Method Comparison of AquaCHROM ECC vs. Colilert for the Detection and Enumeration of Coliforms and *Escherichia coli* in Water Samples

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Objective

To compare the AquaCHROM™ ECC method from CHROMagar™ and the Colilert methods for qualitative and quantitative detection of *E. coli* and non-*E. coli* coliforms with different water matrixes.

Principle of the Method

The AquaCHROM ECC from CHROMagar is a chromogenic medium for the detection of *E. coli* and coliforms in water samples. Coliforms are *Enterobacteriaceae* able to ferment lactose and are present in human and warm-blooded animals' intestinal flora, in the soil and water. This method is intended for laboratory use and should be used by personnel in compliance with good laboratory practices.

The product is composed of a powder medium and is supplied in ready-to-use, pre-weighed doses. Each dose is for a 100 mL water sample. The product is stored at 15 – 30 °C. For presence absence testing, the pre-weighed dose is added to a sterile transparent vessel containing a 100 mL water sample and then incubated at 35 – 37 °C for 18 – 24 h. *E. coli* results are green to bleu green, and non-*E. coli* coliform results are yellow. If a mixture of *E. coli* and non-*E. coli* coliforms are present, the medium will appear green. The product can also be used for MPN analysis. For this method, the 100 mL water sample is poured into a dispenser, and then the dose of AquaCHROM ECC is added. After shaking to dissolve the AquaCHROM ECC powder, the 100 mL sample is dispensed into the wells of a 48-well Deep well sample plate. The plate is incubated at 35 – 37 °C for 18 – 24 h. *E. coli* results are green, and non-*E. coli* coliform results are yellow. If a mixture of *E. coli* and non-*E. coli* coliforms are present, the medium will appear green. The wells are counted based on color, and then compared to the AquaCHROM ECC MPN Table (Annex 1).

Definitions

(a) *Probability of Detection (POD).* —The proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent. There are several POD measures that can be calculated, e.g., POD_R (reference method POD), POD_C (confirmed candidate method POD), POD_{CP} (candidate method presumptive result POD), POD_{CC} (candidate method confirmation result POD) and dPOD, the difference between any two POD values.

(b) *Difference of probabilities of detection (dPOD).* —Difference of probabilities of detection is the difference between any two POD values. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

(c) *Difference of Means.* —Difference in Log_{10} of the average results between the candidate and reference method for one level of contamination.

(d) *Repeatability.* —Precision where independent test results are obtained with the same method on equivalent test items in the same laboratory by the same operator using the same equipment within a short interval of time.

(e) *Confidence interval (CI)*. —A confidence interval displays the probability that a parameter will fall between a pair of values around the mean. Confidence intervals are calculated at the 90% and 95% levels.

(f) *Statistical equivalence*. —The acceptance criterion for statistical equivalence is that the 90% CI on the bias between the methods falls within -0.5, 0.5.

(g) *Most Probable Number (MPN)*. —An estimate of the level of viable microbial contamination of a sample based on probability statistics.

Materials and Method

Test Kit Information

(a) *AquaCHROM ECC*. —box, 100 vials

(b) *Catalog number.* — AQ056

(c) *AquaCHROM ECC MPN 48-Well Plate*

(d) *Ordering information.* —http://www.chromagar.com/

Additional Supplies and Reagents

(a) *MI Agar Plates*

(b) *m-Endo Medium*

(c) *Lauryl Tryptose (LST) Broth Tubes.*—10 mL per test tube

(d) *Brilliant Green Bile Lactose Broth (BGLB) Tubes*.—10 mL per test tube

(e) *Escherichia coli (EC) Broth Tubes.*—10 mL per test tube

(f) *Levine's Eosin-Methylene Blue (L-EMB) Agar Plates*

(g) *Plate Count Agar (PCA) Slant Tubes.*—10 mL per test tube

(h) *Sterile polypropylene bottles*.—Capable of holding 100 mL of water

Apparatus

(a) *Incubators.*—Capable of maintaining 35 ± 0.5 °C, 36 ± 1 °C

(b) *Water Bath*.—Capable of maintaining 46 ± 1 °C

(c) *Refrigerator*.—Capable of maintaining 2 – 8 °C

(d) *Long wave UV lamp, 366 nm*

Sample Preparation

A pre-weighed dose of AquaCHROM ECC is added to each sterile transparent vessel containing a 100 mL water sample. The vessels are then closed, shaken, and incubated at 35 – 37 °C for 18 –24 h. After incubation, the results are determined based on the change in color of the liquid.

Analysis

Presence of *E. coli* will turn the water sample green to blue-green. If the water turns yellow, non-*E. coli* coliform bacteria are present.

Confirmation

Streak all AquaCHROM ECC enriched portions, regardless of result, onto either MI agar (tap water, well water and lake water) or onto m-Endo medium or LES Endo Agar (bottled water) and incubate at 35 ± 0.5 °C for 24 h. On MI agar, blue colonies under normal/ambient light are typical for *E. coli*. When exposed to longwave ultraviolet light (366 nm), *E. coli* will fluoresce blue/green, while blue/white fluorescence indicates coliforms other than *E. coli*. On m-Endo medium, colonies will be pink to dark red with a green metallic surface sheen.

Transfer typical coliform colonies into tubes of LST and incubate at 35 ± 0.5 °C for 48 h. Subculture any gas positive LST tubes to BGLB and incubated t 35 ± 0.5 °C for 48 h. Gas production in BGLB within 48 h is a confirmed coliform test. Report results as number of coliform colonies per 100 mL. *Note*: If typical colonies are not present, pick atypical colonies to screen for the presence of atypical reacting coliforms. To confirm that the colonies are *E. coli*, subculture to EC broth. Incubate EC tubes for 24 ± 2 h at 44.5 °C and examine for gas production. If negative, reincubate and examine again at 48 ± 2 h. To complete *E. coli* confirmation, gently agitate each gassing EC tube, remove a loopful of broth and streak for isolation on a L-EMB agar plate and incubate for 18 – 24 h at 35 ± 0.5 °C. Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer up to 5 suspicious colonies from each L-EMB plate to PCA slants, incubate them for 18 – 24 h at 35 °C ± 0.5 °C and use for further testing. ***Note*:** Identification of any 1 of the 5 colonies as *E. coli* is sufficient to regard that EC tube as positive; hence, not all 5 isolates may need to be tested. Confirm one colony from all test portions via Bruker MALDI Biotyper following AOAC OMA 2017.09 [1].

Method Comparison Study

The AquaCHROM ECC method was compared to the Colilert method for tap water, well water, lake water, and bottled water.

The method comparison consisted of a matrix study for qualitative analysis following an unpaired study design at three levels of contamination (0 CFU/100 mL), (0.2 - 2 CFU/100 mL), (5 - 10 CFU/100 mL) and a matrix study for quantitative analysis following an unpaired study design at four levels of contamination (0 CFU/100 mL), (1 - 50 CFU/100 mL), (51 - 100 CFU/100 mL), (101 - 150 CFU/100 mL).

Preparing of Levels for Natural Contamination

Well water and lake water were found to have natural coliform contamination after screening; however, the levels were too high for the levels specified in the protocol. The contaminated water for both matrixes were diluted with sterile DI water to decrease the coliform count to the range of levels required for the study.

Organism Preparation and Inoculation for Artificial Contamination

Natural contamination of coliforms or *E. coli* were not found in tap water or bottled water matrixes; therefore, artificial contamination was conducted. The waters were artificially contaminated as follows: *E. coli* ATCC 25922 was used to inoculate tap water (qualitative & high level of quantitative), *Citrobacter freundii* ATCC 8090 was used to inoculate tap water (low and medium levels of quantitative), and *E. coli* QL 41411.1 was used to inoculate bottled water.

All cultures were propagated on Tryptic Soy Agar with 5% Sheep Blood (SBA) from a stock culture stored at -70 °C. The SBA was incubated at 35 ± 1 °C for 24 ± 2 h before transferring a single colony to Brain Heart Infusion (BHI) broth and incubating at 35 ± 1 °C for 24 ± 2 h. Using BHI broth as the diluent, the cultures were diluted to the proper contamination levels.

Colilert Method

For each water matrix, a 100 mL test portion was placed into a transparent vessel. A pre-weighed dose (1 packet) of Colilert reagent was added to each portion. The vessel was closed and shaken until dissolved. It was then incubated at 35 ± 0.5 °C for 24 h. Total coliforms are present if a yellow color change is observed. *E. coli* is present is a yellow color change is observed and fluoresces under a long wave UV lamp (366 nm). Negative results produced no color change.

Colilert Quanti-Tray MPN Method

For each water matrix, a 100 mL test portion was placed into a transparent vessel. A pre-weighed dose (1 packet) of Colilert was added to each portion. The vessel was closed and shaken until dissolution. The 100 mL portion was poured into a 51-Well Quanti-Tray® and sealed in an IDEXX Quanti-Tray Sealer. The sealed tray was incubated at 35 ± 0.5 °C for 24 h. Total coliforms are present if a yellow color change is observed. *E. coli* is present if a yellow color change is observed and fluoresces under a long wave UV lamp (366 nm). Negative results produced no color change. The MPN was determined by counting the number of positive wells and referring to the Colilert MPN table (Annex 2).

AquaCHROM ECC Method

For each water matrix, a 100 mL test portion was placed into a transparent vessel. A pre-weighed dose (1 vial) of AquaCHROM ECC was added to each portion. The vessel was closed and shaken until dissolved. It was then incubated at 35 - 37 °C for 18 - 24 h. *E. coli* was present is a green to blue-green color change was observed. Non-*E. coli* coliform bacteria were present if a yellow color change was observed. A negative result produced no color change.

AquaCHROM ECC – MPN Method

For tap water, well water and lake water matrixes, each 100 mL test portion was poured into a dispenser. A pre-weighed dose (1 vial) of AquaCHROM ECC was added to each portion. The vessel was closed and shaken until dissolved. The 100 mL portion was poured into the wells of a 48 well plate. It was then incubated at 35 - 37 °C for 18 - 24 h. The presumptive results were read and recorded at 18 h and at 24 h. Presence of *E. coli* turned the water sample green to blue green. Non-*E. coli* coliform bacteria were present if the water turned yellow. The MPN was determined by referring to the AquaCHROM ECC MPN table (Annex 1).

Qualitative Results

The probability of detection (POD) was calculated as the number of positive outcomes divided by the total number of trials [2]. The POD was calculated for the candidate presumptive results, $POD_{CP,}$ the candidate confirmatory results, POD_{CC}, the difference in the candidate presumptive and confirmatory results, $dPOD_{CP,}$ presumptive candidate results that confirmed positive, $POD_{C,}$ the reference method, POD_{R}, and the difference in the confirmed candidate and

reference methods, $dPOD_C$. The POD analysis between the AquaCHROM ECC method and the reference method indicated that there was no significant difference, with 95% confidence, between the number of positive results for the method. The POD analysis between the AquaCHROM ECC presumptive and confirmed results indicated that there was no significant difference, with 95% confidence for the method. A summary of POD analyses is presented in Table 1.

Quantitative Results

Statistical analysis was conducted for each *E. coli* and other coliform contamination level for tap water, well water and lake water matrixes. Logarithmic transformation of the counts (CFU/100 mL) was performed and the difference of means, with 90 and 95% confidence intervals, between the AquaCHROM method and the Colilert method was determined for each matrix and each contamination level. The difference of means and confidence intervals were calculated using the Independent Laboratory Study Workbook for Unpaired Method Analysis for Micro Testing supplied by the AOAC Research Institute [3]. The 90% confidence interval of the bias between the two methods fell between - 0.5 to 0.5 Log_{10} for each concentration indicating equivalence between the two methods [4].

The repeatability (S_r) calculated as standard deviation (SD) of the AquaCHROM ECC MPN and the Colilert methods was determined for tap water, well water, and lake water. A Cochran and Grubbs outlier test was performed for both methods. The analysis between the two methods indicated that there were no outliers, and the methods were statistically equivalent, with 95% CIs, between the counts achieved by the method for all matrixes. A summary of the study data,

statistical analysis and 95% CIs are presented in Table 2. Figures 1 - 3 display graphs of the Log_{10} values of the AquaCHROM method and the Colilert method.

Discussion

The AquaCHROM ECC method evaluated in this study is statistically equivalent to the Colilert method for determining both the presence/absence as well as the MPN of *E. coli* and other coliforms in 100 mL water samples.

The method allows the user to obtain accurate results within 24 h in the matrixes evaluated for the presence of coliforms in water samples incubated at 35 - 37 °C. The non-agar based medium was easy to interpret based on a color change to green (*E. coli*) or yellow (other coliforms) that can be read under normal lighting conditions. The AquaCHROM ECC method required no additional steps, i.e., the use of UV light to check for fluorescence. It is recommended to loosely close the propylene dispenser bottles when autoclaved for decontamination to ensure multiple use.

Conclusion

The data from this study supports the product claim that the AquaCHROM ECC is an effective one step method for the detection of *E. coli* and coliforms in 100 mL water samples for all matrixes evaluated (tap water, well water, lake water, and bottled water) and for enumeration of *E. coli* and coliforms in 100 mL water samples in tap water, well water, and lake water. The method allows the end user to acquire accurate results within 24 h of incubation without any extra step, obtaining results which are statistically equivalent to those obtained with the compared method.

Conflict of Interest

All authors declare no conflict of interest.

References

[1] Confirmation and Identification of *Salmonella* spp., *Cronobacter* spp. and other Gram-Negative organisms by MALDI Biotyper Method: Collaborative Study. First Action OMA 2017.09.

[2] Wehling, P., LaBudde, R. A., Brunelle, S. L., and Nelson, M. T. 2011. *Probability of Detection (POD) as a Statistical Model for the Validation of Qualitative Methods.* Journal of AOAC International. **94**, 335-47.

[3] AOAC Independent Laboratory Study Workbook for Unpaired Method Analysis for Micro Testing Version 1.2.

[4] AOAC SMPR 2020, Version 4.0: Standard Method Performance Requirements (SMPRs) for Quantitative Microbiology Methods for Food and Environmental Samples.

[5] Blodgett, R. (Content current as of: 10/09/2020). BAM Appendix 2: Most Probable Number from Serial Dilutions. *U.S. Food & Drug*. https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions.

Table 1. AquaCHROM ECC Method vs. Colilert Method – POD Results

Matrix	N[a]	AquaCHROM ECC			Colilert			dPOD$_C$[e]	95% CI[f]
		x[b]	POD$_C$[c]	95% CI	x	POD$_R$[d]	95% CI		
Tap Water (100 mL) *E. coli* ATCC[g] 25922	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
	20	11	0.55	0.34, 0.74	12	0.60	0.39, 0.78	-0.05	-0.33 0.24
	5	5	1.00	0.57, 1.00	5	1.00	0.57,1.00	0.00	-0.43, 0.43
Well Water (100 mL)	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
	20	13	0.65	0.43, 0.82	14	0.70	0.48, 0.86	-0.05	-0.32 0.23
	5	5	1.00	0.57, 1.00	5	1.00	0.57,1.00	0.00	-0.43, 0.43
Lake Water (100 mL)	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
	20	15	0.75	0.53, 0.89	14	0.70	0.48, 0.86	0.05	-0.22 0.31
	5	5	1.00	0.57, 1.00	5	1.00	0.57,1.00	0.00	-0.43, 0.43
Bottled Water (100 mL) *E. coli* QL[h] 41411.1	5	0	0.00	0.00, 0.43	0	0.00	0.00, 0.43	0.00	-0.43, 0.43
	20	17	0.85	0.64, 0.95	15	0.75	0.53, 0.89	0.00	-0.15, 0.34
	5	5	1.00	0.57, 1.00	5	1.00	0.57,1.00	0.00	-0.43, 0.43

[a]N = Number of test portions
[b]x = Number of positive test portions
[c]POD$_C$ = Candidate method confirmed positive outcomes divided by the total number of trials
[d]POD$_R$ = Reference method confirmed positive outcomes divided by the total number of trials
[e]dPOD$_C$= Difference between the confirmed candidate method result and reference method confirmed result POD values
[f]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level
[g]ATCC = American Type Culture Collection, Manassas, VA.
[h]QL = Q Laboratories Culture Collection, Cincinnati, OH.

Table 2. Results of AquaCHROM ECC vs. Colilert Quantitative Study

Matrix	Cont. level[a]	N[a]	AquaCHROM ECC			Reference Method[d]			DOM[e]	SE[f]	95% CI[g]		90% CI	
			Log_{10} Mean[b]	s_r	RSD_r[c]	Log_{10} Mean	s_r	RSD_r			LCL[h]	UCL[i]	LCL	UCL
Tap Water (100 mL) *C. freundii* ATCC[j] 8090 & *E. coli* ATCC 25922	Low	5	0.897	0.412	45.931	0.774	0.488	63.049	0.122	0.286	-0.537	0.781	-0.409	0.654
	Medium	5	1.740	0.066	3.793	1.695	0.085	5.015	0.045	0.048	-0.066	0.156	-0.044	0.135
	High	5	2.008	0.134	6.673	2.017	0.063	3.123	-0.009	0.066	-0.162	0.143	-0.132	0.114
Well Water (100 mL) Naturally contaminated	Low	5	0.414	0.243	58.696	0.438	0.229	52.283	-0.024	0.149	-0.368	0.320	-0.302	0.254
	Medium	5	1.621	0.109	6.724	1.553	0.043	2.769	0.068	0.052	-0.053	0.189	-0.030	0.166
	High	5	1.983	0.048	2.421	1.927	0.053	2.750	-0.009	0.032	-0.082	0.064	-0.068	0.050
Lake Water (100 mL) Naturally contaminated	Low	5	0.859	0.120	13.970	0.292	0.283	96.918	0.567	0.138	0.250	0.885	0.312	0.823
	Medium	5	1.734	0.049	2.826	1.384	0.039	2.818	0.350	0.028	0.285	0.415	0.298	0.402
	High	5	2.052	0.096	4.678	1.940	0.041	2.113	0.112	0.047	0.005	0.220	0.026	0.199

[a]N = Number of test portions

[b]Mean of five replicate portions, after logarithmic transformation: $Log_{10}[CFU/g + (0.1)f]$.

[c]RSD_r = Relative standard deviation of repeatability. $RSD_r = \frac{SD}{MEAN} \times 100$

[d]Reference method is Idexx Colilert Method.

[e]DOM = Difference of means; $mean_{cand}$ - $mean_{ref}$

[f]SE = Standard Error of DOM

[g]CI = Confidence interval for DOM

[h]LCL = Lower confidence limit for DOM

[i]UCL = Upper confidence limit for DOM

[j]ATCC = American Type Culture Collection, Manassas, VA.

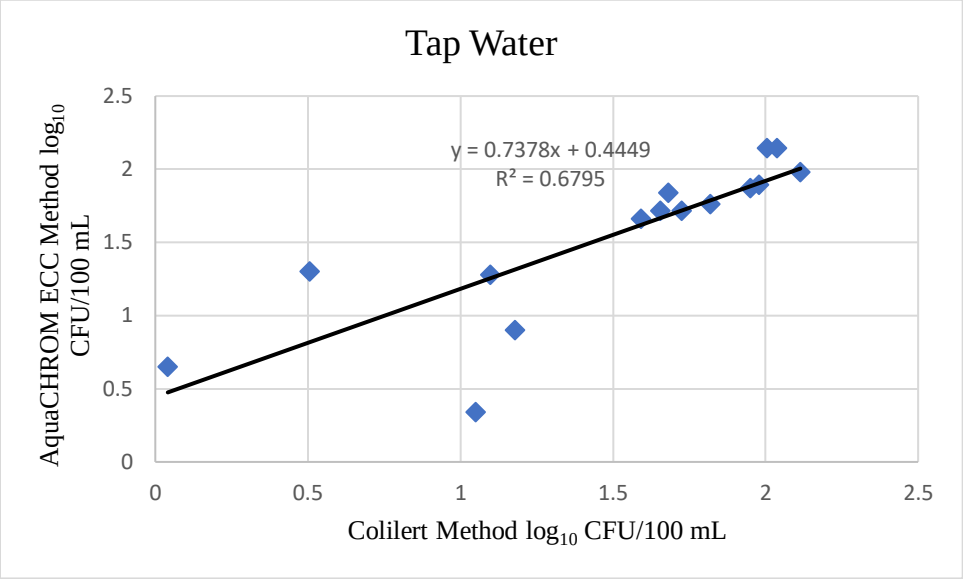

Figure 1: Method Comparison Results of AquaCHROM ECC vs. Colilert for Tap Water

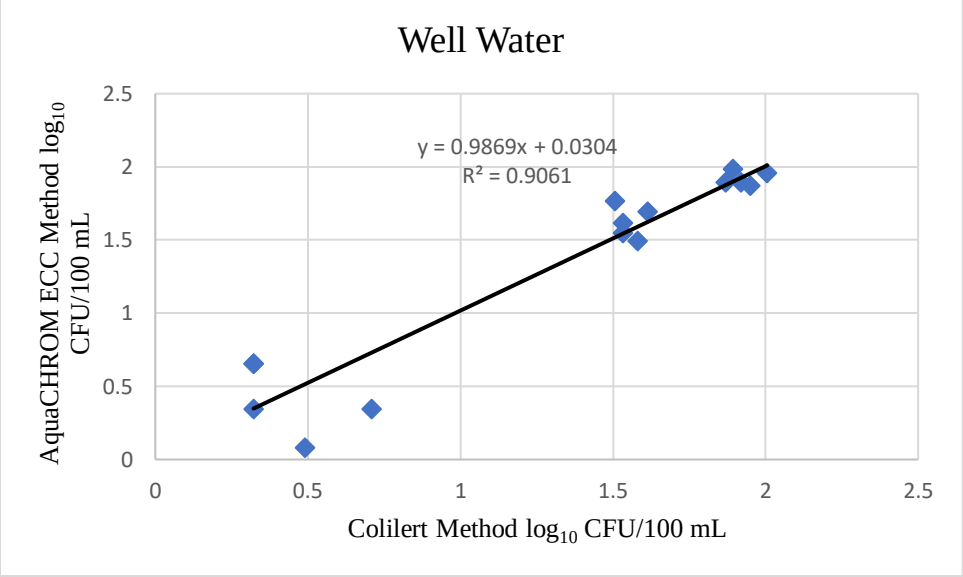

Figure 2: Method Comparison Results of AquaCHROM ECC vs. Colilert for Well Water

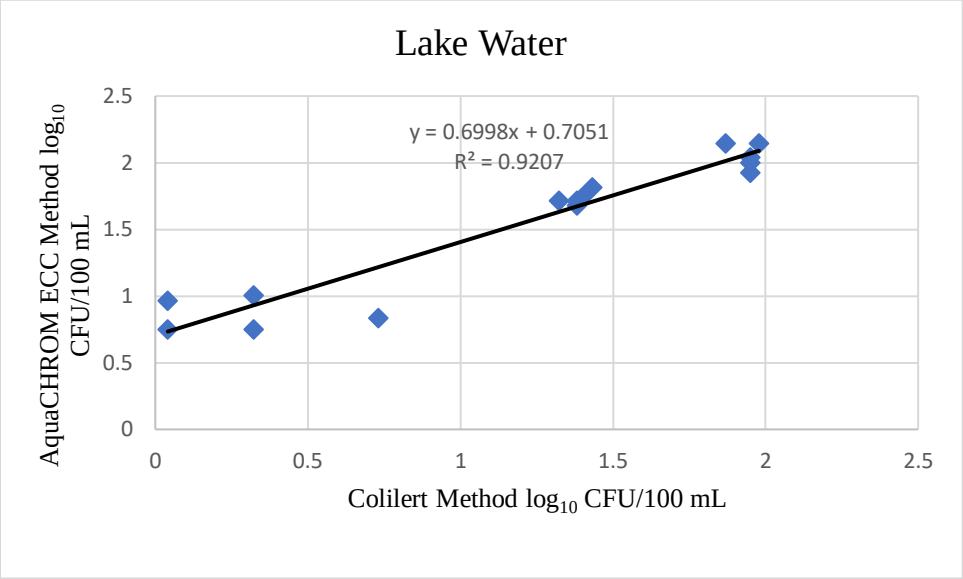

Figure 3: Method Comparison Results of AquaCHROM ECC vs. Colilert for Lake Water

Annex 1

AquaCHROM ECC MPN Table[a]

Positive wells	MPN	Lower 95 %	Upper 95 %
0	0	0	3.8
1	1.1	0.14	7.8
2	2.1	0.52	8.8
3	3.2	1	10
4	4.4	1.6	12
5	5.5	2.2	13
6	6.7	2.9	15
7	7.9	3.7	17
8	9.1	4.5	19
9	10	5.3	20
10	12	6.2	22
11	13	7.1	24
12	14	8.1	26
13	16	9	28
14	17	10	30
15	19	11	31
16	20	12	34
17	22	13	36
18	24	15	38
19	25	16	40
20	27	17	42
21	29	18	45
22	31	20	47
23	33	21	50
24	35	23	53
25	37	24	55
26	39	26	58
27	41	28	61
28	44	30	65
29	46	32	68
30	49	34	72
31	52	36	76
32	55	38	80
33	58	40	84
34	62	43	89
35	65	45	94
36	69	48	99
37	74	51	110
38	78	55	110
39	84	58	120
40	90	62	130
41	96	67	140
42	100	72	150
43	110	78	160
44	120	85	180
45	140	93	210
46	160	100	250
47	190	120	320
48	>190	120	-

[a]Based on BAM Appendix 2 (5).

Annex 2

IDEXX
51-Well Quanti-Tray®
MPN Table

No. of wells giving positive reaction	MPN per 100 ml sample	95% Confidence Limits	
		Lower	Upper
0	<1.0	0.0	3.7
1	1.0	0.3	5.6
2	2.0	0.6	7.3
3	3.1	1.1	9.0
4	4.2	1.7	10.7
5	5.3	2.3	12.3
6	6.4	3.0	13.9
7	7.5	3.7	15.5
8	8.7	4.5	17.1
9	9.9	5.3	18.8
10	11.1	6.1	20.5
11	12.4	7.0	22.1
12	13.7	7.9	23.9
13	15.0	8.8	25.7
14	16.4	9.8	27.5
15	17.8	10.8	29.4
16	19.2	11.9	31.3
17	20.7	13.0	33.3
18	22.2	14.1	35.2
19	23.8	15.3	37.3
20	25.4	16.5	39.4
21	27.1	17.7	41.6
22	28.8	19.0	43.9
23	30.6	20.4	46.3
24	32.4	21.8	48.7
25	34.4	23.3	51.2
26	36.4	24.7	53.9
27	38.4	26.4	56.6
28	40.6	28.0	59.5
29	42.9	29.7	62.5
30	45.3	31.5	65.6
31	47.8	33.4	69.0
32	50.4	35.4	72.5
33	53.1	37.5	76.2
34	56.0	39.7	80.1
35	59.1	42.0	84.4
36	62.4	44.6	88.8
37	65.9	47.2	93.7
38	69.7	50.0	99.0
39	73.8	53.1	104.8
40	78.2	56.4	111.2
41	83.1	59.9	118.3
42	88.5	63.9	126.2
43	94.5	68.2	135.4
44	101.3	73.1	146.0
45	109.1	78.6	158.7
46	118.4	85.0	174.5
47	129.8	92.7	195.0
48	144.5	102.3	224.1
49	165.2	115.2	272.2
50	200.5	135.8	387.6
51	> 200.5	146.1	infinite