IDEXX Summary 16A

Topic	Colilert* 250 vs ISO** 9308-1:2000(E) membrane filtration method
Title	"A Performance Comparison of Colilert 250 Presence/Absence Method and ISO 9308-1:2000(E) in the Detection of *Escherichia coli* and Total Coliform Bacteria in Bottled Water Samples"
Source	IDEXX Laboratories, Inc.
Date	September 2011

Highlights

- Colilert is approved for use in 24 countries worldwide and has been adopted by over 90% of US State laboratories.
- The Colilert 250 test provides bottled water facilities with a more straightforward and easy-to-use method over the ISO 9308-1:2000(E) membrane filtration method.
- Colilert 250 test provides a definitive result after 24 hours of incubation and performed equivalently to ISO9308-1:2000(E) in the detection of *E. coli* and total coliform introduced into various bottled water types
- In this study ISO9308-1:2000(E) falsely identified the total coliform, Klebsiella oxytoca (ATCC 13182), as an *E. coli* (confirmation results: indole positive and oxidase negative) after 48 hours of total incubation and multiple confirmation steps. Colilert 250 provided a definitive total coliform result after 24 hours of incubation (yellow only reaction).
- Colilert 250 is extremely proficient in the suppression of high concentrations of non-target organisms. ISO9308-1:2000(E) required the user to perform multiple confirmation steps, which included an additional 24 hour incubation period, and further delayed definitive results.

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** International Organization for Standardization.

A Performance Comparison of Colilert 250* Presence/Absence Method and ISO 9308-1:2000(E) in the Detection of *Escherichia coli* and Total Coliform Bacteria in Bottled Water Samples

September 2011

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The VITEK® 2 system is a trademark of bioMérieux, Inc.

Introduction

IDEXX entered the water-testing market in 1993 with Colilert; this innovative microbiological test kit quickly became a very popular water testing system which is now adopted by over 90% of US State laboratories[1]. The IDEXX Colilert reagent is approved for use in 24 countries around the world (**Table 1**) for the detection of *Escherichia coli* and total coliform bacteria in drinking water[2]. Colilert has been included for use with drinking and source waters in *Standard Methods for Examination of Water and Wastewater*[3], a joint publication of the Water Environment Federation (WEF), American Water Works Association (AWWA) and American Public Health Association (APHA). The United States Environmental Protection Agency (EPA) has approved Colilert for use with both drinking[4] and source[5] waters. The International approvals of Colilert include the Association of Official Analytical Chemists International[6] (AOAC), International Bottle Water Association[7] (IBWA), and European Bottled Watercooler Association[8] (EBWA).

Table 1
Worldwide Product Approvals/Acceptances listed by Country

Country	Products Approved†	Water Types
Argentina	Colilert*	Drinking water
Australia	Colilert, Colilert*-18, Quanti-Tray*, Quanti-Tray*/2000	Drinking water
Belgium	Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water
Brazil	Colilert, Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water
Canada	Colilert, Colilert-18, Colisure*, Quanti-Tray, Quanti-Tray/2000, SimPlate for HPC	Drinking water
Chile	Colilert, Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water
China	Colilert, Filta-Max*, Quanti-Tray, Quanti-Tray/2000	Drinking water, surface water, ground water, drinking water for poultry and livestock
Colombia	Colilert, Colilert-18, Colisure, Quanti-Tray, Quanti-Tray/2000	Drinking water
Costa Rica	Colilert, Colilert-18, Colisure, Quanti-Tray, Quanti-Tray/2000	Drinking water
Croatia	Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water, bottled water
Czech Republic	Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water
Denmark	Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water
El Salvador	Colilert, Colilert-18, Colisure, Quanti-Tray, Quanti-Tray/2000, SimPlate for HPC	Drinking water
Finland	Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water, fresh bathing water
Germany	Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water, spas and pools
Greece	Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water
Greenland (Denmark)	Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water
Guatemala	Colilert, Colilert-18, Colisure, Quanti-Tray, Quanti-Tray/2000	Drinking water, source water
Hungary	Colilert, Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water, bathing water
Iceland	Colilert, Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water
Ireland	Colilert	Drinking water
Italy	Colilert-18, Filta-Max, Quanti-Tray, Quanti-Tray/2000	Drinking water, surface water, spas and pools
Jamaica	Colilert, Colilert-18, Colisure, Quanti-Tray, Quanti-Tray/2000	Drinking water, source/surface water, wastewater, recreational waters
Japan	Colilert	Drinking water
Mexico	Colilert	Drinking water, bottled water
New Zealand	Colilert, Colilert-18, Colisure, Enterolert*, Quanti-Tray, Quanti-Tray/2000	Drinking water, fresh and marine bathing water
Norway	Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water, bottled water
Panama	Colilert, Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water
Philippines	Colilert	Drinking water
Poland	Colilert-18, Enterolert*-E, Quanti-Disc* YEA, Quanti-Tray, Quanti-Tray/2000	Drinking water, bathing water, source/surface water
Slovakia	Colilert, Colilert-18, Colisure, Quanti-Tray, Quanti-Tray/2000	Drinking water, bottled water, ground water, source/surface water, wastewater
South Africa	Colilert, Quanti-Tray, Quanti-Tray/2000, Quanti-Disc YEA, SimPlate for HPC	Drinking water, wastewater effluent
South Korea	Colilert	Drinking water
Spain	Colilert-18, Quanti-Tray, Quanti-Tray/2000	Drinking water
Sweden	Colilert-18, Enterolert-E, Quanti-Tray	Bathing water
Ukraine	Colilert-18, Quanti-Tray, Quanti-Tray/2000, Quanti-Disc	Drinking water
United Kingdom	Colilert-18, Filta-Max, Quanti-Tray, Quanti-Tray/2000, Quanti-Disc	Drinking water
United States	Colilert, Colilert-18, Colisure, Enterolert, Filta-Max, Filta-Max xpress*, Quanti-Tray, Quanti-Tray/2000, SimPlate for HPC	Drinking water, ambient water, ground water, source/surface water, LT2 testing, wastewater, marine water, dairy water (FDA)
Zimbabwe	Colilert	Drinking water, ambient water, ground water, wastewater

†Approvals/Acceptances vary per region effective as of May 2011. Contact IDEXX for references or updated information.

As indicated above, Colilert has been thoroughly evaluated within the drinking-water community – an industry that requires accurate, rapid and cost-effective *E. coli* and total coliform testing options. The bottled water community has recently become interested in adopting the Colilert test for their routine testing regiment. Therefore, IDEXX conducted the following study to provide additional information on the use of Colilert 250 presence/absence method in bottled water samples and compare its performance against the ISO 9308-1:2000(E) Standard Test method.

1. http://www.idexx.com/view/xhtml/en_us/water/colilert.jsf
2. http://www.idexx.com/pubwebresources/pdf/en_us/water/water-acceptances-worldwide.pdf
3. http://www.idexx.com/pubwebresources/pdf/en_us/water/5L.pdf
4. http://www.idexx.com/pubwebresources/pdf/en_us/water/096221400L.pdf
5. http://www.idexx.com/pubwebresources/pdf/en_us/water/096221800L.pdf
6. http://www.idexx.com/pubwebresources/pdf/en_us/water/096223000L.pdf
7. http://www.idexx.com/pubwebresources/pdf/en_us/water/096359000L.pdf
8. http://www.idexx.com/pubwebresources/pdf/en_us/water/096328000L.pdf

The Colilert 250 presence/absence method uses the IDEXX patented Defined Substrate Technology* (DST*) to simultaneous detect of *E. coli* and total coliform bacteria in 250mL volumes of drinking and bottled water samples after 24 – 28 hours of incubation at 36°C ± 2°C. The two nutrient-indicators, *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl- β-D-glucuronidase (MUG) are the primary nutrients in Colilert and can be metabolized by the total coliform enzyme β-galactosidase and the *E. coli* enzyme β-glucuronidase, respectively. As total coliform bacteria grow in Colilert, they use the enzyme β-galactosidase to metabolize ONPG and change the reagent from colorless to yellow (see **Figures 1a and 1b**). *As E. coli* grow in Colilert, they use the enzyme β-glucuronidase to metabolize MUG and create fluorescence when placed under 365-nm ultraviolet (UV) light (see **Figures 2a and 2b**). The Colilert 250 presence/absence method is also specifically formulated to selectively suppress the few non-*E. coli* and non-total coliform bacteria that have the β-galactosidase and β-glucuronidase enzymes present.

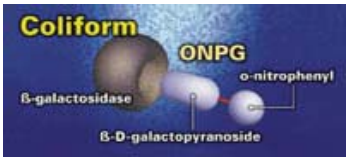


Figure 1a

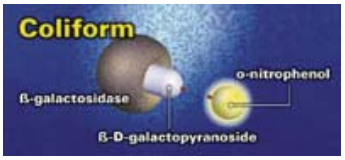


Figure 1b

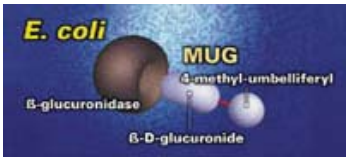


Figure 2a

Figure 2b

Method

Bottled Water Sample Types

The thirty bottled water samples included in this study originated from twelve different countries around the world and were made up of the following bottled water types – spring water brands, artesian water brands, drinking water brands, still mineral water brands, and sparkling mineral water brands (**Table 2**). Colilert 250 requires that sparkling water types be thoroughly degassed prior to testing. Therefore, all sparkling bottled water samples were manually degassed, by shaking, prior to testing.

Table 2
Bottled Water types, total number of brands and geographical locations used in this study

	Total # of waters	Total # of brands	Water source location			
Bottled Spring	9	6	4 x USA	3 x France	1 x Iceland	1 x Scotland
Bottled Artesian	8	5	3 x Croatia	3 x New Zealand	2 x South Pacific Islands	
Bottled Drinking	7	5	7 x USA			
Bottled Sparkling Mineral	5	5	1 x Germany	3 x Italy	1 x United Kingdom	
Bottled Still Mineral	1	1	1 x United Kingdom			
Totals	30	22				

Analytical Methods

It is optimal, whenever possible, to use natural samples with the target organisms when comparing methods. Due to the limited availability of bottled water samples containing *E. coli* and total coliform bacteria, it was necessary to perform the comparison by the inoculation of relevant target and non-target reference organisms into the water matrix in question. ISO/CD 11133:2009 was used as guidance when selecting the target and non-target organisms for the spike challenge study (**Table 3**). Quality control strains listed in Annex F, Table F 3 of the ISO/CD 11133:2009 were included, along with other relevant strains listed within the ISO/CD 11133:2009 document. Pure cultures of the reference strains were sourced from American Type Culture Collection (ATCC) and were analyzed by bioMérieux's VITEK 2 system prior to use (**Appendix B, Table B-1**).

The target organism panel consisted of 9 relevant organisms; 3 strains of *E. coli* and 6 strains of total coliform bacteria. Each organism was inoculated into a bottled water sample at a concentration of 1 – 15 cfu / 250mL. Each bottled water sample was then split and analyzed in duplicate using the Colilert 250 presence/absence method and the reference method ISO 9308-1:2000(E).
The non-target organism panel consisted of 6 relevant organisms; 6 strains of common, non-*E.*coli, non-coliform, gram positive and gram negative water bacteria. Non-target organisms were inoculated into a bottled water sample as a cocktail, each at a concentration of 10 - 50 cfu / 1mL for a total of 250 cfu / 1mL. Each bottled water sample was then split and analyzed in duplicate using the Colilert 250 presence/absence method and the reference method ISO 9308-1:2000(E).

Table 3
Reference strains of *E. coli*, total coliform and non-targets selected for pure culture spike

Bacterium	Source	Relevance
Escherichia coli	ATCC[9] 11775[10]	IDEXX recommended QC[11] Strain
Escherichia coli	ATCC 25922[10]	IDEXX recommended QC Strain ; QC Strain for ISO 9308-1 (ISO 11133:2009)
Escherichia coli	ATCC 8739	QC Strain for ISO 9308-1 (ISO 11133:2009)
Klebsiella pneumoniae	ATCC 13883	IDEXX recommended QC Strain ; QC Strain for ISO 9308-1 (ISO 11133:2009)
Enterobacter aerogenes	ATCC 13048	QC Strain for ISO 9308-1 (ISO 11133:2009)
Enterobacter cloacae	ATCC 13047	Listed in ISO 11133:2009
Citrobacter freundii	ATCC 43864	Listed in ISO 11133:2009
Klebsiella oxytoca	ATCC 13182	Relevant total coliform
Hafnia alvei	ATCC 13337	Listed in ISO 11133:2009
Enterococcus faecalis	ATCC 29212	QC Strain for ISO 9308-1 (ISO 11133:2009)
Staphylococcus aureus	ATCC 25923	QC Strain for ISO 9308-1 (ISO 11133:2009)
Pseudomonas aeruginosa	ATCC 27853[10]	QC Strain for ISO 9308-1 (ISO 11133:2009)
Aeromonas hydrophila	ATCC 7966	Listed in ISO 11133:2009
Bacillus licheniformis	ATCC 14580	Listed in ISO 11133:2009
Pseudomonas putida	ATCC 12633	Listed in ISO 11133:2009

9. American Type Culture Collection (ATCC)
10. ATCC 11775, ATCC 25922, and ATCC 27853 are listed as quality control organisms in the Colilert 250* insert
11. Quality Control (QC)

Colilert 250 presence/absence method was performed as described in the supplied product insert. As previously described, each inoculated bottled water sample was split between the two methods. For Colilert 250, duplicate 250mL samples were dispensed into IDEXX sterile plastic 290mL vessels and a blister pack of Colilert 250 medium was added to each vessel. Colilert 250 samples were agitated until medium was completely dissolved and then placed into an air-circulating incubator set at 36°C ± 1°C. Colilert 250 samples were observed for color and fluorescence under a 365nm UV lamp after 24 and 28 hours of incubation. After the 28 hour observation all Colilert 250 samples, positive and negative, were confirmed by the confirmation methods listed in **Appendix A, Flowcharts A-1 & A-2**.

Reference method ISO 9308-1:2000(E) was performed according to the Standard Test method described in the related ISO documentation. As previously described, each inoculated bottled water sample was split between the two methods. For ISO 9308-1:2000(E), duplicate 250mL samples were concentrated by membrane filtration, membrane was carefully placed on the Lactose TTC agar and finally agar plates were placed into an air-circulating incubator set at 36°C ± 1°C. Samples were observed after 21±3 hours and lactose-positive bacteria were counted and confirmed through the Oxidase and Indole confirmation tests described in ISO 9308-1:2000(E). The confirmed ISO 9308-1:2000(E) membrane filtration count was used to determine the actual *E. coli* or total coliform inoculum during testing.

Statistical Analysis

The confirmed ISO 9308-1:2000(E) membrane filtration count was used to determine the actual *E. coli* or total coliform inoculum, only data sets with an average count falling between 1-15 cfu / 250mL were used in the data analysis. Once used for the determination of actual inoculum, the ISO 9308-1:2000(E) quantitative data sets were converted to presence/absence data sets so that they could be directly compared to the Colilert 250 presence/absence data sets. Finally, all the Colilert 250 and ISO 9308-1:2000(E) results were then populated into a simple 2 x 2 summary table as depicted in **Table 4**. The results in each table were analyzed using the McNemar's test[12,13], which has a final output of a p-value. Summary of the McNemar's test will be shown in the form of a table as depicted in **Table 5.** The p-value was assigned using the chi-square result calculated, with one degree of freedom, from the number of unmatched pairs represented by *b* and *c* in **Table 4**. It was determined that a significance level α of 0.05 would be assigned, therefore requiring a 95% confidence of the null hypothesis. Therefore, it can be said, with a 95% confidence level, that data sets with a p-value equal to or greater than 0.05 would be considered statistically similar while data sets with a p-value less than 0.05 would be considered statistically different, as seen in **Table 5**.

Table 4
Example of 2 x 2 table used in McNemar's test

ISO 9308-1:2000(E) Confirmed result	Colilert 250 Confirmed result	#	Comments
+	+	*a*	Number of matching results
+	-	*b*	ISO method more sensitive
-	+	*c*	Colilert 250 more sensitive
-	-	*d*	Number of matching results
		n = *a* + *b* + *c* + *d*	

12. McNemar, Quinn (June 18, 1947). "Note on the sampling error of the difference between correlated proportions or percentages". Psychometrika 12 (2): 153–157.
13. McNemar's test is listed as a suitable method for the statistical evaluation of Presence/Absence methods in ISO/DIS 17994:2002.

Table 5
Example of McNemar's test output based on Table 4

Study	# data points	Chi squared (1 degree of freedom)	P-value (α = 0.05)	Conclusion
Colilert 250 Confirmed Result vs. ISO 9308-1:2000(E) Confirmed Result	n = *a* + *b* + *c* + *d*	x	y	y ≥ 0.05 = Methods equivalent y < 0.05 = Methods different

Results and Discussion

E.coli Recovery

Fifty eight water samples were inoculated with the 3 different *E. coli* reference strains. Of the 58 samples, 52 samples fell within the required spike concentration of 1 – 15 cfu / 250mL (determined by ISO 9308-1:2000(E) colony count). These 52 sample pairs (104 actual data points) were used to populate **Table 6** representing the 24 hour Colilert 250 results compared to the ISO 9308-1:2000(E) results**.**

Table 6
***E. coli* Recovery of ISO 9308-1:2000(E) vs. Colilert 250 after 24 hour**

ISO 9308-1:2000(E) Confirmed result	Colilert 250 Confirmed result	#
+	+	100
+	-	4[14,15]
-	+	0
-	-	0
		n = 104

Of the 104 data points, the number of confirmed Colilert 250 positive results matching the ISO 9308-1:2000(E) confirmed positive results was 100 after 24 hours of incubation and increased up to 103 after 28 hours of incubation. The 3 sample difference in the performance of Colilert 250 in the detection of *E. coli* ATCC 8739 was directly related to the use of carbonated mineral bottled water types. The carbonated water types were degassed by manual shaking, but these results may indicate that the presence of residual carbonation may slightly delay the detection of some *E.coli* strains. Results listed in **Table 6** were subsequently used in the statistical analysis by the McNemar's test. The statistical analysis indicated that the Colilert 250 confirmed positive results after 24 hours of incubation were equivalent to the ISO 9308-1:2000(E) confirmed positive results, summarized in **Table 7.**

Table 7
***E. coli* - McNemar's test output based on Table 6**

Study	# data points	Chi squared (1 degree of freedom)	P-value (α = 0.05)	Conclusion
Colilert 250 at 24 hours vs. ISO 9308-1:2000(E)	104	2.250	0.1336	Methods equivalent

14. Delay to detect *E.coli* (ATCC 8739) in three sparkling mineral water type samples, resolved by 28 hour
15. *Klebsiella oxytoca* (ATCC 13182) was spiked into 14 different waters (28 samples per method); presence of this total coliform was correctly identified by Colilert 250 after 24 hours of incubation (yellow only reaction) while ISO9308-1:2000(E) falsely identified this total coliform as an *E. coli* (confirmation results : indole positive and oxidase negative). The false positive *E. coli* results by ISO9308-1:2000(E) are further summarized in **Appendix C – Table C-4** and not included in this summary table.

Total Coliform Recovery

Eighty Four water samples were inoculated with the 6 different total coliform reference strains. Of the 84 samples, 72 samples fell within the required spike concentration of 1 – 15 cfu / 250mL (determined by ISO 9308-1:2000(E) colony count). These 72 sample pairs (144 actual data points) were used to populate **Table 8** representing the 24 hour Colilert 250 results compared to the ISO 9308-1:2000(E) results**.**

Table 8
Total coliform Recovery of ISO 9308-1:2000(E) vs. Colilert 250 after 24 hour

ISO 9308-1:2000(E) Confirmed result	Colilert 250 Confirmed result	#
+	+	140[16]
+	-	3[17]
-	+	1[16]
-	-	0
		n = 144

Of the 144 data points, the number of confirmed Colilert 250 positive results matching the ISO 9308-1:2000(E) confirmed positive results were 140 after 24 hours of incubation. The 4 sample difference in the performance of Colilert 250 and ISO 9308-1:2000(E) was directly related to total coliform reference strain, *Hafnia alvei* (ATCC 13337)[18]. Results listed in **Table 8** were subsequently used in the statistical analysis by the McNemar's test. The statistical analysis indicated that the Colilert 250 confirmed positive results after 24 hours of incubation were equivalent to the ISO 9308-1:2000(E) confirmed positive results, summarized in **Table 9.**

Table 9
Total coliform - McNemar's test output based on Tables 9 & 10

Study	# data points	Chi squared (1 degree of freedom)	P-value ($\alpha = 0.05$)	Conclusion
Colilert 250 at 24 hours vs. ISO 9308-1:2000(E)	144	1.333	0.2482	Methods equivalent

Non-target Suppression

Twenty one water samples were inoculated with a non-target cocktail spike containing 6 different non-total coliform and non-*E. coli* reference strains. Of the 21 samples, 21 samples fell within the required total non-target spike concentration of 200 – 250 cfu / 1mL (determined by spread plate colony count on Tryptic Soy Agar with 5% Sheep Blood). **Appendix D, Table D-1** lists the average concentration of each non-target strain along with the total cocktail concentration. The 21 sample pairs (42 actual data points) were used to populate **Table 10** representing the 24 hour Colilert 250 results compared to the ISO 9308-1:2000(E) results**.**

16. *Klebsiella oxytoca* (ATCC 13182) was spiked into 14 different waters (28 samples per method); presence of this total coliform was correctly identified by Colilert 250 after 24 hours of incubation (yellow only reaction) while ISO9308-1:2000(E) falsely identified this total coliform as an *E. coli* (confirmation results : indole positive and oxidase negative). The false positive *E. coli* results by ISO9308-1:2000(E) are further summarized in **Appendix C – Table C-4** and not included in this summary table.
17. *H. alvei* was spiked into 15 different waters (30 samples) and only cultured on Lactose TTC three times (these are listed in **Appendix C, Table C-4**). Growth on the Lactose TTC was atypical in nature – extremely small clear and red coloured colonies but confirmed as a total coliform (Indole - negative, Oxidase - negative). It should be noted that *H. alvei* does not grow on EMB or MacConkey agar and is a rare environmental total coliform, considered so by its ability to produce low levels of acid by lactose fermentation.

Table 10
Non-target Suppression of ISO 9308-1:2000(E) vs. Colilert 250 after 24 hour

ISO 9308-1:2000(E) Confirmed result	Colilert 250 Confirmed result	#
+	+	0
+	-	0
-	+	0
-	-	42
		n = 42

Of the 42 data points, the number of confirmed Colilert 250 negative results matching the ISO 9308-1:2000(E) confirmed negative results were 42 after 24 hours of incubation. All samples analyzed using ISO 9308-1:2000(E) resulted in confluent colony growth on membrane filters and required additional confirmation steps which included an additional 24 hour incubation period, delaying the completion of final result. Colilert 250 product significantly outperformed the ISO 9308-1:2000(E) by providing a definitive and confirmed result after 24 hours of incubation, completely suppressing the non-target cocktail spike.

Conclusions

The following study showed that the detection capabilities of Colilert 250, for *E. coli* and total coliform bacterium in various bottled water matrices is equivalent to that of ISO9308-1:2000(E), and is further summarized in **Table 11**.

Table 11
Summary of Colilert 250 performance in detect of target organisms

Study	# data points	P-value ($\alpha = 0.05$)	Conclusion
E. coli - Colilert 250 vs. ISO 9308-1:2000(E)	104	0.1336	Methods equivalent
Total coliform - Colilert 250 vs. ISO 9308-1:2000(E)	144	0.2482	Methods equivalent

In this study the presence of total coliform, *Klebsiella oxytoca* (ATCC 13182)[18], was correctly identified by Colilert 250 after 24 hours of incubation (yellow only reaction) while ISO9308-1:2000(E) falsely identified this total coliform as an *E. coli* (confirmation results: indole positive and oxidase negative) after 48 hours of total incubation and multiple confirmation steps. The false positive *E. coli* result by ISO9308-1:2000(E) is further summarized in **Appendix C – Table C-4.**

This study also showed that the Colilert 250 product is extremely proficient in the suppression of high concentrations of non-target organisms. This is another area that ISO9308-1:2000(E) falls short; requiring the user to perform multiple confirmation steps which includes an additional 24 hour incubation that further delays definitive results. Additionally, the excessive non-target growth on the membrane filter may interfere with the method's ability to detect target organisms.

18. *Klebsiella oxytoca* (ATCC 13182), along with all other reference strains used in this study, were analyzed by bioMérieux's VITEK 2 system prior to use (**Appendix B, Table B-1**).

References

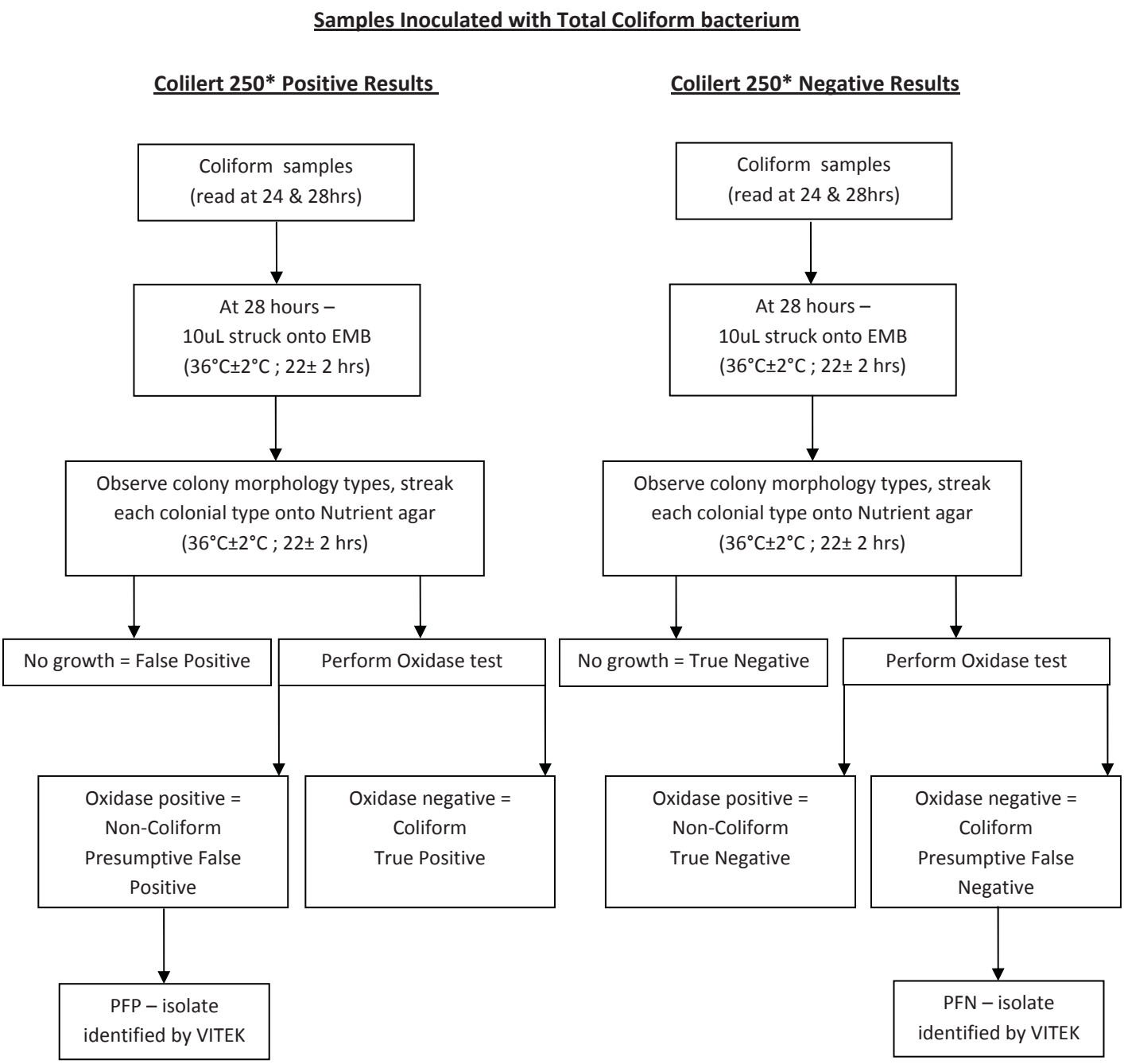
- ISO/TR 13843:2000(E) Water Quality – Guidance on validation of microbiological methods. Geneva: International Organization for Standardization.

- ISO 17994:2004 Water Quality – Criteria for establishing equivalence between microbiological methods. Geneva: International Organization for Standardization.

- ISO 9308-1:2000(E) Water Quality – Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method. Geneva: International Organization for Standardization.

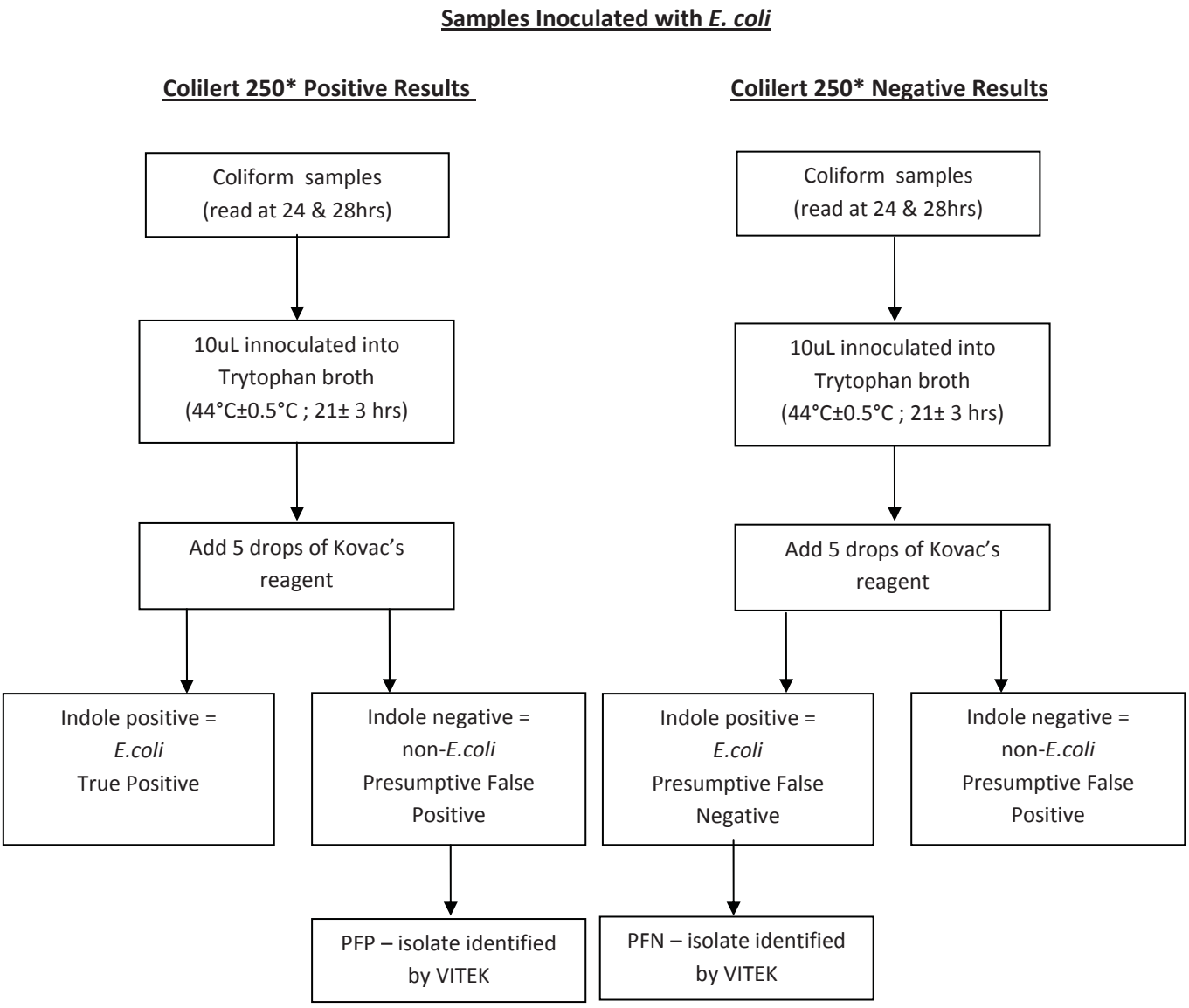
Appendix A
Methods used to confirm Colilert 250 presence/absence samples

Flowchart A-1
Confirmation steps performed on positive and negative Colilert 250 samples inoculated with total coliform



Appendix A (continued)
Methods used to confirm Colilert 250 presence/absence samples

Flowchart A-2
Confirmation steps performed on positive and negative Colilert 250 samples inoculated with *E. coli*



Appendix B
bioMérieux's VITEK 2 System Analysis of Pure Culture Reference Strains

Table B-1
Results from VITEK 2 System on Reference Strains used in this Study

Bacterium	Source	VITEK 2 Analysis		
		Selected Organism	Probability	Bionumber
Escherichia coli	ATCC 11775	*Escherichia coli*	99%	0405610540566601
Escherichia coli	ATCC 25922	*Escherichia coli*	98%	0405411560567601
Escherichia coli	ATCC 8739	*Escherichia coli*	99%	0405611540564611
Klebsiella pneumoniae	ATCC 13883	*Klebsiella pneumoniae*	99%	6605734773564010
Enterbacter aerogenes	ATCC 13048	*Enterbacter aerogenes*	99%	6625734553577010
Enterobacter cloacae	ATCC 13047	*Enterobacter cloacae*	95%	0627715553573210
Citrobacter freundii	ATCC 43864	*Citrobacter freundii*	99%	4415610555460211
Klebsiella oxytoca	ATCC 13182	*Klebsiella oxytoca*	99%	6707734677164011
Hafnia alvei	ATCC 13337	*Hafnia alvei*	89%	1421711342546201
Enterococcus faecalis	ATCC 29212	*Enterococcus faecalis*	99%	114002761773471
Staphylococcus aureus	ATCC 25923	*Staphylococcus aureus*	99%	050402022763271
Pseudomonas aeruginosa	ATCC 27853	*Pseudomonas aeruginosa*	98%	0003053303500252
Bacillus licheniformis	ATCC 14580	*Bacillus licheniformis*	96%	0333371555476261
Pseudomonas putida	ATCC 12633	*Pseudomonas putida*	99%[19]	0003051103500352

19. VITEK 2 reported Low Discrimination (*Pseudomonas aeruginosa*(50%) / *Pseudomonas putida*(50%)). Tests to separate, indicated by VITEK 2 report, were completed and resulted in a final determination of *P. putida (*99%). Results of the tests to separate included a lack of growth by organism at 42°C and lack of pyocyanin production during growth.

Appendix C
ISO 9308-1:2000(E) with confirmation, and Colilert 250 after 24 & 28 hours

Table C-1 : Legend for bacterium codes

Spike Code	Bacterium	Source
E1	*E. coli*	ATCC 11775
E2	*E. coli*	ATCC 25922
E3	*E. coli*	ATCC 8739
EA	*Enterbacter aerogenes*	ATCC 13048
EC	*Enterobacter cloacae*	ATCC 13047
KO	*Klebsiella oxytoca*	ATCC 13182
KP	*Klebsiella pneumoniae*	ATCC 13883
HA	*Hafnia alvei*	ATCC 13337
CF	*Citrobacter freundii*	ATCC 43864
NT	Enterococcus faecalis Staphylococcus aureus Pseudomonas aeruginosa Aeromonas hydrophila Bacillus licheniformis Pseudomonas putida	ATCC 29212 ATCC 25923 ATCC 278532 ATCC 7966 ATCC 14580 ATCC 12633

Table C-2 : Colilert 250 presence/absence method and ISO 9308-1:2000(E) results for *E. coli* spike

Sample ID			Lactose TTC agar		INDOLE TEST	Average Confirmed ISO *E. coli* count	Colilert 250 @ 24 hours		Colilert 250 @ 28 hours	
Spike Code[20]	Bottled Water Code[21]	Duplicate	Yellow / Orange colonies	Red colonies	+ / -		Yellow	FLR[22]	Yellow	FLR
E3	21	A	14		+	13.5	+	-	+	+
		B	13				+	-	+	+
E3	23	A	9		+	11.5	+	+	+	+
		B	14				+	-	+	+
E3	5	A	2		+	1.5	-	-[23]	-	-[23]
		B	1				+	+	+	+

Table C-3 : Colilert 250 presence/absence method and confirmation results for *E. coli* spike

Sample ID			Colilert 250 @ 24hours		Colilert 250 @ 28hours		INDOLE TEST	Confirmed Result
Spike Code	Bottled Water Code	Duplicate	Yellow	FLR	Yellow	FLR	+ / -	
E3	**5**	A	-	-	-	-	-	Yes
		B	+	+	+	+	+	Yes

20. See **Appendix C, Table C-1** for legend of Spike Codes
21. Each of the bottled waters was assigned a unique identity number; please see **Table 2** for more information on the bottled water types used
22. FLR = Fluorescent under a 365nm Ultraviolet lamp
23. Average inoculum was 1.5 cfu / 250mL; Colilert 250 replicate 1 - negative, Colilert 250 replicate 2 - positive (uneven distribution of low bacterial concentration may have been the cause); Colilert 250 replicate 1 confirmed to be absent of *E.coli* by confirmation testing (See Appendix D, Tables D-1 & D-3)

Appendix C (continued)
ISO 9308-1:2000(E) with confirmation, and Colilert 250 after 24 & 28 hours

Table C-4
Colilert 250 presence/absence method and ISO 9308-1:2000(E) for total coliform spike, *Klebsiella oxytoca* (ATCC 13182)

Sample ID			Lactose TTC agar		INDOLE TEST	OXIDASE TEST	Average Confirmed ISO *E. coli* count[24]	Colilert 250 @ 24 hours		Colilert 250 @ 28 hours	
Spike Code	Bottled Water Code	Duplicate	Yellow / Orange colonies	Red colonies	+ / -	+ / -		Yellow	FLR	Yellow	FLR
KO	1	A	10		+	-	7	+	-	+	-
		B	4		+	-		+	-	+	-
KO	2	A	13		+	-	11.5	+	-	+	-
		B	10		+	-		+	-	+	-
KO	3	A	6		+	-	6.5	+	-	+	-
		B	7		+	-		+	-	+	-
KO	4	A	9		+	-	9	+	-	+	-
		B	9		+	-		+	-	+	-
KO	6	A	9		+	-	8.5	+	-	+	-
		B	8		+	-		+	-	+	-
KO	7	A	5		+	-	7	+	-	+	-
		B	9		+	-		+	-	+	-
KO	8	A	14		+	-	10	+	-	+	-
		B	6		+	-		+	-	+	-
KO	9	A	7		+	-	5.5	+	-	+	-
		B	4		+	-		+	-	+	-
KO	10	A	7		+	-	4.5	+	-	+	-
		B	2		+	-		+	-	+	-
KO	11	A	10		+	-	8	+	-	+	-
		B	6		+	-		+	-	+	-
KO	12	A	9		+	-	9	+	-	+	-
		B	9		+	-		+	-	+	-
KO	13	A	9		+	-	8	+	-	+	-
		B	7		+	-		+	-	+	-
KO	14	A	8		+	-	10.5	+	-	+	-
		B	13		+	-		+	-	+	-
KO	15	A	11		+	-	12	+	-	+	-
		B	13		+	-		+	-	+	-

24. False Positive Reaction – *Klebsiella oxytoca* is incorrectly confirmed as an *E. coli* by ISO 9308-1:2000(E)

Appendix C (continued)
ISO 9308-1:2000(E) with confirmation, and Colilert 250 after 24 & 28 hours

Table C-4
Colilert 250 presence/absence method and ISO 9308-1:2000(E) for total coliform spike, *Hafnia alvei* (ATCC 13337)

Sample ID			Lactose TTC agar		INDOLE TEST	OXIDASE TEST	Average Confirmed ISO TC[25] count	Colilert 250 @ 24 hours		Colilert 250 @ 28 hours	
Spike Code	Bottled Water Code	Duplicate	Yellow / Orange colonies	Red colonies	+ / -	+ / -		Yellow	FLR	Yellow	FLR
HA	11	A		0	-	-	3	-	-	-	-
		B		6	-	-		-	-	-	-
HA	15	A		4	-	-	5	-	-	-	-
		B		6	-	-		-	-	-	-

25. TC = Total coliform

Appendix D
Non-target concentrations

Table D-1
Non-target spike inoculum was determined by a spreadplate count

Bacterium	Set 1 (n = 10) cfu / 1mL	Set 2 (n = 10) cfu / 1mL	Set 3 (n = 22) cfu / 1mL
S. aureus	38	59	45
A. hydrophila	71	63	90
E. faecalis	26	49	33
B. licheniformis	7	9	34
P. aeruginosa	70	72	18
P. putida	18	11	33
Total (all strains combined)	230	263	253