REPORT NO. 1169

Cryptosporidium and *Giardia*

(Round 44)

Proficiency Testing Program

February 2020

ACKNOWLEDGMENTS

PTA wishes to gratefully acknowledge the technical assistance provided for this program by J Smith. This assistance included providing input into the design of the program and the technical commentary for the report. Further appreciation is extended to Ms B Ferrari of the School of Biotechnology and Bimolecular Sciences, University of New South Wales, for the supply of QC mud and to Ms L Millican and the staff at BioPoint Pty Ltd for sample preparation, distribution and homogeneity testing.

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CONTENTS

1. FOREWORD

This report summarises the results of the forty-forth round of a planned series of proficiency testing rounds involving the analysis of water samples for the detection and enumeration of *Cryptosporidium* and *Giardia*. This program is accredited to ISO/IEC 17043:2010 "*Conformity assessment - General requirements for proficiency testing"* by International Accreditation New Zealand (IANZ).

The proficiency round was conducted in October 2019 by Proficiency Testing Australia (PTA). The Technical Adviser was J Smith. The Program Coordinator was Mrs Y Christie. This report was authorised by Mrs K Cividin, PTA Quality Manager.

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 four laboratories (two from Australia and two from 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's 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 44 were produced in line with EasySeed™ batch number 683, which are certified reference samples. The preparation of these certified reference samples is considered to have satisfied the homogeneity testing requirements (see Appendix B).

(e) 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 44 Sample Design

Sample	***Cryptosporidium*** **(Count)**	***Giardia*** **(Count)**	***Amount of QC mud added***
A	0	100	500 µL
B	60	60	150 µL
C	160	140	250 µL
D	140	120	100 µL
E	90	0	200 µL
F (Trip control)	70	80	50 µL

Notes for Table A:
1. QC mud was added to samples to simulate an environmental sample.
2. One nominated laboratory (Code 8) was provided with F, as trip control.

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

Sample preparation

BioPoint 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. 6)*.

Seed samples were prepared on 10 October 2019. Seed samples were dispensed in IsoFlow™ and the sterilisation method was gamma irradiation.

Cryptosporidium parvum (Iowa strain) oocysts were of bovine origin, excreted on 29 September 2019. 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 26 September 2019. 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 11 October 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 28 October 2019.

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 was added to selected water concentrate samples at a concentration of 50, 100, 250 or 500 µL 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 (2 ml packed pellet analysed by IMS-IFA in 0.5 mL aliquots), and particulates characterised and quantified using microscopic particulate analysis (USEPA 1996).

4. FORMAT OF APPENDICES

Appendix A (A1.1 - A1.3) contains the total count and confirmed count results reported by participating laboratories for each of the four water concentrate samples. Percentage recovery rates and charts are also presented (A1.4 - A1.9). 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 the *Instructions to Participants* and the *Results Sheet* (C1.1 – C1.3).

5. FALSE RESULTS

Results were examined for false positive and false negative results with all testing methods pooled. No false results were reported 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.

No *Cryptosporidium* or *Giardia* results were outside the acceptable recovery range.

7. PTA AND TECHNICAL ADVISER'S COMMENTS

A total of 38 Total Count results were received for this program.

Percentage Recovery Rate

- Overall Round 44 performance was satisfactory with mean recoveries, variability and measurement uncertainty (for those within the acceptable recovery limit range) generally typical of these measurands and test procedures.
- Overall the pooled average recoveries of both *Cryptosporidium* oocysts and *Giardia* cysts increased compared to the previous round (43) (Figs. 1A & 1B.)

Recovery variability:

-*Intra-sample*: The greatest recovery variability for *Cryptosporidium* occurred for sample C (250 µL matrix, 160 *Cryptosporidium* oocysts; MU = 142%). The greatest recovery variability for *Giardia* occurred for sample A (500 µL matrix, 100 *Giardia* cysts; MU = 78%). The lowest recovery variability for *Cryptosporidium* occurred for sample D (100 µL matrix, 140 *Cryptosporidium* oocysts; MU = 50%), while lowest recovery variability for *Giardia* occurred for sample B (150 µL matrix, 60 *Giardia* oocysts; MU = 40%) (Table B.)

-*Intra-laboratory*: Laboratory code 8 had the greatest *Cryptosporidium* recovery variability (53%). This variability was substantially higher than the other participating laboratories. Laboratory code 7 had the greatest *Giardia* recovery variability (25%.) Laboratory codes 6 and 8 had the least *Cryptosporidium* and *Giardia* recovery variability (15%, 5% RSD, respectively).

- Recovery medians: Median recoveries were generally similar to those reported for other proficiency schemes and published literature (14-83% *Cryptosporidium*; 32-83% *Giardia*). However, laboratory code 9 consistently had the lowest *Giardia* (32-52%) recoveries, and the lowest *Cryptosporidium* recovery of this round (14%.) *Giardia* cyst recoveries below approximately 40%, and *Cryptosporidium* below approximately 30% should warrant investigation and potential corrective action.

- Recovery maxima: Maximum recoveries were fairly consistent for both *Cryptosporidium* (68-77%) and *Giardia* (76-83%) in this round, and much less variable for *Cryptosporidium* compared to those in PTA round 43 (*Cryptosporidium* 48-90%; *Giardia* 72-79%).

- Recovery minima: Minimum recoveries were more variable for *Cryptosporidium* (14-38%), and consistently higher for *Giardia* (32-52%), compared to those in PTA round 43 (*Cryptosporidium* 14-18%; *Giardia* 16-34%).

- Control Samples: Counts of trip control samples (F_T) were lower than those of the sample kept on premises (F_{NoT}) for *Cryptosporidium,* but essentially the same for *Giardia* (B1.3). Control sample recoveries were also lower than their respective samples analysed by participant laboratories in terms of oocysts per unit matrix; sample D for *Cryptosporidium* (controls 17-37%; participant laboratories median 60%.).

Impact of Matrix

- Considering test measurement uncertainty, median and average recoveries of *Cryptosporidium* and *Giardia* were generally similar regardless of matrix amount. However, lowest median and average recoveries of *Cryptosporidium* were obtained for samples with the greatest amount of matrix material; sample C for *Cryptosporidium* (250 µL) and sample A for *Giardia* (500 µL.)

- This is in contrast to rounds 39, 40, 41, and the majority of round 43 samples in which lower mean *Cryptosporidium* recoveries were *generally* associated with *lower* matrix material levels. *Giardia* recoveries also contrasted with previous rounds 39-40, 43 in which lower matrix levels were *generally* associated with higher recoveries.

Impact of Reference Count

- There was no substantial general trend in *Cryptosporidium or Giardia* reference count observed in relation to recoveries (Figs. 2A and 3A.)

- There was no general trend in *Giardia* recoveries in relation to matrix amount (Fig 3B.)

- There was a general *decreasing* trend observed with respect to reference count *per-unit-matrix* for *Cryptosporidium* - and *increasing* average mean *Giardia* - recoveries up to approximately 0.6 (Figs. 2C and 3C).

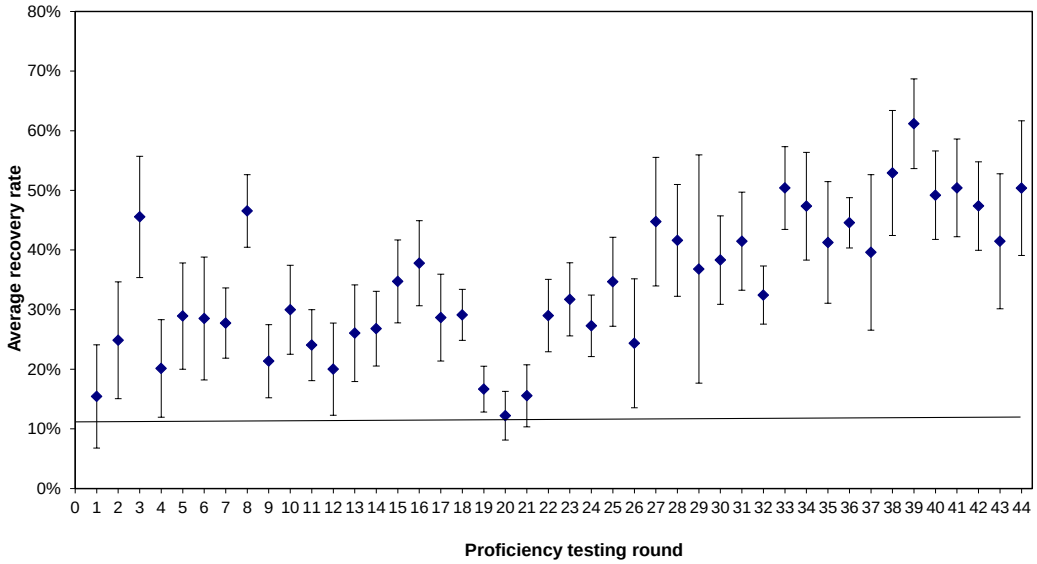
Confirmation

- Percent confirmed (DAPI[+]) *Cryptosporidium* oocysts in F_{NoT} (75%) was lower than that of rounds 40, 41, and the participant labs in the current round (ca. 95-100%), Percentages of DAPI(+) *Giardia* cysts in F_T and F_{NoT} samples (88%) were generally higher than current round participants.

- Laboratory code 9 consistently had the lowest levels of confirmed (DAPI[+]) *Giardia* cysts (with the exception of sample B) and the lowest lab average (54%.) Laboratory code 6 also had lower *Giardia* cyst confirmation levels (58-77%) for samples A-C.

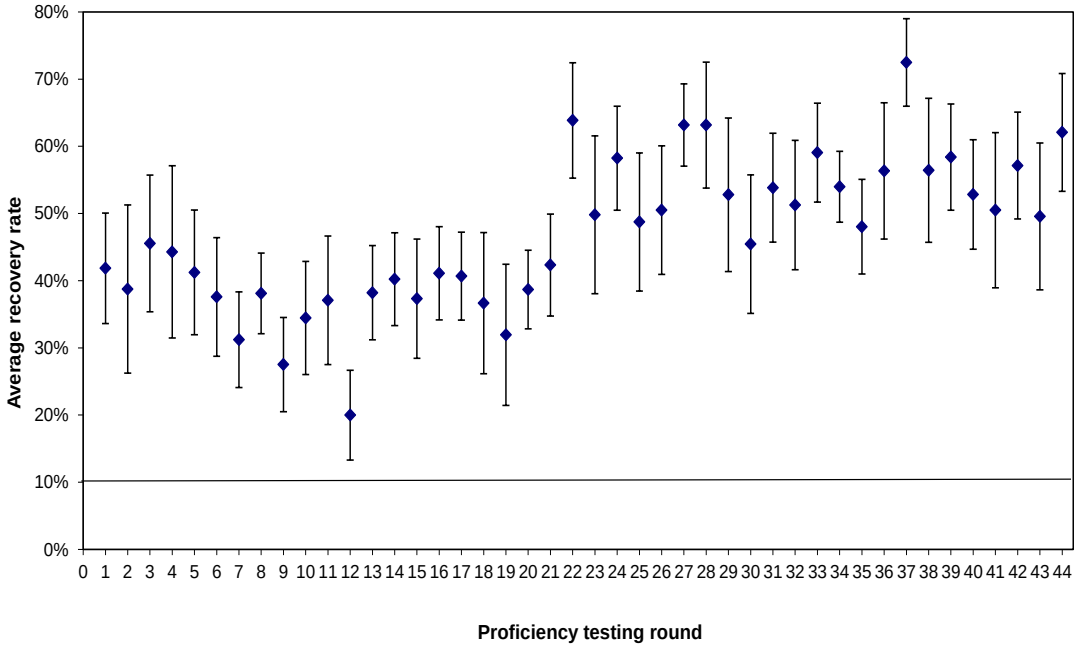
Total average *Cryptosporidium* recovery rate (50.4%) has increased compared to the previous round. Figure 1A shows the average percent recovery rate for *Cryptosporidium* for each round (refer to notes below).

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



Total average *Giardia* recovery rate (62.1%) has increased 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 up to Round 37, 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); for Round 15, one sample (Sample D); and for Round 34, one sample (Sample C) out of the five samples analysed by each laboratory did not contain QC mud. Round 42, 43 and 44 contained QC mud only.
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.
4. Regarding Round 37, selected samples contained QC mud or Drinking Water Plant Filter Backwash (DWPFBW).
5. Rounds 38 - 41 samples contained DWPFBW only.

Figure 2A: Reference Count vs % Recovery for *Cryptosporidium*

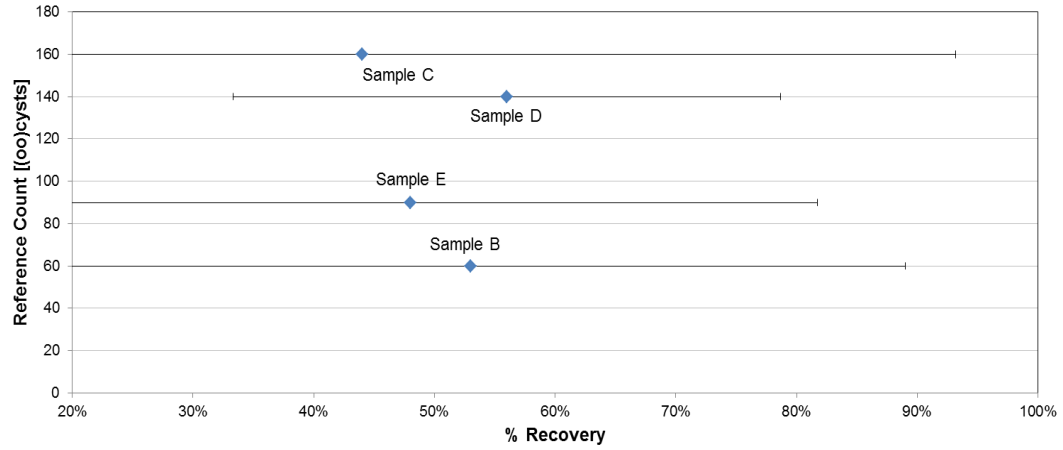


Figure 2B: Added Matrix vs % Recovery for *Cryptosporidium*

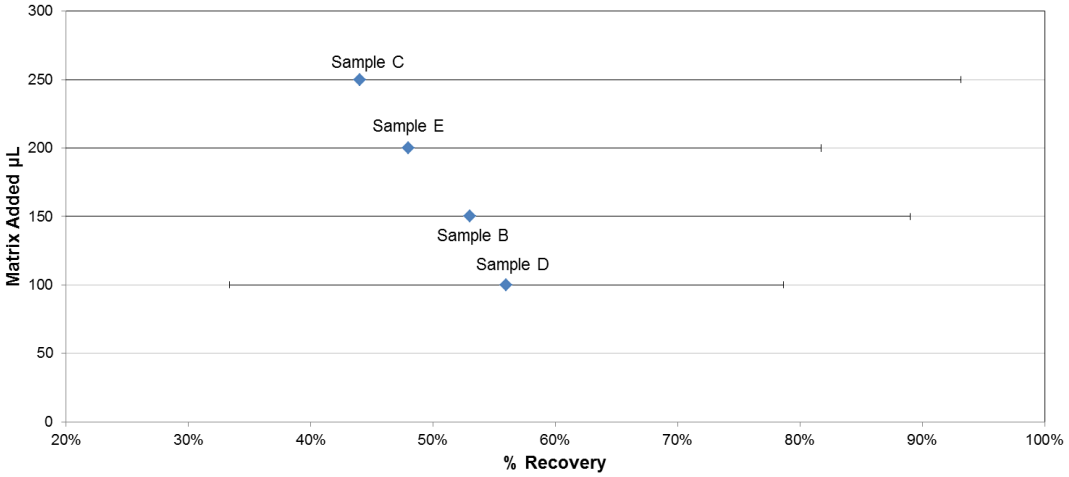
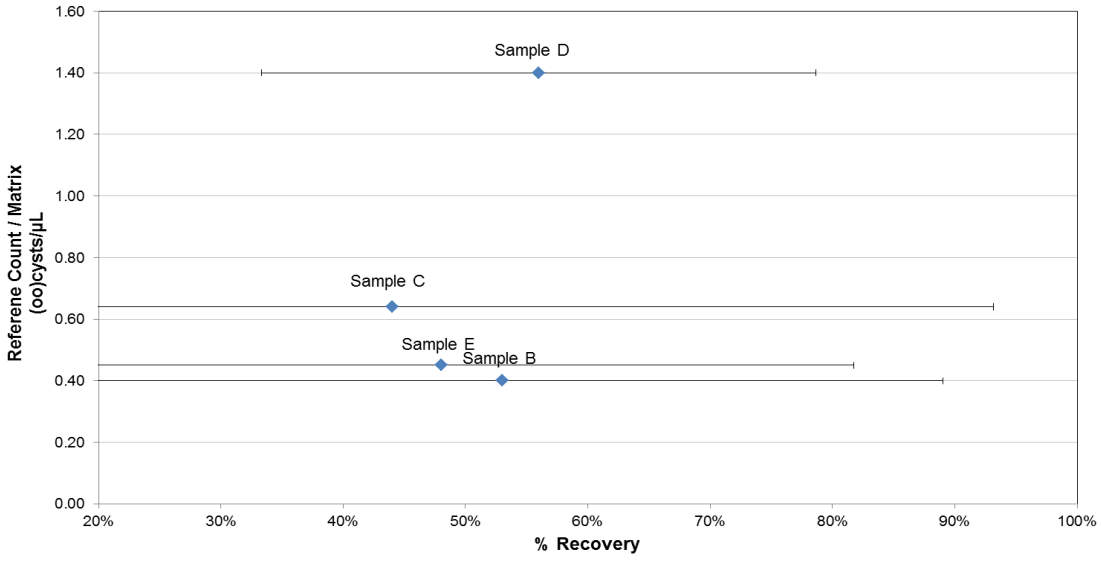


Figure 2C: Reference Count/Matrix vs % Recovery for *Cryptosporidium*



1. The blue diamonds represent the mean recoveries for each sample.
2. The horizontal bars (error bars) represent measurement uncertainties.

Figure 3A: Reference Count vs % Recovery for *Giardia*

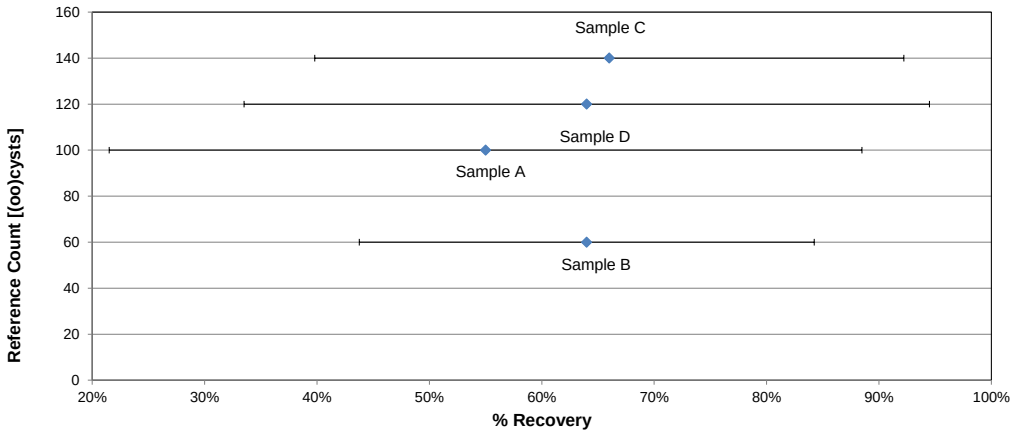


Figure 3B: Added Matrix vs % Recovery for *Giardia*

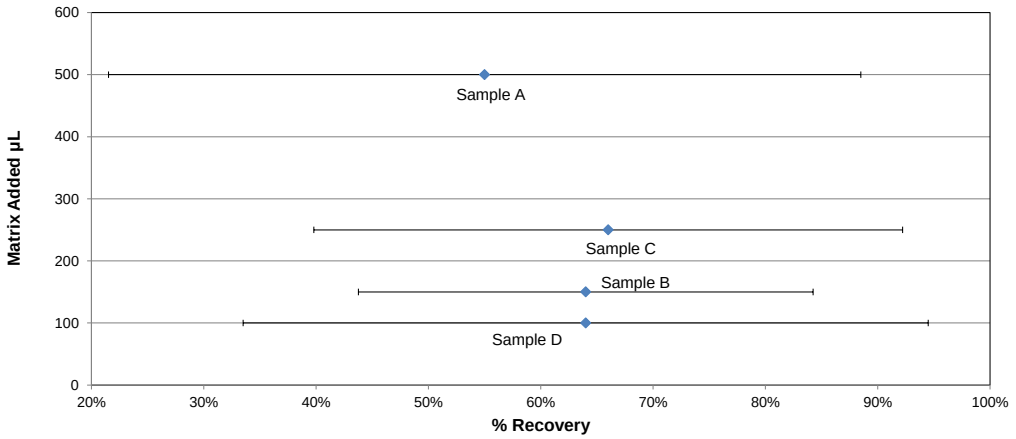
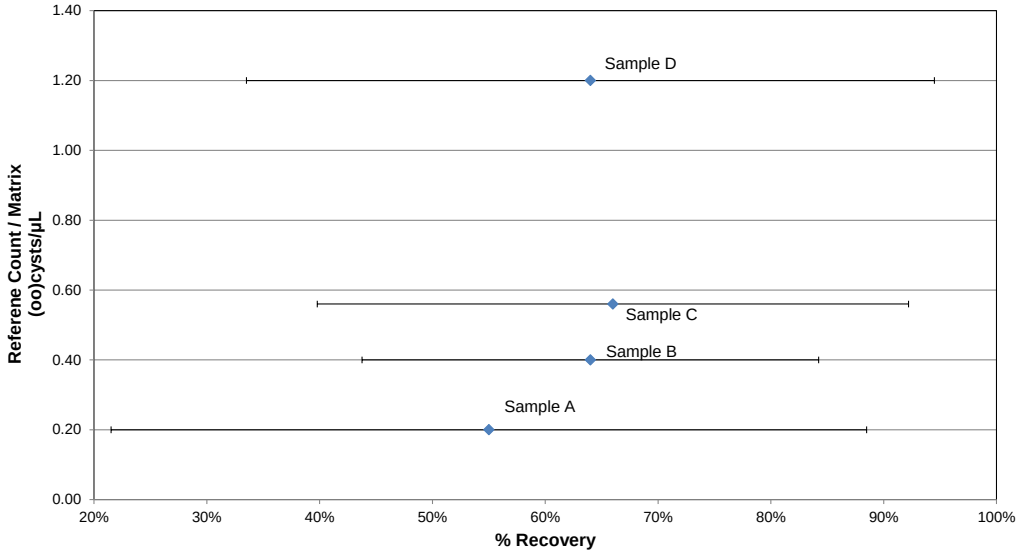


Figure 3C: Reference Count/Matrix vs % Recovery for *Giardia*



1. The blue diamonds represent the mean recoveries for each sample.
2. The horizontal bars (error bars) represent measurement uncertainties.

Measurement Uncertainty (MU) Estimation

Results including MU calculations are presented as relative % recoveries, as seen in Table B below. This table and comments are provided for information purposes only, and do not affect the evaluation of participants' results.

TABLE B: *Cryptosporidium* and *Giardia* Round 44 Recovery - Measurement Uncertainty

SAMPLE	ORGANISM	MEDIAN RECOVERY (%)	STANDARD DEVIATION	RELATIVE STANDARD DEVIATION (RSD - %)	✦MEASUREMENT UNCERTAINTY (RSD - %)	REFERENCE COUNT
A	*Giardia*	53	21	39	78	100
	Cryptosporidium					
B	*Giardia*	61	14	20	40	60
	Cryptosporidium	53	8	43	83	60
C	*Giardia*	74	17	25	50	140
	Cryptosporidium	38	31	71	142	160
D	*Giardia*	63	19	30	60	120
	Cryptosporidium	60	14	25	50	140
E	*Giardia*					
	Cryptosporidium	43	21	44	88	90

Notes for Table B:
1. ✦ = All measurement uncertainty values are at the 95% level of confidence.
2. Sample A did not include *Cryptosporidium*.
3. Sample E did not contain *Giardia.*

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

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

Round	*Cryptosporidium* levels (Counts)	Round	*Cryptosporidium* levels (Counts)
1	50-200	23	50-250
2	50-200	24	50-200
3	50-300	25	50-250
4	110	26	50-200
5	50-200	27	50-200
6	25-75	28	50-250
7	50-100	29	50-140
8	65-140	30	80-135
9	125	31	70-140
10	110-235	32	50-130
11	50-200	33	50-170
12	110-235	34	50-130
13	90-205	35	50-120
14	55-135	36	50-130
15	55-135	37	50-120
16	55-120	38	50-110
17	90-310	39	50-110
18	50-300	40	90-180
19	50-300	41	70-160
20	50-200	42	50-160
21	100-200	43	60-120
22	50-300	44	60-160

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

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

Round	*Giardia* levels (Counts)	Round	*Giardia* levels (Counts)
1	50-200	23	50-300
2	50-200	24	50-200
3	50	25	50-250
4	40	26	50-250
5	50-200	27	50-200
6	75-120	28	50-200
7	50	29	50-150
8	65-140	30	85-150
9	55	31	50-140
10	70-85	32	50-170
11	50-200	33	50-130
12	110-125	34	50-170
13	90-145	35	50-120
14	55-200	36	50-170
15	55-200	37	60-120
16	120-255	38	60-170
17	135-310	39	60-170
18	150-300	40	60-160
19	150-300	41	50-140
20	50-120	42	50-170
21	90-200	43	80-170
22	50-250	44	60-140

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.

With respect to the bulk water concentration method, all four laboratories indicated the use of cartridge filtration. All laboratories used IMS as their purification method and immunofluorescence microscopy as their presumptive ID and total count enumeration method. Two laboratories reported the use of DAPI staining only for confirmation, with the other two indicating the additional use of DIC microscopy.

Laboratory code 9 had the lowest average recovery of both *Giardia* and *Cryptosporidium* (Tables A1.4, A1.7). This laboratory used the same method as laboratory code 6, and also had the lowest average confirmation percentages for both measurands.

TABLE E: Recovery and recovery variability by bulk water concentration method

Bulk Water Concentration Method	Average *Cryptosporidium* Recovery (%) and Variability (RSD)	Average *Giardia* Recovery and Variability (RSD)	Number of laboratories using method
Cartridge-Filtration	50% (38%)	62% (25%)	4

Overall Laboratory Performance

Overall, the pooled average recoveries of *Cryptosporidium* oocysts and *Giardia* cysts increased when compared to the previous round (43.)

Laboratory code 9 had the lowest average recoveries, and also had the lowest average confirmation percentages for b both *Giardia* cysts and *Cryptosporidium* oocysts (Tables A1.4, A1.6, A1.7, A1.9). This laboratory should investigate potential causal factors, including failure to concentrate from bulk water and/or disperse (oo)cysts from matrix materials prior to IMS; and/or failure to add, capture or dissociate IMS beads; and/or status of associated reagents.

This laboratory may also want to review confirmation procedures including DAPI staining procedures in light of relative performance and potentially investigate corrective action(s) of low relative confirmation during sample analysis. Some laboratories experience issues with low percentages of DAPI stained cysts and oocysts. Several methods suggest use of heat and/or acid for dissociation of oocysts from IMS beads, as well as permeabilisation for subsequent DAPI staining. These laboratories may find the following publication of interest if such techniques to optimise DAPI staining are not already employed: Ware, MW, Wymer, L, Lindquist, and Schaefer, FW. (2003).

Measurement Uncertainty (MU)

Laboratory code 6 did not report MU. Laboratory code 9 did not report the sample number (*n*) used for MU estimation. Estimated uncertainties of measurement between laboratories reporting MU were not atypical for these measurands.

The highest reported variability (142% RSD) was associated with generally moderate dose and matrix combination (160 *Cryptosporidium* oocysts, 250 µL matrix, sample C). The range of variability in recoveries for *Cryptosporidium* (14-83%) was also larger than that for *Giardia* (32-83%.)

TABLE F: 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
32	Concentrate samples - QC mud - Labs. add to 10 Litres water	0.0%	0	3.5%	1
33	Concentrate samples - QC mud - Labs. add to 10 Litres water	0.0%	0	0.0%	0
34	Concentrate samples - QC mud - Labs. add to 10 Litres water	0.0%	0	0.0%	0
35	Concentrate samples - QC mud - Labs. add to 10 Litres water	3.3%	1	0.0%	0
36	Concentrate samples - QC mud - Labs. add to 10 Litres water	0.0%	0	0.0%	0
37	Concentrate samples - QC mud - Labs. add to 10 Litres water	10%	2	9.38%	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
38	Concentrate samples - QC mud - Labs. add to 10 Litres water	1.25%	1	7.81%	5
39	Concentrate samples - DWPFBW - Labs. add to 10 Litres water	1.11%	1	0.0%	0
40	Concentrate samples - DWPFBW - Labs. add to 10 Litres water	0.0%	0	0.0%	0
41	Concentrate samples - DWPFBW - Labs. add to 10 Litres water	6.06%	4	1.51%	1
42	Concentrate samples - QC Mud - Labs. add to 10 Litres water	0.0%	0	0.0%	0
43	Concentrate samples - QC Mud - Labs. add to 10 Litres water	8.0%	1	8.0%	1
44	Concentrate samples - QC Mud - Labs. add to 10 Litres water	0%	0	0%	0

Notes for Table F:
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. For Round 34, QC mud was only added to Samples A, B, D and E.
3. For Round 35, a combination of QC mud or Drinking Water Plant Filter Backwash (DWPFBW) was added to samples.
4. Rounds 38 - 41 samples contained DWPFBW only.
5. Round 42, 43 and 44 samples contained QC Mud only.

Conclusions

Performance was satisfactory for all laboratories
It was noted that one laboratory (Code 6) neglected to report MU, while one laboratory (Code 9) neglected to report the *n* used to estimate MU.

8. REFERENCES

[1] *Guide to Proficiency Testing Australia,* 2019 (this document can be found on the PTA website, www.pta.asn.au).

[2] *Evaluation of an alternative IMS dissociation procedure for use with Method 1622: detection of Cryptosporidium in water*. Ware, MW, Wymer, L, Lindquist, and Schaefer, FW (2003).

[3] USEPA (1996) *Microscopic particulate analysis (MPA) for filtration plant optimization.* EPA 910-R-96-001.

[4] USEPA (2011) *Method 1623 Improvements*. *Cryptosporidium* Lab Approval Program Technical Support Center, Standards and Risk Management Division, Office of Ground Water and Drinking Water. Miller, C. https://www.epa.gov/sites/production/files/2016-12/documents/method1623improvements.pdf

APPENDIX A

Summary of Results

Results *Cryptosporidium* (total counts)

REFERENCE COUNTS	0	60	160	140	90	
QC Mud per vial	500µL	150µL	250 µL	100µL	200µL	
Lab Code No.	**Sample A**	**Sample B**	**Sample C**	**Sample D**	**Sample E**	**Method Codes**
Code 6 - Total Count	0	41	84	94	47	
Confirmed Count	-	41	84	94	47	3, 6, 7, 8, 9
MU	-	-	-	-	-	
Code 7 - Total Count	0	46	133	73	69	
Confirmed Count	0	41	121	64	65	3, 6, 7, 8, 9, 10
MU	-	32 ± 21 n=6	47 ± 78 n=7	50 ± 75 n=8	52 ± 71 n=9	
Code 8 - Total Count	0	18	39	95	27	
Confirmed Count	0	18	39	95	27	3, 7, 8, 9, 10
MU	0	12-28 (n=50)	25-60 (n=50)	62-146 (n=50)	18-41 (n=50)	
Code 9 - Total Count	0	23	23	53	31	
Confirmed Count	0	23	23	52	25	3, 7, 8, 9
MU	-	23 ± 15	23 ± 15	53 ± 34	31 ± 20	

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	4	4	4	4	4
Minimum	0	18	23	53	27
Maximum	0	46	133	95	69
Average	0	32	70	79	44
Median	0.0	32.0	61.5	83.5	39.0
SD	0.0	13.6	49.4	19.9	19.1
Median Absolute Deviation (%)	NA	42.5	70.6	25.2	43.4

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	100	60	140	120	0	
QC Mud	**500µL**	**150µL**	**250 µL**	**100µL**	**200µL**	
Lab Code No.	**Sample A**	**Sample B**	**Sample C**	**Sample D**	**Sample E**	**Method Codes**
Code 6 - Total Count	60	36	102	93	0	
Confirmed Count	35	23	79	83		3, 6, 7, 8, 9
MU						
Code 7 - Total Count	45	37	106	56	0	
Confirmed Count	43	32	98	49	0	3, 6, 7, 8, 9, 10
MU	36 ± 18 n=6	36 ± 17 n=7	45 ± 52 n=8	46 ± 49 n=9	-	
Code 8 - Total Count	81	49	104	100	0	
Confirmed Count	81	49	104	100	0	3, 7, 8, 9, 10
MU	42-154 (n=50)	26-93 (n=50)	55-198 (n=50)	52-191 (n=50)		
Code 9 - Total Count	32	31	58	58	0	
Confirmed Count	11	21	30	36	0	3, 7, 8, 9
MU	32 ± 18	31 ± 17	58 ± 32	58 ± 32	-	

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	4	4	4	4	4
Minimum	32	31	58	56	0
Maximum	81	49	106	100	0
Average	55	38	93	77	0
Median	52.5	36.5	103.0	75.5	0.0
SD	21.0	7.6	23.1	23.0	0.0
Median Absolute Deviation (%)	38.2	20.0	24.8	29.8	NA

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

Recovery Results for *Cryptosporidium* (%)

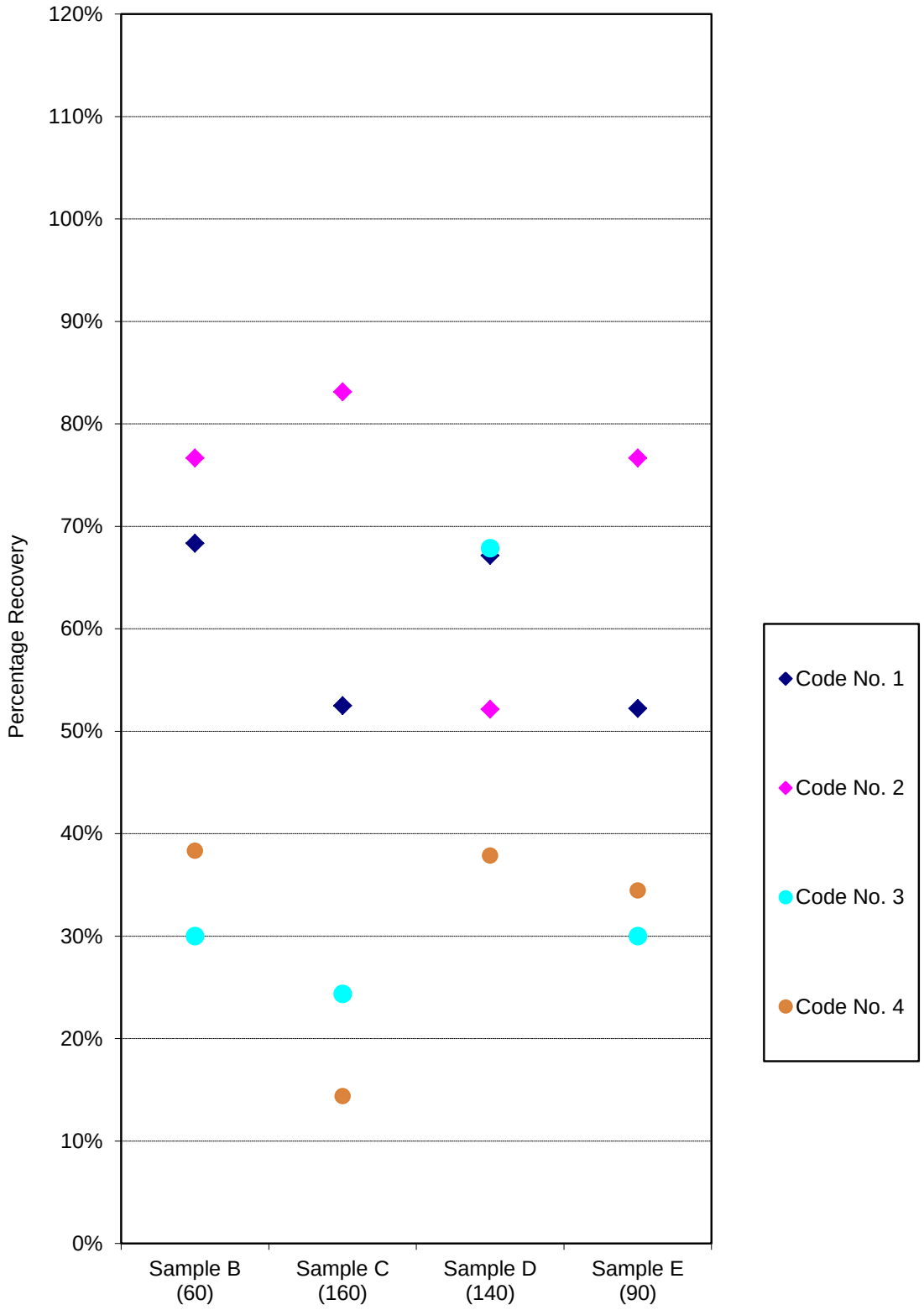
Calculated as a % of TOTAL Counts / Reference Counts

Reference Counts	60	160	140	90	Lab Average	Lab SD	Lab %RSD
QC Mud	150 µL	250 µL	100 µL	200 µL			
Code No.	Sample B	Sample C	Sample D	Sample E			
6	68%	53%	67%	52%	60%	9%	15%
7	77%	83%	52%	77%	72%	14%	19%
8	30%	24%	68%	30%	38%	20%	53%
9	38%	14%	38%	34%	31%	11%	36%
No. of Results	4	4	4	4			
Minimum	30%	14%	38%	30%			
Maximum	77%	83%	68%	77%			
Average	53%	44%	56%	48%			
Median	53%	38%	60%	43%			

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 outlier results.
3. "-" refers to no result returned.

Results *Cryptosporidium* **(% Recovery Rate)**



Note:

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

Confirmed Results for *Cryptosporidium* (%)

Calculated as a % of Confirmed Counts / Total Counts

Reference Counts	60	160	140	90	
QC Mud	150 µL	250 µL	100 µL	200 µL	Lab Average
Code No.	Sample B	Sample C	Sample D	Sample E	
6	100%	100%	100%	100%	100%
7	89%	91%	88%	94%	91%
8	100%	100%	100%	100%	100%
9	100%	100%	98%	81%	95%
No. of Results	4	4	4	4	
Minimum	89%	91%	88%	81%	
Maximum	100%	100%	100%	100%	
Average	97%	98%	96%	94%	
Median	100%	100%	99%	97%	

Note:

1. "-" refers to no result returned.

Recovery Results for *Giardia* (%)

Calculated as a % of TOTAL Counts / Reference Counts

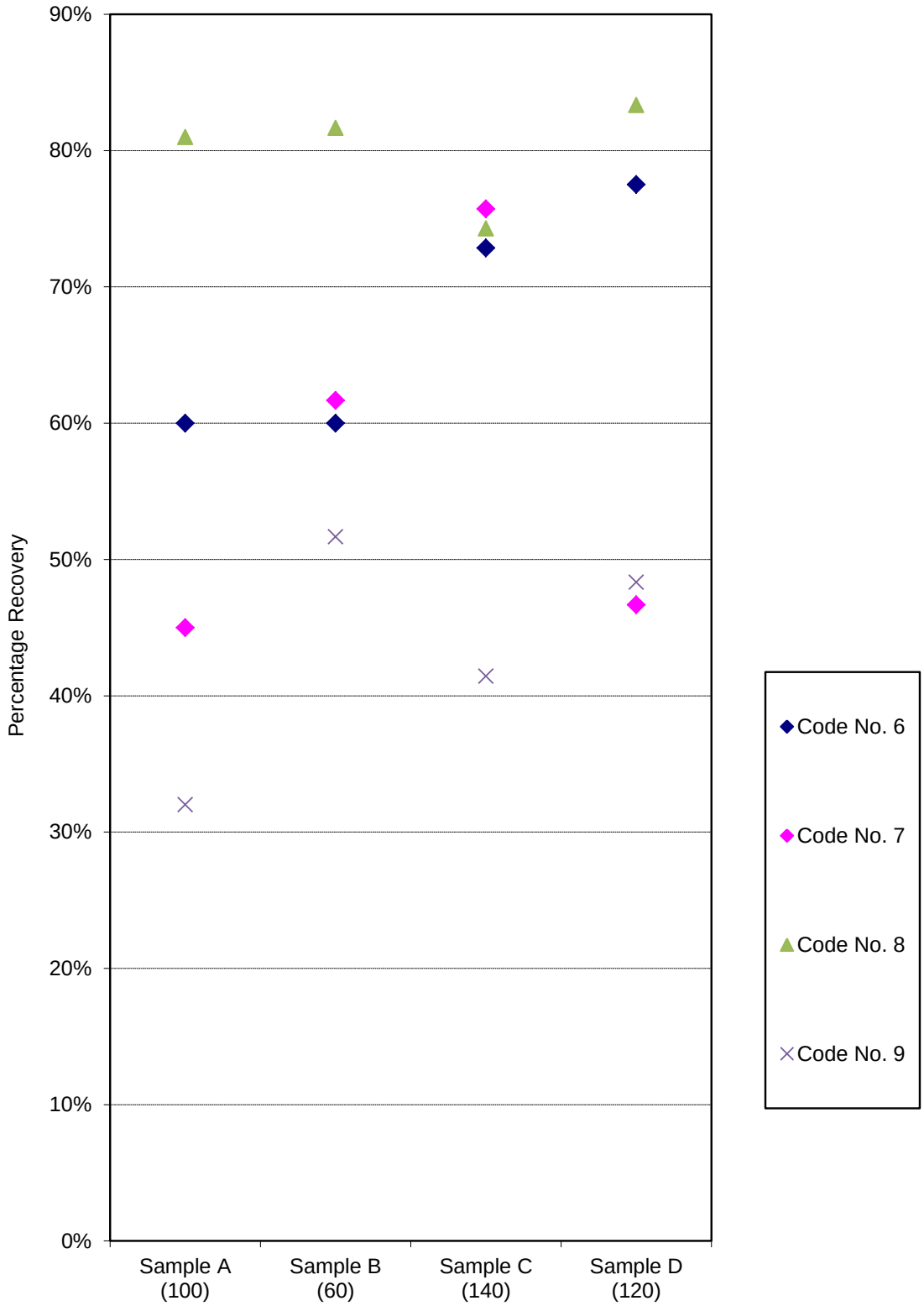
Reference Counts	100	60	140	120	Lab Average	Lab SD	Lab %RSD
QC Mud	500 µL	150 µL	250 µL	100 µL			
Code No.	Sample A	Sample B	Sample C	Sample D			
6	60%	60%	73%	78%	68%	9%	13%
7	45%	62%	76%	47%	57%	14%	25%
8	81%	82%	74%	83%	80%	4%	5%
9	32%	52%	41%	48%	43%	9%	20%
No. of Results	4	4	4	4			
Minimum	32%	52%	41%	47%			
Maximum	81%	82%	76%	83%			
Average	55%	64%	66%	64%			
Median	53%	61%	74%	63%			

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 outlier results.
3. "-" refers to no result returned.

A1.8

Results *Giardia* (% Recovery Rate)



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

A1.9

Confirmed Results for *Giardia* (%)

Calculated as a % of Confirmed Counts / Total Counts

Reference Counts	100	60	140	120	
QC Mud	500 µL	150 µL	250 µL	100 µL	Lab Average
Code No.	Sample A	Sample B	Sample C	Sample D	
6	58%	64%	77%	89%	72%
7	96%	86%	92%	88%	90%
8	100%	100%	100%	100%	100%
9	34%	68%	52%	62%	54%
No. of Results	4	4	4	4	
Minimum	34%	64%	52%	62%	
Maximum	100%	100%	100%	100%	
Average	72%	80%	80%	85%	
Median	77%	77%	85%	88%	

Note:

1. "-" refers to no result returned.

APPENDIX B

Homogeneity Testing and Trip Control

Homogeneity Testing and Trip Control

Samples for Round 44 were produced in line with EasySeed batch number 683, 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 G: Relative Standard Deviation for Various Sample Doses (Round 44)

ORGANISM	DOSE	RSD (%)	MU as RSD (Absolute)	Resultant dose with absolute uncertainty
Cryptosporidium	60	2.8	4	60 ± 4
Cryptosporidium	90	1.7	4	90 ± 3
Cryptosporidium	140	1.7	5	140 ± 5
Cryptosporidium	160	1.7	5	160 ± 5
Giardia	60	2.4	3	60 ± 3
Giardia	100	2.1	4	100 ± 4
Giardia	120	2.1	5	120 ± 5
Giardia	140	2.1	6	140 ± 6

Notes for Table G:

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 70 *Cryptosporidium* oocysts and 80 *Giardia* cysts was used as the trip control.

BioPoint Pty Ltd retained a 3.5 mL water concentrate samples F (F_{NoT}), on their premises after preparation. Sample F (F_{NoT}) was added to 10 L of distilled water, concentrated and analysed on 21 November 2019.

One nominated laboratory (Code 8) was provided with a 3.5 mL water concentrate samples F (F_T) and was requested to return the sample immediately upon receipt. Sample F (F_T) was subsequently added to 10 L of distilled water and analysed by BioPoint Pty Ltd on 21 November 2019. 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
21 November 2019 (Sample kept on premises)	24	75%	51	88%
21 November 2019 (Sample sent to laboratory and returned)	12	100%	52	88%
Actual counts	**70**		**80**	
F_{NoT} % Recovery Rate	37%		64%	
F_T % Recovery Rate	17%		65%	

The trip controls 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 44

INSTRUCTIONS TO PARTICIPANTS

To ensure 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 that may contain matrix material/s (added to simulate an environmental water sample). Samples *may* have been spiked with *Cryptosporidium* oocysts and/or *Giardia* cysts at various concentrations.
   Your laboratory may receive an additional 3.5 mL bulk-water-concentrate sample that will be utilised as the proficiency-testing program *Trip Control*. If you receive this sample (labelled "PTA Sample F"), please refer to the included associated covering letter for further instructions.

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

3. Mix the 3.5 mL tube containing the bulk-water-concentrate sample by inversion, then immediately place the bottom of the tube on a vortex mixer 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 bulk 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 bulk water used for dilution 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 the concentrate thoroughly dispersed throughout 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 sec. Empty contents into 10 L water sample.
   - **iv)** Repeat steps ii-iii.

   *Laureth-12 Envirocheck® elution buffer or other Tween®-containing solutions for rinsing filters may alternatively be used to rinse bulk-water-concentrate sample tubes.

4. A Senior QA/QC Officer (or similar) must sign the *Results Sheet* declaration to confirm your facility has diluted the bulk-water-concentrate samples to 10 L.

5. Laboratories must then proceed to analyse the 10 L samples using their **routine test method** (that 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 results in the *Final Report*, your facility

has been allocated a laboratory code number, which appears on your *Results Sheet.*

6. PTA is aware of the internal positive control reference material ColorSeed™. Although PTA understands the advantage of this material 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.

7. Record the results for each sample on the *Results Sheet* provided. Participants must report both *Total* and *Confirmed* 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 or non-discreet non-whole number values (i.e. less than/greater than values, presence/absence, detected/not detected, decimal places such as 0.5 or 55.4 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 unless a true internal standard is employed for every routine sample as described in (6) above. If such internal standard correction is used, this must be indicated.

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

9. Commence testing as soon as possible after samples are received. IMPORTANT: All participants must return completed *Results Sheets* no later than **Friday 15 November 2019** 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

PTA would like to thank you for participating in this *Cryptosporidium* and *Giardia* proficiency-testing program.

Proficiency Testing Australia

Cryptosporidium and *Giardia* Round 44 - Proficiency Testing Program

Results Sheet

Laboratory Code:

Date / Time of Sample Receipt: _____

Condition of Samples 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) _____

Filtration Type (please tick): Sponge ☐ Flat Bed ☐ Cartridge ☐ Sponge ☐ *Other ☐
*Describe_____

Purification (e.g. IMS) _____

Enumeration (e.g. Microscopy) _____

Confirmation method(s) (e.g. DAPI, DIC) _____

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 10 L of water prior to analysis.

Print Name: _____ **Date:** _____

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

Return no later than **Friday 15 November 2019** 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, DWPFBW 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 ---------