Validation for *Listeria* Right Now™

Revision 1: March 2019

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Listeria Right Now™

ASSAY DESCRIPTION

Neogen's enrichment-free ANSR® Right Now platform is a major advancement in pathogen control. By having results one hour after taking an environmental sample from a visibly clean and free of excess sanitizer surface, users can have significant improvements in their environmental pathogen monitoring and control program.

This validation book is designed to orient users to the dynamic shift in the approach to monitoring for environmental pathogens by allowing users to take immediate action on enrichment-free results in under one hour after sampling. This is made possible by the assay's targeting of highly specific ribosomal RNA (rRNA), which has thousands of copies per cell, compared to a single-copy DNA.

This ANSR *Listeria* Right Now (LRN) Validation Book is a reference manual for the food industry. The data is compiled from testing performed by Neogen, as well as third parties, for environmental samples from visibly clean surfaces that are free of excess sanitizers.

Neogen's *Listeria* Right Now system is a molecular-based, environmental *Listeria* detection system that requires no enrichment and features a total time to result of under one hour for verification of sanitization. The environmental sampling procedure follows recommended cross-hatch or site-specific sampling technique.

ASSAY TECHNOLOGY

The system is based on Neogen's ANSR platform and features an isothermal amplification system. The equipment is comprised of a reader, a computer with accompanying software, dry block heaters, thermometers, a vortex, pipettors with accompanying tips, and autoclavable test tube rack. The assay kit contains environmental sampling swabs, lysis buffer and sealed reaction reagent tubes.

Neogen's *Listeria* Right Now system is able to detect very low levels of *Listeria* spp., including *L. monocytogenes*, from environmental samples without enrichment.

- The system uses an amplified nucleic acid-based reaction to target rRNA.
- Amplification occurs through a polymerization mechanism by a specific endonuclease.
- Detection occurs in real-time using a fluorescent, molecular beacon.

rRNA is present in much greater numbers in *Listeria* cells than a traditional DNA target (~1000 – 10,000 copies per cell vs. one copy per cell for DNA). This can result in a 1,000 to 10,000-fold increase in target analyte concentration.

The isothermal reaction within the ANSR instrument produces a constant cycle of molecular replication, allowing faster analyte production compared to traditional PCR reactions that use a thermocycler, which involves a series of heating and cooling cycles.

USING *LISTERIA* RIGHT NOW TO STAY WITHIN REGULATORY COMPLIANCE

North American regulatory guidance for *Listeria* is focused primarily on environmental control of *Listeria monocytogenes*[1,2]. Until recently, tests used for *Listeria* detection took one to three days to complete and centered around growing *Listeria* to a high titer before being able to determine whether *Listeria* was present in the sample. Food production companies are often unwilling to risk growing *Listeria* in their own facility for fear of accidental cross-contamination, meaning samples need to be shipped out for testing. The one-to-three day delay in obtaining results poses a problem. The recleaning of the environmental site, if necessary, could not be accomplished immediately after sampling. The lack of immediate action allows for the uncontrolled movement of *Listeria* in a facility, making environmental vectoring a challenging.

The introduction of *Listeria* Right Now has changed how environmental monitoring can be done. For the first time, food manufacturers can test, without a time-consuming enrichment step, and take immediate action on positive samples to effectively reduce the spread of *Listeria*.

The *Listeria* regulatory guidance found in Health Canada's *Listeria* policy[1] and the U.S. Food and Drug Administration's (FDA) Food Safety Modernization Act (FSMA) *Listeria* policy[2] state that the environment is the predominant source for *Listeria* contamination of food. Sites within food production and handling facilities that harbor *Listeria* act as sources for continuous contamination and transfer of *Listeria*. If *Listeria* is allowed to transition around a food production facility, it may contaminate food products. This undesirable situation is further magnified if the product supports the growth of *Listeria* prior to product consumption.

The focus on environmental *Listeria* control has led food producers to adopt *Listeria* Right Now as a process control tool for immediate action on environmental samples. When immediate action takes place, the tracking and transfer of *Listeria* in the environment can be controlled in ways never previously demonstrated.

By taking immediate action, *Listeria* Right Now users are at the forefront of *Listeria* control in food production facilities. This assay allows you to meet or exceed regulatory guidance to prevent the presence, and spread, of *Listeria* within a food production facility by actively monitoring and verifying sanitization of *Listeria*.

1 https://www.canada.ca/en/health-canada/services/food-nutrition/legislation-guidelines/policies/policy-*listeria*-monocytogenes-ready-eat-foods-2011.html
2 https://www.fda.gov/downloads/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/UCM535981.pdf

LISTERIA RIGHT NOW INCLUSIVITY FOR *LISTERIA* SPP.

Neogen conducted an in-house study to evaluate the *Listeria* Right Now assay's ability to accurately detect various strains of *Listeria* spp. Table 1 below lists the various strains detected from various sources.

Neogen Table 1. Inclusivity for *Listera spp.* testing on the ANSR *Listeria* Right Now system.

	Organism	Origin (if known)
1	*L. aquatica*	x
2	*L. booriae*	x
3	*L. cornellensis*	x
4	*L. fleischmanni*	x
5	*L. floridensis*	x
6	*L. grandensis*	x
7	*L. grayi*	Environmetal
8	*L. grayi*	Chinchilla feces
9	*L. grayi subsp. murrayi*	x
10	*L. innocua*	Cheese
11	*L. innocua*	Cow brain
12	*L. innocua*	Cheese
13	*L. innocua*	Cheese
14	*L. innocua*	Cheese
15	*L. innocua*	Cheese
16	*L. innocua*	x
17	*L. innocua*	Raw milk
18	*L. ivanovii*	Mouse
19	*L. ivanovii*	Human
20	*L. ivanovii*	Watercress
21	*L. ivanovii*	Sheep
22	*L. marthii*	x
23	*L. monocytogenes*	Human blood
24	*L. monocytogenes*	Fish
25	*L. monocytogenes*	Human blood
26	*L. monocytogenes*	Cheese
27	*L. monocytogenes*	Cheese
28	*L. monocytogenes*	Cheese
29	*L. monocytogenes*	Human blood
30	*L. monocytogenes*	x
31	*L. monocytogenes*	Human blood
32	*L. monocytogenes*	x
33	*L. monocytogenes*	Raw milk
34	*L. monocytogenes*	Raw milk
35	*L. monocytogenes*	Brie cheese
36	*L. monocytogenes*	Chocolate milk
37	*L. monocytogenes*	Human CSF
38	*L. monocytogenes*	Cheese
39	*L. monocytogenes*	x
40	*L. monocytogenes*	Brie cheese
41	*L. monocytogenes*	Human blood
42	*L. monocytogenes*	Cheese
43	*L. monocytogenes*	Brie cheese
44	*L. monocytogenes*	Ruminant brain
45	*L. monocytogenes*	x
46	*L. monocytogenes*	x
47	*L. monocytogenes*	x
48	*L. monocytogenes*	Human
49	*L. newyorkensis*	x
50	*L. riparia*	x
51	*L. rocourtiae*	x
52	*L. seeligeri*	Sewage
53	*L. seeligeri*	Cheese
54	*L. seeligeri*	Vole
55	*L. seeligeri*	Cheese
56	*L. welshimeri*	Cheese
57	*L. welshimeri*	Environmental isolate
58	*L. welshimeri*	Plant material
59	*L. welshimeri*	Soil
60	*L. welshimeri*	Environmental isolate

LISTERIA RIGHT NOW EXCLUSIVITY FOR *LISTERIA* SPP.

An in-house study to evaluate the *Listeria* Right Now assay's ability to accurately exclude non-*Listeria* bacterial strains from detection with the assay. Table 2 below lists the various strains detected from various sources.

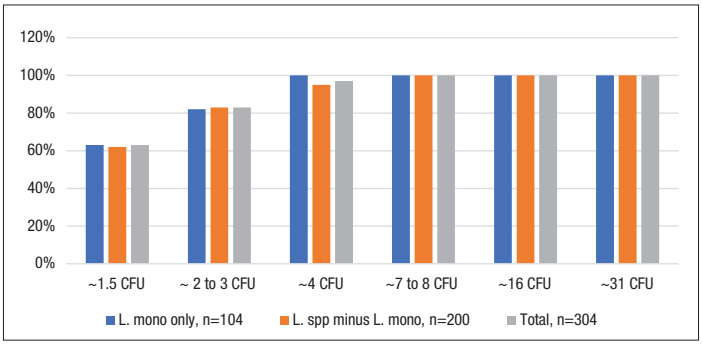
Neogen Table 2. Exclusivity for *Listera spp.* testing on the ANSR *Listeria* Right Now system.

	Organism	Source (ATCC#)	Origin (if known)
1	*Bacillus cereus*	25621	Cow dung
2	*Bacillus megaterium*	14581	x
3	*Bacillus subtilis*	21556	x
4	*Brevibacillus parabrevis*	8186	Dairy product
5	*Brochothrix thermosphacta*	11509	Pork sausage
6	*Enterococcus durans*	6056	Human feces
7	*Enterococcus faecalis*	27275	x
8	*Enterococcus faecium*	6057	Cheese
9	*Enterococcus hirae*	35220	Cow dung
10	*Geobacillus stearothermophilus*	12980	x
11	*Gordonia sputi*	29627	Human
12	*Kocuria rosea*	185	x
13	*Kocuria varians*	15306	Milk
14	*Kurthia gibsonii*	43195	Meat
15	*Kurthia zopfii*	33403	Turkey cecum
16	*Lactobacillus acidophilus*	4356	Human
17	*Lactobacillus buchneri*	11307	Beer
18	*Lactobacillus casei*	393	Cheese
19	*Lactobacillus fermentum*	9338	x
20	*Lactococcus lactis*	11454	x
21	*Micrococcus luteus*	381	Water
22	*Rhodococcus equi*	6939	Horse
23	*Rhodococcus fascians*	12974	x
24	*Staphylococcus aureus*	12600	Human pleural fluid
25	*Staphylococcus epidermidis*	14990	Human
26	*Staphylococcus saprophyticus*	15305	Human urine
27	*Streptococcus equi*	33398	x
28	*Streptococcus agalactiae*	13813	x
29	*Streptococcus mutans*	25175	Human mouth
30	*Streptococcus pneumoniae*	6303	x
31	*Streptococcus sanguinis*	10556	Human

LISTERIA RIGHT NOW'S LIMIT OF DETECTION OF *LISTERIA MONOCYTOGENES* AND OTHER SPECIES

A series of dilutions were performed and inoculated directly onto the test surface. The surfaces were tested by Neogen with *Listeria* Right Now. A total of 304 positive samples were tested (104 positive for *L. mono* only and 200 *Listeria* spp., excluding *L. mono*). Organisms tested included: *L. monocytogenes, L. innocua, L. welshimeri, L. grayi, L. ivanovii, L. seeligeri.* Figure 1 below displays the limit of detection per CFU per swab.

Figure 1. Results indicate a probable limit of detection at between 1.5 CFU and 2~3 CFU per swab. This would be considered a partial detection. A 95% confidence limit of detection can be determined at ~4 CFU per swab.



IN-HOUSE SURFACE VALIDATIONS

Listeria with and without background organisms were tested on a variety of surfaces. The background cocktail of *Pseudomonas aeruginosa, Bacillus subtilis, Enterococcus faecium* were present at about 3 x 10^3 CFU/mL with the Trial *Listeria* seen in Table 3.

Surface inoculation levels to get partials by *Listeria* Right Now/culture method (semi-paired) where as follows with sampling sizes between 1"X1" and 4"X4":

Neogen Table 3. Neogen study on five common surface types. The results show that the LRN system is as sensitive or more sensitive than culture methods. The exception is on sealed concrete at very low inoculation. This is not considered to be a significant difference and the study by AOAC showed favorable comparisons as well.

Surface Type	Trial	*Listeria* CFU/ Swab	N	LRN Positive	Culture Positive	dPOD (95% CI)
Stainless steel	*L. mono* (4b) only	1	15	3	2	0.07 (-0.21, 0.34)
		2	15	10	7	0.20 (-0.14, 0.48)
		2438	5	5	5	0 (-0.43, 0.43)
		0	5	0	0	0 (-0.43, 0.43)
Stainless steel	*L. mono* (4b) + background	1.8	20	8	7	0.05 (-0.23, 0.32)
		1800	5	5	5	0 (-0.43, 0.43)
		0	5	0	0	0 (-0.43, 0.43)
Plastic	*L. innocua* + background	2.3	20	9	9	0 (-0.28, 0.28)
		2250	5	5	5	0 (-0.43, 0.43)
		0	5	0	0	0 (-0.43, 0.43)
Sealed concrete	*L. welshimeri* + background	1.2	20	6	11	-0.25 (-0.5, 0.05)
		1550	5	5	5	0 (-0.43, 0.43)
		0	5	0	0	0 (-0.43, 0.43)
Ceramic tile	*L. mono* (1/2a) + background	1.93	20	14	9	0.25 (-0.05, 0.50)
		130	5	5	5	0 (-0.43, 0.43)
		0	5	0	0	0 (-0.43, 0.43)

NSF INTERNATIONAL SURFACE VALIDATIONS

The surfaces of 4" x 4" squares of food-grade stainless steel were inoculated with different levels of *Listeria monocytogenes* and a consortium of competing organisms, including *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Enterococcus faecium*. No false negatives, false positives or invalids were observed during this study. The evaluation determined that under the conditions employed in this study, the enrichment-free *Listeria* Right Now method is as sensitive as the enrichment-based culture reference method for detection of *L. monocytogenes* on a stainless steel surface. The results from this study have been included. For a copy of the full report, please contact your Neogen representative.

NSF Table 1: Method development results to determine the most suitable method for culture preparation by comparing bacterial titers between the uses of a McFarland standard and historical expected titer. TNTC= too numerous to count.

Round One		
Inoculum Prep type	**Theoretical CFU/Plate**	**Actual CFU/Plate**
McFarland Standard	15	TNTC
Historical Density		201
Round Two		
Inoculum Prep type	**Theoretical CFU/Plate**	**Actual CFU/Plate**
McFarland Standard	15	3
Historical Density		46
Round Three		
Inoculum Prep type	**Theoretical CFU/Plate**	**Actual CFU/Plate**
McFarland Standard 1	15	15
McFarland Standard 2		13
Historical Density		8

NSF Table 2: Environmental surface study inoculum counts.

Inoculum Count	CFU/mL
L. monocytogenes (McFarland std)	4.60E+08
P. aeruginosa (McFarland std)	1.20E+09
B. subtilis (McFarland std)	2.50E+07
E. faecium (McFarland std)	3.14E+09
Background Cocktail 10X stock	5.10E+04
Inoculum Count (pre-spike)	**CFU/mL**
L. monocytogenes (target 60 CFU/mL)	34
L. monocytogenes (target 60 CFU/mL) + Background	1.00E+03
Inoculum Count (post-spike)	**CFU/mL**
L. monocytogenes (target 60 CFU/mL)	56
L. monocytogenes (target 60 CFU/mL) + Background	7.10E+03

*Possible lab accident occurred during the enumeration of "L. monocytogenes (target 60 CFU/mL) + Background".

**The concentration of the Background Cocktail in the inoculum suspension was 5.10E+03 CFU/mL; 10-fold less than the Background Cocktail 10X stock (5.10E+04 CFU/mL).

NSF Table 3: Environmental surface study results for *Listeria monocytogenes* and background organisms.

Listeria monocytogenes +Background Organisms				
	Swab 1		Swab 2	
	LRN	If Retest	MOX	CONF
Negative				
1	Negative	N/A	Negative	N/A
2	Negative	N/A	Negative	N/A
3	Negative	N/A	Negative	N/A
4	Negative	N/A	Negative	N/A
5	Negative	N/A	Negative	N/A
Level 1: 6 CFU/coupon (Theoretical 3 CFU/swab)				
1	Positive	N/A	Negative	N/A
2	Positive	N/A	Positive	Positive
3	Positive	N/A	Positive	Positive
4	Positive	N/A	Positive	Positive
5	Positive	N/A	Positive	Positive
6	Positive	N/A	Positive	Positive
7	Positive	N/A	Negative	N/A
8	Positive	N/A	Negative	N/A
9	Positive	N/A	Positive	Positive
10	Positive	N/A	Negative	N/A
11	Negative	N/A	Positive	Positive
12	Positive	N/A	Positive	Positive
13	Positive	N/A	Negative	N/A
14	Positive	N/A	Positive	Positive
15	Positive	N/A	Negative	N/A
Level 3: 45 CFU/coupon (Theoretical 22.5 CFU/swab)				
1	Positive	N/A	Positive	Positive
2	Positive	N/A	Positive	Positive
3	Positive	N/A	Positive	Positive
4	Positive	N/A	Positive	Positive
5	Positive	N/A	Positive	Positive
6	Positive	N/A	Positive	Positive
7	Positive	N/A	Positive	Positive
8	Positive	N/A	Positive	Positive
9	Positive	N/A	Positive	Positive
10	Positive	N/A	Positive	Positive
11	Positive	N/A	Positive	Positive
12	Positive	N/A	Positive	Positive
13	Positive	N/A	Positive	Positive
14	Positive	N/A	Positive	Positive
15	Positive	N/A	Positive	Positive

Listeria monocytogenes +Background Organisms				
	Swab 1		Swab 2	
	LRN	If Retest	MOX	CONF
Positive: 4.8E+4 CFU/coupon (Theoretical 2.4E+4 CFU/swab)				
1	Positive	N/A	Positive	Positive
2	Positive	N/A	Positive	Positive
3	Positive	N/A	Positive	Positive
4	Positive	N/A	Positive	Positive
5	Positive	N/A	Positive	Positive
Level 2: 18 CFU/coupon (Theoretical 9 CFU/swab)				
1	Positive	N/A	Positive	Positive
2	Positive	N/A	Positive	Positive
3	Positive	N/A	Positive	Positive
4	Positive	N/A	Positive	Positive
5	Positive	N/A	Positive	Positive
6	Positive	N/A	Positive	Positive
7	Positive	N/A	Positive	Positive
8	Positive	N/A	Positive	Positive
9	Positive	N/A	Positive	Positive
10	Positive	N/A	Positive	Positive
11	Positive	N/A	Positive	Positive
12	Positive	N/A	Positive	Positive
13	Positive	N/A	Positive	Positive
14	Positive	N/A	Positive	Positive
15	Positive	N/A	Positive	Positive

Note: Inoculum spikes for levels 1, 2, and 3 contained 1.5E+03 CFU per coupon (theoretical 7.6E+02 CFU per swab) of background cocktail.

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NSF Table 4: Environmental surface study results for *Listeria monocytogenes* and background organisms on stainless steel.

Level	Theoretical Inoculum (CFU/swab)	Sample Number	LRN Positive	% LRN Positive	Culture Positive	% Culture Positive
Negative	0	5	0	0%	0	0%
Positive	2.4E+4	5	5	100%	5	100%
L1	3	15	14	93%	9	60%
L2	9	15	15	100%	15	100%
L3	22.5	15	15	100%	15	100%

Note: NSF Table 4 presents the results for the environmental surface study using a challenge inoculum of *L. monocytogenes* plus a consortium of competing organisms. Three different inoculation levels were evaluated on the stainless steel carriers: Level 1 = 3 CFU, Level 2 = 9 CFU and Level 3 = 22.5 CFU (theoretical CFU/swab). At Level 1, the detection rates for LRN and the reference enrichment-based culture method were 93% and 60%, respectively. At Levels 2 and 3, the detection rates for LRN and the reference enrichment-based culture method were 100%. No false negatives, false positives or invalids were observed during this study. The data illustrates that under the conditions employed in this study *Listeria* Right Now is as sensitive as the enrichment-based culture reference method for detection of *L. monocytogenes* on a stainless steel surface.

AOAC RESEARCH INSTITUTE PERFORMANCE TEST

Awarded on August 14, 2018, AOAC validated the *Listeria* Right Now assay on stainless steel, sealed concrete, plastic, rubber and ceramic tile. Following is the certificate and results on the different surfaces. For a copy of the full report, please contact your Neogen representative.

AOAC Table 2. Results of exclusivity testing for the *Listeria* Right Now test (1)

Organism	Strain #	Source (ATCC #)	Origin (if known)	Culture Conditions[a]	Result
Bacillus cereus	A208	25621	Cow dung		Negative
Bacillus megaterium	GT2128	14581	–		Negative
Bacillus subtilis	GT4402	21556	–		Negative
Brevibacillus parabrevis	GT803	8186	Dairy product		Negative
Brochothrix thermosphacta	GT664	11509	Pork sausage	BHI broth[b], 5% CO_2, 48 h, 25°C	Negative
Enterococcus durans	GT407	6056	Human feces		Negative
Enterococcus faecalis	GT3242	27275	–		Negative
Enterococcus faecium	GT919	6057	Cheese		Negative
Enterococcus hirae	GT923	35220	Cow dung		Negative
Geobacillus stearothermophilus	GT4373	12980	–		Negative
Gordonia sputi	GT3474	29627	Human	Nutrient broth, 5% CO_2, 48 h, 37°C	Negative
Kocuria rosea	GT1944	185	–	BHI broth, 48 h, 26°C	Negative
Kocuria varians	GT4404	15306	Milk		Negative
Kurthia gibsonii	GT2129	43195	Meat		Negative
Kurthia zopfii	GT1941	33403	Turkey cecum	Negative	
Lactobacillus acidophilus	GT256	4356	Human		Negative
Lactobacillus buchneri	GT4082	11307	Beer	MRS broth[c], 48 h, 30°C	Negative
Lactobacillus casei	GT805	393	Cheese		Negative
Lactobacillus fermentum	GT4063	9338	–		Negative
Lactococcus lactis	GT3516	11454	–		Negative
Micrococcus luteus	GT1943	381	Water		Negative
Rhodococcus equi	GT665	6939	Horse		Negative
Rhodococcus fascians	GT3524	12974	–	BHI broth, 48 h, 26°C	Negative
Staphylococcus aureus	A179	12600	Human pleural fluid		Negative
Staphylococcus epidermidis	A183	14990	Human		Negative
Staphylococcus saprophyticus	A185	15305	Human urine		Negative
Streptococcus equi	GT3596	33398	–		Negative
Streptococcus agalactiae	GT405	13813	–		Negative
Streptococcus mutans	GT412	25175	Human mouth		Negative
Streptococcus pneumoniae	GT408	6303	–		Negative
Streptococcus sanguinis	GT411	10556	Human		Negative

[a]If other than TSB, 24 h, 36°C.
[b]Brain heart infusion broth.
[c]DeMan, Rogosa & Sharpe broth

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AOAC Table 3. *Listeria* Right Now Results: Presumptive vs. Confirmed per BAM Ch. 10 cultural confirmation procedure (1)

				Listeria Right Now presumptive			*Listeria* Right Now confirmed by culture				
Matrix	**Strain**	**Inoculation level[a]**	**N[b]**	**x[c]**	**POD_{CP}[d]**	**95% CI**	**x**	**POD_{CC}[e]**	**95% CI**	**dPO D_{CP}[f]**	**95% CI[g]**
Stainless steel, 1" × 1" (swab)	*L. monocytogenes* 1/2a UVM[h] CWD1620/ 10X *E. faecalis* ATCC[i] 29212	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.47, 0.47
		86/1,200	20	9	0.45	0.26, 0.66	9	0.45	0.26, 0.66	0	-0.13, 0.13
		160/10,000	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.47, 0.47
Sealed concrete, 1" x 1" (swab)	*L. seeligeri* ATCC 35967	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.47, 0.47
		74	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0	-0.13, 0.13
		230	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.47, 0.47

[a]Inoculation level = CFU applied to each 1" x 1" surface area (test portion).
[c]x = Number of positive test portions.
[d]PODCP = Candidate method presumptive positive outcomes divided by the total number of trials.
[e]PODCC = Candidate method confirmed positive outcomes (per BAM Ch. 10 cultural confirmation procedure) divided by the total number of trials.
[f]dPODCP = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h]University of Vermont collection, Burlington,VT.
[i]American Type Culture Collection, Manassas, VA.

AOAC Table 4. Method Comparison Results: *Listeria* Right Now (Culture Confirmation) vs. BAM Ch. 10 (1)

				Listeria Right Now results			BAM Ch. 10 results				
Matrix	**Strain**	**Inoculation level[a]**	**N[b]**	**x[c]**	**POD_{CP}[d]**	**95% CI**	**x**	**POD_{CC}[e]**	**95% CI**	**dPO D_{CP}[f]**	**95% CI[g]**
Stainless steel, 1" × 1" (swab)	*L. monocytogenes* 1/2a UVMh CWD1620/ 10X E. faecalis ATCCi 29212	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.43, 0.43
		86/1,200	20	9	0.45	0.26, 0.66	7	0.35	0.18, 0.57	0.10	-0.19, 0.37
		160/10,000	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.43, 0.43
Sealed concrete, 1" x 1" (swab)	*L. seeligeri* ATCC 35967	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.43, 0.43
		74	20	8	0.40	0.22, 0.61	6	0.30	0.15, 0.52	0.10	-0.18, 0.36
		230	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.43, 0.43

[a]Inoculation level = CFU applied to each 1" x 1" surface area (test portion).
[b]N = Number of test potions.
[c]x = Number of positive test portions.
[d]PODC = Candidate method presumptive positive outcomes confirmed positive per BAM Ch. 10 cultural confirmation procedure.
[e]PODR = Reference method confirmed positive outcomes divided by the total number of trials.
[f]dPODC = Difference between the candidate method and reference method POD values.
[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h]University of Vermont collection, Burlington, VT.
[i]American Type Culture Collection, Manassas, VA

AOAC Table 5. *Listeria* Right Now Results: Presumptive vs. Confirmed by PCR (1)

				Listeria Right Now presumptive			*Listeria* Right Now confirmed by PCR				
Matrix	**Strain**	**Inoculation level[a]**	**N[b]**	**x[c]**	**POD_{CP}[d]**	**95% CI**	**x**	**POD_{CC}[e]**	**95% CI**	**dPO D_{CP}[f]**	**95% CI[g]**
Stainless steel, 1" × 1" (swab)	*L. monocytogenes* 1/2a UVMh CWD1620/ 10X *E. faecalis* ATCCi 29212	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.47, 0.47
		86/1,200	20	9	0.45	0.26, 0.66	9	0.45	0.26, 0.66	0	-0.13, 0.13
		160/10,000	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.47, 0.47
Sealed concrete, 1" x 1" (swab)	*L. seeligeri* ATCC 35967	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.47, 0.47
		74	20	8	0.40	0.22, 0.61	8	0.40	0.22, 0.61	0	-0.13, 0.13
		230	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.47, 0.47

[a]Inoculation level = CFU applied to each 1" x 1" surface area (test portion).
[c]x = Number of positive test portions.
[d]PODCP = Candidate method presumptive positive outcomes divided by the total number of trials.
[e]PODCC = Candidate method confirmed positive outcomes (per PCR) divided by the total number of trials.
[f]dPODCP = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h]University of Vermont collection, Burlington, VT.
[i]American Type Culture Collection, Manassas, VA

AOAC Table 6. Method Comparison Results: *Listeria* Right Now (PCR Confirmation) vs. BAM Ch. 10 (1)

				Listeria Right Now results			BAM Ch. 10 results				
Matrix	**Strain**	**Inoculation level[a]**	**N[b]**	**x[c]**	**POD_{CP}[d]**	**95% CI**	**x**	**POD_{CC}[e]**	**95% CI**	**dPO D_{CP}[f]**	**95% CI[g]**
Stainless steel, 1" × 1" (swab)	*L. monocytogenes* 1/2a UVMh CWD1620/ 10X E. faecalis ATCCi 29212	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.43, 0.43
		86/1,200	20	9	0.45	0.26, 0.66	7	0.35	0.18, 0.57	0.10	-0.19, 0.37
		160/10,0 00	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.43, 0.43
Sealed concrete, 1" x 1" (swab)	*L. seeligeri* ATCC 35967	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.43, 0.43
		74	20	8	0.40	0.22, 0.61	6	0.30	0.15, 0.52	0.10	-0.18, 0.36
		230	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.43, 0.43

[a]Inoculation level = CFU applied to each 1" x 1" surface area (test portion).
[b]N = Number of test potions.
[c]x = Number of positive test portions.
[d]PODC = Candidate method presumptive positive outcomes confirmed positive per PCR.
[e]PODR = Reference method confirmed positive outcomes divided by the total number of trials.
[f]dPODC = Difference between the candidate method and reference method POD values.
[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h]University of Vermont collection, Burlington, VT.
[i]American Type Culture Collection, Manassas, VA

Listeria Right Now™

Discussion of the modification approved November 2018 (9)

Results of this study show that the LRN method is an effective procedure for detection of *Listeria* spp. in swab samples from ceramic tile, plastic, and rubber surfaces. There were no significant differences in performance between the LRN and reference culture methods for any of the three matrixes tested as determined by POD analysis.

There were no false negative results by the LRN method. Traditional culture confirmation and the in-house reverse transcriptase PCR method were in complete agreement. There were a total of five unconfirmed positive results by the LRN (ANSR) assay for the three matrixes combined. All of these occurred on low-level inoculated test portions. It is possible, even likely, that these results represent detection of residual nucleic acid from non-viable cells in these test portions. In this case, one would conclude that the ANSR assay is more sensitive than the confirmatory PCR assay.

The data provide support for extension of the original claims for stainless steel and sealed concrete. The enrichment-free LRN test provides food industry personnel with a powerful tool for monitoring of environmental surfaces for *Listeria* contamination in real time.

AOAC Nov, 2018. Table 2. Method Comparison Results: *Listeria* Right Now (Culture Confirmation) vs. BAM Ch. 10 (9)

				***Listeria* Right Now results**			**BAM Ch. 10 results**				
Matrix	**Strain**	**Inoculation level[a]**	**N[b]**	**x[c]**	**POD_{CP}[d]**	**95% CI**	**x**	**POD_{CC}[e]**	**95% CI**	**dPO D_{CP}[f]**	**95% CI[g]**
Ceramic tile, 1" × 1" (swab)	*L. monocytogenes* 4b ATCCh 19115	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.43, 0.43
		56	20	10	0.50	0.30, 0.70	7	0.35	0.18, 0.57	0.15	-0.15, 0.41
		140	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.43, 0.43
Plastic, 1" x 1" (swab)	*L. innocua* ATCC 33091	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.43, 0.43
		44	20	9	0.45	0.26, 0.66	8	0.40	0.22, 0.61	0.05	-0.24, 0.33
		110	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.43, 0.43
Rubber, 1" x 1" (swab)	*L. monocytogenes* 1/2b ATCC BAA-751	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.43, 0.43
		72	20	14	0.70	0.48, 0.85	1 2	0.60	0.39, 0.78	0.10	-0.18, 0.36
		210	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.43, 0.43

[a]Inoculation level = CFU applied to each 1" x 1" surface area (test portion).
[b]N = Number of test potions.
[c]x = Number of positive test portions.
[d]PODC = Candidate method presumptive positive outcomes confirmed positive per BAM Ch. 10 cultural confirmation procedure.
[e]PODR = Reference method confirmed positive outcomes divided by the total number of trials.
[f]dPODC = Difference between the candidate method and reference method POD values.
[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h]American Type Culture Collection, Manassas, VA.

AOAC Nov, 2018. Table 3. *Listeria* Right Now Results: Presumptive vs. Confirmed by PCR (9)

				Listeria Right Now presumptive			*Listeria* Right Now confirmed by PCR				
Matrix	**Strain**	**Inoculation level[a]**	**N[b]**	**x[c]**	**POD_{CP}[d]**	**95% CI**	**x**	**POD_{CC}[e]**	**95% CI**	**dPO D_{CP}[f]**	**95% CI[g]**
Ceramic tile, 1" × 1" (swab)	*L. monocytogenes* 4b ATCCh 19115	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.47, 0.47
		56	20	13	0.65	0.43, 0.82	10	0.50	0.30, 0.70	0.15	-0.05, 0.35
		140	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.47, 0.47
Plastic, 1" x 1" (swab)	*L. innocua* ATCC 33091	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.47, 0.47
		44	20	10	0.50	0.30, 0.70	9	0.45	0.26, 0.66	0.05	-0.11, 0.21
		110	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.47, 0.47
Rubber, 1" x 1" (swab)	*L. monocytogenes* 1/2b ATCC BAA-751	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.47, 0.47
		72	20	15	0.75	0.53, 0.89	14	0.70	0.48, 0.85	0.05	-0.11, 0.21
		210	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.47, 0.47

[a]Inoculation level = CFU applied to each 1" x 1" surface area (test portion).
[c]x = Number of positive test portions.
[d]PODCP = Candidate method presumptive positive outcomes divided by the total number of trials.
[e]PODCC = Candidate method confirmed positive outcomes (per PCR) divided by the total number of trials.
[f]dPODCP = Difference between the candidate method presumptive result and candidate method confirmed result POD values.
[g]95% CI = If the confidence interval of a dPOD does not

Listeria Right Now™

AOAC Nov, 2018. Table 4. Method Comparison Results: *Listeria* Right Now (PCR Confirmation) vs. BAM Ch. 10 (9)

				***Listeria* Right Now results**			**BAM Ch. 10 results**				
Matrix	**Strain**	**Inoculation level[a]**	**N[b]**	**x[c]**	**POD_{CP}[d]**	**95% CI**	**x**	**POD_{CC}[e]**	**95% CI**	**dPO D_{CP}[f]**	**95% CI[g]**
Ceramic tile, 1" × 1" (swab)	*L. monocytogenes* 4b ATCCh 19115	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.43, 0.43
		56	20	10	0.50	0.30, 0.70	7	0.35	0.18, 0.57	0.15	-0.15, 0.41
		140	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.43, 0.43
Plastic, 1" x 1" (swab)	*L. innocua* ATCC 33091	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.43, 0.43
		44	20	9	0.45	0.26, 0.66	8	0.40	0.22, 0.61	0.05	-0.24, 0.33
		110	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.43, 0.43
Rubber, 1" x 1" (swab)	*L. monocytogenes* 1/2b ATCC BAA-751	0	5	0	0	0, 0.43	0	0	0, 0.43	0	-0.43, 0.43
		72	20	14	0.70	0.48, 0.85	12	0.60	0.39, 0.78	0.10	-0.18, 0.36
		210	5	5	1	0.57, 1	5	1	0.57, 1	0	-0.43, 0.43

[a]Inoculation level = CFU applied to each 1" x 1" surface area (test portion).
[b]N = Number of test potions.
[c]x = Number of positive test portions.
[d]PODC = Candidate method presumptive positive outcomes confirmed positive per PCR.
[e]PODR = Reference method confirmed positive outcomes divided by the total number of trials.
[f]dPODC = Difference between the candidate method and reference method POD values.
[g]95% CI = If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.
[h]American Type Culture Collection, Manassas, VA.

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SANITIZER VALIDATIONS

A variety of sanitizers can be used in different facilities. An internal study was conducted with commonly used sanitizers at recommended working concentrations. Our studies did not display interference from these sanitizers when the excess was rinsed free from the surfaces as seen in Table 4 and 5 below.

Several requests around PeroxyAcetic Acid (PAA) were also addressed. PAA is used at different concentrations (0.1% to 2%) for cleaning and sanitation at food processing facilities. For some applications, a rinse is required prior to resuming operations. Different concentrations of PAA were prepared (~0.25 to 2.5%) to evaluate the impact of PAA on the LRN assay. No invalid results were found when PAA was used at <1% concentrations. Higher concentrations of PAA (>1%) could sometimes result in invalids (data not shown). More tests were conducted by preparing PAA at 0.1 and 1% for use with half of the samples being spiked with *L. monocytogenes* (~10^2 CFU/mL).

Neogen Table 4. Varying concentration of Peroxyacetic Acid (PAA), half, as indicated in the above table, with a spike of *L. mono* (~10^2 CFU/mL). All control tests showed a negative result, and all spiked samples showed a positive result, indicating no interference from the PAA on the *Listeria* Right Now test.

PAA Concentration	Sample	LRN Result	PAA Concentration	Sample	LRN Result
1.0% PAA	Swab 1	Negative	**0.15% PAA**	Swab 1	Negative
	Swab 2	Negative		Swab 2	Negative
	Swab 3	Negative		Swab 3	Negative
	Swab 4	Negative		Swab 4	Negative
1.0% PAA + *L. mono*	Swab 1	Positive	**0.15% PAA + *L. mono***	Swab 1	Positive
	Swab 2	Positive		Swab 2	Positive
	Swab 3	Positive		Swab 3	Positive
	Swab 4	Positive		Swab 4	Positive
0.5% PAA	Swab 1	Negative	**0.1% PAA**	Swab 1	Negative
	Swab 2	Negative		Swab 2	Negative
	Swab 3	Negative		Swab 3	Negative
	Swab 4	Negative		Swab 4	Negative
0.5% PAA + *L. mono*	Swab 1	Positive	**0.1% PAA + *L. mono***	Swab 1	Positive
	Swab 2	Positive		Swab 2	Positive
	Swab 3	Positive		Swab 3	Positive
	Swab 4	Positive		Swab 4	Positive

Neogen Table 5. Varying sanitizers were tested with and without an inoculum of *L. mono*. All tests showed expected results indicating no interference from manufacturer-recommended dosage use on the *Listeria* Right Now test.

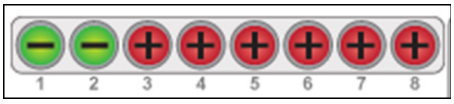
The *Listeria* Right Now assay was not affected by concentrations at 0.1–0.15%, which is the commonly used working concentration range. A 1:10 dilution of the sample will be recommended in these situations if rinsing does not reduce the concentration enough.

Sample ID	Number of Swabs	Duplicate Swab Sample	LRN Assays	Positive	Negative	Invalid
No *Listeria* organism on the surface						
10% Bleach	7	Yes	14	0	14	0
Mandate Plus	7	Yes	14	0	14	0
Ster-Bact	7	Yes	14	0	14	0
PeroxyAcetic Acid 1%	4	No	4	0	4	0
PeroxyAcetic Acid 2%	4	No	4	0	4	0
Negative Control	3	Yes	6	0	6	0
With *L. mono* of the surface						
XY-12	4	Yes	8	8	0	0
Mandate Plus	4	Yes	8	8	0	0
PeroxyAcetic Acid 1%	4	No	4	4	0	0
PeroxyAcetic Acid 2%	4	No	4	4	0	0
Water Control	4	Yes-1	7	7	0	0

ANSR TEST RESULT EXAMPLE CHARTS

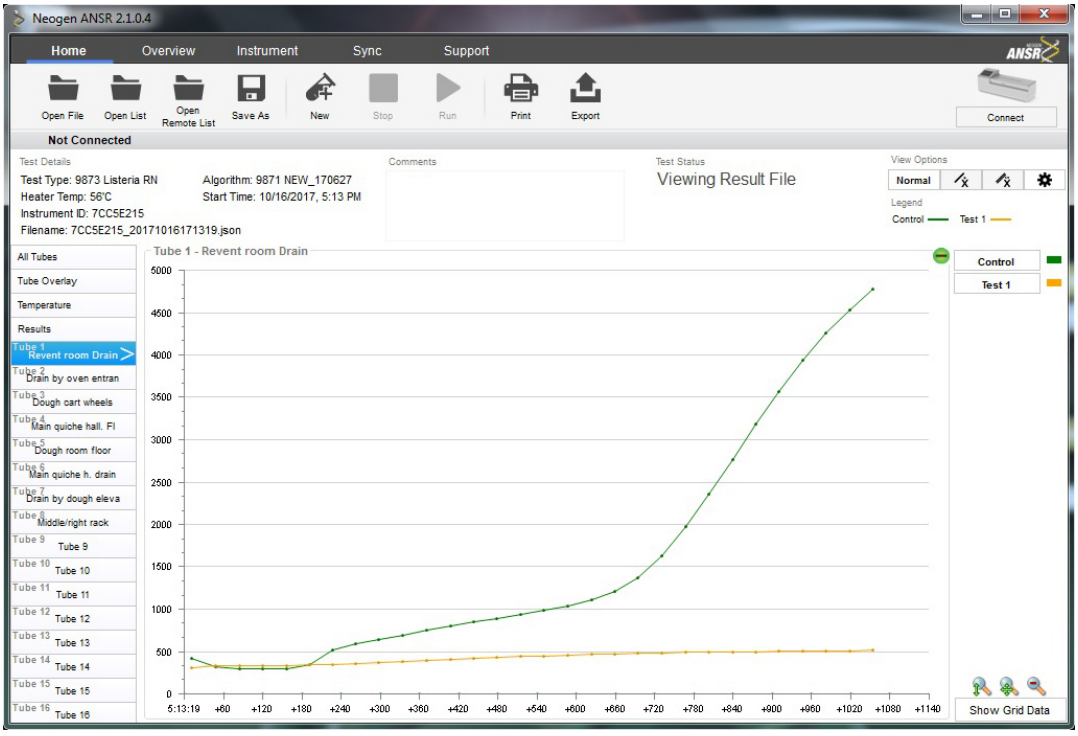
The ANSR reader will interpret the results and give a clear indication if the result is positive, negative or indeterminate as seen in figure 2 below. **We do not recommend interpreting the graphs**. Figures 3, 4, and 5 below are examples of potential graph results demonstrating a presumptive positive result, a negative result and an indeterminate result respectively. These are just examples, so your resulting graphs could look different.

Neogen Figure 2. The image below is an example of the results screen for running eight (8) tests.The negative result, indicated by the green negative symbols in the first two wells reflect that those samples were negative for *Listeria spp*. The positive result, indicated by the red positive symbols in the last 6 wells, reflect that those samples were a presumptive positive for *Listeria spp*.

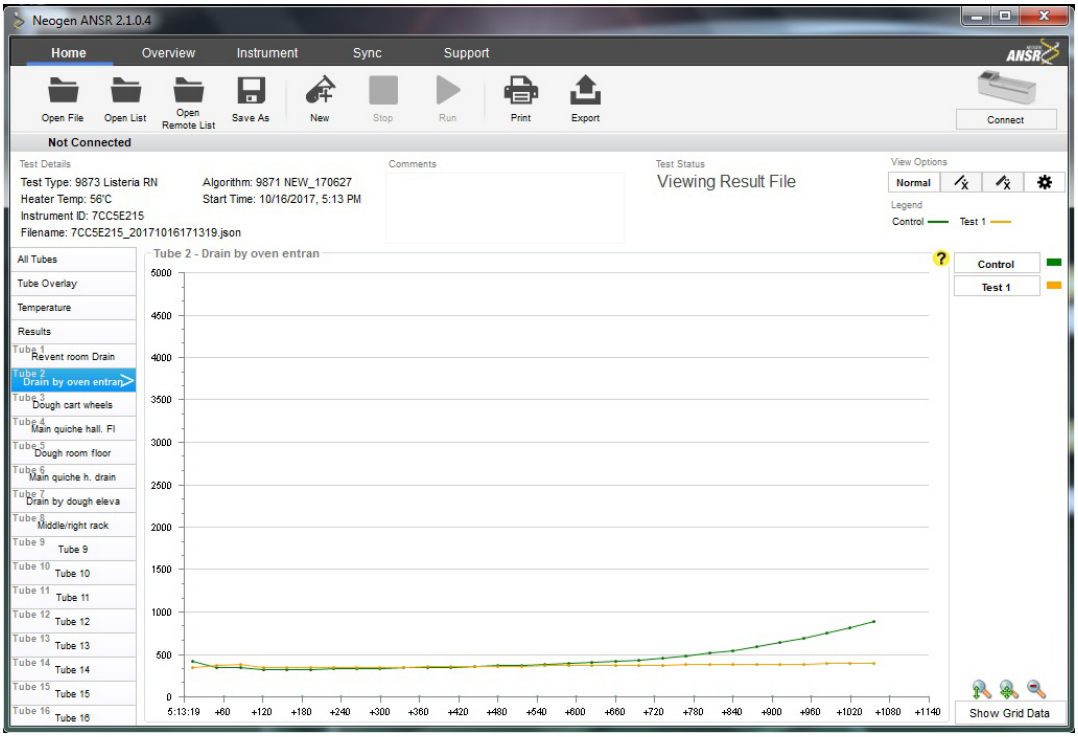


Neogen Figure 3. The image below is an example of a presumptive positive result, indicated by the red plus sign in the upper right corner, for *Listeria* using the ANSR *Listeria* Right Now system. The green line represents the internal positive control that is not based on nucleic acid. The yellow line represents the test line for *Listeria spp*. based on nucleic acid targets.

Neogen Figure 4. The image below is an example of a negative result, indicated by the green negative symbol in the upper right corner, for *Listeria* using the ANSR *Listeria* Right Now system. The green line represents the internal positive control that is not based on nucleic acid. The yellow line represents the test line for *Listeria spp.* based on nucleic acid targets.



Neogen Figure 5. The image below is an example of an indeterminate result, indicated by the yellow question mark in the upper right corner, for *Listeria* using the ANSR *Listeria* Right Now system. The green line represents the internal positive control that is not based on nucleic acid. The yellow line represents the test line for *Listeria spp.* based on nucleic acid targets. In cases of indeterminates, we recommend re-running the test as described in the kits insert.



TROUBLESHOOTING INDETERMINATES

Follow the *Listeria* Right Now kit insert instructions. Each ANSR reaction tube contains reagents for the development of an Internal Positive Control (IPC) curve and a Test curve. The IPC must meet specific curve amplitude criteria for the test to be considered valid; otherwise, the test will report an indeterminate result. Several situations could potentially lead to an indeterminate results.

- **Pipetting technique** – This is critical to the assay to minimize the amount of potential debris making it to the final sample. We recommend the following steps in pipetting for ANSR assays.
    - Always know where your first stop and second stop are.
    - Before entering your sample container, push the plunger to the first stop.
    - Find the bottom of the sample container and come off of the bottom. If there is sedimentation already forming, avoid it.
    - While in the sample and without moving your hand, release your thumb in one smooth motion. Don't overfill.
    - When you dispense, do it in one smooth motion pushing all the way through the first and second stop.
    - Keep your thumb down as you withdraw from the sample container. Release your thumb when over the disposal container to eject the used tips.
    - When going into cluster tubes on the 80°C block, line the bottom of the filter in the tips to the top of the cluster tube. Come up slightly to draw from the top of the sample. Be sure not to disturb the sedimentation at the bottom of the tubes.
- **Debris in the assay** – Making sure to sample a visibly clean surface that is free of excess sanitizer is important. Too much debris will interfere with the IPC curve being generated. Allowing debris to settle can often overcome this issue. If a sample has debris we recommend using 30 minutes at the 80°C step of the assay. If the sample is invalid, go back to the sample on the 80°C block and retest. By that point it would have 50 to 55 minutes to settle. If it is still invalid after that, the sample has too much debris for the assay to operate as needed.
- **Clear Lipids, Fats and Proteins** – These structures can interfere with tests. Typically these are found around drains. If a drain appears to be clean and the sample looks clear, but still comes up invalid twice in the same sample, you may need to scrub the drain with a degreaser. This typically has resolved the issue for the indeterminates as it removes the potential clear biofilm.
- **Excess Sanitizer** – We recommend sampling on visibly clean areas that are free of excess sanitizer. As described above, the assay does not have issues when sampling from areas that are rinsed free from working strength sanitizers. If a sample is taken from an area with excess sanitizer it can come back as indeterminate. The area should be rinsed free of the sanitizer and retested.
- **Vortexing** – We can not stress enough the importance of making sure the caps are secure on the ANSR reaction tubes prior to vortexing the sample. Once they are secure, use two hands, on either side of the reaction tubes and vortex for about one (1) to two (2) seconds. Quickly check each well for bubbles in the middle or bottom of the reaction wells. If there are bubbles, gently flick the tubes to release the bubbles to the top of the well.
- **Empty Well** – Occasionally an empty well has been run. Before starting the assay, visually check for a pellet in each of the wells. While checking for bubbles, after vortexing, make sure each well has fluid at the appropriate level in it and the pellet is dissolved. If the levels are not sufficient to dissolve the pellet, the test will give an invalid.
- **Reader Lid Open** – Before pressing start, the lid to the reader needs to be closed and remain closed for the test to operate properly. Opening the lid, or not closing it, during a test can cause the results to become invalid.
- **Maintenance** – Make sure that each of the pieces of equipment are cleaned properly using diluted commercially available household strength bleach (9 parts water:1 part bleach) after use. Periodically calibrate the instruments to assure proper results. Follow the manufacturer's recommendations for the equipment as seen in the ANSR Preventative Maintenance guide.

If at any time you feel that the results from the ANSR unit are not correct, please contact our Technical Services group. Always go by the results generated by the ANSR unit. It is **not** recommended that you interpret curves.

NEOGEN® TECHNICAL SERVICES CONTACT INFORMATION

620 Lesher Place
Lansing, MI 48912 USA
800/234-5333 (USA)
517/372-9200
Fax: 517/372-2006

DISPOSAL OF REACTION TUBES, CLUSTER TIPS, CAPS AND TIPS

DO NOT AUTOCLAVE any of the reaction tubes, sampling tubes, cluster tubes, caps or pipette tips.

A wide mouth container lined with a disposal ziplock-style bag should be used for collecting of materials. This container should have a 10% bleach solution (9 parts water:1 part commercially available household strength bleach) that is made fresh daily. It should be sufficient enough to submerge the reaction tubes, caps, cluster tubes and pipette tips.

- **Sampling swabs** – When the lysis buffer is added, the cells contained in the sampling swabs are lysed. We recommend securing the caps and disposing of with the regular refuse.
- **Cluster tubes, used reaction cap tubes, pipette tips, reaction tubes with unopened permanent caps** – Each of these items should be placed into the wide mouth container containing the fresh 10% bleach solution. After the container is full, remove the ziplock style bag and seal it. It can be disposed of with the regular refuse. The permanent caps should **NEVER** be removed from the reaction tubes. The reaction tubes should be submerged in the bleach solution.

Other Documents of Interest

- Neogen's *Listeria* Right Now Kit Insert
- Neogen's Process Control for *Listeria* Guide
- Neogen's ANSR Preventative Maintenance Guide
- Neogen's ANSR Reader User Manual
- Neogen's ANSR Software/Firmware Update Guide
- Neogen's ANSR Test Type Package Update Guide
- Neogen's Tech Guide for Amplicon

Please contact your Neogen sales associate with any inquiries about ANSR *Listeria* Right Now test kit and about the ANSR system.