REPORT NO. 822

Cryptosporidium and *Giardia*

(Round 31)

Proficiency Testing Program

September 2013

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1. FOREWORD

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

The exercise was conducted in June 2013 by PTA. The Technical Advisor was Professor J Smith, Queensland University of Technology. The Program Coordinator was Ms Y Christie. This report was authorised by Dr M Bunt, PTA Statistician.

The program aim was to assess laboratories' ability to competently detect and report levels of *Cryptosporidium* and *Giardia* (oo)cysts in water.

2. FEATURES OF THE PROGRAM

(a) A total of seven laboratories (five Australia, two New Zealand) received samples, of which all returned results for inclusion in the report.

(b) Participating laboratories were requested to report both total and confirmed count results. Participants were also requested to calculate and report an estimate of measurement uncertainty (MU) for each reported result.

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

(d) In addition to the samples, laboratories were provided with the *Instructions to Participants* and a *Results Sheet* (see Appendix C). Laboratories were instructed to perform the tests according to their routine methods (method most frequently employed). Laboratories were reminded that PTA is aware of the internal positive control ColorSeed™, developed by BTF Pty Ltd. Although PTA can see the advantage of ColorSeed™ as an internal positive control, participants were instructed to note that it is not acceptable for laboratories to adjust results obtained with the PTA proficiency testing samples on the basis of recoveries obtained using ColorSeed™. An exception to this would be if the respective laboratory routine practice/standard operating procedure routinely uses ColorSeed™ as a true internal standard, i.e. addition to *every* sample, *and* correction of observed count using internal standard recovery during routine sample reporting.

(e) The samples for Round 31 were produced in line with EasySeed batch number 482, which are certified reference samples. The preparation of these certified reference samples is considered to have satisfied the homogeneity testing requirements (see Appendix B).

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

3. DESIGN OF THE PROGRAM

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

TABLE A: Round 31 Sample Design

Sample	***Cryptosporidium*** **(Count)**	***Giardia*** **(Count)**	***Amount of QC mud added***
A	110	50	500µL
B	120	140	1000µL
C	140	120	100µL
D	0	90	500µL
E	70	0	500µL
F (Trip control)	120	120	1000µL

Notes for Table A:
1. QC mud was added to these samples to simulate an environmental sample.
2. One nominated laboratory (Code 5) was provided with a sixth sample, as a trip control.

The sample design did not include a blank sample for Round 31.

All samples were added to Milli-Q™ water to make a final volume of approximately 3.5 mL.

Sample preparation

BTF Pty Ltd, NSW, prepared different water concentrate samples for this program, using PTA in-house method *PTPM 11.1 Sample Preparation – Cryptosporidium and Giardia (Version No. 4)*.

Seed samples were prepared on 24 May 2013. Seed samples were dispensed in IsoFlow™ and the sterilisation method was gamma irradiation.

Cryptosporidium parvum (Iowa strain) oocysts were of bovine origin, excreted on 1 May 2013. Oocysts were purified by discontinuous sucrose and caesium chloride gradient centrifugation.

Giardia lamblia (H3 strain) cysts were obtained from experimentally-infected gerbils and were excreted on 9 May 2013. Cysts were purified by sucrose and Percoll™ density gradient centrifugation, followed by water washes.
The seed samples were prepared using flow cytometry and an automated dispensing method. *Cryptosporidium* and *Giardia* (suspended in IsoFlow™ solution) were dispensed into 4 mL tubes.

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

On 24 May 2013 each of the seed samples were spiked with QC mud (see 'Confounding materials' below) and then made up to approximately 3.5 mL with Milli-Q™ water to produce the water concentrate samples sent to participants on 17 June 2013.

Participating laboratories were asked to add each of the water concentrate samples to 10 L of water of their choice prior to analysis. The laboratories were also instructed to take care to ensure that the water used did not contain any cysts or oocysts and could, for example, use reverse osmosis or membrane filtered (suggested pore size ≤ 45 µm) water.

Confounding materials

QC mud:

QC mud was added to all water concentrate samples at a concentration of 100, 500 or 1000 µL QC mud per water concentrate sample (see Table A).

Quality assurance of QC mud

To ensure the QC mud did not contain *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 (EasyStain™ – BTF Pty Ltd), screened and purified using flow cytometry then examined using epifluorescence microscopy. No *Cryptosporidium* oocysts or *Giardia* cysts were found in 10 mL of QC mud.

4. FORMAT OF APPENDICES

Appendix A (A1.1 - A1.8) contains the total count and confirmed count results reported by participating laboratories for each of the five water concentrate samples. Percentage recovery rates and charts are also presented. Please note that recovery rates are calculated using total counts only.

Appendix B contains details of homogeneity testing, quality control and trip control results (B1.1 - B1.2).

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

5. FALSE RESULTS

Results were examined for false-positive and false-negative results with all testing methods pooled. There were no false results for *Cryptosporidium* or *Giardia*.

6. LOW/HIGH RECOVERIES

The acceptable range set for this program is a recovery between 10-110%. This has been determined to be an appropriate acceptability range by technical experts in this area of testing. 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 for *Cryptosporidium.*

TABLE B: *CRYPTOSPORIDIUM* LOW RECOVERY RATES

Test for the enumeration of *Cryptosporidium*	
Sample	Low Recoveries (by laboratory code number)
B	5

No low/high recoveries were seen regarding the analysis of *Giardia* results.

7. PTA AND TECHNICAL ADVISOR'S COMMENTS

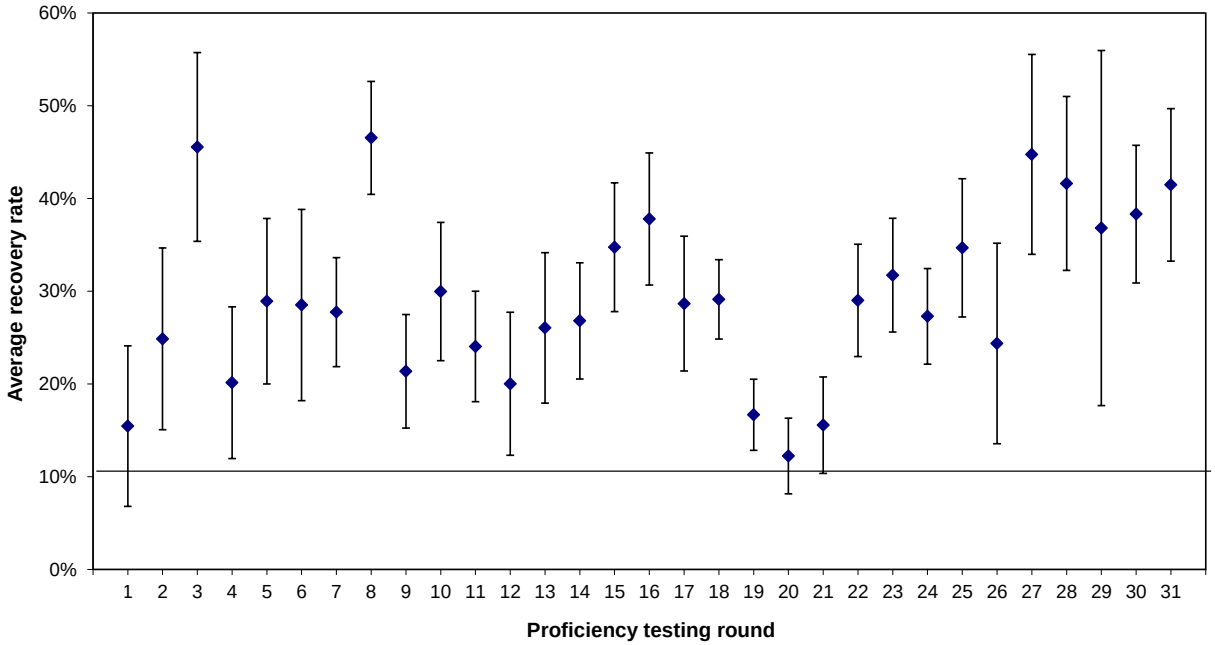
A total of 68 Total Count results were received for this program.

Percentage Recovery Rate

Overall recovery rates for *Cryptosporidium* were lower than for *Giardia*.

Total average *Cryptosporidium* recovery rate (41.5%) has increased compared to the previous round. Figure 1A shows the average percent recovery rate for *Cryptosporidium* for each round (refer to notes on page 6).

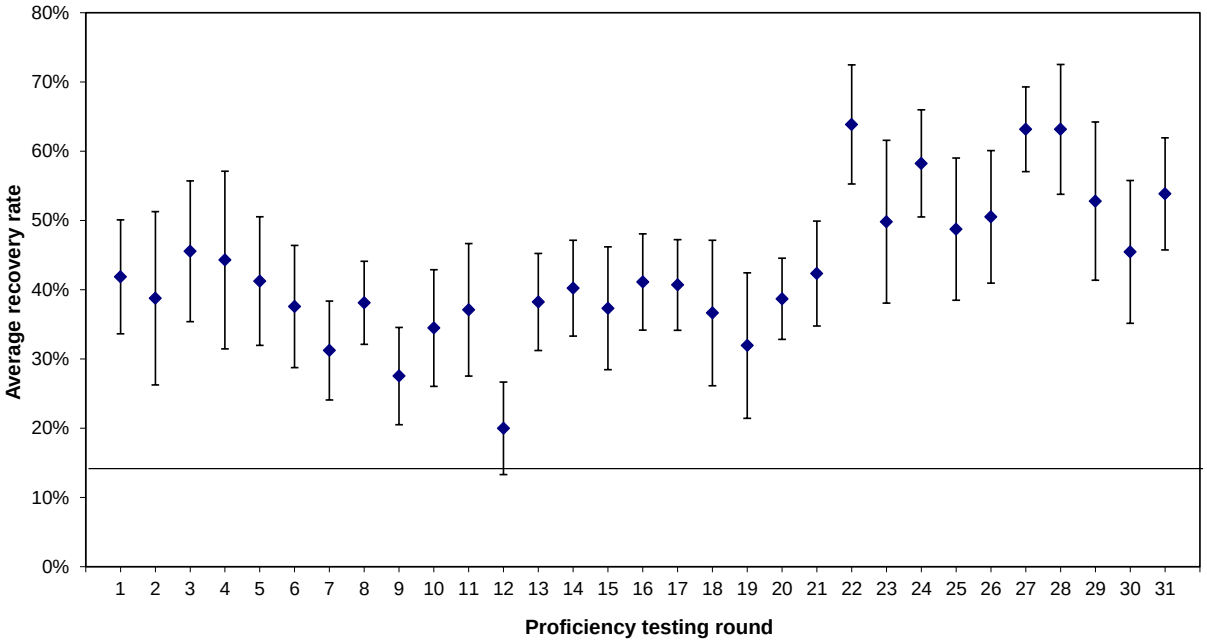
Figure 1A: Comparison of total average recovery rates for *Cryptosporidium*



Some apparent cyclic trending in median *Giardia* recoveries has been observed over successive proficiency testing rounds (see below). For example, generally increasing recoveries from rounds 19-28, but progressively decreasing from rounds 28-30, with the current round results showing an increased recovery rate. Also observed is a decreasing trend from rounds 3-7. Such trends do not appear as clearly for *Cryptosporidium* recoveries. This may be due to methodological changes or lability and/or aging of common batches of critical reagents, such as those incorporating anti-*Giardia* antibodies, for example IMS beads and/or immunofluorescent antibodies. Laboratories may wish to examine their internal positive control recovery to see if similar trends are apparent.

Total average *Giardia* recovery rate (53.8%) has increased in this round when compared to the previous round. The graph below displays this (refer to notes below figure).

Figure 1B: Comparison of total average recovery rates for *Giardia*



Notes to Average Recovery Rates Graphs:

1. The vertical bars in the graphs represent 95% confidence intervals.
2. All rounds, except rounds 1, 2, 3 and 8, contain QC mud (see table on pages 11 through to 14). 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.
3. From Rounds 14-21, average recovery rates are calculated on confirmed counts only. For rounds excluding Rounds 14-21, participants reported either total or confirmed counts, and therefore the average recovery rates presented in this table prior to Round 14 may include both total and confirmed counts. From Round 22 onwards, only total counts are presented.

Measurement Uncertainty (MU) Estimation

Results including MU calculations are presented in two formats, including absolute oocyst numbers (so that participants can compare numeric recoveries against seed doses) and relative % recoveries, as seen in Tables E and F below. These tables and comments are provided for information purposes only, and do not affect the evaluation of participants' results.

TABLE C: *Cryptosporidium* and *Giardia* Round 31 (Oo)cyst Recovery - % Measurement Uncertainty

SAMPLE	ORGANISM	(OO)CYST MEDIAN RECOVERY	STANDARD DEVIATION	RELATIVE STANDARD DEVIATION (RSD - %)	✦MEASUREMENT UNCERTAINTY (± [OO]CYSTS)	REFERENCE COUNT
A	*Giardia*	25	11	44	88	50
	Cryptosporidium	65	27	41	42	110
B	*Giardia*	74	30	41	82	140
	Cryptosporidium	44	28	64	128	120
C	*Giardia*	62	30	48	96	120
	Cryptosporidium	67	28	42	84	140
D	*Giardia*	42	17	41	82	90
E						
	Cryptosporidium	23	13	57	114	70

Notes for Table C:
1. ✦ = All measurement uncertainty values are at the 95% level of confidence.
2. Sample D did not include *Cryptosporidium*.
3. Sample E did not contain *Giardia.*
4. Values with low recoveries have been included.

TABLE D: *Cryptosporidium* and *Giardia* Round 31 Recovery - % Measurement Uncertainty

SAMPLE	ORGANISM	(OO)CYST MEDIAN RECOVERY	STANDARD DEVIATION	RELATIVE STANDARD DEVIATION (RSD - %)	✦MEASUREMENT UNCERTAINTY (RSD-%)	REFERENCE COUNT
A	*Giardia*	50	22	44	89	50
	Cryptosporidium	59	25	22	45	110
B	*Giardia*	53	22	18	36	140
	Cryptosporidium	37	23	17	33	120
C	*Giardia*	52	25	21	41	120
	Cryptosporidium	48	20	14	28	140
D	*Giardia*	47	18	21	41	90
E						
	Cryptosporidium	33	18	26	51	70

Notes for Table D:
1. ✦ = All measurement uncertainty values are at the 95% level of confidence.
2. Sample D did not include *Cryptosporidium*.
3. Sample E did not contain *Giardia.*
4. Values with low recoveries have been included.

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

TABLE E: Comparison of *Cryptosporidium* Oocyst Levels for Each Round

Round	*Cryptosporidium* levels (Counts)	Round	*Cryptosporidium* levels (Counts)
1	50-200	17	90-310
2	50-200	18	50-300
3	50-300	19	50-300
4	110	20	50-200
5	50-200	21	100-200
6	25-75	22	50-300
7	50-100	23	50-250
8	65-140	24	50-200
9	125	25	50-250
10	110-235	26	50-200
11	50-200	27	50-200
12	110-235	28	50-250
13	90-205	29	50-140
14	55-135	30	80-135
15	55-135	31	70-140
16	55-120		

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

TABLE F: Comparison of *Giardia* Cyst Levels for Each Round

Round	*Giardia* levels (Counts)	Round	*Giardia* levels (Counts)
1	50-200	17	135-310
2	50-200	18	150-300
3	50	19	150-300
4	40	20	50-120
5	50-200	21	90-200
6	75-120	22	50-250
7	50	23	50-300
8	65-140	24	50-200
9	55	25	50-250
10	70-85	26	50-250
11	50-200	27	50-200
12	110-125	28	50-200
13	90-145	29	50-150
14	55-200	30	85-150
15	55-200	31	50-140
16	120-255		

Method Commentary

- *Analysis of Results by Method Groups*

In order for methods to be grouped for analysis, PTA requires at least 11 sets of results from the same method group. As there were less than 11 results submitted for each method, reliable conclusions cannot be drawn from analysing grouped methods on this occasion. Therefore, results from all method groups have been pooled for analysis.

Of the seven laboratories returning results, all provided details of the methods used to determine their results. Of these seven laboratories, two used membrane filtration as the method of concentration. Three laboratories used centrifugation (laboratories 1, 2 and 5). Laboratory 4 used Envirocheck, whilst laboratory 7 used $CaCO_3$ floculation.

Of the seven laboratories, four laboratories indicated the use of cartridges for their filtration method, whilst one laboratory reported the use of Sponge filtration and one used Flat Bed filtration. All laboratories used IMS as their purification method and fluorescent microscopy as the enumeration method. All seven laboratories reported the use of DAPI staining as the confirmation method, whilst two laboratories reported the additional use of DIC and FITC 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

Table G that appears on pages 11-14 illustrates the comparison of overall laboratory performance for rounds 1 - 31.

There was one low recovery for *Cryptosporidium* (laboratory 5, sample B) reported for this round. There were lower recoveries of *Cryptosporidium* compared to *Giardia*. Recoveries of < 10% for *Cryptosporidium* suggest significant non-conformance requiring corrective actions.

The low *Cryptosporidium* recovery obtained by laboratory 5 may have resulted from one of several sources. Failure to add *Cryptosporidium* IMS beads, or the status of associated reagents are possibilities. Laboratory code 5 uses the most common (4/7) bulk water concentration technique of the participating laboratories (cartridge filtration). It is recommended this laboratory investigate sources of error and possible oocyst losses/failure to stain during sample processing; including filter elution, IMS and IFA staining (if on slides, particularly associated wash steps) as applicable.

Pooled laboratory data indicated broad range of median recoveries for each measurand; 8-79%-*Cryptosporidium* and 22-92% *Giardia*. However, recoveries are generally higher than those obtained in PTA round 30 (19-42%-*Cryptosporidium*, 38-56% *Giardia*) with substantially higher maximum recoveries. These results are typical of recoveries obtained using the methods employed. Overall, the median recoveries of *Cryptosporidium* oocysts and *Giardia* cysts were higher than the previous round (round 30).

For equivalent matrix amounts (samples A and E; 0.5 mL) but different *Cryptosporidium* doses (110, 70, respectively) median recoveries were 59% and 33%, respectively, with higher dose showing slightly higher median recovery. For equivalent matrix amounts (samples A and D; 0.5 mL) but different *Giardia* doses (50, 90, respectively) median recoveries were similar at 50% and 47%. Highest variability in median recoveries were 89% RSD for *Giardia* in sample A and 51% RSD for *Cryptosporidium* in sample E (assessed using % RSD MU.) Variability in median recoveries was generally greater for *Giardia* than *Cryptosporidium*.

Laboratories 1 and 6 obtained substantially lower confirmation of (oo)cysts than other participants as follows:

- laboratory code 1 all samples, ca. 47% DAPI (+) *Giardia*.
- laboratory code 6, samples A and C, ca. 60 and 20% DAPI (+) *Cryptosporidium*, respectively.

All facilities used DAPI staining for confirmation, including laboratories 1 and 6. Some laboratories experience issues with low percentages of DAPI stained cysts and oocysts. Several methods indicate use of heat or acid for both dissociation of (oo)cysts from IMS beads, as well as permeabilisation for subsequent DAPI staining.

Measurement Uncertainty (MU)

Six of the seven participating laboratories provided MU information with their results. Laboratory 5 is encouraged to report MU values in future rounds.

Recoveries found to be “close”, yet lower than the acceptable minimum recovery of 10% should be evaluated in light of the sample dose MU (see page B1.1). In this case Laboratory 5 had low *Cryptosporidium* recovery (8%) for sample B (120 ± 3) when the mean dose of 120 is used in recovery calculation. There is, however, a 95% probability the true value could be 120 ± 3 (range 117-123). However, taking dose uncertainty into consideration, there is *not* a significant probability the recovery reported might fall within the acceptable range. In the case above, the laboratory 5, sample B recovery of 9/117 = 8% is outside the acceptable range. This result does *not* have a significant probability of falling within the acceptable range and should warrant investigation/corrective action(s) by the respective laboratory.

TABLE G: Overall Laboratory Performance

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. add to 10 Litres distilled water	8.2%	4	6.4%	5
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. add to 10 Litres distilled water & 10 Litres - RO water + QC mud	2.3%	1	3.5%	2

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
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. add to 10 Litres distilled water	1.3%	1	5.0%	2
16	Concentrate samples - QC mud - Labs. add to 10 Litres distilled water	0.0%	0	3.3%	2
17	Concentrate samples - QC mud - Labs. add to 10 Litres distilled water	1.5%	1	2.9%	1
18	Concentrate samples - QC mud - Labs. add to 10 Litres water	0.0%	0	0.0%	0
19	Concentrate samples - QC mud - Labs. add to 10 Litres water	6.0%	1	11.4%	1
20	Concentrate samples - QC mud - Labs. add to 10 Litres water	10.0%	4	7.1%	3

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
21	Concentrate samples - QC mud - Labs. add to 10 Litres water	5.4%	1	10.7%	2
22	Concentrate samples - QC mud - Labs. add to 10 Litres water	1.4%	1	1.4%	1
23	Concentrate samples - QC mud - Labs. add to 10 Litres water	0.0%	0	1.7%	1
24	Concentrate samples - QC mud - Labs. add to 10 Litres water	1.4%	1	0.0%	0
25	Concentrate samples - QC mud - Labs. add to 10 Litres water	0.0%	0	0.0%	0
26	Concentrate samples - QC mud - Labs. add to 10 Litres water	1.4%	1	4.3%	2
27	Concentrate samples - QC mud - Labs. add to 10 Litres water	0.0%	0	0.0%	0
28	Concentrate samples - QC mud - Labs. add to 10 Litres water	0.0%	0	3.3%	1

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
29	Concentrate samples - QC mud - Labs. add to 10 Litres water	10.0%	2	18.8%	3
30	Concentrate samples - QC mud - Labs. add to 10 Litres water	2.5%	1	3.75%	3
31	Concentrate samples - QC mud - Labs. add to 10 Litres water	0.0%	0	1.4%	1

Notes for Table G:
1. RO = reverse osmosis.
2. * = 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.

Conclusions

Variable results were reported for Round 31, with a decreased number of low recoveries and false results reported when compared to previous rounds.

Laboratories reporting low recoveries should perform corrective actions to investigate sources of error, which may include possible oocyst losses during sample processing; including filter elution, IMS and IFA staining (if on slides, particularly associated wash steps), and/or failure to IFA stain.

8. REFERENCE

[1] *Guide to Proficiency Testing Australia, 2012.*

APPENDIX A

Summary of Results

Results *Cryptosporidium* (total counts)

REFERENCE COUNTS	110	120	140	0	70	
Lab Code No.	**Sample A**	**Sample B**	**Sample C**	**Sample D**	**Sample E**	**Method Codes**
Code 1 - Total Count	23	50	65	0	23	3, 6, 7, 8, 9
Confirmed Count	20	49	61	-	18	
MU	19-27, n=102	41-60, n=102	54-78, n=102	#	19-27, n=102	
Code 2 - Total Count	65	44	78	0	37	1, 6, 7, 8, 9, 10
Confirmed Count	54	37	65	0	34	
MU	+/- 35 n=6	+/- 35, n=7	+/-42, n=8	-	+/-36, n=9	
Code 3 - Total Count	70	85	110	0	37	2, 7, 8, 9
Confirmed Count	68	69	108	-	34	
MU	70±26 oocysts at 95%Cl (600)	85±31 oocysts at 95% Cl (600)	110+/-41 oocysts at 95% Cl (600)	-	37±14 oocysts at 95% Cl (600)	
Code 4 - Total Count	26	15	26	0	12	3, 7, 8, 9
Confirmed Count	18	8	19	-	7	
MU	10% / 25	10% / 25	10% / 25	10% / 25	10% / 25	
Code 5 - Total Count	22	9	39	-	14	3, 6, 7, 8, 9
Confirmed Count	21	7	38	-	13	
MU	-	-	-	-	-	
Code 6 - Total Count	74	32	79	0	15	3, 7, 8, 9
Confirmed Count	47	23	12	0	10	
MU	27 - 190, n=17	17 - 120, n=17	41 - 290, n=17	n=17	8 - 85, n=17	
Code 7 - Total Count	84	72	67	0	45	5, 7, 8, 9, 10
Confirmed Count	84	72	67	0	45	
MU	RSD = 3.9%, n=>500	RSD = 3.9%, n=>500	RSD = 3.9%, n=>500	RSD = 3.9%, n=>500	RSD = 3.9%, n=>500	

Note:
1. A "-" indicates that no result was returned for this sample/test.

Summary Statistics for *Cryptosporidium* (total counts)

	Sample A	Sample B	Sample C	Sample D	Sample E
No. of Results	7	7	7	6	7
Minimum	22	9	26	0	12
Maximum	84	85	110	0	45
Average	52	44	66	0	24
Median	65.0	44.0	67.0	0.0	23.0
SD	27.1	28.0	27.6	0.0	12.5

Summary Statistics for *Cryptosporidium* (total counts) with outliers removed (for information purposes)

	Sample A	Sample B	Sample C	Sample D	Sample E
No. of Results	7	6	7	6	7
Minimum	22	15	26	0	12
Maximum	84	85	110	0	45
Average	52	50	66	0	24
Median	65.0	47.0	67.0	0.0	23.0
SD	27.1	30.0	27.6	0.0	12.5

Note:
1. Please note that outlier results have been removed when calculating these statistics. The above is presented for information purposes only.

Method Codes

Analysis	**Method used to obtain results**	**Code**
Concentration	Filtration (Sponge)	1
	Filtration (Flat Bed)	2
	Filtration (Cartridge)	3
	Filtration (Tangential Flow)	4
	Flocculation	5
	Centrifugation	6
Purification	IMS	7
Enumeration	Immunofluorescence Microscopy	8
Confirmation	DAPI Staining	9
	DIC Microscopy	10
Methods not defined	-	11

Results *Giardia* (total counts)

REFERENCE COUNTS	50	140	120	90	0	
Lab Code No.	**Sample A**	**Sample B**	**Sample C**	**Sample D**	**Sample E**	**Method Codes**
Code 1 - Total Count	46	115	89	69	0	3, 6, 7, 8, 9
Confirmed Count	25	47	40	32	-	
MU	40-53, n=102	99-133, n=102	77-103, n=102	59-80, n=102	#	
Code 2 - Total Count	25	52	62	55	0	1, 6, 7, 8, 9, 10
Confirmed Count	25	50	57	49	0	
MU	+/- 28, n=6	+/- 33, n=7	+/- 36, n=8	+/- 34, n=9	-	
Code 3 - Total Count	43	116	103	64	0	2, 7, 8, 9
Confirmed Count	42	94	101	27	-	
MU	43±18 cysts at 95% Cl (600)	94±39 cysts at 95% Cl (600)	103+/-42 cysts at 95% Cl (600)	103+/-42 cysts at 95% Cl (600)	-	
Code 4 - Total Count	18	38	27	30	0	3, 7, 8 ,9
Confirmed Count	14	33	20	30	-	
MU	18% / 26	18% / 26	18% / 26	18% / 26	18% / 26	
Code 5 - Total Count	30	74	70	28	-	3, 6, 7, 8, 9
Confirmed Count	15	26	32	8	-	
MU	-	-	-	-	-	
Code 6 - Total Count	19	84	42	42	0	3, 7, 8, 9
Confirmed Count	12	62	23	34	0	
MU	12 - 52, n=17	53 - 230, n=17	26 - 120, n=17	26 - 120, n=17	8 - 55, n=17	
Code 7 - Total Count	25	62	26	36	0	5, 7, 8, 9, 10
Confirmed Count	21	62	16	34	0	
MU	RSD=5.7%, n=>500	RSD=5.7%, n=>500	RSD=5.7%, n=>500	RSD=5.7%, n=>500	RSD=5.7%, n=>500	

Note:
1. A "#" indicates that no result was returned for this sample/test.

Summary Statistics for *Giardia* (total counts)

	Sample A	Sample B	Sample C	Sample D	Sample E
No. of Results	7	7	7	7	6
Minimum	18	38	26	28	0
Maximum	46	116	103	69	0
Average	29	77	60	46	0
Median	25.0	74.0	62.0	42.0	0.0
SD	11.1	30.0	29.9	16.5	0.0

Method Codes

Analysis	**Method used to obtain results**	**Code**
Concentration	Filtration (Sponge)	1
	Filtration (Flat Bed)	2
	Filtration (Cartridge)	3
	Filtration (Tangential Flow)	4
	Flocculation	5
	Centrifugation	6
Purification	IMS	7
Enumeration	Immunofluorescence Microscopy	8
Confirmation	DAPI Staining	9
	DIC Microscopy	10
Methods not defined	-	11

Summary of Percentage Recovery Rates and Charts

Results *Cryptosporidium* (% Recovery Rate)

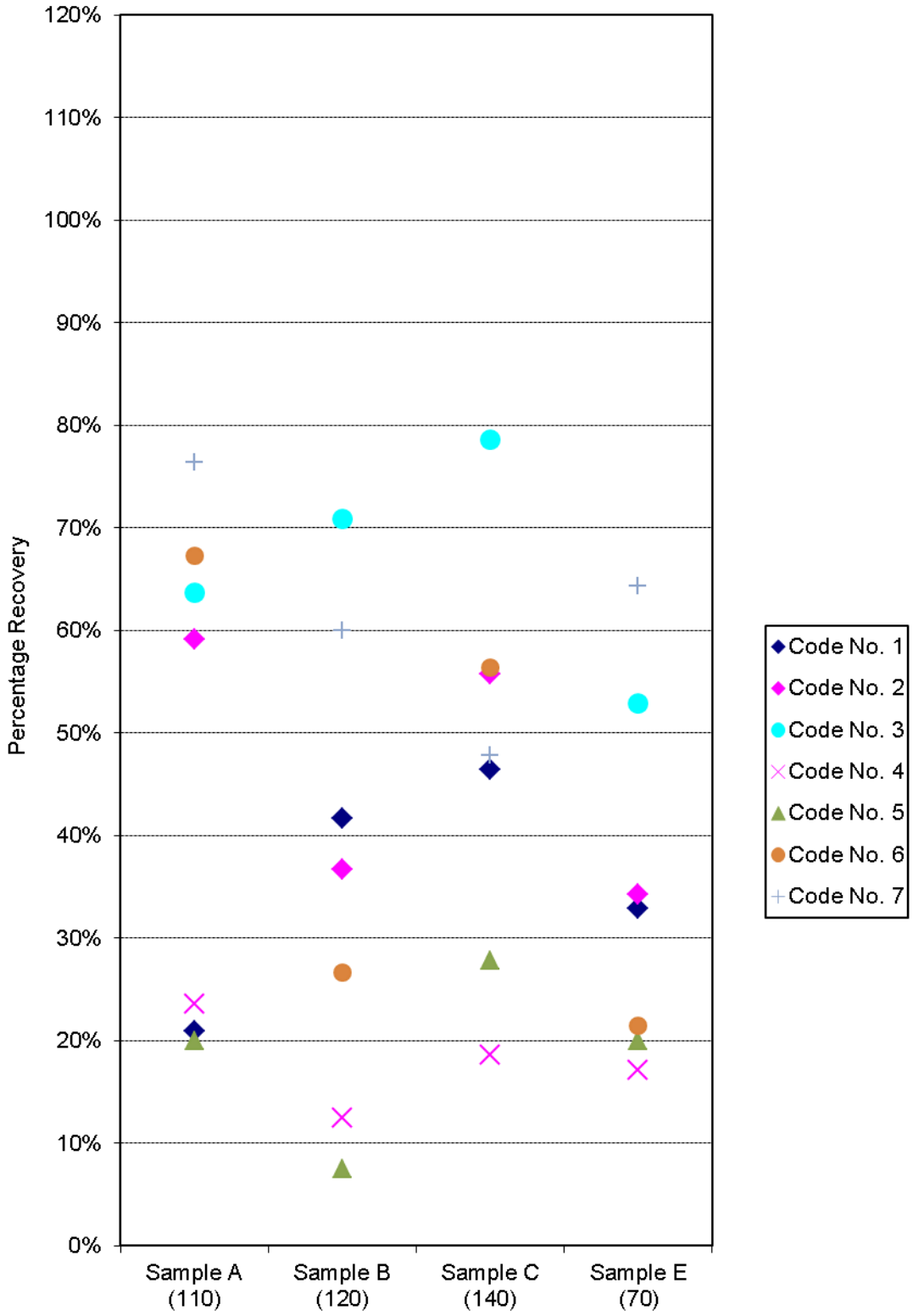
Calculated on TOTAL Counts only

Code No.	Sample A	Sample B	Sample C	Sample E
1	21%	42%	46%	33%
2	59%	37%	56%	34%
3	64%	71%	79%	53%
4	24%	13%	19%	17%
5	20%	8% ₸	28%	20%
6	67%	27%	56%	21%
7	76%	60%	48%	64%
Minimum	20%	8%	19%	17%
Maximum	76%	71%	79%	64%
Average	47%	37%	47%	35%
Median	59%	37%	48%	33%

Notes:

1. The acceptable percentage recovery rate range is 10-110%.
2. A "₸" indicates a result outside of the acceptable recovery rate of 10-110%.
3. 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), however, is less subject to extreme results.

Results *Cryptosporidium* (% Recovery Rate)



Note:
1. *Cryptosporidium* reference count included in brackets alongside corresponding sample name.

Results *Giardia*
(% Recovery Rate)

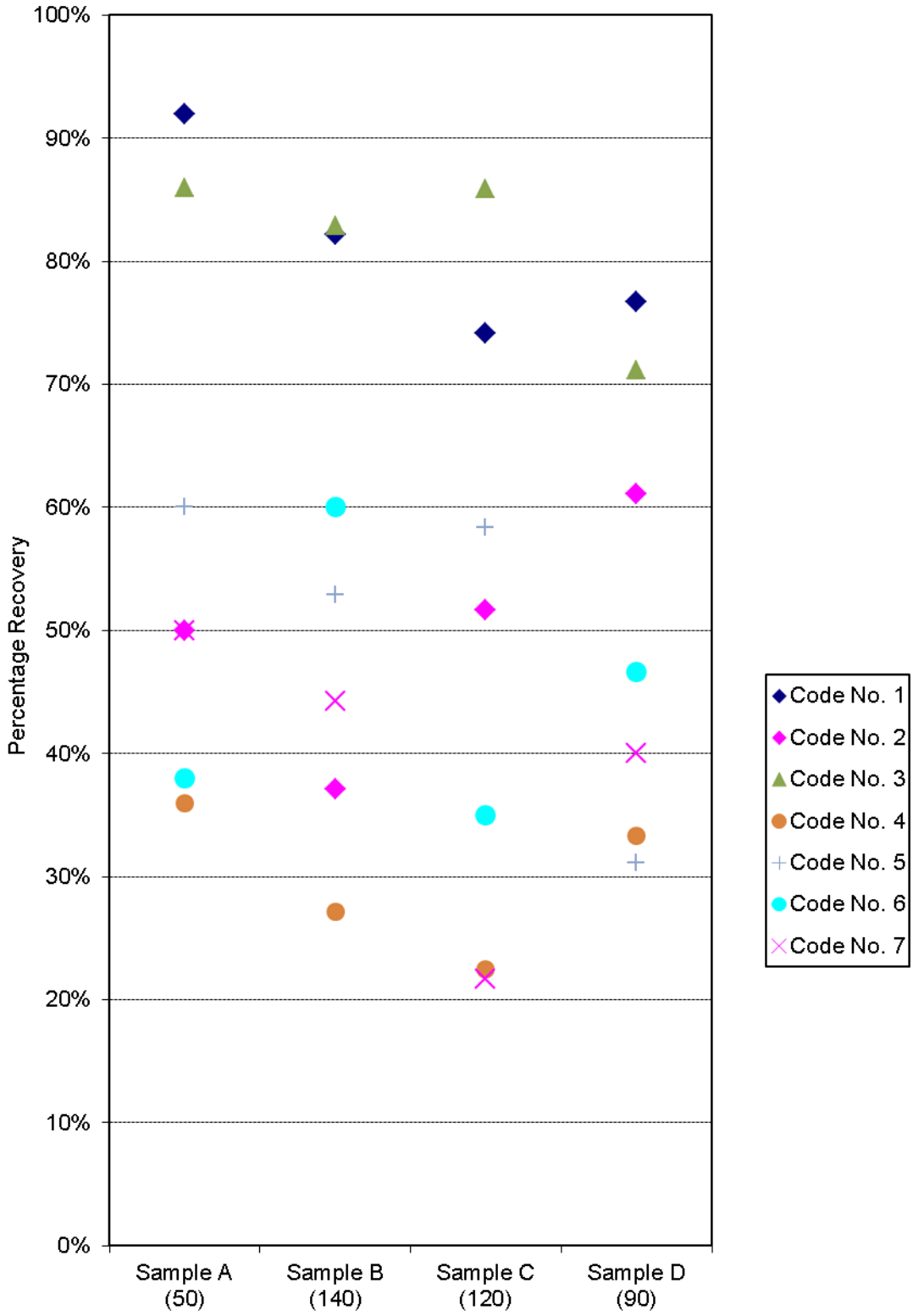
Calculated on TOTAL Counts only

Code No.	Sample A	Sample B	Sample C	Sample D
1	92%	82%	74%	77%
2	50%	37%	52%	61%
3	86%	83%	86%	71%
4	36%	27%	23%	33%
5	60%	53%	58%	31%
6	38%	60%	35%	47%
7	50%	44%	22%	40%
Minimum	36%	27%	22%	31%
Maximum	92%	83%	86%	77%
Average	59%	55%	50%	51%
Median	50%	53%	52%	47%

Notes:

1. The acceptable percentage recovery rate range is 10-110%.
2. 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), however, is less subject to extreme results.

Results *Giardia* (% Recovery Rate)



Note:
1. *Giardia* reference count included in brackets alongside corresponding sample name.

APPENDIX B

Homogeneity Testing
and
Trip Control

Homogeneity Testing

The samples were produced in line with EasySeed batch number 482, which are certified reference samples. The preparation of these certified reference samples is considered to have satisfied the homogeneity testing requirements.

An estimate of uncertainty, expressed as Relative Standard Deviation (RSD), for each organism for the *Cryptosporidium* and *Giardia* proficiency testing program was calculated for each dose within the sample set. These are presented in the table below:

TABLE H: Relative Standard Deviation for Various Sample Doses (Round 31)

ORGANISM	DOSE	RSD (%)	MU % as RSD	Resultant dose with absolute uncertainty
Cryptosporidium	70	0.6	1.2	70 ± 1
Cryptosporidium	110	1.2	2.4	110 ± 3
Cryptosporidium	120	1.2	2.4	120 ± 3
Cryptosporidium	140	1.4	2.7	140 ± 4
Giardia	50	0.4	0.8	50 ± 1
Giardia	90	2.0	4.0	90 ± 4
Giardia	120	2.0	4.0	120 ± 5
Giardia	140	2.0	4.1	140 ± 6

Notes for Table H:

1. Historical QC data and homogeneity testing data have been used to calculate the information in the above table.
2. * = All measurement uncertainty estimates are at the 95% level of confidence.
3. All numbers have been rounded to whole numbers. Although it may appear that the "MU as RSD is always 2 x RSD%" rule has been ignored, the rule itself ignores the impact of the continuous data used to calculate each value (the impact of rounding up/down).

Trip Control

Water concentrate sample F, spiked with 120 *Cryptosporidium* oocysts and 120 *Giardia* cysts was used as the trip control.

BTF Pty Ltd retained a 3.5 mL water concentrate sample F (F_{NoT}), on their premises after preparation. Sample F_{NoT} was added to 10 L of distilled water, concentrated and analysed on 9 July 2013.

One nominated laboratory (Code 5) was provided with a 3.5 mL water concentrate sample F (F_T) and was requested to return the sample to BTF Pty Ltd immediately upon receipt. Sample F_T was subsequently added to 10 L of distilled water and analysed by BTF Pty Ltd on 9 July 2013. Trip control samples were concentrated using membrane filtration, and then analysed using the Dynal IMS system and epifluorescence microscopy.

Results for Control Samples F_{NoT} , F_T

Date Analysed	*Crypto.* Counts	No. DAPI positive	*Giardia* Counts	No. DAPI positive
9 July 2013 (Sample kept on premises)	75	98%	103	86%
9 July 2013 (Sample sent to laboratory and returned)	49	98%	112	94%
Actual counts	**120**		**120**	
F_{NoT} % Recovery Rate	63%		86%	
F_T % Recovery Rate	41%		93%	

The trip control sent to the laboratory indicated sample stability during transport. Percentage recovery rates for 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 31

INSTRUCTIONS TO PARTICIPANTS

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

1. For this round each participant will be supplied with a sample set consisting of five 3.5 mL bulk water concentrate samples. Each sample contains reverse osmosis water which may contain matrix materials from reservoir water (added to simulate an environmental water sample). Samples may have been spiked with *Cryptosporidium* and/or *Giardia* at various concentrations. In addition, confounding organisms may have been added to some samples.

   Your laboratory *may* receive an additional 3.5 mL bulk water concentrate sample which will be utilised as the proficiency testing program trip control. If you receive this sample (labelled PTA Sample F), refer to the associated covering letter for further instructions.

2. On receipt at your facility, samples should be refrigerated at 1-8ºC. The date and time of sample receipt *must* be recorded on the *Results Sheet.*

3. Mix the 3.5 mL tube by inversion then immediately place the bottom of the tube on a vortex and mix such that the vortex extends to the bottom of the tube. Add each of the 3.5 mL bulk water concentrates to individual, respective 10 L water samples of your choice, taking care not to mix-up the order of the sample vials in relation to their respective 10 L water samples. Ensure the water used does not contain any *Giardia* cysts or *Cryptosporidium* oocysts. For example, use reverse osmosis or membrane-filtered (suggested pore size ≤ 45 µm) water. Ensure the bulk water concentrate sample vial is effectively rinsed and thoroughly dispersed into the 10 L bulk water. The following rinse procedure is recommended to ensure optimal sample transfer:
   - **i)** Carefully add the contents of the proficiency testing sample (bulk water concentrate) tube to respective 10 L water samples.
   - **ii)** Add 3 mL 0.05% (v/v) Tween 20* to the empty sample tube, recap and vortex for 20 sec. Empty contents into the 10 L water sample.
   - **iii)** Add 3 mL reagent grade water to the empty sample vial, recap and vortex for 20 seconds. Empty contents into 10L water sample.
   - **iv)** Repeat steps ii)-iii).

   *Laureth-12 Envirocheck® elution buffer or other Tween-containing solutions for rinsing filters may be used to rinse tubes if preferred.

4. A Senior QA/QC Officer (or similar) must sign the results sheet to declare your laboratory has diluted the concentrate samples to 10 L.

5. Laboratories must then proceed to analyse the 10 L samples using their **routine method** (method most frequently employed). Samples are to be tested in the respective order on the *Results Sheet*. One-hundred percent (100%) of each sample supplied must be analysed. Participants are advised that analytical methods used will be noted in the final report. To allow

for confidential treatment of your results in the final report, your facility has been allocated a code number, which appears on your *Results Sheet.*

PTA is aware of the internal positive control ColorSeed™, developed by BTF Pty Ltd. Although PTA understands the advantage of ColorSeed™ use as an internal positive control, laboratories should note that it is not acceptable for laboratories to adjust results obtained with the PTA proficiency testing samples on the basis of recoveries obtained using ColorSeed™ unless the respective laboratory routine practice/standard operating procedure uses ColorSeed™ as a true internal standard, i.e. addition to *every* sample, *and* correction of observed count using internal standard recovery during routine sample reporting.

6. Record the results for each sample on the *Results Sheet* provided. Participants must report both *Total* and C*onfirmed* Counts on the PTA *Results Sheet* and specify the method(s) used for confirmation. **Please be advised** that PTA uses Total Counts (rather than Confirmed Counts) in data analysis. Participants must **not** report non-numerical values (i.e. less than/greater than values, presence/absence, detected/not detected etc) on the PTA *Results Sheet*. Actual counts observed under the microscope must be reported. Participants must not use conversion (recovery) factors derived from quality control to adjust raw data.

7. Participants are requested to calculate and report an estimate of **measurement uncertainty (MU)** for each reported Total Count result. All MU must be reported as a 95% confidence interval (coverage factor $k \approx 2$). Estimates must be reported as either relative (% RSD – e.g. 100 +/- 10% [oo/cysts] at 95% CI) or absolute (e.g. 100 +/- 10 [oo/cysts] at 95% CI) including the number (*n*) of determinations used to generate the MU estimate.

8. Commence testing as soon as possible after samples are received. IMPORTANT: All participants must return *Results Sheets* no later than **Monday 24 June 2013** to:

Yvette Christie
Proficiency Testing Australia
PO Box 7507
Silverwater NSW 2128.

phone: +61 2 9736 8397, fax: +61 2 9743 6664, email: yvette.christie@pta.asn.au

Proficiency Testing Australia

Cryptosporidium and *Giardia* Round 31 - Proficiency Testing Program

Results Sheet

Laboratory Code: ☐

Date / Time of Sample Receipt: _____

Condition of Shipment Upon Receipt: _____

Sample	*Cryptosporidium* Counts			*Giardia* Counts			Date & time of testing
	Total Count	MU and **n*	*Confirmed Count*	*Total Count*	MU and **n*	*Confirmed Count*	
A							
B							
C							
D							
E							

n – number of determinations used to generate MU estimate.

Methods used:

Concentration (e.g. Flocculation Method) _____

Filtration Method (please tick): Sponge ☐ Flat Bed ☐ Cartridge ☐ Tangential Flow ☐

Purification (e.g. IMS) _____

Enumeration (e.g. Microscopy) _____

Details of confirmation methods _____

Please be advised that methods used to obtain results will be noted in the final report.

Print Name: _____ **Date:** _____

Signed: _____ **(Analyst/s)**

I confirm that the concentrate was added to 10L of water prior to analysis.

Print Name: _____ **Date:** _____

Signed: _____ **(Senior QA/QC Officer or similar)**

Return no later than **Monday 24 June 2013**, to:

Yvette Christie
Proficiency Testing Australia, PO Box 7507, Silverwater NSW 2128.
Email: yvette.christie@pta.asn.au, Phone: +61 2 9736 8397, Fax: +61 2 9743 6664

GLOSSARY

Trip Control	A sample used to monitor the effect(s) of sample set transport. Sent to a nominated laboratory and returned.
Seed Sample	Sample containing *Cryptosporidium* oocysts and/or *Giardia* cysts in various doses, prior to dispensing into the PTA sample container.
Water Concentrate Sample	Final proficiency testing sample, containing *Cryptosporidium* oocysts and/or *Giardia* cysts, QC mud and Milli-Q water.
IMS	Immunomagnetic separation
DAPI	4',6-diamidino-2-phenylindole
DIC	Differential Interference Contrast (Microscopy)
IFA	Immunofluorescent Antibody
FITC	Fluorescein isothiocyanate

---------- End of report ---------