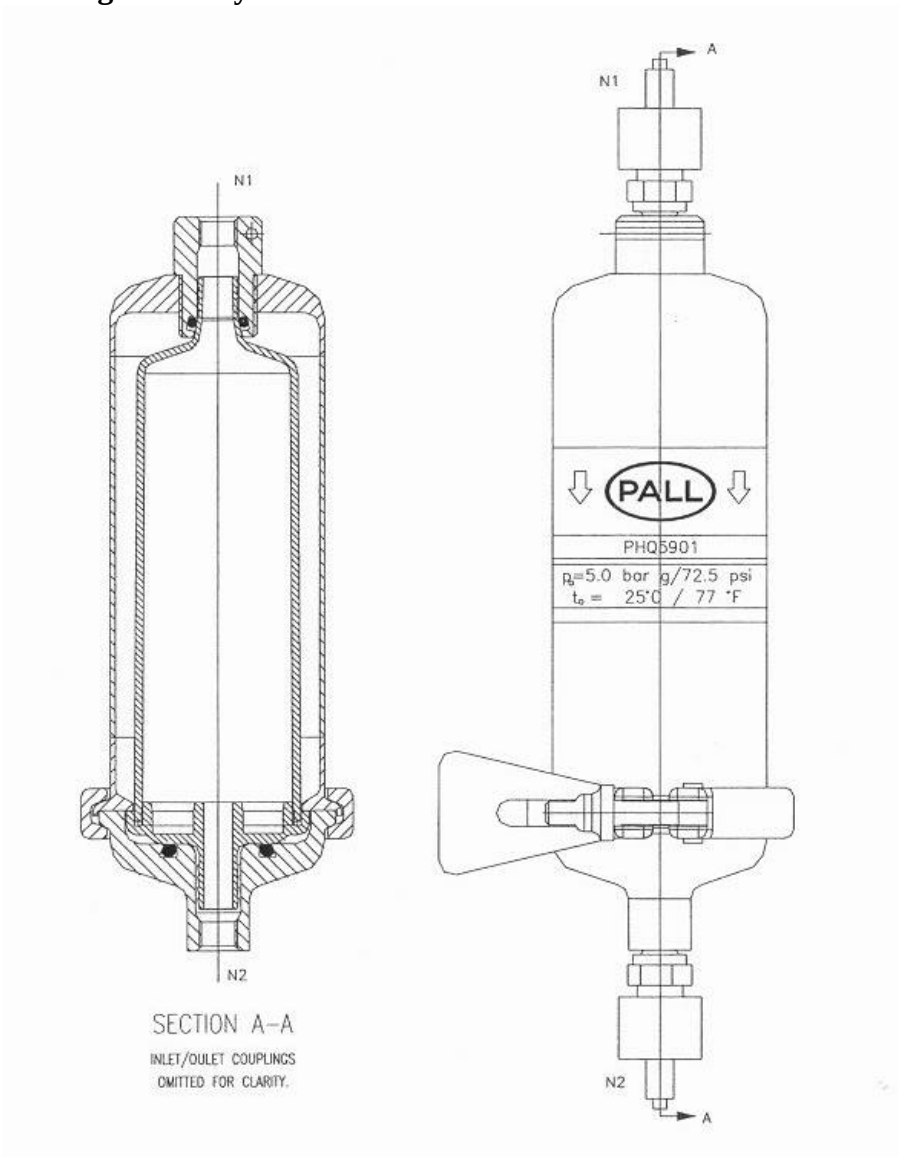


Photo 1: Envirochek™ HV Capsule With Tamper Evident Label

Photo 2: Stainless Steel Housing Assembly with Quick Connect Couplings

APPENDIX B SPECIFIC EQUIPMENT, MATERIALS & REAGENTS

B.1 IDEXX Filta-Max® Equipment[9]

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. Each housing so dedicated must have a label firmly fixed to the housing showing the site location code at which the housing is intended to be used. The use of the housing at only one 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, see section 5.5.10.

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 Pall Life Sciences Envirochek™ HV *Cryptosporidium* Filter

B.2.1 The Pall Life Sciences Envirochek™ HV filter unit is a specified sampling device enclosed in a stainless steel case. Each stainless steel case (Product No. ENKG 7153J) must be supplied with a unique number for identification on both halves. It is advisable to have at least three of these housings specifically dedicated to each site. Each housing so dedicated must have a label firmly fixed to the housing showing the site location code at which the housing is intended to be used. The use of the housing at only one site will minimise the risk of cross-contamination or equivalent device validated in house to demonstrate equivalent performance.

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

B.2.2 Filter module must be the Pall Life Sciences Envirochek^{TM} HV filter module. Each module must be supplied with a unique number for identification purposes (Product No. PN 12097 or 12096 - 25 in a pack).

B.2.3 Pall Life Sciences laboratory shaker (Product No. PNs 4822). This is the only sample shaker to have been validated. Laboratories would have to validate an alternative to ensure its performance is satisfactory.

B.2.4 Pre-treatment conditioner, 0.5% sodium polyphosphate. Prepared by dissolving 5 g of sodium polyphosphate ($(NaPO_3)_n$ (Aldrich Cat. No. 30.555-3)) in 1 litre of reagent water. The solution should be kept at room temperature and used within one week.

B.2.5 Elution buffer. The ingredients are-

1 g Laureth 12 (Pall Life Sciences PN 4820).
10 ml 1M Tris pH 7.4.
2 ml 0.5 M Ethylenediaminetetraacetic acid 2Na dihydrate (EDTA), pH 8.0.
150 µl antifoam Sigma catalogue No. A 5758
1 litre reagent water.

The elution buffer is prepared as follows: Weigh out 1.0 g of Laureth into a glass bottle and add 100 ml of reagent water. Warm on a hot plate or in a microwave oven to dissolve the Laureth. Add 10 ml of Tris solution, 2 ml of EDTA solution and 150 µl of Antifoam A. Bring the final volume of the buffer up to 1000 ml with reagent water. The buffer will have an opaque appearance.

Preparation of 1 M Tris pH 7.4. Dissolve 121.1 g of Tris in 700 ml of reagent water and adjust the pH to 7.4 using 1 N HCl or NaOH. Bring the volume to 1000 ml using reagent water.

This reagent is available commercially e.g. Sigma catalogue No. T 2663.

Preparation of 0.5 M EDTA. Dissolve 186.1 g of EDTA (Sigma catalogue No. ED 2SS) in 900 ml of reagent water by heating. Cool to room temperature. Adjust the pH to 8.0 using 10 N sodium hydroxide. Bring the volume to 1000 ml with reagent water.

B.2.6 The elution buffer can be obtained commercially. The product has a two month storage life and should be clearly indicated on the container. The containers if not black should be stored in the dark. Any buffer removed for the purpose of filter elution and not subsequently used should not be returned to its original container.

B.3 Dynal Biotech Equipment

B.3.1 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.3.2 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.3.3 Dynal Spot-On, 9 mm 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.

B.3.4 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.3.5 Dynal Dynabeads, Anti-*Cryptosporidium* kit for 10 tests, contains 1 ml 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 a temperature between +2 ^{o}C to +8 ^{o}C. Ensure that they are equilibrated to room temperature (15-22 ^{o}C).

B.4 TCS Isolate *Cryptosporidium* IMS Equipment

B.4.1 Variable speed rotator, with angle of tilt set at 20 ^{o} from horizontal (Product No. ZAICS1).

B.4.2 Isolate Rotator Disc and Hub for use with variable speed rotator. (Product No. ZAICS2).

B.4.3 6 x Incu-Clip Leighton Tube Clip for Incubation (Product No. ZAICS3 each).

NB: As an alternative, the Dynal MX1 rotating mixer can be used to incubate the Leighton tube. After an hour at room temperature, transfer the Leighton tube from the mixer to a Magna-Clip (white) and proceed from 5.10.2.4 (E).

B.4.4 6 x Magna-Clip, Magnetic Leighton Tube Clip for Separation (Product No. ZAICS4 each).

B.4.5 6 x Micro-Clip, Magnetic Micro-centrifuge Tube Clip for Separation (Product No. ZAICS5 each).

B.4.6 Dissociation Block (Product No. ZAICS8).

B.4.7 Glass Leighton tubes, flat sided tubes with cap, window (flat area) size 11 mm x 37 mm, 36 in a case (Product No ZAICS6) or equivalent tubes validated in house to demonstrate equivalent performance.

B.4.8 Hendley (Essex) Ltd 9mm single well blue-coated microscope slides (Product No. PH246 Hendley).

B.4.9 TCS Isolate *Cryptosporidium* IMS Kit for 100 tests containing 10ml antibody coated paramagnetic beads plus 110ml Reagent A and 100ml Reagent B (Product No. ZAICS100).

B.5 Cellab FITC stain

B.5.1 Monoclonal antibody/FITC reagent Cellabs Cryptocel IF antibody (5 ml, Product Code Z1RR1).

B.6 Microgen Bio-Products FITC

B.6.1 Monoclonal antibody/FITC reagent (Microgen FITC conjugated anti-*Cryptosporidium* antibody, product No. M85).

B.7 Mounting Medium

B.7.1 IFA mounting medium (e.g. TCS DABCO IF mounting fluid, 10 ml, Product Code Z1MM10) or equivalent medium validated in house to demonstrate equivalent performance.

B.8 DAPI reagent

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B.8.1 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.8.2 DAPI (stock) solution, 2 mg/ml DAPI per millilitre of methanol. Prepared by weighing out 1 mg of freeze-dried DAPI reagent and adding 0.5 ml methanol. This stock solution is stable for 1 month if stored in a refrigerator at 4 ^{O}C.

B.8.3 DAPI (working) solution, 10 µl DAPI stock solution in 50 ml of PBS, prepared daily.

B.9 Phosphate Buffered Saline/Tween (PBST)

B.9.1 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.

B9.2 Phosphate buffered saline (PBS) pH 7.4 at 25 ºC. 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 25 ºC. Each PBS sachet makes one litre, use ultra pure or distilled water to make 0.01M PBS, 0.138M NaCl, 0.0027M KCl, pH7.4 at 25 degrees centigrade. Supplied by SIGMA (product Code = P-3813) in sachets.

B.9.5 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.9.6 Ready for use elution buffer (PBS/Tween), each carboy labelled with a use by date (Vickers).

B.9.7 To make up PBS from separate Analar ingredients use the Oxoid formula given in the current catalogue for Product No. BR0014G.

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

B.9.8 To make Phosphate buffered saline/Tween to each litre of phosphate buffered saline add 100 µl of Tween and stir for 10 minutes.

B.10 Approved sources of oocysts

B.10.1 Supply of *C. parvum* oocysts (Moredun Animal Health).

B.10.2 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 2 ml 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 3 ml 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.10.3 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.10.4 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.

B10.5 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).

B11 Equipment required for the ChemScan®RDI Analyser

B11.1 ChemScan®RDI analyser (supplied by Chemunex). The ChemScanRDI should be used in accordance with the instructions in the user manual.

B11.2 Vortex mixer suitable for 1 - 40 ml tubes - Fisons Whirlmixer or equivalent.

B11.3 Epifluorescence Microscope with Prior motorised stage and 20x, 50x and 100x (facultative) objectives - Zeiss Axioscope, Leica DMRB, Olympus BX 50 or equivalent. This system should include excitation/band pass filters for fluorescein and DAPI and DIC equipment.

B11.4 A humidity chamber capable of holding slides.

B11.5 Incubator - Fisher Scientific isotemp or equivalent.

B11.6 Sample mixer - Stuart Scientific Tube Rotator SB1 or equivalent.

B11.7 Dynal Magnetic Particle Concentrator, MPC-1, (code: 200-B0120-01). Available from Chemunex.

B11.8 Dynal Magnetic Particle Concentrator, MPC-M, (code: 200-B0121-01), for 1.5 ml Eppendorf tubes. Available from Chemunex.

B11.9 Dynal L10 tubes (code : 200-C1014-01), Leighton tubes 16 x 125 mm, available from Chemunex.

B11.10 1.5 ml Microcentrifuge tubes from Eppendorf.

B12 Reagents required for the ChemScan®RDI Analyser

Chemunex reagents required to perform the assay:

B12.1 Cryptosporidium Test Kit with IMS :

A **ChemSol B12 (code: 200-R2045-04)**, store at room temperature to avoid precipitation of salts. This reagent is used as a labelling buffer. It is provided as a concentrated solution, which MUST be diluted in the ratio of 1:5 with distilled or deionised water before use.

B **ChemId 2 (code: 200-R1024-01)**, store at 2-8°C, this reagent contains the monoclonal antibody used to specifically label the Cryptosporidium oocysts. MUST be filtered before use (Polycarbonate filtration unit).

B12.2 10 X SL –buffer A, store at 2-8°C. (Dynal Kit)

B12.3 10 X SL – buffer B, store at 2-8°C. (Dynal Kit)

B12.4 Dynabeads anti-Cryptosporidium, store at 2-8°C. (Dynal Kit)

B12.5 Mounting Medium (code : 200-R3074-01) store at room temperature.

General reagents :

B12.6 Deionised / distilled water - water free of oocysts and interfering materials.

B12.7 Hydrochloric Acid (HCl) - Adjusted to 0.1N made in deionised or distilled water - Sigma H7020 or equivalent.

B12.8 Sodium peroxide (NaOH)- Adjusted to 1N in deionised or distilled water –

B12.9 Hydrogen peroxide (H_2O_2) - Adjusted to 4% with deionised or distilled water prior to use.

B12.10 Methanol (99% vol.)

B12.11 DAPI (4',6-diamidino-2-phenylindole).

Consumable items required to perform the assay, are as follows:

B12.12 Chemunex Slide (code : 200-C2015-01)Gilson type pipettes (P100, P200 and P1000).

B12.13 Disposable 10 ml and 1 ml syringes.

B12.14 Syringe filters (0.2 µm) e.g. Costar Syrfil MF or equivalent.

B12.15 Pasteur pipettes and teats

B13 BTF PTY LTD

B13.1 FITC Easystain obtainable direct from BTF PTY LTD see DWI website for details

APPENDIX C GENERAL EQUIPMENT & MATERIALS

C.1 Centrifuge to take up to 50 ml 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 250 ml 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 +/- 0.5 ml.

C.3 High speed vortex mixer.

C.4 Epifluorescence microscope with UV (340-380 nm excitation, 450 nm emission) and FITC (450-490 nm 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 10 ml.

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

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

C.8 Micro-pipettors and tips, variable volume dispensers, Eppendorf or similar, with calibration certification, 5-50 µl and 50-200 µl 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, 0.1 M, oocyst-free.

C.17 Sodium hydroxide, 1.0 M, 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 a requirement for this SOP, that any results should be admissible to a Court of Law, in the event of a prosecution by the DWI. It is thus imperative that a chain of evidence can be demonstrated and that the procedures used are scientifically defensible in such an event so that the water company and/or laboratory 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 DWI.

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" December 2000, Drinking Water Inspectorate, DWI 70/2/107. 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. Calibration check to include calibration/calibration check or other operational check as appropriate.

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 DWI 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 In order that the results are admissible in a Court of Law, in the event of a prosecution by the DWI, the following laboratory quality assurance tests are considered necessary to ensure the validity of the results.

E.1.2 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. There is a one hour allowance in the sampling period to allow for changing the sample and any maintenance that may be required (the same as specified in the Regulations). The sample shall be taken in accordance with the Regulations 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 SECURE CONDITIONS FOR THE SAMPLE

E.3.1 The sample taken for internal quality control shall be taken under 'secure' conditions. That is:

(a) if the validation sampling rig is in an open access area then it must be in a locked cabinet with the key only available to approved *Cryptosporidium* laboratory staff; or

(b) if the validation sampling rig is in in a secure room with a unique key available only to approved *Cryptosporidium* laboratory staff, including the *Cryptosporidium* laboratory then a board mounted validation sampling rig may be used.

E.4 THE VALIDATION SAMPLING RIG

E.4.1 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.4.2 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.4.2 Specification of the Validation Rig

E.4.2.1 Technical Specification

Dimensions of 2 line rig (mm) 850 h x 850 w x 250 d
(optional enclosure 1000 x 1000 x 320)
1 line rig (mm) 625 h x 625w x 250 d
(optional enclosure 1000 x 1000 x 320)
Weight (kg) of 2 line rig approx 15 Kg
1 line rig approx 8 Kg
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.4.2.2 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.4.2.3 The equipment is specifically designed to provide a means of introducing a known ammount of cryptosporidium into a flow of water upstream of a proprietory cryptosporidium filter.

E.4.2.4 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.4.2.5 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.4.2.6 Each line consists of a check valve followed by a ball valve and then an injection port.

E.4.2.7 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.4.2.8 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.

N.B. 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 it's 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.4.2.9 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 septa in to the injection port. Open the needle valve, and the ball valve on the line to be used. Purge the lines of air. Check that a minimum pressure of 4 bar is available on the inlet gauge.

E.4.2.10 When injecting the sample of cryptosporidium through the septa first reduce the line pressure using the needle valve.
N.B.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 liter 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.4.2.11 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.

N.B. 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 septum part **5183-4757-50** (11mm) 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

A supplier is: 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 1 ml 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 Inspectorate 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 are 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 Regulations.] 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 24 hrs. Record the time, date, analyst and meter readings. Check the septum for leakage and replace if necessary.

E.7.2 Procedure for use of Pall Envirochek™ HV filter module in the Validation Sampling Rig

E.7.2.1 It is important that the same batch of filters are 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. Insert a Pall Life Sciences Envirochek™ HV filter module into the filter housing 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 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 Regulations.] 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 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 an appropriate volume filtered over 24 hrs. 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 Inspectorate. 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 with a known tolerance: with Easyseed and with 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 in an AQC analysis book, 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 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 DWI 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 DWI 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

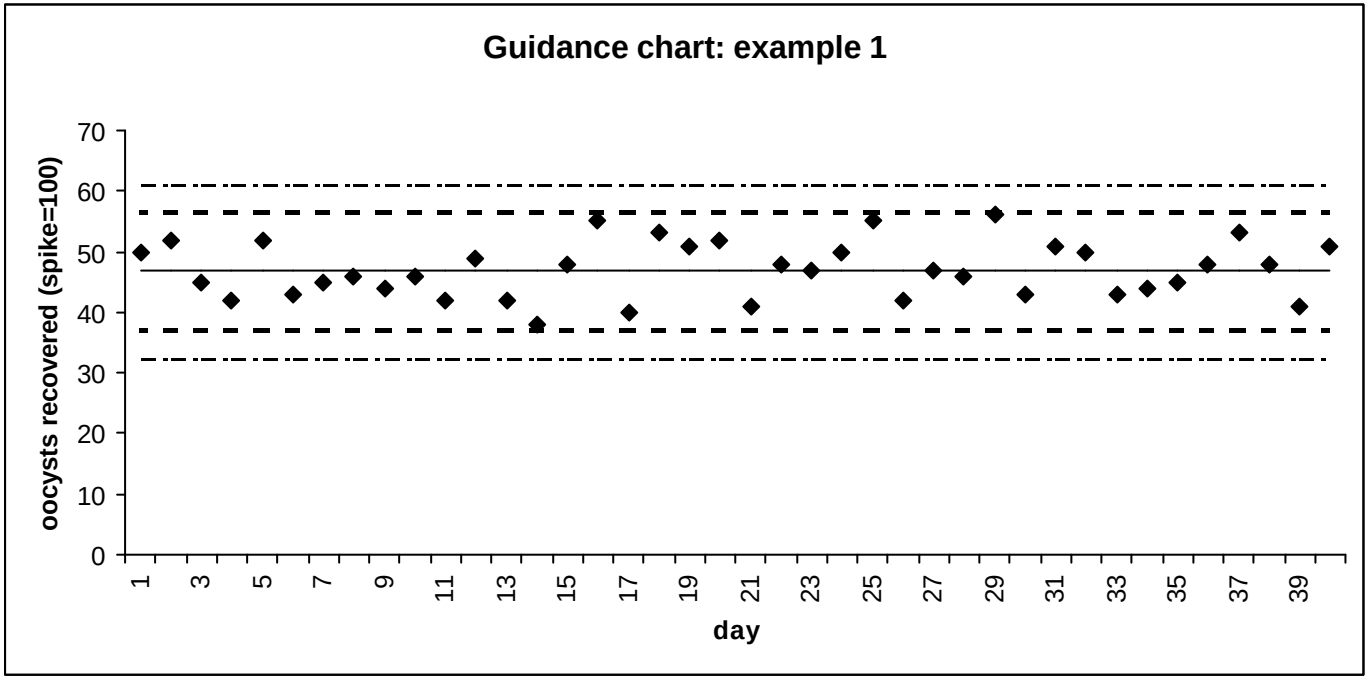
(e) after the scheme has been running for about a year this document will be reviewed. Further worked examples will be provided using real data.

E.9.8 Examples of QC Charts for Information and Guidance

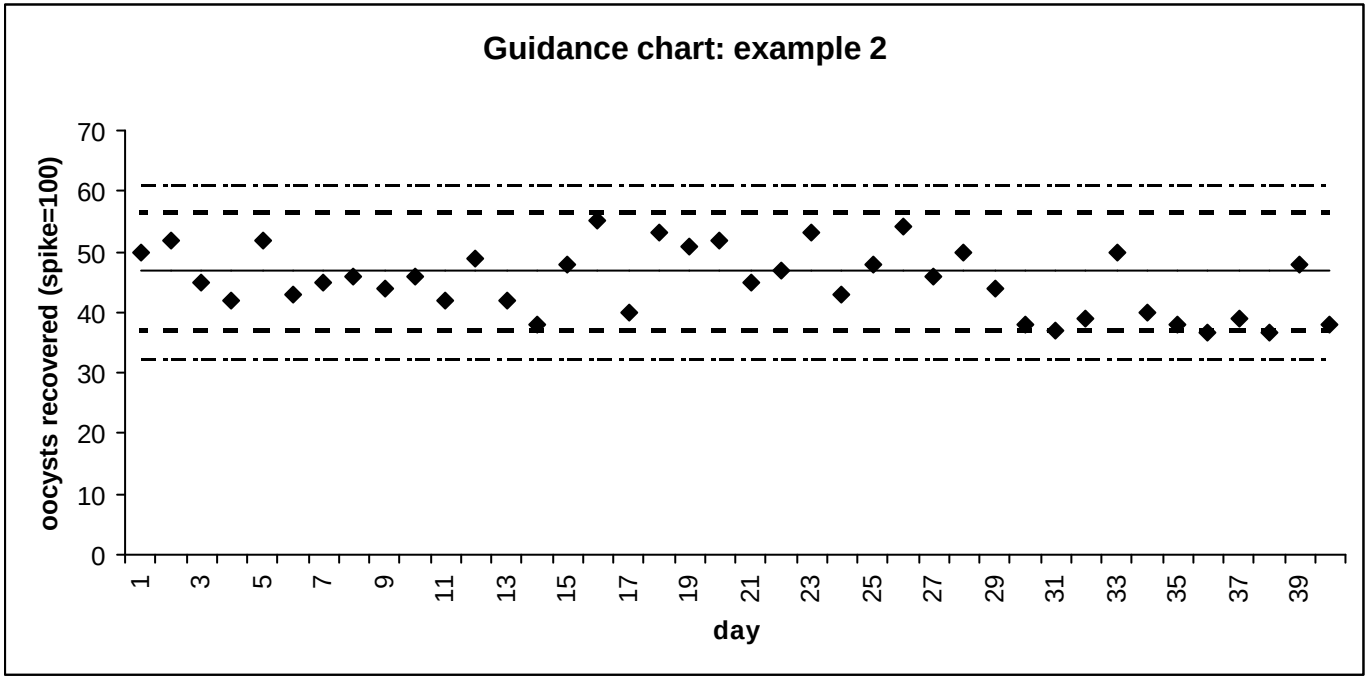
E.9.8.1 Example 1 shows a 40-day series. The first 20 observations were taken from real data and their mean (46.8) and standard deviation (4.83) were used to calculate warning and response lines. Because no further data were available for this example, the next 20 results were computer-generated to have 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 existing approved method of analysis is that method which is posted on the DWI website, thus over time the method will change as improvements and alternatives are brought in.] 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 DWI. 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 DWI. 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 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 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 a 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.

E.11 NEW SITE VALIDATIONS

E.11.1 Site validations will only be required for new sources that have not previously used as a potable water source. Prior to regulatory sampling of a new source 10 site validation tests will be undertaken using a 10 litre grab sample following the procedure detailed in section 10.5. The results should be recorded and checked for equivalence and recovery from the new site should not be dissimilar to recoveries obtained within the laboratories.

E.12 MOTHBALLED OR SEASONAL SOURCES OR SOURCES TAKEN OUT OF SERVICE FOR GREATER THAN SIX MONTHS

E.12.1 Any source which has been taken out of service for a period longer than six months shall have three site validations using 10 litre grab samples shall be taken to determine that the method is still applicable for that source. The samples shall either be taken before the source is returned to service or as soon as possible after. The sampling may be undertaken at the same time as samples would be taken to comply with section 15 of the Water Supply (Water Quality Regulations) 2000 [2001 for Wales].

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) details of training undertaken on the significance of chain of custody and security in the laboratory;

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

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

(v) results of inter-laboratory proficiency tests on samples and slides provided by DWI 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

LIST OF CRYPTOSPORIDIUM COMPANY REFERENCE NUMBERS

Company	Company Number
Anglian	33
Bournemouth and West Hants	73
Bristol	4
Cambridge	5
Cholderton	7
Dee Valley	31
Dwr Cymru	40
Essex and Suffolk	71
Folkstone and Dover	15
Hartlepool	16
Mid Kent	18
Northumbrian	34
Portsmouth	23
Severn Trent	36
South East	72
South Staffs	25
South West	38
Southern	37
Sutton and East Surrey	27
Tendring Hundred	28
Thames	39
Three Valleys	66
United Utilities	35
Wessex	41
Yorkshire	42
Inset appointment: Albion Water	76

LIST OF CRYPTOSPORIDIUM LABORATORY CODES

Laboratory	Laboratory
AES	AES
Alcontrol	ALC
Anglian	ANG
Severn Trent	STE
South West	SWE
South East	SAU
Southern	SOU
Thames	THA
Wessex	WES
United Utilities	NWE
Vivendi Water Partnership	VWP

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
Envirochek™ HV	Proprietary name for Pall Life Sciences filter and wash station assemblies
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
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

APPENDIX I USEFUL LINKS ON THE DWI WEBSITE

An updated list of approved suppliers is maintained on the DWI website: www.dwi.gov.uk

An updated list of approved products for Regulatory analysis for Cryptosporidium oocysts is maintained on the DWI website: www.dwi.gov.uk

All the forms are now located in the Cryptosporidium Forms Cabinet on the DWI Website: www.dwi.gov.uk

APPENDIX J - REVISED ANOMALY NOTIFICATION

New anomaly notification

Notification timescale and reporting requirements

Priority	Initial Notification Timescale	Reports required
1	Immediate by telephone[10], using cascade system if necessary. Confirm details by email	72 hour event report. 30 day incident report may be required.
2	By email[11] on the next working day	Written report of investigation[12] within seven working days of notification.
3	Not required	Written report of investigation[13] within seven working days of notification
4	Not required	Written report of investigation[14] with monthly data return

[10] The anomaly must be notified directly to an Inspector. A voice mail message, Email message or Fax will not suffice.

[11] The email should be sent to tony.hallas@defra.gsi.gov.uk with a copy to the DWIDoc.Controller@defra.gsi.gov.uk

[12] Use the form in the DWI Cryptosporidium website forms cabinet

[13] Use the form in the DWI Cryptosporidium website forms cabinet

[14] Use the form in the DWI Cryptosporidium website forms cabinet

Anomalies to be reported and priority

Notification Code	Anomaly	Comment	Priority
1	Preliminary or confirmed result above regulatory limit	Will be handled as a water quality event.	1
2	No sample taken or sample lost before receipt at laboratory.	May be handled as a water quality event.	2
3	No result obtained by laboratory or inability to obtain a valid result	May be handled as a water quality event. Eg partial loss of sample during analysis. May be handled as a water quality event	2
4	Failure to sample for part of the sampling period	Including maintenance work carried out outside changeover period or overrunning work scheduled to be completed during changeover period. May be handled as a water quality event.	2
5	Sample container transit bag not sealed or evidence of tampering		3
6	Open evidence bag, incorrectly sealed evidence bag or evidence of tampering with evidence bag after sealing	Up to receipt at laboratory	3
7	Evidence of tampering with sample after receipt at laboratory		3
8	Unauthorised breaking of security devices on sampling lines and units		3
9	Failure to record correctly authorised security device changes		3
10	Sampling cabinet found unlocked or otherwise in an insecure condition		3
11	Incorrect white tag fitted to sampling unit door	Report incorrect recording of number on evidence bag by issuing laboratory as code 17	3

Notification Code	Anomaly	Comment	Priority
12	Key to sampling cabinet or key safe lost or stored insecurely or security padlocks replaced on the sampling cabinet or Swagelok KO75 fitting replaced in cabinet	Report any loss, transfer or replacement of either key, security padlock or Swagelok KO75 fitting	3
13	Any gap in the chain of evidence or failure of security not covered above	Including missing signature, or correction not initialled, timed or dated	3
14	Sample not analysed within specified timescale or result not reported within specified timescale	3 days for routine samples 1 day for express analysis	4
15	Volume filtered less than 40 litres per hour over sampling period	Only to be used when actual average rate of flow is less than 40l/hr	4
16	Deviations from approved sampling procedure		4
17	Incorrect recording of filter unit numbers or other information on the evidence bag by the laboratory or failure of laboratory to record information	Include tag numbers and insecure green tag	4
18	Deviations from approved analysis procedure	Not covered by codes 3, 7, 13, 14 or 17 above.	4
19	Sampling information missing from evidence bag other than chain of evidence records.	Use code 17 for laboratory omissions. Use code 13 for missing signature or initials or continuity block not completed.	4
20	Sampling information incorrectly recorded on evidence bag other than chain of evidence records	If no correct written contemporaneous record exists. Use code 17 for laboratory errors. Use code 13 for chain of evidence information.	4

Notification Code	Anomaly	Comment	Priority
21	Minor errors or inconsistencies in recording sampling information on the evidence bag other than chain of evidence records	For use only if a contemporaneous written record of the correct information exists (eg sampler's log). If information inferred or deduced from other records eg preceding or subsequent evidence bags use code 20. Use code 17 for laboratory errors. Use code 13 for chain of evidence information.	4
22	Any contravention of part 1 of the protocol (sampling) not included in this list		4
23	Any contravention of part 2 of the protocol (analysis) not included in this list	Also parts 2a and 2b if appropriate	4

Each event must be recorded as a separate anomaly.
Eg if an analysts drops a tray of slides, this is one anomaly affecting a number of samples.
If a sampler fails to sign all the evidence bags on a particular day, each failure is a separate event and an anomaly reported for each sample affected.
If less than 40l/hr is filtered at a site for 2 or 3 days while a replacement part is obtained and fitted, this is one anomaly affecting a number of samples.

Comparison Of Superseded Anomaly Codes With The New Codes

IL 28/2000 Anomaly code	Proposed new code
01	01
02	02 , 03 and 04
03	05
04	06 and 07
05	08
06	09 and 17
07	10
08	11 and 13
09	13
10	15
11	02 or 04 (only if sampling interrupted)
12	02 or 04 (only if sampling interrupted)
13	None
14	None
15	None
16	None
17	02 or 04
18	04
19	14, 16, 17 and 18
20	12
21	17
22	17 (broken in lab) 16 (broken by sampler) 13 (broken while fitted to sampling device and not done by sampler)
23	17
24	17 (by laboratory) 19 and 20 (by sampler) 13 (if chain of evidence broken)
25	22 and 23
26	None
27	21
28	17
29	22
30	None
31	None
32	None
33	None
34	None
35	None