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Performance Evaluation of HiCap™ Neutralizing Broth; Phase 2. Inter-Laboratory Study

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1.0 PURPOSE

To evaluate the performance of HiCap Neutralizing Broth for an environmental monitoring program.

2.0 SCOPE

This study assessed the ability of HiCap™ Neutralizing Broth (or "HiCap")[1] to meet the dual requirements for a collection broth. These are to (i) neutralize sanitizers (quat, chlorine or peroxide-based) that are commonly used in the production facility and (ii) preserve the viability of low levels of microorganisms for a period of 72 hours on EZ Reach Sponge Samplers pre-dosed with no, low, or high levels of sanitizer.

3.0 PROJECT SUMMARY

The first part of the Inter-Laboratory Study was a qualitative study that determined whether EZ Reach with HiCap is able to maintain the viability of *Listeria spp.* in the presence of three different types of sanitizers for a 72 hour period. The final determination of survival over 72 hours was assessed qualitatively using the *Listeria* detection method chosen by each participating laboratory.

Cherney Microbiological Services pre-dosed two types of EZ Reach Samplers; EZ Reach units hydrated with 10ml of neutralizing broth and EZ Reach units hydrated with 10 ml of HiCap Neutralizing Broth, with no, low or high amounts of sanitizer. The sanitizers included Whisper™ V (a mixed quat), XY-12® (a sodium hypochlorite solution) or Vortexx™ (peroxide/peroxyacetic acid/organic acid)[2]. The sponges were then inoculated with low levels of a cocktail of different *Listeria* strains and immediately refrigerated at $4^{o}C \pm 1^{o}C$. The sponges were blind-coded and packed into coolers with time/temperature indicators inside each cooler and shipped next day to participating laboratories. At 72 hours ± 2 hours after inoculation at Cherney, the participating laboratories proceeded with the enrichment and detection protocol employed by the laboratory's detection kit and reported *Listeria* presence/absence results back to Cherney Microbiological.

The second part of the Inter-Laboratory Study was a quantitative study that determined whether EZ Reach with HiCap is able to maintain the viability of *E. coli* in the presence of each sanitizer for a 72 hour period. The final determination of survival over 72 hours was assessed quantitatively using 3M™ Petrifilm™ Enterobacteriaceae Count Plates[3].

[1] HiCap is a trademark of Worldbioproducts, Bothell, WA
[2] Whisper V, XY-12, and Vortexx are trademarks of Ecolab USA Inc., St. Paul, MN
[3] 3M and Petrifilm are trademarks of 3M, St. Paul, MN

Similar to the qualitative study with *Listeria spp*., Cherney Microbiological Services pre-dosed two types of EZ Reach Samplers; EZ Reach units hydrated with 10ml of letheen broth and EZ Reach units hydrated with 10ml of HiCap Neutralizing Broth, with no, low or high amounts of sanitizers. The sponges were then inoculated with low levels of *Escherichia coli* and immediately refrigerated at $4^{o}C \pm 1^{o}C$. The sponges were blind-coded, packed into coolers with a time/temperature indicator and next day shipped to participating laboratories. At 72 hours ± 2 hours after inoculation, the participating laboratories inoculated 3M Enterobacteriaceae Petrifilm Count Plates with a 1 ml aliquot from each sample bag. The inoculated plates were incubated for 24 hours ± 2 hours at 35°C ± 1°C. Upon completion of incubation, the quantitative assessment of *E. coli* was determined by counting the colonies displaying a yellow acid zone and a gas bubble. The total number of *E. coli* was reported back to Cherney Microbiological.

Throughout this study, a pour plate procedure using Standard Methods Agar was utilized to confirm inoculum levels. In addition, sanitizer levels were verified using titration and test strip methods as recommended by the manufacturer of the sanitizer.

4.0 MATERIALS AND METHODS

The following ATCC microorganisms were grown according to Microbiologics' recommended procedure[1]: *Listeria ivanovii* ATCC#19119, *Listeria monocytogenes* ATCC#19114, *Listeria monocytogenes* ATCC#7644, *Listeria innocua* ATCC#33090 and *Escherichia coli* ATCC#25922.

Overnight cultures of the *Listeria* strains and *Escherichia coli (E. coli)* were diluted to cell densities of approximately 5000-8000 cells per milliliter with the aid of a DensiCHEK™ Plus instrument[2], which provides values in McFarland units proportional to microorganism density. To prepare an inoculum cocktail of *Listeria* organisms, the diluted cultures were combined to give an approximately equal concentration for each strain. Cell densities of each cocktail were determined using a pour plate procedure on Standard Methods Agar, following the procedure described in the Compendium of Methods for the Microbiological Examination of Foods 4th Edition. The results of the pour plate test gave an average of 817 colony forming units per 0.1 milliliter of inoculum for the *Listeria spp.* cocktail and an average of 527 colony forming units per 0.1 milliliter of inoculum for *E. coli.*

For the qualitative study with *Listeria spp*., EZ Reach units hydrated with 10 ml of neutralizing buffer and EZ Reach units hydrated with 10 ml of HiCap Neutralizing Broth (World Bioproducts, Bothell, Washington, Item No. EZ-10HC-PUR) were pre-dosed with one of the three sanitizers at the concentrations shown in Table 7. The three sanitizers included in this study were Whisper V (a fifth generation mixed quaternary ammonium sanitizer), XY-12 (a sodium hypochlorite sanitizer) and Vortexx (a peroxide/peroxyacetic acid/organic acid sanitizer).

[1] Microbiologics, St. Cloud, Minnesota Technical Information Bulletin TIB.081 Revision B
[2] bioMerieux, Hazelwood, Missouri

Prior to the addition of the sanitizer and inoculum, the EZ Reach units were refrigerated for a minimum of 2 hours. The sponges were kept at 4°C ± 1°C immediately prior to and following the dosing of sanitizer and the addition of organisms.

The sanitizer was delivered to the EZ Reach unit solution by aseptically adding 0.1 ml of the specified level of sanitizer to the liquid squeezed from the sponge and collected in the bottom corner of the bag. One milliliter of sterile deionized water was added for the control (no sanitizer level). The sanitizer or deionized water was evenly distributed throughout the sponge and solution by gently massaging the sponge through the bag for 10 seconds. The solution was aseptically squeezed from the sponge a second time and inoculated with 0.1 ml of *Listeria spp.* cocktail or 0.1 ml of *E. coli* to the solution collected in the corner of the bag. The inoculum level was 820 cells per 0.1 ml of the *Listeria* cocktail or 530 cells per 0.1 ml of *E. coli* as measured with a pour plate procedure using Standard Methods Agar. The inoculum was evenly distributed throughout the sponge by massaging the sponge through the bag for 10 seconds. The inoculated EZ Reach Samplers were then immediately refrigerated at 4°C ± 1°C.

The following day, the inoculated sponges were packed into coolers with ice bricks and shipped overnight to participating laboratories. Each box contained a temperature/time indicator with instructions to read immediately upon opening to ensure the contents were not exposed to temperatures above 8°C. The participating laboratories immediately unpacked and refrigerated the sponges. Each sample bag received by the participating laboratory was blind coded with reference on the type of study (qualitative or quantitative), organism used, type of EZ Reach unit and level of sanitizer.

At 72 hours ± 2 hours from inoculation time, the EZ Reach Samplers containing *Listeria spp.* for the qualitative study were enriched and tested for the presence or absence of *Listeria* according to the protocol specified by the participating laboratory and the manufacturer of each pathogen kit.

At 72 hours ± 2 hours from inoculation time, the EZ Reach Samplers containing *E. coli* for the quantitative study were massaged for 10 seconds and then squeezed to release the collection solution. One ml of solution was aseptically removed and pipetted directly onto 3M Petrifilm Enterobacteriaceae Count Plates. An additional 1 ml aliquot of solution was transferred to a 9 ml Butterfield's phosphate buffer dilution blank for a 1:10 dilution. One milliliter from the 1:10 dilution blank was pipetted onto a second 3M Petrifilm Enterobacteriaceae Count Plate. The inoculated plates were incubated for 24 h ± 2 h at 35°C ± 1°C. Colonies displaying a yellow acid zone and a gas bubble were counted as *E. coli*. Results were sent back to Cherney Microbiological.

Prior to the initiation of the Inter-Laboratory Study, a study verifying the lethality of the sanitizers in the absence of neutralizing agents was performed. EZ Reach Samplers, hydrated with Butterfield's phosphate buffer, were pre-dosed with one of the three sanitizers at the

concentrations chosen for this study using the procedure described above. Butterfield's phosphate buffer was used to hydrate the sponges because it contains no components that are capable of neutralizing sanitizers. Each pre-dosed sponge was inoculated as described above with 0.1 ml aliquots from diluted cultures with concentrations of 600-800 cells per milliliter for the *Listeria* cocktail or 600-800 cells per milliliter for the *E. coli* culture. The sponges were evaluated at the point of inoculation (time=0) and after 72 hours of refrigerated storage (time=72) by squeezing each sponge, aseptically collecting the expressed solution and performing a pour plate procedure using Standard Methods Agar.

5.0 RESULTS

Table 1 shows that EZ Reach Samplers hydrated with HiCap were able to maintain the viability of *Listeria spp*. when stored at refrigerated temperatures over a 72 hour holding time for all sanitizers and concentrations tested. For HiCap, no negative results were obtained for any of the 81 samples tested. In contrast, EZ Reach Samplers hydrated with neutralizing buffer produced 16 negative results out of 81 samples tested, for a negative rate of 19.7%.

Table 1. Qualitative Assessment of *Listeria spp.* Survival – Summary of All Sanitizers Tested

All Sanitizers Tested	Level of Sanitizer Tested						Totals No. Samples Tested	Totals No. Positive Samples	Totals No. Negative Samples	% Negative Samples
EZ Reach Type	None		Low		High					
	No. Pos. Samples	No. Neg. Samples	No. Pos. Samples	No. Neg. Samples	No. Pos. Samples	No. Neg. Samples				
EZ Reach with HiCap Neutralizing Broth	27	0	27	0	27	0	81	81	0	0.00%
EZ Reach with Neutralizing Buffer	27	0	22	5	16	11	81	65	16	19.75%
Totals	54	0	49	5	43	11	162	146	16	9.88%

With further analysis of these data by type of sanitizer, the EZ Reach Samplers hydrated with HiCap produced no negative results out of 18 samples that were pre-dosed with Vortexx sanitizer (Table 2). EZ Reach Samplers hydrated with neutralizing buffer produced 6 negative samples out of 18 samples, for a 33.3% negative rate. Two of the 6 negative results were seen when a low amount of Vortexx (approximately 0.6 mg) was added to the sponge whereas 4 negative results were obtained when a high amount of Vortexx (approximately 3 mg) was added to the sponge.

Table 2. *Listeria spp.* Survival – HiCap & Neutralizing Buffer with Vortexx™ (peroxide/peroxyacetic acid/organic acid) Sanitizer

Vortexx Only	Level of Sanitizer Tested				Totals No. Samples Tested	Totals No. Positive Samples	Totals No. Negative Samples	% Negative Samples
EZ Reach Type	Low		High					
	No. Pos. Samples	No. Neg. Samples	No. Pos. Samples	No. Neg. Samples				
EZ Reach with HiCap Neutralizing Broth	9	0	9	0	18	18	0	0.00%
EZ Reach with Neutralizing Buffer	7	2	5	4	18	12	6	33.33%
Totals	16	2	14	4	36	30	6	16.67%

No negative results were obtained for EZ Reach Samplers hydrated with either HiCap or neutralizing buffer and pre-dosed with the Whisper V sanitizer, when tested at low (approximately 0.6 mg) and high (approximately 3 mg) concentrations (Table 3).

Table 3. *Listeria spp.* Survival – HiCap & Neutralizing Buffer with Whisper™ V (a mixed quat) Sanitizer

Whisper Only	Level of Sanitizer Tested				Totals No. Samples Tested	Totals No. Positive Samples	Totals No. Negative Samples	% Negative Samples
EZ Reach Type	Low		High					
	No. Pos. Samples	No. Neg. Samples	No. Pos. Samples	No. Neg. Samples				
EZ Reach with HiCap Neutralizing Broth	9	0	9	0	18	18	0	0.00%
EZ Reach with Neutralizing Buffer	9	0	9	0	18	18	0	0.00%
Totals	18	0	18	0	36	36	0	0.00%

EZ Reach Samplers hydrated with HiCap produced no negative results out of 18 samples that were pre-dosed with XY-12 (Table 4). EZ Reach Samplers hydrated with neutralizing buffer produced 10 negative results out of 18 samples, producing a 55.6% negative rate. Three of the 10 negative results were obtained when the sponges were pre-dosed with a low concentration (approximately 0.8 mg) of XY-12 while 7 negative results were seen with a high amount (approximately 3 mg) of this sanitizer.

Table 4. *Listeria spp.* Survival – HiCap & Neutralizing Buffer with XY-12® (a sodium hypochlorite solution) Sanitizer

XY-12 Only	Level of Sanitizer Tested				Totals No. Samples Tested	Totals No. Positive Samples	Totals No. Negative Samples	% Negative Samples
EZ Reach Type	Low		High					
	No. Pos. Samples	No. Neg. Samples	No. Pos. Samples	No. Neg. Samples				
EZ Reach with HiCap Neutralizing Broth	9	0	9	0	18	18	0	0.00%
EZ Reach with Neutralizing Buffer	6	3	2	7	18	8	10	55.56%
Totals	15	3	11	7	36	26	10	27.78%

The participating laboratories followed the protocol of the pathogen assay performed in their laboratory for the positive and negative determination of *Listeria spp.* The diagnostic tests consisted of VIDAS™[1], RapidChek™[2], BAX System Q7™[3], iQ-Check™[4], ROKA Bioscience ATLAS™[5], and Assurance GDS®[6] . The *Listeria spp.* results by type of diagnostic test are shown in Table 5 below.

Table 5. *Listeria spp.* Results by Type of Diagnostic Test

		EZ Reach with **HiCap Neutralizing Broth**				EZ Reach with **Neutralizing Buffer**			
Company	Diagnostic Test Used	No. of Negative Results with No Sanitizer Added	No. of Negative Results with Low or High Amounts of Vortexx Added	No. of Negative Results with Low or High Amounts of Whisper V Added	No. of Negative Results with Low or High Amounts of XY-12 Added	No. of Negative Results with No Sanitizer Added	No. of Negative Results with Low or High Amounts of Vortexx Added	No. of Negative Results with Low or High Amounts of Whisper V Added	No. of Negative Results with Low or High Amounts of XY-12 Added
1	RapidChek	0	0	0	0	0	2	0	2
2	BAX	0	0	0	0	0	1	0	1
3	VIDAS	0	0	0	0	0	1	0	0
4	VIDAS	0	0	0	0	0	0	0	1
6	iQ-Check	0	0	0	0	0	1	0	2
7	GDS	0	0	0	0	0	0	0	1
8	ROKA	0	0	0	0	0	0	0	1
9	VIDAS	0	0	0	0	0	0	0	1
11	VIDAS	0	0	0	0	0	1	0	1
Totals		0	0	0	0	0	6	0	10

[1] VIDAS is a registered trademark of 3M, St.Paul, MN
[2] RapidChek is a registered trademark of bioMerieux SA, Marcy l'Etoile
[3] BAX is a registered trademark of Strategic Diagnostics Inc., Newark, DE
[4] iQ-Check is a registered trademark of BioRad Laboratories, Hercules, CA
[5] ROKA Bioscience ATLAS is a registered trademark of Gen-Probe, Inc., San Diego, CA
[6] Assurance GDS is a registered trademark of BioControl Systems, Inc., Bellevue, WA

Each participating laboratory reported at least 1 negative result with the EZ Reach Samplers hydrated with neutralizing buffer (Table 5). Four out of the nine participating laboratories used VIDAS as their detection test for *Listeria spp*. Of the 5 negative results received by the laboratories using VIDAS, 3 negatives were obtained with sponges pre-dosed with XY-12 and 2 negatives were obtained with sponges pre-does with Vortexx. Five different diagnostic tests were represented with the remaining 5 laboratories. The laboratory using RapidChek obtained 4 negative results with EZ Reach Samplers hydrated with neutralizing buffer. One negative result for Vortexx and 1 negative result for XY-12 was obtained by the laboratory using BAX with sponges hydrated with neutralizing buffer. The laboratory using iQ-Check reported 2 negative results for XY-12 and 1 negative result for Vortexx when working with EZ Reach sponges with neutralizing buffer. One negative result was obtained by each laboratory using ROKA or GDS when testing EZ Reach Samples with neutralizing buffer that were pre-dosed with XY-12 sanitizer.

EZ Reach Samplers, without the addition of sanitizer, gave counts that averaged 54 cfu/ml for sponges hydrated with HiCap and 50 cfu/ml with sponges hydrated with letheen broth over the 72 hour refrigerated storage (Table 6). (Please note the data reported by each participating laboratory for the quantitative part of this Inter-Laboratory Study is shown in Tables 9-16 in the Appendix section of this report.)

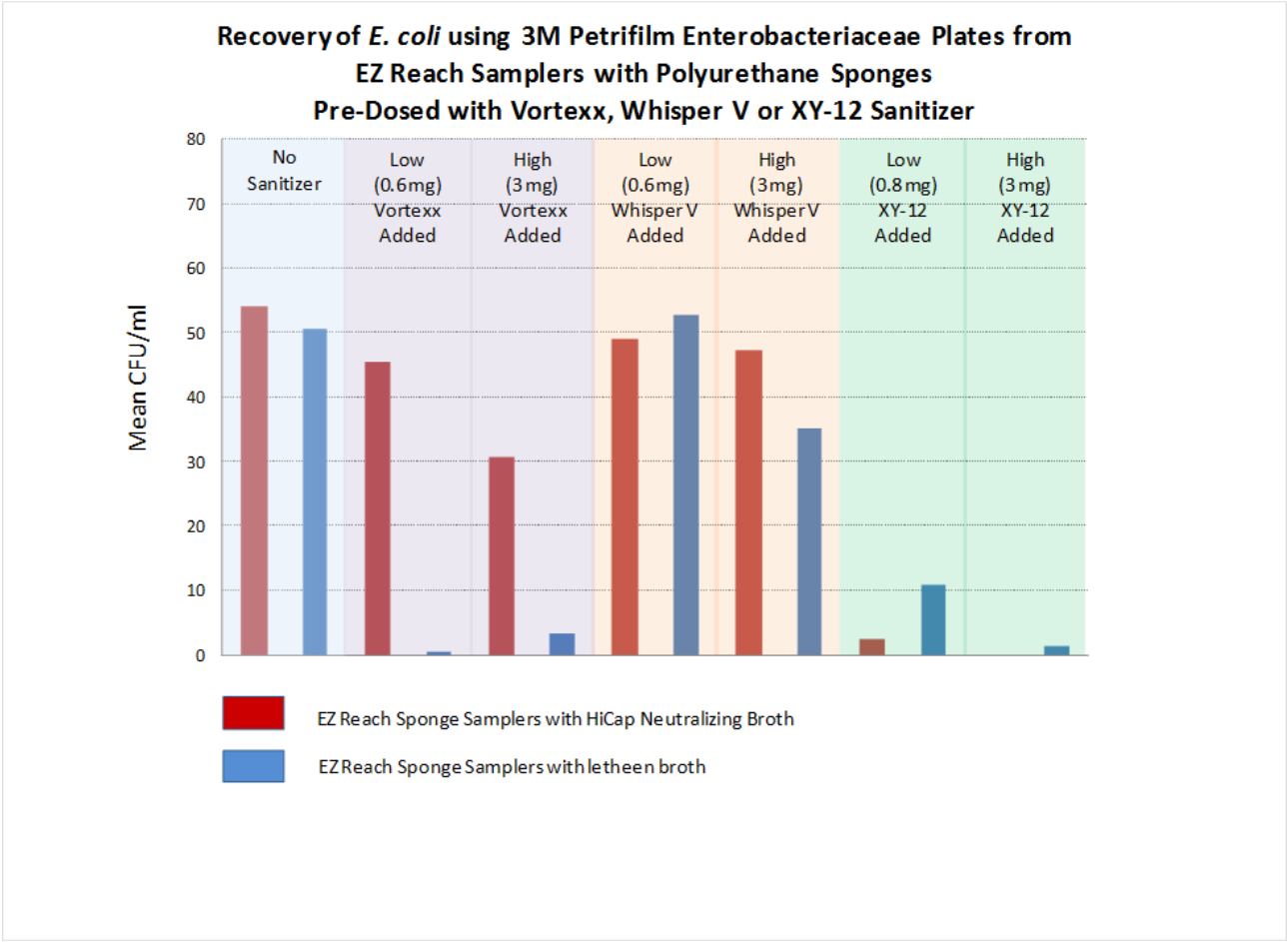
As seen in Table 6, the recovery of *E. coli* was very low for EZ Reach Samplers hydrated with letheen broth, averaging 0 and 3 cfu/ml respectively for sponges receiving low (approximately 0.6 mg) and high (approximately 3 mg) of Vortexx. In contrast, much higher recoveries of *E. coli* were obtained with EZ Reach Samplers hydrated with HiCap and pre-dosed with low and high concentrations of Vortexx. High levels of *E. coli* recovery were seen with both types of EZ Reach Samplers that were pre-dosed with Whisper V, although a lower mean count was obtained with EZ Reach Samplers hydrated with letheen broth that were pre-dosed with high levels (approximately 3 mg) of Whisper V. Poor recoveries of *E. coli* were seen with EZ Reach Samplers hydrated with HiCap or letheen broth and tested using low and high amounts of the XY-12 sanitizer.

Table 6. Recovery of *E. coli* using 3M Petrifilm from EZ Reach Samplers Pre-Dosed with Sanitizer

Organism	EZR Type	Sanitizer Type	Level of Sanitizer (as calculated, in milligrams)	Total No. of Samples Tested	Average CFU/ml
E. coli	EZR HC	None	None	33	54
E. coli	EZR LET	None	None	33	50
E. coli	EZR HC	Vortexx	0.6	11	45
E. coli	EZR LET	Vortexx	0.6	11	0
E. coli	EZR HC	Vortexx	3.0	11	31
E. coli	EZR LET	Vortexx	3.0	11	3
E. coli	EZR HC	Whisper V	0.6	11	49
E. coli	EZR LET	Whisper V	0.6	11	53
E. coli	EZR HC	Whisper V	3.0	11	47
E. coli	EZR LET	Whisper V	3.0	11	35
E. coli	EZR HC	XY-12	0.8	11	2
E. coli	EZR LET	XY-12	0.8	11	11
E. coli	EZR HC	XY-12	3.0	11	0
E. coli	EZR LET	XY-12	3.0	11	1

The data in Table 6 is presented graphically in Figure 1 to show the recovery differences for *E. coli* between samples receiving no sanitizer and units receiving low and high concentrations of the three sanitizers.

Figure 1. Recovery of *E.coli* using 3M Petrifilm Enterobacteriaceae Count Plates from EZ Reach Samplers with Polyurethane Sponges Pre-Dosed with Vortexx, Whisper V or XY-12 Sanitizer



The sanitizer concentrations selected for this study are believed to represent levels that are routinely encountered on production surfaces. Table 7 shows the approach for preparing the sanitizers for addition to the EZ Reach Samplers. The sanitizer amounts reported throughout this study represent the amount of sanitizer, expressed in milligrams, added to each sponge. The amounts are calculated using information published by Ecolab on the concentrations of active ingredients in an undiluted sanitizer preparation. To confirm levels of sanitizer applied to the sponges, sanitizer test strips or titration methods were employed. However, generally poor agreement was seen between the calculated sanitizer values and the values as measured with the sanitizer strips and titration kit. These differences are seen in Table 7. Others have reported on difficulties using test strips to discern small differences in sanitizer concentrations (see Tech Talk: Measuring Disinfectant/Sanitizer Concentration, http://solutions.3m.com/wps/portal/3M/en_US/Facilities-Care-Cleaning-NA/commercial-cleaning/facility-management-news-and-views/news-archive/?PC_7_U00M8B1A0G7SD0170B99930SI7000000_assetId=1319238400139).

Table 7. Dilution Scheme for Sanitizers Added to EZ Reach Sponges

Type of Sanitizer:	Active Ingredients	Concentration of Active Ingredients - Grams per 100 ml (undiluted)	Concentration of Active Ingredients - Milligrams per 1 ml (undiluted)	Dilution of Sanitizer Used in the Assay	Concentration Active Ingredients - Milligrams per 1 ml (after dilution)	Amount (ml) of Diluted Sanitizer Added to Sponge	Amount (mg) of Diluted Sanitizer Added to Sponge	Amount (mg) in 10 ml of Neutralizing Solution in Sponge	Concentration of Sanitizer in Neutralizing Solution (milligrams per 1 L or PPM)	As Measured with Titration or Strips (ppm)
Whisper V	Alkyl dimethyl benzyl ammonium chloride 3.00%; Octyl decyl dimethyl ammonium chloride 2.25%; Didecyl dimethyl ammonium chloride (1.35%); Dioctyl dimethyl ammonium chloride 0.9%	7.5	75	Not Added (Control)		0			0	
				1:12.5	6.0	0.1	0.60	0.06	60	100-200 [1]
				1:2.5	30.0	0.1	3.00	0.30	300	1000 [2]
Vortex	Hydrogen peroxide (6.9%); Peroxyacetic acid (4.4%); Octanoic Acid (3.3%)	14.6	146	Not Added (Control)		0			0	
				1:25	5.84	0.1	0.58	0.06	58	80 [3]
				1:4.8	30.42	0.1	3.04	0.30	304	160 [3]
XY-12	Sodium Hypochlorite	8.4	84	Not Added (Control)		0.000			0	
				1:10	8.4	0.1	0.84	0.08	84	380 [4]
				1:2.8	30.0	0.1	3.00	0.30	300	1060 [4]

[1] As measured with Titration or Test Strips (ppm) as noted in the footnotes below

To confirm that lethal concentrations of each sanitizer were being used in this study, sponges were inoculated and refrigerated following the protocol used in the Inter-Laboratory Study, with the exception that the sponges were hydrated with Butterfield's phosphate buffer that contains no neutralizing agents. Counts at inoculation and after 72 hours of refrigerated storage were made using a pour plate procedure employing Standard Methods Agar. As can be seen in Table 8, all concentrations of the sanitizers used in this study were lethal to the *E. coli* and *Listeria spp.* used to inoculate samples for the Inter-Laboratory Study.

1[1] pHydrion® Papers QT-10, Micro Essential Laboratory, Brooklyn, NY

1[2] Hydrion®Quat Chek, Micro Essential Laboratory, Brooklyn, NY

1[3] pHydrion® Peracetic Acid Test Strips 0-160ppm, Micro Essential Laboratory, Brooklyn, NY

1[4] Chlorine Test Kit #321, Ecolab Inc., St. Paul, MN

Table 8. Lethality of Sanitizers to the Test Organisms in the Absence of Neutralizing Agents

	Time 0	Time 72 hours
Sanitizer Type	**APC Pour CFU/ml**	**APC Pour CFU/ml**
Whisper V *E. coli* Control	64	10
Whisper V *E. coli* Low	0	0
Whisper V *E. coli* High	0	0
Vortexx *E. coli* Control	71	14
Vortexx *E. coli* Low	0	0
Vortexx *E. coli* High	0	0
XY-12 *E. coli* Control	56	11
XY-12 *E. coli* Low	0	0
XY-12 *E. coli* High	0	0
Whisper V *Listeria* Control	84	21
Whisper V *Listeria* Low	0	0
Whisper V *Listeria* High	0	0
Vortexx *Listeria* Control	129	22
Vortexx *Listeria* Low	1	0
Vortexx *Listeria* High	0	0
XY-12 *Listeria* Control	120	8
XY-12 *Listeria* Low	0	0
XY-12 *Listeria* High	0	0

6.0 CONCLUSIONS

The solutions used in the collection of surface samples play a critical role in the success of an environmental monitoring program. The solutions must have the capacity to neutralize sanitizers that may be present on a production surface and be able to maintain microorganism viability until the sample is processed. This Inter-Laboratory Study evaluated the efficacy of HiCap Neutralizing Broth (or “HiCap”) both qualitatively and quantitatively when used in an environmental monitoring program.

EZ Reach Sponges hydrated with HiCap and pre-dosed with low and high levels of Vortexx (peroxyacetic acid), Whisper V (quat) or XY-12 (sodium hypochlorite) sanitizers resulted in superior recovery of *Listeria spp.* when compared to EZ Reach Sponges hydrated with neutralizing buffer. In this blind Inter-Laboratory Study, the participating laboratories reported no negative results using EZ Reach Samplers hydrated with HiCap compared to a 19.75% negative rate for EZ Reach Samplers hydrated with neutralizing buffer (Table 1). These data suggest that *Listeria* contamination of a production surface might be missed if sanitizer is present, even at a low level, and a sampling device with neutralizing buffer is used to collect the sample. HiCap showed a greater capacity to neutralize all of the sanitizers employed in this study even at high and low concentrations.

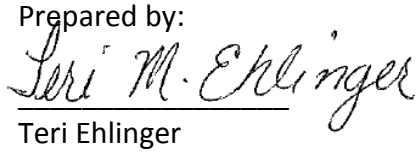
In the quantitative study, it was observed that the survival of *E. coli* on sponges hydrated with HiCap was superior or equal to that observed on sponges hydrated with letheen broth for the Vortexx and Whisper V sanitizers, as shown in Figure 1. *E. coli* showed minimal to no survival on sponges hydrated with either HiCap or letheen broth and pre-dosed with low and high levels of the XY-12 sanitizer, a sodium hypochlorite solution. This finding is surprising in light of the fact that HiCap showed a capacity to fully neutralize the XY-12 sanitizer in the *Listeria* part of this Inter-Laboratory Study. The discrepancy in results between the quantitative study with *E. coli* and the qualitative study with *Listeria spp.* with the XY-12 sanitizer may be explained by differences in recovery methods. Studies of chlorine injured *E. coli* are numerous and have consistently shown the disadvantages of using a selective agar media to recover these stressed organisms. (For further information on this topic, the reader is referred to this excellent review article: Gordon A. McFeters and Mark W. LeChevallier, Chapter 15: Chemical Disinfection and Injury of Bacteria in Water. Pp. 255-275, In "Nonculturable Microorganisms in the Environment" (Ed. R. R. Colwell and D. J. Grimes), 2000, ASM Press, Washington, D.C.) A follow-up study comparing *E. coli* recovery methods is warranted. Such a study may help to elucidate the best approach to recover *E. coli* and other organisms from surfaces with free chlorine residues.

The results obtained in this study lead to additional opportunities for future studies. For example, the EZ Reach Samplers hydrated with letheen broth showed a lower recovery for E. coli compared to EZ Reach Samplers hydrated with HiCap when 3 mg of quat was added to the sponge. It would be useful to know whether slightly higher levels of quat (e.g. 4 to 6 mgs) would confirm differences in the neutralization capacities of these two solutions. It would also be valuable in a separate study to measure the actual levels of sanitizers remaining on production surfaces to establish the neutralization capacity requirements of any sampling device in any setting where a sanitizer residue is encountered. Such a study would need to consider different surface types, application approaches and topography.

HiCap Neutralizing broth was evaluated for its appropriateness for use in an environmental monitoring program. This study has confirmed the ability of HiCap to meet the dual requirements for a collection broth. These are to (i) neutralize sanitizers (quat, chlorine or peroxide-based) that are commonly used in the production facility and (ii) preserve the viability of low levels of microorganisms inoculated onto EZ Reach Sponge Samplers that have been pre-dosed with none, low and high level of sanitizer for a period of at least 72 hours.

7.0 Final Report Approval

Prepared by:



5-13-2013

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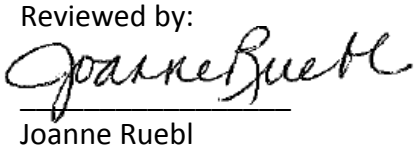
Cherney Microbiological Services, LTD

Reviewed by:



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Appendix 1

Inter-Laboratory Study Results of *E. coli* Recovery from Pre-dosed EZ Sponge Samplers with Sanitizer using EZ Reach Sponge Samplers Hydrated with HiCap or Letheen Broth

Table 9. *E. coli* Recovery from EZ Reach Sponge Samplers Hydrated with HiCap and Pre-dosed with No Sanitizer

Company	Organism	EZ Reach Type	Sanitizer Type	Level of Sanitizer	CFU/ml	CFU/0.1ml
1	E. coli	EZR HC	None	None	77	2
	E. coli	EZR HC	None	None	50	2
	E. coli	EZR HC	None	None	40	1
2	E. coli	EZR HC	None	None	65	4
	E. coli	EZR HC	None	None	61	3
	E. coli	EZR HC	None	None	53	2
3	E. coli	EZR HC	None	None	67	6
	E. coli	EZR HC	None	None	65	6
	E. coli	EZR HC	None	None	39	4
4	E. coli	EZR HC	None	None	68	6
	E. coli	EZR HC	None	None	59	8
	E. coli	EZR HC	None	None	50	2
5	E. coli	EZR HC	None	None	50	3
	E. coli	EZR HC	None	None	48	4
	E. coli	EZR HC	None	None	40	2
6	E. coli	EZR HC	None	None	44	2
	E. coli	EZR HC	None	None	74	3
	E. coli	EZR HC	None	None	56	9
7	E. coli	EZR HC	None	None	45	2
	E. coli	EZR HC	None	None	51	2
	E. coli	EZR HC	None	None	50	3
8	E. coli	EZR HC	None	None	45	2
	E. coli	EZR HC	None	None	48	1
	E. coli	EZR HC	None	None	41	4
9	E. coli	EZR HC	None	None	65	1
	E. coli	EZR HC	None	None	49	3
	E. coli	EZR HC	None	None	52	1
10	E. coli	EZR HC	None	None	52	3
	E. coli	EZR HC	None	None	76	4
	E. coli	EZR HC	None	None	67	5
11	E. coli	EZR HC	None	None	51	6
	E. coli	EZR HC	None	None	29	1
	E. coli	EZR HC	None	None	55	3
Totals	E. coli	EZR HC	None	Total No. of Samples	33	33
				Mean Counts	54.00	3.33
				Log Mean Counts	1.73	0.52
				Std. Dev	11.52	2.03

Table 10. *E. coli* Recovery from EZ Reach Sponge Samplers Hydrated with Letheen Broth and Pre-dosed with No Sanitizer

Company	Organism	EZ Reach Type	Sanitizer Type	Level of Sanitizer	CFU/ml	CFU/0.1ml
1	E. coli	EZR LET	None	None	49	7
	E. coli	EZR LET	None	None	47	5
	E. coli	EZR LET	None	None	49	6
2	E. coli	EZR LET	None	None	65	2
	E. coli	EZR LET	None	None	47	3
	E. coli	EZR LET	None	None	42	4
3	E. coli	EZR LET	None	None	49	6
	E. coli	EZR LET	None	None	50	5
	E. coli	EZR LET	None	None	56	2
4	E. coli	EZR LET	None	None	50	4
	E. coli	EZR LET	None	None	45	10
	E. coli	EZR LET	None	None	39	8
5	E. coli	EZR LET	None	None	70	5
	E. coli	EZR LET	None	None	59	2
	E. coli	EZR LET	None	None	40	8
6	E. coli	EZR LET	None	None	55	1
	E. coli	EZR LET	None	None	58	1
	E. coli	EZR LET	None	None	37	1
7	E. coli	EZR LET	None	None	36	4
	E. coli	EZR LET	None	None	33	7
	E. coli	EZR LET	None	None	45	8
8	E. coli	EZR LET	None	None	40	4
	E. coli	EZR LET	None	None	40	5
	E. coli	EZR LET	None	None	38	8
9	E. coli	EZR LET	None	None	60	4
	E. coli	EZR LET	None	None	41	1
	E. coli	EZR LET	None	None	46	0
10	E. coli	EZR LET	None	None	50	9
	E. coli	EZR LET	None	None	155	10
	E. coli	EZR LET	None	None	54	4
11	E. coli	EZR LET	None	None	39	2
	E. coli	EZR LET	None	None	48	4
	E. coli	EZR LET	None	None	32	3
Totals	E. coli	EZR LET	None	Total No. of Samples	33	33
				Mean Counts	50.42	4.64
				Log Mean Counts	1.70	0.67
				Std. Dev	20.82	2.78

Table 11. *E. coli* Recovery - Low Concentration Vortexx Sanitizer and HiCap (EZR HC) or Letheen Broth (EZR LET)

Company	Organism	EZ Reach Type	Sanitizer Type	Level of Sanitizer	CFU/ml	CFU/0.1ml
1	E. coli	EZR HC	Vortexx	Low - 0.6 mg	56	3
2	E. coli	EZR HC	Vortexx	Low - 0.6 mg	39	1
3	E. coli	EZR HC	Vortexx	Low - 0.6 mg	40	1
4	E. coli	EZR HC	Vortexx	Low - 0.6 mg	60	4
5	E. coli	EZR HC	Vortexx	Low - 0.6 mg	49	4
6	E. coli	EZR HC	Vortexx	Low - 0.6 mg	28	5
7	E. coli	EZR HC	Vortexx	Low - 0.6 mg	23	0
8	E. coli	EZR HC	Vortexx	Low - 0.6 mg	52	3
9	E. coli	EZR HC	Vortexx	Low - 0.6 mg	51	2
10	E. coli	EZR HC	Vortexx	Low - 0.6 mg	56	1
11	E. coli	EZR HC	Vortexx	Low - 0.6 mg	46	2
Totals	E. coli	EZR HC	Vortexx - Low (0.6 mg)	Total No. of Samples	11	11
				Mean Counts	45.45	2.36
				Log Mean Counts	1.66	0.37
				Std. Dev	11.84	1.57
1	E. coli	EZR LET	Vortexx	Low - 0.6 mg	0	0
2	E. coli	EZR LET	Vortexx	Low - 0.6 mg	0	0
3	E. coli	EZR LET	Vortexx	Low - 0.6 mg	0	1
4	E. coli	EZR LET	Vortexx	Low - 0.6 mg	0	0
5	E. coli	EZR LET	Vortexx	Low - 0.6 mg	0	0
6	E. coli	EZR LET	Vortexx	Low - 0.6 mg	0	0
7	E. coli	EZR LET	Vortexx	Low - 0.6 mg	0	0
8	E. coli	EZR LET	Vortexx	Low - 0.6 mg	0	0
9	E. coli	EZR LET	Vortexx	Low - 0.6 mg	0	0
10	E. coli	EZR LET	Vortexx	Low - 0.6 mg	3	1
11	E. coli	EZR LET	Vortexx	Low - 0.6 mg	0	0
Totals	E. coli	EZR LET	Vortexx - Low (0.6 mg)	Total No. of Samples	11	11
				Mean Counts	0.27	0.18
				Log Mean Counts	-0.56	-0.74
				Std. Dev	0.90	0.40

Table 12. *E. coli* Recovery - High Concentration Vortexx Sanitizer and HiCap (EZR HC) or Letheen Broth (EZR LET)

Company	Organism	EZ Reach Type	Sanitizer Type	Level of Sanitizer	CFU/ml	CFU/0.1ml
1	E. coli	EZR HC	Vortexx	High - 3 mg	28	2
2	E. coli	EZR HC	Vortexx	High - 3 mg	17	5
3	E. coli	EZR HC	Vortexx	High - 3 mg	45	3
4	E. coli	EZR HC	Vortexx	High - 3 mg	24	2
5	E. coli	EZR HC	Vortexx	High - 3 mg	63	4
6	E. coli	EZR HC	Vortexx	High - 3 mg	23	1
7	E. coli	EZR HC	Vortexx	High - 3 mg	15	0
8	E. coli	EZR HC	Vortexx	High - 3 mg	34	1
9	E. coli	EZR HC	Vortexx	High - 3 mg	30	9
10	E. coli	EZR HC	Vortexx	High - 3 mg	21	4
11	E. coli	EZR HC	Vortexx	High - 3 mg	38	4
Totals	E. coli	EZR HC	Vortexx - High (3 mg)	Total No. of Samples	11	11
				Mean Counts	30.73	3.18
				Log Mean Counts	1.49	0.50
				Std. Dev	13.97	2.48
1	E. coli	EZR LET	Vortexx	High - 3 mg	0	0
2	E. coli	EZR LET	Vortexx	High - 3 mg	0	0
3	E. coli	EZR LET	Vortexx	High - 3 mg	0	0
4	E. coli	EZR LET	Vortexx	High - 3 mg	0	0
5	E. coli	EZR LET	Vortexx	High - 3 mg	0	0
6	E. coli	EZR LET	Vortexx	High - 3 mg	0	0
7	E. coli	EZR LET	Vortexx	High - 3 mg	0	0
8	E. coli	EZR LET	Vortexx	High - 3 mg	0	0
9	E. coli	EZR LET	Vortexx	High - 3 mg	0	0
10	E. coli	EZR LET	Vortexx	High - 3 mg	27	2
11	E. coli	EZR LET	Vortexx	High - 3 mg	0	0
Totals	E. coli	EZR LET	Vortexx - High (3 mg)	Total No. of Samples	11	11
				Mean Counts	2.45	0.18
				Log Mean Counts	0.39	-0.74
				Std. Dev	8.14	0.60

Table 13. *E. coli* Recovery - Low Concentration Whisper V Sanitizer and HiCap (EZR HC) or Letheen Broth (EZR LET)

Company	Organism	EZ Reach Type	Sanitizer Type	Level of Sanitizer	CFU/ml	CFU/0.1ml
1	E. coli	EZR HC	Whisper V	Low - 0.6 mg	49	1
2	E. coli	EZR HC	Whisper V	Low - 0.6 mg	54	6
3	E. coli	EZR HC	Whisper V	Low - 0.6 mg	55	7
4	E. coli	EZR HC	Whisper V	Low - 0.6 mg	36	0
5	E. coli	EZR HC	Whisper V	Low - 0.6 mg	42	6
6	E. coli	EZR HC	Whisper V	Low - 0.6 mg	41	5
7	E. coli	EZR HC	Whisper V	Low - 0.6 mg	50	4
8	E. coli	EZR HC	Whisper V	Low - 0.6 mg	38	2
9	E. coli	EZR HC	Whisper V	Low - 0.6 mg	56	4
10	E. coli	EZR HC	Whisper V	Low - 0.6 mg	70	4
11	E. coli	EZR HC	Whisper V	Low - 0.6 mg	48	6
Totals	E. coli	EZR HC	Whisper V - Low (0.6 mg)	Total No. of Samples	11	11
				Mean Counts	49.00	4.09
				Log Mean Counts	1.69	0.61
				Std. Dev	9.78	2.26
1	E. coli	EZR LET	Whisper V	Low - 0.6 mg	51	5
2	E. coli	EZR LET	Whisper V	Low - 0.6 mg	60	3
3	E. coli	EZR LET	Whisper V	Low - 0.6 mg	46	3
4	E. coli	EZR LET	Whisper V	Low - 0.6 mg	50	6
5	E. coli	EZR LET	Whisper V	Low - 0.6 mg	49	4
6	E. coli	EZR LET	Whisper V	Low - 0.6 mg	58	4
7	E. coli	EZR LET	Whisper V	Low - 0.6 mg	52	3
8	E. coli	EZR LET	Whisper V	Low - 0.6 mg	49	5
9	E. coli	EZR LET	Whisper V	Low - 0.6 mg	61	4
10	E. coli	EZR LET	Whisper V	Low - 0.6 mg	66	7
11	E. coli	EZR LET	Whisper V	Low - 0.6 mg	38	6
Totals	E. coli	EZR LET	Whisper V - Low (0.6 mg)	Total No. of Samples	11	11
				Mean Counts	52.73	4.55
				Log Mean Counts	1.72	0.66
				Std. Dev	7.91	1.37

Table 14. *E. coli* Recovery - High Concentration Whisper V Sanitizer and HiCap (EZR HC) or Letheen Broth (EZR LET)

Company	Organism	EZ Reach Type	Sanitizer Type	Level of Sanitizer	CFU/ml	CFU/0.1ml
1	E. coli	EZR HC	Whisper V	High - 3 mg	33	2
2	E. coli	EZR HC	Whisper V	High - 3 mg	42	5
3	E. coli	EZR HC	Whisper V	High - 3 mg	40	5
4	E. coli	EZR HC	Whisper V	High - 3 mg	35	4
5	E. coli	EZR HC	Whisper V	High - 3 mg	56	6
6	E. coli	EZR HC	Whisper V	High - 3 mg	52	2
7	E. coli	EZR HC	Whisper V	High - 3 mg	52	5
8	E. coli	EZR HC	Whisper V	High - 3 mg	46	1
9	E. coli	EZR HC	Whisper V	High - 3 mg	63	5
10	E. coli	EZR HC	Whisper V	High - 3 mg	57	0
11	E. coli	EZR HC	Whisper V	High - 3 mg	43	5
Totals	E. coli	EZR HC	Whisper V - High (3 mg)	Total No. of Samples	11	11
				Mean Counts	47.18	3.64
				Log Mean Counts	1.67	0.56
				Std. Dev	9.58	2.01
1	E. coli	EZR LET	Whisper V	High - 3 mg	38	1
2	E. coli	EZR LET	Whisper V	High - 3 mg	33	1
3	E. coli	EZR LET	Whisper V	High - 3 mg	22	1
4	E. coli	EZR LET	Whisper V	High - 3 mg	34	6
5	E. coli	EZR LET	Whisper V	High - 3 mg	32	4
6	E. coli	EZR LET	Whisper V	High - 3 mg	30	2
7	E. coli	EZR LET	Whisper V	High - 3 mg	37	3
8	E. coli	EZR LET	Whisper V	High - 3 mg	45	3
9	E. coli	EZR LET	Whisper V	High - 3 mg	47	4
10	E. coli	EZR LET	Whisper V	High - 3 mg	40	0
11	E. coli	EZR LET	Whisper V	High - 3 mg	29	2
Totals	E. coli	EZR LET	Whisper V - High (3 mg)	Total No. of Samples	11	11
				Mean Counts	35.18	2.45
				Log Mean Counts	1.55	0.39
				Std. Dev	7.25	1.75

Table 15. *E. coli* Recovery - Low Concentration XY-12 Sanitizer and HiCap (EZR HC) or Letheen Broth (EZR LET)

Company	Organism	EZ Reach Type	Sanitizer Type	Level of Sanitizer	CFU/ml	CFU/0.1ml
1	E. coli	EZR HC	XY-12	Low - 0.8 mg	2	0
2	E. coli	EZR HC	XY-12	Low - 0.8 mg	0	0
3	E. coli	EZR HC	XY-12	Low - 0.8 mg	0	0
4	E. coli	EZR HC	XY-12	Low - 0.8 mg	<1	0
5	E. coli	EZR HC	XY-12	Low - 0.8 mg	3	0
6	E. coli	EZR HC	XY-12	Low - 0.8 mg	4	0
7	E. coli	EZR HC	XY-12	Low - 0.8 mg	2	0
8	E. coli	EZR HC	XY-12	Low - 0.8 mg	1	0
9	E. coli	EZR HC	XY-12	Low - 0.8 mg	1	1
10	E. coli	EZR HC	XY-12	Low - 0.8 mg	11	2
11	E. coli	EZR HC	XY-12	Low - 0.8 mg	0	0
Totals	E. coli	EZR HC	XY-12 - Low (0.8 mg)	Total No. of Samples	11	11
				Mean Counts	2.40	0.27
				Log Mean Counts	0.38	-0.56
				Std. Dev	3.31	0.65
1	E. coli	EZR LET	XY-12	Low - 0.8 mg	37	5
2	E. coli	EZR LET	XY-12	Low - 0.8 mg	1	1
3	E. coli	EZR LET	XY-12	Low - 0.8 mg	0	3
4	E. coli	EZR LET	XY-12	Low - 0.8 mg	0	2
5	E. coli	EZR LET	XY-12	Low - 0.8 mg	0	0
6	E. coli	EZR LET	XY-12	Low - 0.8 mg	0	0
7	E. coli	EZR LET	XY-12	Low - 0.8 mg	20	3
8	E. coli	EZR LET	XY-12	Low - 0.8 mg	0	2
9	E. coli	EZR LET	XY-12	Low - 0.8 mg	14	1
10	E. coli	EZR LET	XY-12	Low - 0.8 mg	24	7
11	E. coli	EZR LET	XY-12	Low - 0.8 mg	1	2
Totals	E. coli	EZR LET	XY-12 - Low (0.8 mg)	Total No. of Samples	11	11
				Mean Counts	8.82	2.36
				Log Mean Counts	0.95	0.37
				Std. Dev	12.99	2.11

Table 16. *E. coli* Recovery - High Concentration XY-12 Sanitizer and HiCap (EZR HC) or Letheen Broth (EZR LET)

Company	Organism	EZ Reach Type	Sanitizer Type	Level of Sanitizer	CFU/ml	CFU/0.1ml
1	E. coli	EZR HC	XY-12	High - 3 mg	0	0
2	E. coli	EZR HC	XY-12	High - 3 mg	0	0
3	E. coli	EZR HC	XY-12	High - 3 mg	0	0
4	E. coli	EZR HC	XY-12	High - 3 mg	0	0
5	E. coli	EZR HC	XY-12	High - 3 mg	0	0
6	E. coli	EZR HC	XY-12	High - 3 mg	0	0
7	E. coli	EZR HC	XY-12	High - 3 mg	0	0
8	E. coli	EZR HC	XY-12	High - 3 mg	0	0
9	E. coli	EZR HC	XY-12	High - 3 mg	0	0
10	E. coli	EZR HC	XY-12	High - 3 mg	0	0
11	E. coli	EZR HC	XY-12	High - 3 mg	0	0
Totals	E. coli	EZR HC	XY-12 - High (3 mg)	Total No. of Samples	11	11
				Mean Counts	0.00	0.00
				Log Mean Counts	0.00	0.00
				Std. Dev	0.00	0.00
1	E. coli	EZR LET	XY-12	High - 3 mg	0	0
2	E. coli	EZR LET	XY-12	High - 3 mg	0	0
3	E. coli	EZR LET	XY-12	High - 3 mg	0	0
4	E. coli	EZR LET	XY-12	High - 3 mg	0	0
5	E. coli	EZR LET	XY-12	High - 3 mg	0	0
6	E. coli	EZR LET	XY-12	High - 3 mg	0	0
7	E. coli	EZR LET	XY-12	High - 3 mg	0	0
8	E. coli	EZR LET	XY-12	High - 3 mg	0	0
9	E. coli	EZR LET	XY-12	High - 3 mg	0	0
10	E. coli	EZR LET	XY-12	High - 3 mg	10	0
11	E. coli	EZR LET	XY-12	High - 3 mg	0	0
Totals	E. coli	EZR LET	XY-12 - High (3 mg)	Total No. of Samples	11	11
				Mean Counts	0.91	0.00
				Log Mean Counts	0.00	0.00
				Std. Dev	3.02	0.00