REPORT NO. 508

Cryptosporidium and *Giardia* (Round 17) Proficiency Testing Program

May 2006

ACKNOWLEDGMENTS

PTA wishes to acknowledge gratefully the technical assistance provided for this program by Professor Duncan Veal of Department of Biological Sciences, Macquarie University. This assistance included providing input into the design of the program and technical commentary for the report.

PO Box 7507, SILVERWATER NSW 2128

CONTENTS

1. FOREWORD

This report summarises the results of the seventeenth round of a planned series of proficiency testing programs involving the analysis of water samples for the detection and enumeration of *Cryptosporidium* and *Giardia*.

The exercise was conducted in March 2006 by PTA. The aim of the program was to assess laboratories' ability to competently detect and report levels of the protozoan parasites in water.

2. NATA LABORATORY TESTING PERFORMANCE

Individual laboratory testing performance will be monitored by NATA for each round of this program. Laboratories that have reported an extreme result (false positive/false negative) or reported low/high recoveries (percentage recovery rates outside the acceptable range of 10% - 110%) will be required to instigate investigative action to identify the cause. Details of this investigative action and any associated corrective action will be required to be reported in writing to NATA by a stated date. The laboratory's investigation will be reviewed and any technical comments will be returned.

Note that the acceptable range for percentage recovery rates, **in the first instance** will be 10% - 110%. This range has the possibility of changing once the confidence levels based on a history of data have been established.

For these rounds all participating laboratories' performance will be graded as:

- ***Satisfactory*** - Laboratories that do not report any extreme results (false positives and/or false negatives) and/or low/high recoveries for any sample tested in two consecutive rounds will be graded as satisfactory.

- ***Questionable*** - If any laboratory has been identified as having reported extreme results and/or low/high recoveries in one round of this program then their performance will be graded as questionable.

- ***Unsatisfactory*** - If any laboratory has been identified as having reported extreme results and/or low/high recoveries in two consecutive rounds of this program then their performance will be graded as unsatisfactory. NATA laboratories graded as unsatisfactory will become suspended for this testing. These laboratories will need to perform to a satisfactory standard in two consecutive rounds before their accreditation status for this testing is reinstated.

3. FEATURES OF THE PROGRAM

(a) A total of 7 laboratories (comprising five Australian and two New Zealand) received samples of which 7 returned results.

(b) Participating laboratories were requested to report both total and confirmed counts for their results.

(c) The results as reported by participants are presented in Appendix A.

(d) Along with the samples, laboratories were provided with the *Instructions to Participants* and a *Results Sheet* (see Appendix C). The laboratories were requested to perform the tests according to their routine methods. Laboratories were also reminded that the use of ColorSeed™ in PTA proficiency testing samples is not acceptable.

(e) Prior to the time of sample distribution, a number of randomly selected seed samples were analysed for homogeneity. Based on the results of this testing it is considered that the samples utilised for this program were sufficiently homogenous (see Appendix B).

(f) Each laboratory was randomly allocated a unique code number for the program to ensure confidentiality of results. Reference to each laboratory in this report is by its code number.

4. DESIGN OF THE PROGRAM

Participants were requested to provide quantitative results for the presence of *Cryptosporidium* and *Giardia* in 5 x concentrate water samples. The sample design of the water samples is presented below.

Sample type	***Cryptosporidium*** **(Count)**	***Giardia*** **(Count)**	***400 µL QC mud***
A	310	310	✓
B	0	0	✓
C	90	0	✓
D	0	135	✓
E	135	135	✓
F (Trip control)	310	310	✓

✓ indicates that QC mud was added to these samples to simulate an environmental sample.

All samples were added to reverse osmosis water to make approximately 50mL. One nominated laboratory (Code 7) was provided with a sixth sample, to represent the trip control.

Preparation of samples

BTF Pty Ltd, NSW, prepared different spikes for this program.

Seed samples were prepared on 7 March 2006. The suspension media was phosphate buffered saline and the sterilisation and preservation method performed was gamma irradiation.

The *Cryptosporidium* oocysts were of bovine origin and were excreted on 10 January 2006. The organism is *Cryptosporidium parvum* (Iowa strain). The oocysts were purified by discontinuous sucrose and caesium chloride centrifugation gradients.

The *Giardia* cysts were sourced from experimentally infected gerbils and were excreted on 20 January 2006. The organism is *Giardia lamblia* (H3 strain). The cysts were purified by sucrose and percoll gradients, followed by water washes.

The samples were prepared by using flow cytometry and an automated dispensing method. The *Cryptosporidium* and *Giardia* were dispensed into 5mL test tubes containing saline solution.

The samples were then sealed, labelled and exposed to a controlled dose of gamma irradiation. The *Cryptosporidium* were also heat treated to prevent excystation. Quality Control was performed on the seed dose.

Each of the seed samples were then poured into approximately 50mL of reverse osmosis water. The test tube containing the seed sample was washed with *0.05% Tween 80* and then two rinses of water. All washes were poured into the 50mL samples.

The participating laboratories were asked to add each of the seed samples to 10 Litres of reverse osmosis water, prior to analysis.

Confounding materials

QC mud:

QC mud was added to all samples at a concentration of 400µL of QC mud per 50mL sample.

Quality Assurance of the seed samples and the reverse osmosis water
(refer to Appendix B)

BTF Pty Ltd provided counts for *Cryptosporidium* and *Giardia* on a minimum of 8 sets of randomly selected spiked samples for homogeneity. The counts were performed prior to adding to the reverse osmosis water.

As a quality control check, randomly selected samples from each of the spike material type were analysed to determine the percent DAPI positive. The internal structures of the seeds were analysed by the flow cytometry method to determine the percentage full.

The reverse osmosis water was analysed prior to adding it to the seed doses to ensure that no *Cryptosporidium* or *Giardia* were present.

10 Litres of the reverse osmosis water was concentrated using membrane filtration and then analysed using the Dynal IMS system and epifluorescence microscopy. No *Cryptosporidium* or *Giardia* were detected.

Quality Assurance of the QC mud

To ensure that the QC mud did not contain either *Cryptosporidium* oocysts or *Giardia* cysts, QC mud samples were analysed prior to addition to proficiency samples.

Briefly, samples of QC Mud were stained using FITC-labelled monoclonal antibodies (*Cry 104* for *Cryptosporidium* and *G203* for *Giardia*), purified using flow cytometry then examined using fluorescence microscopy. No *Cryptosporidium* oocysts or *Giardia* cysts were found in 10mL of QC mud, which is equivalent to 50 samples.

5. FORMAT OF APPENDICES

Appendix A (A1.1 - A1.7) contains the results reported (total counts and confirmed counts) by participating laboratories for each of the five samples. The percentage recovery rates and charts are also presented (calculated using confirmed counts only).

Appendix B contains details of homogeneity testing, quality control and the trip control results (B1.1 - B1.6).

Appendix C contains the *Instructions to Participants* and *Results Sheet* (C1.1 – C1.3).

Appendix D contains the DAPI staining protocol used by BTF Pty Ltd (D1.1 – D1.2).

6. FALSE RESULTS

The results were examined for false positive and false negative results with all testing methods pooled. Table A below summarises the false results detected.

TABLE A: *CRYPTOSPORIDIUM* FALSE NEGATIVE RESULTS

Test for the detection of *Cryptosporidium*	
Sample	False Negatives (by laboratory code number)
C	Code 3

7. LOW/HIGH RECOVERIES

The results were examined for low/high recoveries (recovery rates that lie outside the acceptable range of 10-110%) with all testing methods pooled. Table B below summarises the low recovery results detected.

TABLE B: *CRYPTOSPORIDIUM* LOW RECOVERY RATES

Test for the enumeration of *Cryptosporidium*	
Sample type	Low Recoveries (by laboratory code number)
A	Code 3
C	Code 3

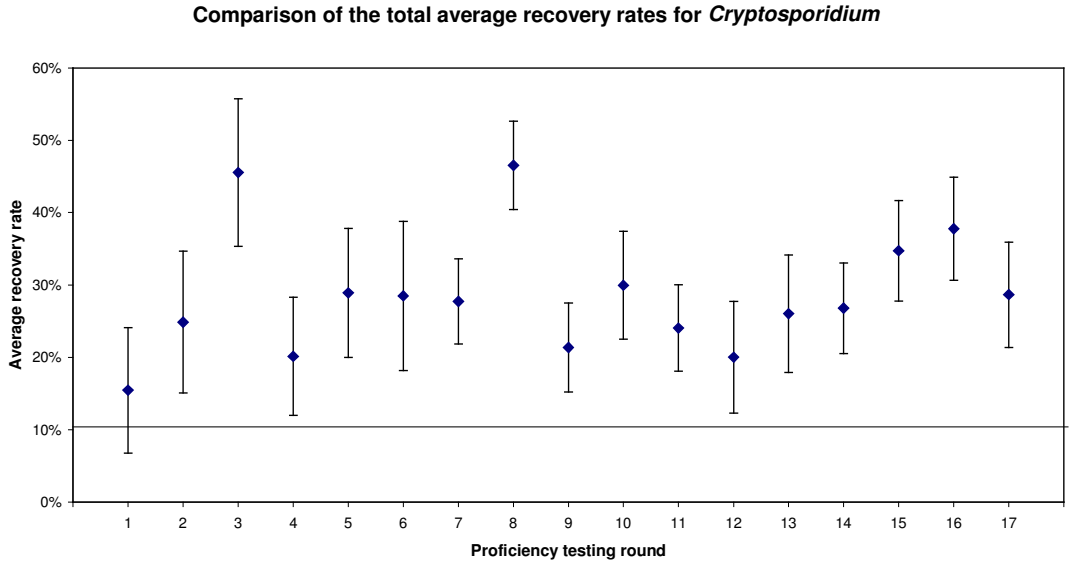
8. PTA AND TECHNICAL ADVISER'S COMMENTS

Of the 7 laboratories that returned results for the program, one laboratory (Code 3) was identified with extreme results (ie. false positive/false negative). One laboratory (Code 3) was identified as having low recoveries. Of the 68 confirmed count results received, 1.5% were identified as extreme (ie. false positive/false negative) and 2.9% were identified as low/high recoveries.

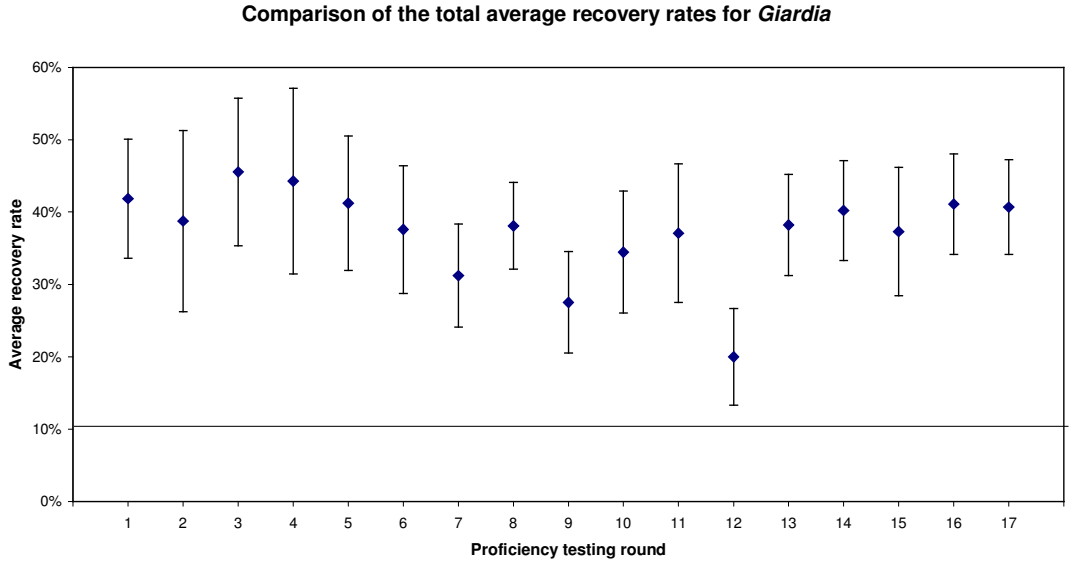
Comments on the Percentage Recovery Rate

The overall recovery rates for *Cryptosporidium* were lower than the recovery rates for *Giardia*.

The total average *Cryptosporidium* recovery rate has decreased in this round compared to the previous round. The graph below shows the average percent recovery rate for *Cryptosporidium* for each round (Refer to Notes on page 7).



The total average *Giardia* recovery rate has remained constant in this round compared to the previous round. The graph below displays this (refer to notes on page 7).



Notes to Average Recovery Rates Graphs:

The vertical bars in the graphs represent the 95% confidence intervals.

All rounds, except for rounds 1, 2, 3 & 8, contain QC mud (see table on pages 9, 10 and 11). For Round 5, one sample (Sample type 4), for Round 14, one sample (Sample C) and for Round 15, one sample (Sample D) out of the five samples analysed by each laboratory did not contain QC Mud.

From Round 14 onwards, the average recovery rates are calculated on confirmed counts only. Prior to this round, participants reported either total or confirmed counts, and therefore the average recovery rates presented in this table from Rounds 1 to 13 may include both total and confirmed counts.

The table below shows the *Cryptosporidium* levels for each round.

Round	*Cryptosporidium* levels (Counts)	Round	*Cryptosporidium* levels (Counts)
1	50-200	17	90-310
2	50-200		
3	50-300		
4	110		
5	50-200		
6	25-75		
7	50-100		
8	65-140		
9	125		
10	110-235		
11	50-200		
12	110-235		
13	90-205		
14	55-135		
15	55-135		
16	55-120		

The table below shows the *Giardia* levels for each round.

Round	*Giardia* levels (Counts)	Round	*Giardia* levels (Counts)
1	50-200	17	135-310
2	50-200		
3	50		
4	40		
5	50-200		
6	75-120		
7	50		
8	65-140		
9	55		
10	70-85		
11	50-200		
12	110-125		
13	90-145		
14	55-200		
15	55-200		
16	120-255		

Of all the results received by the laboratories, two results were calculated as low/high recoveries, that is, percentage recovery rates outside the acceptable range of 10%-110% (refer to A1.4 and Table B, page 5).

Samples A and C for *Cryptosporidium*: One laboratory (Code 3) had a percentage recovery rate below the acceptable range.

Laboratories which obtained a percentage recovery rate outside the acceptable range of 10%-110% were advised to take investigative action and provide NATA with their comments.

False Results

It has been 6 months since the previous proficiency testing program had been conducted. Of all the results received by the laboratories, one result was calculated as a false result (refer to A1.1 and Table A, page 5).

Method Commentary

Laboratories used a variety of concentration methods, including membrane filtration and flocculation. All laboratories used IMS as their purification method. All laboratories used fluorescent microscopy in their method. All laboratories reported the use of DAPI staining as the confirmation method. Two laboratories also reported the use of DIC microscopy.

No standard method has been prescribed in Australia. The variety of methods and modifications used by the participating laboratories reflected this lack of standardisation.

Overall Laboratory Performance

The table that appears below over the next two pages shows the comparison of the overall laboratory performance for rounds 1 - 17.

The number of laboratories reporting false results has generally declined over the 17 rounds. Likewise, the percentage of false results reported has decreased over time.

Round	Sample Type	Percentage false positive and false negative results reported	Number of laboratories reporting false results	Percentage low/high recovery results reported	Number of laboratories reporting low/high percentage recovery rates
1	10 Litres - tap water	11.0%	6	11.0%	7
2	10 Litres - tap water	6.7%	1	7.8%	3
3	10 Litres -Milli-Q water	3.8%	3	4.7%	3
4	10 Litres - RO water + QC mud + confounding organisms	10.3%	3	11.8%	4
5	10 Litres - RO water + QC mud*	7.0%	4	11.0%	5
6	10 Litres - RO water + QC mud	8.3%	4	8.3%	5
7	Concentrate samples - QC mud - labs. added to 10 Litres distilled water	8.2%	4	6.4%	5

Round	Sample Type	Percentage false positive and false negative results reported	Number of laboratories reporting false results	Percentage low/high recovery results reported	Number of laboratories reporting low/high percentage recovery rates
8	10 Litres - RO water	1.2%	1	1.2%	1
9	10 Litres - RO water + QC mud	2.7%	1	7.3%	4
10	Concentrate samples - QC mud - labs. added to 10 Litres distilled water & 10 Litres - RO water + QC mud	2.3%	1	3.5%	2
11	10 Litres - RO water + QC mud	0.0%	0	6.8%	4
12	10 Litres - RO water + QC mud	5.5%	2	17.5%	6
13	10 Litres - RO water + QC mud	0.0%	0	10.0%	4
14	10 Litres - RO water + QC mud*	2.6%	1	2.6%	1
15	Concentrate samples - QC mud* - Labs. added to 10 Litres distilled water	1.3%	1	5.0%	2
16	Concentrate samples - QC mud - Labs. added to 10 Litres distilled water	0.0%	0	3.3%	2
17	Concentrate samples - QC mud - Labs. added to 10 Litres distilled water	1.5%	1	2.9%	1

Note: RO = reverse osmosis.
* = For Round 5, QC Mud was only added to Sample types 1, 2, 3 and 5. For Round 14, QC Mud was only added to Samples A, B, D and E. For Round 15, QC Mud was only added to Samples A, B, C and E.

NATA Laboratory Testing Performance

After the completion of Rounds 1 - 17 the testing performance of the laboratories have been graded according to the criteria given on page 1. Six laboratories have been graded as satisfactory (one of these laboratories did not participate in round 17). Two laboratories are questionable and there are no laboratories that have been graded as unsatisfactory.

9. REFERENCE

[1] *Guide to Proficiency Testing Australia, 2006.*

APPENDIX A

Summary of Results

Results *Cryptosporidium*

ACTUAL COUNTS	310	0	90	0	135
Code No.	**Sample type A**	**Sample type B**	**Sample type C**	**Sample type D**	**Sample type E**
Code 1 - Total Count	99	0	54	0	81
Code 1 - Confirmed Count	99	0	54	0	81
Code 2 - Total Count	141	0	27	0	41
Code 2 - Confirmed Count	141	0	27	0	41
Code 3 - Total Count	20	0	0	0	36
Code 3 - Confirmed Count	19	0	0	0	28
Code 4 - Total Count	#	0	13	0	26
Code 4 - Confirmed Count	#	0	12	0	26
Code 5 - Total Count	98	0	36	0	62
Code 5 - Confirmed Count	83	0	33	0	51
Code 6 - Total Count	72	0	11	0	34
Code 6 - Confirmed Count	72	0	11	0	34
Code 7 - Total Count	110	0	33	0	30
Code 7 - Confirmed Count	109	0	33	0	30
Number Results*	6	7	7	7	7
Minimum*	19	0	0	0	26
Maximum*	141	0	54	0	81
Average*	87	0	24	0	42
Median*	91.0	0.0	27.0	0.0	34.0

* Refer to notes following on page A1.3

Results *Giardia*

ACTUAL COUNTS	310	0	0	135	135
Code No.	**Sample type A**	**Sample type B**	**Sample type C**	**Sample type D**	**Sample type E**
Code 1 - Total Count	89	0	0	97	68
Code 1 - Confirmed Count	89	0	0	97	67
Code 2 - Total Count	126	0	0	16	31
Code 2 - Confirmed Count	126	0	0	16	31
Code 3 - Total Count	228	0	0	88	77
Code 3 - Confirmed Count	130	0	0	51	54
Code 4 - Total Count	#	0	0	60	52
Code 4 - Confirmed Count	#	0	0	60	51
Code 5 - Total Count	131	0	0	73	67
Code 5 - Confirmed Count	87	0	0	69	52
Code 6 - Total Count	111	0	0	52	36
Code 6 - Confirmed Count	109	0	0	52	36
Code 7 - Total Count	153	0	0	88	83
Code 7 - Confirmed Count	144	0	0	85	79
Number Results*	6	7	7	7	7
Minimum*	87	0	0	16	31
Maximum*	144	0	0	97	79
Average*	114	0	0	61	53
Median*	117.5	0.0	0.0	60.0	52.0

* Refer to notes following on page A1.3

Notes to Results Tables :

[#] - Analytical failure.

* - Statistics are presented for Confirmed Counts only. Total counts are provided for information only.

The median is provided for information only. It is the middle result. It is a measure of the centre of the data and is similar to the mean (or average).

Results *Cryptosporidium* (% Recovery Rate)

Calculated on Confirmed Counts only

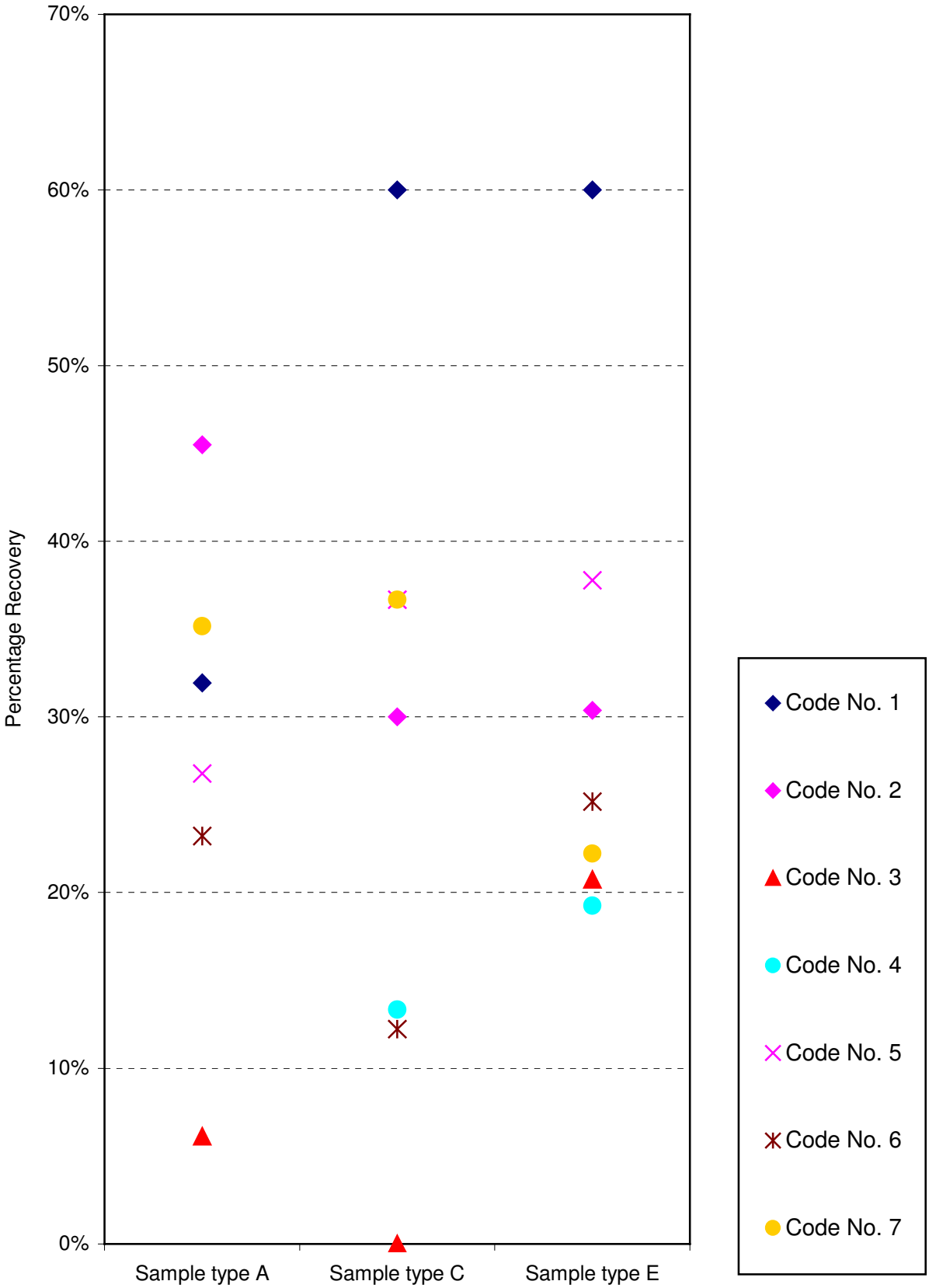
Code No.	Sample type A	Sample type C	Sample type E
1	32%	60%	60%
2	45%	30%	30%
3	6%●	0%●	21%
4	#	13%	19%
5	27%	37%	38%
6	23%	12%	25%
7	35%	37%	22%
Minimum	6%	0%	19%
Maximum	45%	60%	60%
Average	28%	27%	31%
Median	29%	30%	25%

● Below minimum acceptable recovery rate of 10%.

\# Analytical failure.

The median is provided for information only. It is the middle result. It is a measure of the centre of the data and is similar to the mean (or average).

Results Cryptosporidium (% Recovery Rate)



Results *Giardia* (% Recovery Rate)

Calculated on Confirmed Counts only

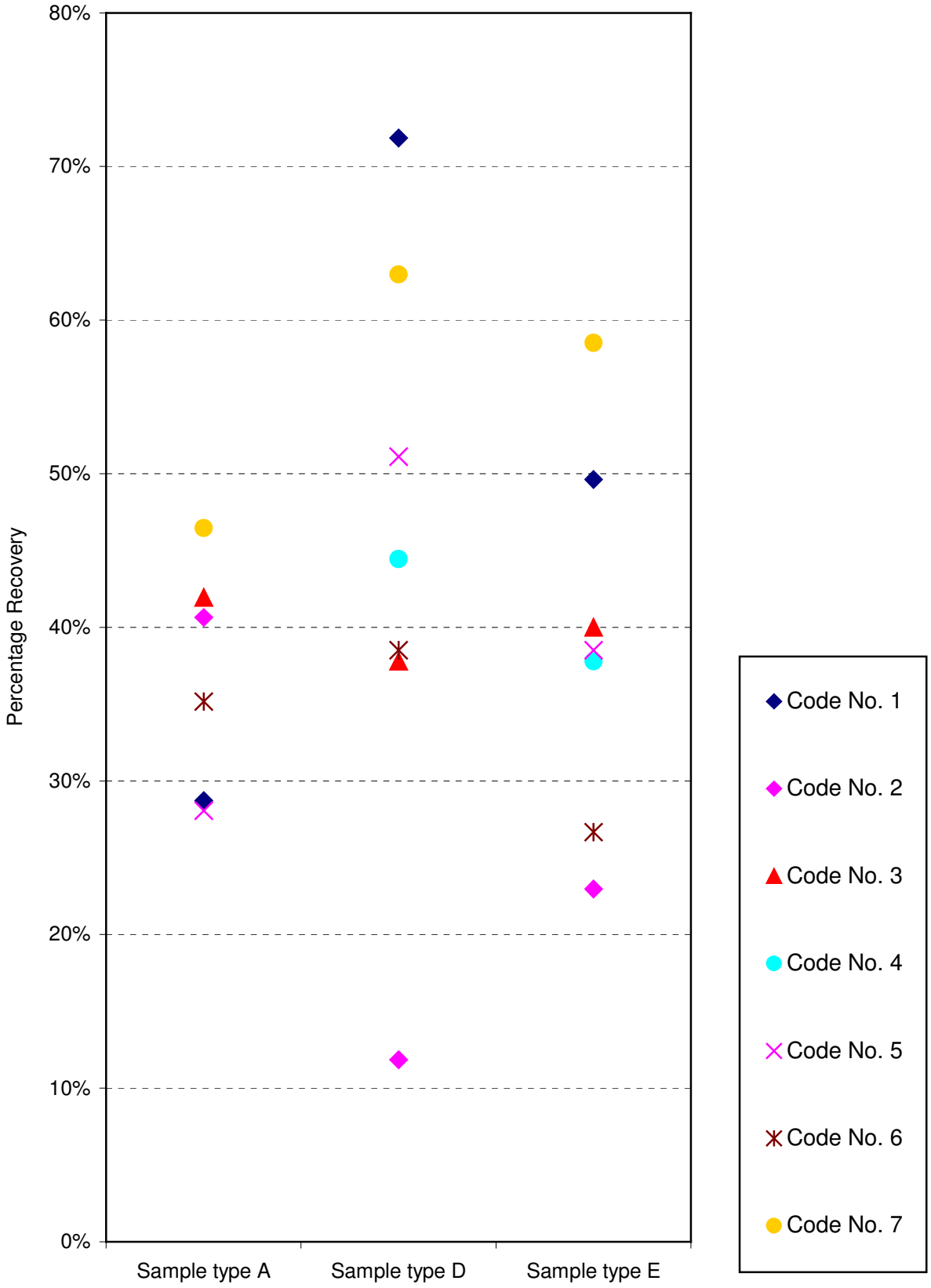
Code No.	Sample type A	Sample type D	Sample type E
1	29%	72%	50%
2	41%	12%	23%
3	42%	38%	40%
4	#	44%	38%
5	28%	51%	39%
6	35%	39%	27%
7	46%	63%	59%
Minimum	28%	12%	23%
Maximum	46%	72%	59%
Average	37%	46%	39%
Median	38%	44%	39%

● Below minimum acceptable recovery rate of 10%.

\# Analytical failure.

The median is provided for information only. It is the middle result. It is a measure of the centre of the data and is similar to the mean (or average).

Results Giardia (% Recovery Rate)



APPENDIX B

Homogeneity Testing
Quality Control
and
Trip Control

Homogeneity Testing

BTF Pty Ltd provided counts for *Cryptosporidium* and *Giardia* on a minimum of 8 sets of randomly selected spiked samples for homogeneity. The counts were performed prior to adding to the reverse osmosis water. This procedure involved the addition of FITC-labelled antibodies to the test tubes and analysing the samples using flow cytometry (Bennett *et al*., 1999. A comparison of enumeration techniques for *Cryptosporidium parvum* oocysts. *Journal of Parasitology*. 85(6):1165-1168).

Results for Samples A & F

Sample No.	*Cryptosporidium* Counts	*Giardia* Counts
A&F1	310	312
A&F2	305	307
A&F3	309	312
A&F4	311	309
A&F5	310	310
A&F6	306	309
A&F7	308	307
A&F8	308	308
No. of Results	8	8
Median	308.5	309.0
Minimum	305	307
Maximum	311	312
Range	6	5
Mean	308.4	309.3
Std Deviation	2.07	1.98
Classic CV	0.67%	0.64%

Results for Sample C

Sample No.	*Cryptosporidium* Counts
C1	90
C2	90
C3	88
C4	91
C5	90
C6	87
C7	87
C8	90

No. of Results	8
Median	90.0
Minimum	87
Maximum	91
Range	4
Mean	89.1
Std Deviation	1.55
Classic CV	1.74%

Results for Sample D

Sample No.	*Giardia* Counts
D1	133
D2	135
D3	134
D4	134
D5	136
D6	133
D7	135
D8	135

No. of Results	8
Median	134.5
Minimum	133
Maximum	136
Range	3
Mean	134.4
Std Deviation	1.06
Classic CV	0.79%

Results for Sample E

Sample No.	*Cryptosporidium* Counts	*Giardia* Counts
E1	135	135
E2	134	132
E3	135	135
E4	135	131
E5	131	136
E6	133	135
E7	134	134
E8	133	134
No. of Results	8	8
Median	134.0	134.5
Minimum	131	131
Maximum	135	136
Range	4	5
Mean	133.8	134.0
Std Deviation	1.39	1.69
Classic CV	1.04%	1.26%

It was determined that an appropriate CV for homogeneity data in this program is less than 5%. Since the CV's for all samples in this round are less than 5%, it was concluded that the samples are homogenous.

Quality Control

As a quality control check, BTF Pty Ltd randomly selected and analysed samples from each of the spike material type to determine the percent DAPI positive (refer to Appendix D for the protocol). The internal structures of the seeds were analysed by the flow cytometry method to determine the percentage full, percentage partially empty and the percentage empty.

Samples A & F

DAPI Staining	
Cryptosporidium percent positive	100%
Giardia percent positive	100%
Internal Structures (Percent full)	
Cryptosporidium	99.80%
Giardia	100%

Sample C

DAPI Staining	
Cryptosporidium percent positive	100%
Internal Structures (Percent full)	
Cryptosporidium	99.80%

Sample D

DAPI Staining	
Giardia percent positive	100%
Internal Structures (Percent full)	
Giardia	100%

Sample E

DAPI Staining	
Cryptosporidium percent positive	100%
Giardia percent positive	100%

Internal Structures (Percent full)	
Cryptosporidium	99.80%
Giardia	100%

In terms of quality control of the samples, it was determined that an appropriate percentage of full internal structures and DAPI positive seeds is >95%. Since these percentages are greater than 95% for this round, the samples are considered to be of an acceptable quality.

Trip Control

Sample type F was used as the trip control.

BTF Pty Ltd kept a 50mL concentrate sample type F on their premises after preparation. The sample was added to 10 Litres of distilled water and was concentrated and analysed on 24.03.06.

One nominated laboratory (Code 7) was provided with a 50mL concentrate sample type F and was requested to return the sample to BTF Pty Ltd immediately upon receipt. This trip control type F sample was subsequently analysed by BTF Pty Ltd on 31.03.06.

The trip control samples were concentrated using membrane filtration, and then analysed using the Dynal IMS system and epifluorescence microscopy.

Results for Trip Control Sample type F

Date Analysed	*Crypto.* Counts	No. DAPI positive	*Giardia* Counts	No. DAPI positive
24.03.06 (Sample kept on premises)	276	45%	265	95%
31.03.06 (Sample sent to laboratory and returned)	275	50%	272	98%
Actual counts	**310**		**310**	
Sample kept on premises % Recovery Rate	89%		85%	
Sample sent to laboratory and returned % Recovery Rate	89%		88%	

The trip control sent to the laboratory shows the stability of the samples during transport. The percentage recovery rates for the trip control samples lie within the acceptable range of 10% - 110%.

APPENDIX C

Instructions to Participants
and
Results Sheet

Proficiency Testing Australia

Proficiency Testing Program

Cryptosporidium and *Giardia* Round 17

INSTRUCTIONS TO PARTICIPANTS

To ensure that results from this program can be analysed properly, participants are asked to adhere carefully to the following instructions.

1. For this round each participant will be supplied a sample set consisting of five spiked 50mL concentrate water samples. Each sample contains reverse osmosis water which may contain concentrated debris from reservoir water (added to simulate an environmental water sample). The samples may have been spiked with *Cryptosporidium* and / or *Giardia* at various concentrations. In addition some confounding organisms may have been added to some samples.

   Your laboratory may receive an additional 50mL concentrate sample, which will be utilised as the trip control. If you do receive this sample, then refer to the covering letter for further instructions.

2. On receipt, the samples should be refrigerated at 4ºC, and the date and time of receipt should be recorded on the *Results Sheet*.

3. Add each of the concentrates to 10 litres of distilled water, taking care not to mix up the sample vials. Ensure the concentrate sample vial is effectively rinsed and thoroughly dispersed into the 10 litres. You are required to sign the results sheet to declare your laboratory has diluted the concentrate samples to 10 litres.

4. Laboratories should then proceed to analyse the samples by the **routine method**. The samples are to be tested in the order on the *Results Sheet*. 100% of each supplied sample is to be analysed.

   Laboratories should adopt methods to all sorts of water types. Reverse osmosis water is used in US EPA method 1622. If required, phosphate buffer could be added to adjust the pH of the samples.

   PTA is aware of the internal positive control ColorSeed ™, which was developed by BTF Pty Ltd. Although PTA can see the advantage of ColorSeed ™ being used as an internal positive control, laboratories should note that it is not acceptable for the use of ColorSeed ™ in the PTA proficiency testing samples.

5. Record the results for each sample on the *Results Sheet* provided. Laboratories are requested to report both total counts and confirmed counts on the PTA Results Sheet. Please specify the method(s) used for confirmation. Confirmed counts only will be used for analysis by PTA. Do **not** report non-numerical values (ie. less than/greater than values, or detected/not detected), on the PTA *Results Sheet*. Actual counts observed under the microscope are to be reported. The use of conversion (recovery) factors derived from quality control to adjust raw data is not acceptable.

If a laboratory submits more than 1 set of results (for more than 1 set of samples), then they must nominate the set of results they wish to submit for analysis. However, a laboratory must submit the set of results which were obtained from the method that they hold accreditation.

6. Commence testing as soon as possible after samples are received.
   IMPORTANT: All laboratories must return results sheets, summary details of your methodology, routine worksheets and test report, no later than **Thursday 6 April 2006** to:

   Germaine Saric

   Proficiency Testing Australia
   PO Box 7507
   Silverwater NSW 2128.

   phone: +61 2 9736 8397, fax: +61 2 9743 6664

Proficiency Testing Australia

Cryptosporidium and *Giardia* Round 17 - Proficiency Testing Program

Results Sheet

Lab Code: «Cod

Date and Time of Sample Receipt: _____

RESULTS

SAMPLE	% of sample analysed	*Cryptosporidium* Counts		*Giardia* Counts		Date & time of testing
		Total Count	*Confirmed Count*	*Total Count*	*Confirmed Count*	
A						
B						
C						
D						
E						

Methods used to obtain results:

Concentration (eg. Flocculation Method) _____

Purification (eg. IMS) _____

Enumeration (eg. Fluorescent Microscopy) _____

Details of methods used for confirmation _____

I / We declare that our laboratory has diluted the concentrate samples to 10 litres prior to analysis for *Cryptosporidium* and *Giardia*.

Print Name: _____

Signed: _____ **Date:** _____

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APPENDIX D

DAPI staining protocol

D1.1

Quality Control Method for Analysing DAPI staining of *Cryptosporidium* and *Giardia*

Revision 1, 9-2-2000

(Developed by BioTechnology Frontiers – www.biotechfrontiers.com)

Background:

The following procedure is used for assessing the DAPI staining of *Easy*Seed *Giardia* cysts and *Cryptosporidium* oocysts for quality control purposes. DAPI staining is utilised for morphological confirmation of presumptive cysts/oocysts during analytical procedures.

Materials:

1. 4',6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI) (Sigma Aldrich, D 9642).
2. DAPI stock – 2 mg/ml DAPI in methanol. (Stable 6 months at ca. -20°C, cap *tightly* to avoid evaporation)
3. DAPI working solution (2 µg/ml in PBS) (i.e., 10 µl DAPI stock in 10mls PBS) (Stable 2 weeks at <5°C in the dark, avoid freezing)
4. Phosphate buffered saline (PBS) (Oxoid – BR14a), or equivalent (2.7 mM KCl, 137 mM NaCl, 10 mM phosphate buffer, pH 7.4)
5. Distilled H_2O (dH_2O)
6. Filtration Membrane (e.g., Whatman Nucleopore – 13 mm, 8 µm pore-size), or equivalent
7. Vacuum source (supplying 5 bar pressure)
8. 50°C waterbath or incubator
9. Anti-*Cryptosporidium* and anti-*Giardia* specific fluorescent antibodies (BTFRGTXX)
10. Ethanol (100%, absolute)
11. Immuno-fluorescence Mounting medium (BTF)
12. Flat-bladed forceps
13. 13mm Swinnex filtration unit. (Millipore)

Sample treatment:

1. Take standard sample vial and add a volume of 100% ethanol equal to volume in tube, *cap tightly*.
2. Vortex for 10 seconds at medium speed
3. Place tube in 50°C water bath for 1 hour

DAPI staining:

1. Prepare membrane by placing on Swinnex holder using flat-bladed forceps
2. Apply vacuum to membrane (5 bar pressure)
3. Apply sample so it is drawn across the membrane slowly, so as not to lose any sample
4. Add 500 µl ethanol to the tube to rinse, and then add rinse to membrane
5. Turn off vacuum to membrane

6. Add 100 µl DAPI working solution to membrane and stain for 2 min. at room temp.
7. Apply vacuum and draw DAPI through membrane; while vacuum is still applied wash membrane with 500 µl of dH_2O
8. Apply 100 µl of working-strength anti-*Cryptosporidium* and *Giardia* fluorescent mAbs (typically between 2 -10 µg/ml)
9. Stain for 15 mins. at room temp.
10. Apply vacuum and draw across membrane

Mounting Membrane:

1. Place 2 µl mounting medium on slide
2. Place membrane on mounting medium, allow medium to perfuse membrane (ca. 1 min)
3. Place 2 µl mounting medium on coverslip
4. Invert slide with membrane and place with membrane centered on coverslip
5. Read as required (see below)

Microscopy

1. Turn on mercury epifluorescence source at least 5-10 min. prior to microscopy.
2. Ensure microscope optics are adjusted correctly and FITC and DAPI filter cubes are in the filter holders for use.
3. Scan the membrane completely (or *a minimum* 50 each cysts and oocysts) in an up-and-down, or side-to-side fashion under FITC-illumination looking for brightly fluorescent-green objects of typical cyst/oocyst morphology.
4. When a cyst and/or oocysts is located, switch the filter cube to DAPI and examine for the presence of bright-aqua-blue nucleii within the cyst or oocyst. Cysts and oocysts will be scored "positive" or "negative". The criteria for a positive scores are as follows: *Giardia* = 2 nucleii, *Cryptosporidium* = 4 nucleii.
5. Count a minimum of 50 cysts and 50 oocysts, keeping track of scores of positives and total numbers for each. Convert these data to percent positive for both cysts and oocysts as follows: (#Positive/Total#) X 100 = % Positive.

Technical notes:

1. Treatment of Easyseed for 1 hour at 50°C is optimal for DAPI staining of both *Cryptosporidium* and *Giardia*

2. Typically, C&G standard samples will show >95% DAPI positive for both *Cryptosporidium* and *Giardia*.
   If not, inform the laboratory quality manager immediately.

3. Be careful when adding reagents such as mAbs and DAPI to the membrane not to excessively flood the membrane such that trapped particles on the membrane are washed off.

GLOSSARY

Trip Control	A sample used to monitor the trip of the sample set sent to a nominated laboratory.
IMS	Immunomagnetic separation
DAPI	4',6-Diamdine-2'-phenylindile dihydrochloride
DIC	Differential Interference Contrast (Microscopy)
IFA	Immunofluorescence assay
PCR	Polymerised Chain Reaction