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Dr. George W Latimer, Jr. (ed.)

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CHAPTER

p. C17-352

17.10.19 AOAC Official Method 2016.08 *Listeria monocytogenes* in a Variety of Foods and Select Environmental Surfaces: Neogen[®] Molecular Detection Assay (MDA)

2–*Listeria monocytogenes* Method

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First Action 2016

Final Action 2019

{Applicable to the detection of *Listeria monocytogenes* in hot dogs (25 and 125 g), salmon (25 g), deli turkey (25 and 125 g), cottage cheese (25 g), chocolate milk (25 mL), vanilla ice cream (25 g), queso fresco (25 g), bagged raw spinach (25 g), romaine lettuce (25 g), melon (whole), raw chicken leg pieces (25 g), and raw chicken breast fillet (25 g), as well as on sealed concrete [Neogen Hydrated Sponge with Dey-Engley (D/E) Neutralizing Broth; 225 and 100 mL], stainless steel (Neogen Hydrated Sponge with D/E Neutralizing Broth; 225 mL), and plastic (high-density polyethylene; Neogen EnviroSwab with Lethen Broth; 10 mL) environmental samples.]

See Tables 2016.08A and B for a summary of results of the interlaboratory study supporting acceptance of the method.

A Principle

The Molecular Detection Assay (MDA) 2 – *Listeria monocytogenes* method is used with the Neogen Molecular Detection System (MDS) for the rapid and specific detection of *L. monocytogenes* in enriched food and on food process environmental samples. The MDA 2–*Listeria monocytogenes* uses loop-mediated isothermal amplification of unique DNA target sequences, with high specificity and sensitivity, combined with bioluminescence to detect the amplification. Presumptive positive results are reported in real-time, whereas negative results are displayed after the assay is completed. Samples are pre-enriched in Demi-Fraser (DF) Broth with ferric ammonium citrate (FAC) broth.

B Apparatus and Reagents

Items (a)–(n), (y), and (z) are available from Neogen Corp. (Lansing, MI, USA, www.neogen.com). Items (b)–(f) are available as the MDA 2–*Listeria monocytogenes* kit from Neogen Corp. (Cat. No. MDA2LMO96; Product SKU 700002149).

- (a) MDS.—Model MDS100; Product SKU 700002195.
- (b) MDA 2–*L. monocytogenes* reagent tubes.—Twelve strips of eight tubes.
- (c) Lysis solution (LS) tubes.—Twelve strips of eight tubes.



Table 2016.08A Summary of results for the detection of *L. monocytogenes* in deli turkey (125 g)^a

Neogen MDA 2- <i>L. monocytogenes</i> results	Inoculation level		
	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	0/132	69/132	132/132
POD _{CP}	0.00 (0.00, 0.03)	0.52 (0.43, 0.61)	1.00 (0.97, 1.00)
s _r	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s _L	0.00 (0.00, 0.16)	0.00 (0.00, 0.16)	0.00 (0.00, 0.16)
s _R	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P _T	1.0000	0.8091	1.0000
Candidate confirmed positive/total No. of samples analyzed	0/132	66/132	132/132
POD _{CC}	0.00 (0.00, 0.03)	0.50 (0.41, 0.59)	1.00 (0.97, 1.00)
s _r	0.00 (0.00, 0.16)	0.51 (0.46, 0.52)	0.00 (0.00, 0.16)
s _L	0.00 (0.00, 0.16)	0.00 (0.00, 0.14)	0.00 (0.00, 0.16)
s _R	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P _T	1.0000	0.9123	1.0000
Candidate confirmed positive/total No. of samples analyzed	0/132	66/132	132/132
POD _C	0.00 (0.00, 0.03)	0.50 (0.41, 0.59)	1.00 (0.97, 1.00)
s _r	0.00 (0.00, 0.16)	0.51 (0.46, 0.52)	0.00 (0.00, 0.16)
s _L	0.00 (0.00, 0.16)	0.00 (0.00, 0.14)	0.00 (0.00, 0.16)
s _R	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P _T	1.0000	0.9123	1.0000
Positive reference samples/total No. of samples analyzed	0/132	60/132	132/132
POD _R	0.00 (0.00, 0.03)	0.45 (0.37, 0.54)	1.00 (0.97, 1.00)
s _r	0.00 (0.00, 0.16)	0.51 (0.46, 0.52)	0.00 (0.00, 0.16)
s _L	0.00 (0.00, 0.16)	0.00 (0.00, 0.11)	0.00 (0.00, 0.16)
s _R	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P _T	1.0000	0.9829	1.0000
dLPOD _C (candidate vs reference) ^b	0.00 (-0.03, 0.03)	0.04 (-0.08, 0.17)	0.00 (-0.03, 0.03)
dLPOD _{CP} (candidate presumptive vs candidate confirmed) ^b	0.00 (-0.03, 0.03)	0.02 (-0.10, 0.15)	0.00 (-0.03, 0.03)

a Results include 95% confidence intervals.

- b A confidence interval for dLPOD that does not contain the value 0 indicates a statistical significant difference between the two methods.



Table 2016.08B Summary of results for the detection of *L. monocytogenes* in raw chicken breast fillet (25 g)^a

Neogen MDA 2 – <i>L. monocytogenes</i> results	Inoculation level		
	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	0/132	86/132	129/132
POD _{CP}	0.00 (0.00, 0.03)	0.65 (0.57, 0.73)	0.98 (0.93, 0.99)
s _r	0.00 (0.00, 0.16)	0.48 (0.43, 0.52)	0.15 (0.13, 0.17)
s _L	0.00 (0.00, 0.16)	0.00 (0.00, 0.17)	0.03 (0.00, 0.08)
s _R	0.00 (0.00, 0.23)	0.48 (0.43, 0.52)	0.15 (0.13, 0.18)
P _T	1.0000	0.7057	0.1089
Candidate confirmed positive/total No. of samples analyzed	0/132	86/132	132/132
POD _{CC}	0.00 (0.00, 0.03)	0.65 (0.57, 0.73)	1.00 (0.97, 1.00)
s _r	0.00 (0.00, 0.16)	0.48 (0.43, 0.52)	0.00 (0.00, 0.16)
s _L	0.00 (0.00, 0.16)	0.00 (0.00, 0.18)	0.00 (0.00, 0.16)
s _R	0.00 (0.00, 0.23)	0.48 (0.43, 0.52)	0.00 (0.00, 0.23)
P _T	1.0000	0.5632	1.0000
Candidate confirmed positive/total No. of samples analyzed	0/132	85/132	129/132
POD _C	0.00 (0.00, 0.03)	0.64 (0.56, 0.73)	0.98 (0.93, 0.99)
s _r	0.00 (0.00, 0.16)	0.48 (0.43, 0.52)	0.15 (0.13, 0.17)
s _L	0.00 (0.00, 0.16)	0.00 (0.00, 0.18)	0.03 (0.00, 0.08)
s _R	0.00 (0.00, 0.23)	0.49 (0.43, 0.52)	0.15 (0.13, 0.18)
P _T	1.0000	0.6228	0.1089
Positive reference samples/total No. of samples analyzed	0/132	64/132	132/132
POD _R	0.00 (0.00, 0.03)	0.48 (0.40, 0.57)	1.00 (0.97, 1.00)
s _r	0.00 (0.00, 0.16)	0.51 (0.46, 0.52)	0.00 (0.00, 0.16)
s _L	0.00 (0.00, 0.16)	0.00 (0.00, 0.14)	0.00 (0.00, 0.16)
s _R	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P _T	1.0000	0.9192	1.0000
dLPOD _C (candidate vs reference) ^b	0.00 (-0.03, 0.03)	0.16 (0.04, 0.28)	-0.02 (-0.06, 0.01)
dLPOD _{CP} (candidate presumptive vs candidate confirmed) ^b	0.00 (-0.03, 0.03)	0.00 (-0.12, 0.12)	-0.02 (-0.06, 0.01)

a Results include 95% confidence intervals.

b A confidence interval for dLPOD that does not contain the value 0 indicates a statistical significant difference between the

two methods.

(d) *Extra caps.*—Twelve strips of eight caps.

(e) *Reagent control.*—Eight reagent tubes.

p. C17-353 (f) *Quick Start Guide.*

(g) *Molecular Detection Speed Loader Tray.*

(h) *Molecular Detection Chill Block Insert.*

(i) *Molecular Detection Heat Block Insert.*

(j) *Molecular Detection Cap/Decap Tool for reagent tubes.*

(k) *Molecular Detection Cap/Decap Tool for lysis tubes.*

(l) *Empty Lysis Tube Rack.*

(m) *Empty Reagent Tube Rack.*

(n) *DF Broth.*

(o) *FAC.*—American Chemical Society grade, 5% sterilized (MP Biomedicals or equivalent).

(p) *Disposable pipet.*—Capable of delivering 20 μ L.

(q) *Multichannel (eight-channel) pipet.*—Capable of delivering 20 μ L.

(r) *Sterile filter-tip pipet tips.*—Capable of delivering 20 μ L.

(s) *Filter Stomacher[®] bags.*—Seward or equivalent.

(t) *Stomacher.*—Seward or equivalent.

(u) *Thermometer.*—Calibrated range to include $100 \pm 1^\circ\text{C}$.

(v) *Dry block heater unit.*—Capable of maintaining $100 \pm 1^\circ\text{C}$.

(w) *Incubator.*—Capable of maintaining $37 \pm 1^\circ\text{C}$.

(x) *Refrigerator.*—Capable of maintaining $2\text{--}8^\circ\text{C}$, for storing the MDA components.

(y) *Computer.*—Compatible with the Neogen Molecular Detection Instrument.

(z) *Neogen EnviroSwab hydrated with Letheen Broth.*

(aa) *Neogen Hydrated-Sponge with 10 mL D/E Neutralizing Broth.*

C General Instructions

- (a) Store the MDA 2–*Listeria monocytogenes* at 2–8°C. Do not freeze. Keep kit away from light during storage. After opening the kit, check that the foil pouch is undamaged. If the pouch is damaged, do not use. After opening, unused reagent tubes should always be stored in the resealable pouch with the desiccant inside to maintain stability of the lyophilized reagents. Store resealed pouches at 2–8°C for no longer than 60 days. Do not use MDA 2 – *Listeria monocytogenes* past the expiration date.
- (b) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

Safety precautions.—The MDA 2–*Listeria monocytogenes* is intended for use in a laboratory environment by professionals trained in laboratory techniques. Neogen has not documented the use of this product in industries other than the food and beverage industries. For example, Neogen has not documented this product for testing drinking water, pharmaceutical, cosmetic, clinical, or veterinary samples. The MDA 2–*Listeria monocytogenes* has not been evaluated with all possible food products, food processes, testing protocols, or with all possible strains of bacteria.

As with all test methods, the source of enrichment medium can influence the results. The MDA 2–*Listeria monocytogenes* has only been evaluated for use with the enrichment media specified in the manufacturer’s “Instructions for Use.”

The Molecular Detection Instrument is intended for use with samples that have undergone heat treatment during the assay lysis step, which is designed to destroy organisms present in the sample. Samples that have not been properly heat-treated during the assay lysis step may be considered a potential biohazard and should NOT be inserted into the MDS instrument.

The user should read, understand, and follow all safety information in the instructions for the MDS and the MDA 2–*Listeria monocytogenes*. Retain the safety instructions for future reference.

To reduce the risks associated with exposure to chemicals and biohazards, the following safety precautions should be taken: Perform pathogen testing in a properly equipped laboratory under the control of trained personnel. Always follow standard laboratory safety practices, including wearing appropriate protective apparel and eye protection while handling reagents and contaminated samples. Avoid contact with the contents of the enrichment media and reagent tubes after amplification. Dispose of enriched samples according to current industry standards.

L. monocytogenes is of particular concern for pregnant women, the aged, and the infirm. It is recommended that these concerned groups avoid handling this organism. After use, the enrichment medium and the MDA 2–*Listeria monocytogenes* tubes can potentially contain pathogenic materials. Periodically decontaminate laboratory benches and equipment (pipets, cap/decap tools, etc.) with a household bleach solution (1–5%, v/v in water; 5250–6500 ppm) or DNA removal solution. When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information and local regulations for disposal.

To reduce the risks associated with environmental contamination: Follow current industry standards for disposal of contaminated waste.

D Sample Enrichment

- (a) *Foods.*—(1) Allow the DF Broth enrichment medium (includes FAC) to equilibrate to ambient laboratory temperature (20–25°C).
- (2) Aseptically combine the enrichment medium and sample according to Table 2016.08C. For all meat and highly particulate samples, the use of filter bags is recommended.
- (3) Homogenize thoroughly by stomaching or hand-mixing for 2 ± 0.2 min. Incubate at $37 \pm 1^\circ\text{C}$ according to Table 2016.08C.
- (b) *Environmental samples.*—(1) Sample collection devices can be a sponge hydrated with a neutralizing solution to inactivate the effects of the sanitizers. Neogen recommends the use of a biocide-free cellulose sponge. The neutralizing solution can be D/E Neutralizing Buffer or Letheen Broth. It is recommended to sanitize the area after sampling.

Caution: Should you select to use D/E Neutralizing Buffer (NB) that contains aryl sulfonate complex as the hydrating solution for the sponge, it is required to perform a 1:2 dilution (one part sample to one part sterile enrichment broth) of the enriched environmental sample before testing to reduce the risks associated with a false-negative result leading to the release of contaminated product. Another option is to transfer 10 μL NB enrichment into the LS tubes.



Table 2016.08C Enrichment protocols using DF Broth at $37 \pm 1^\circ\text{C}$ according to AOAC *Performance Tested Method*SM Certification No. 081501^a

Sample matrix ^b	Sample size	Enrichment broth volume, mL	Enrichment time, h
Beef hot dogs, queso fresco, vanilla ice cream, 4% milk fat cottage cheese, 3% chocolate whole milk, romaine lettuce, bagged raw spinach, and cold smoked salmon	25 g	225	24–30
Raw chicken	25 g	475	28–32
Deli turkey	125 g	1125	24–30
Cantaloupe ^c	Whole melon	Enough volume to allow melon to float	26–30
Environmental samples			
Stainless steel	1 sponge	225	24–30
Sealed concrete	1 sponge	100	24–30
Plastic ^d	1 swab	10	24–30

a See Figure 2016.08A.

b All samples for the AOAC validation were homogenized by stomaching unless otherwise noted.

c Sample homogenized by hand-mixing.

d Sample homogenized by vortex-mixing.

- (2) The recommended size of the sampling area to verify the presence or absence of the pathogen on the surface is at least 100 cm² (10 × 10 cm or 4 × 4 in.). When sampling with a sponge, cover the entire area going in two directions (left to right and then up and down) or collect environmental samples by following the laboratory's current sampling protocol or according to U.S. Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM), USDA/FSIS MLG, or ISO 18593:2004 guidelines.
- (3) Allow the DF Broth enrichment medium (includes FAC) to equilibrate to ambient laboratory temperature (20–25°C).
- (4) Aseptically combine the enrichment medium and sample according to Table 2016.08C.
- (5) Homogenize thoroughly by vortex-mixing (swab) or stomaching (sponge) for 2 ± 0.2 min. Incubate at 37 ± 1°C for 24–30 h.

E Preparation of the Molecular Detection Speed Loader Tray

- (a) Wet a cloth or paper towel with a household bleach solution (1–5%, v/v in water; 5250–6500 ppm) and wipe the Molecular Detection Speed Loader Tray.
- (b) Rinse the Molecular Detection Speed Loader Tray with water.
- (c) Use a disposable towel to wipe the Molecular Detection Speed Loader Tray dry.
- (d) Ensure the Molecular Detection Speed Loader Tray is dry before use.

F Preparation of the Molecular Detection Heat Block Insert

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Place the Molecular Detection Heat Block Insert in a dry double block heater unit. Turn on the dry block heater unit and set the temperature to allow the Molecular Detection Heat Block Insert to reach and maintain a temperature of 100 ± 1°C.

Note: Depending on the heater unit, allow approximately 30 min for the Molecular Detection Heat Block Insert to reach temperature. Using an appropriate, calibrated thermometer (e.g., a partial immersion thermometer or a digital thermocouple thermometer, not a total immersion thermometer) placed in the designated location, verify that the Molecular Detection Heat Block Insert is at 100 ± 1°C.

G Preparation of the Molecular Detection Instrument

- (a) Launch the Molecular Detection Software and log in.
- (b) Turn on the Molecular Detection Instrument.
- (c) Create or edit a run with data for each sample. Refer to the MDS User Manual for details.

Note: The Molecular Detection Instrument must reach and maintain temperature of 60°C before inserting the Molecular Detection Speed Loader Tray with reaction tubes. This heating step takes approximately 20 min and is indicated by an orange light on the instrument's status bar. When the instrument is ready to start a run, the status bar will turn green.

H Lysis

- (a) Allow the LS tubes to warm up by setting the rack at room temperature (20–25°C) overnight (16–18 h). Equilibrating the LS tubes to room temperature may also be accomplished by setting the LS tubes on the laboratory bench for at least 2 h, by incubating the LS tubes in a 37 ± 1°C incubator for 1 h, or by placing them in a dry double block heater for 30 s at 100 ± 1°C.
- (b) Invert the capped tubes to mix. Proceed to the next step within 4 h.
- (c) Remove the enrichment broth from the incubator.
- (d) One LS tube is required for each sample and the negative control (NC; sterile enrichment medium) sample.
 - (1) LS tube strips can be cut to the desired LS tube number. Select the number of individual LS tubes or eight-tube strips needed. Place the LS tubes in an empty rack.
 - (2) To avoid cross-contamination, decap one LS tube strip at a time and use a new pipet tip for each transfer step.
 - (3) Transfer enriched sample to LS tubes as described below:

Note: Transfer each enriched sample into individual LS tube *first*. Transfer the NC *last*.

- (4) Use the Molecular Detection Cap/Decap Tool–Lysis to decap one LS tube strip, one strip at a time.
- (5) Discard the LS tube cap; however, if lysate will be retained for retest, place the caps into a clean container for reapplication after lysis.
- (6) Transfer 20 µL sample into an LS tube.
- (e) Repeat step (d)(6) until each individual sample has been added to a corresponding LS tube in the strip, as illustrated in Figure 2016.08A.
- (f) Repeat steps (d)(1)–(6), as needed, for the number of samples to be tested. When all samples have been transferred, transfer 20 µL NC into an LS tube. Do not recap tubes.
- (g) Verify that the temperature of the Molecular Detection Heat Block Insert is at 100 ± 1°C. Place the rack of LS tubes in the Molecular Detection Heat Block Insert and heat for 15 ± 1 min. During heating, the LS solution will change from pink (cool) to yellow (hot).
- (h) Remove the uncovered rack of LS tubes from the heating block and allow to cool in the Molecular Detection Chill Block Insert for at least 5 min and a maximum of 10 min. The Molecular Chill block Insert, used at ambient temperature (20–25°C) without the Molecular Detection Chill Block Tray, should sit directly on the laboratory bench. When cool, the LS will revert to a pink color.
- (i) Remove the rack of LS tubes from the Molecular Detection Chill Block Insert.

I Amplification

- (a) One reagent tube is required for each sample and the NC.
 - (1) Reagent tube strips can be cut to desired tube number. Select the number of individual reagent tubes or eight-tube strips needed.
 - (2) Place reagent tubes in an empty rack.
 - (3) Avoid disturbing the reagent pellets from the bottom of the tubes.
- (b) Select one reagent control (RC) tube and place in rack.
- (c) To avoid cross-contamination, decap one reagent tube strip at a time and use a new pipet tip for each transfer step.
- (d) Transfer lysate to reagent tubes and RC tube as described below:

Note: Transfer each sample lysate into individual reagent tubes *first*, followed by the NC. Hydrate the RC tube *last*.

- (1) Use the Molecular Detection Cap/Decap Tool–Reagent to decap the reagent tubes, one reagent tube strip at a time. Discard cap.
- (2) Transfer 20 μ L sample lysate from the upper half of the liquid (avoid precipitate) in the LS tube into corresponding reagent tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down five times.
- (3) Repeat step (d)(2) until each individual sample lysate has been added to a corresponding reagent tube in the strip.



Figure 2016.08A

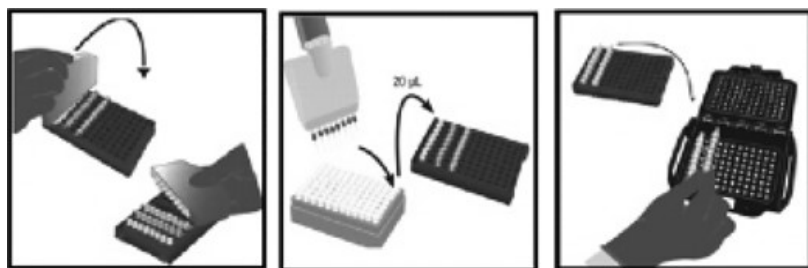


Figure 2016.08B

- (4) Cover the reagent tubes with the provided extra cap and use the rounded side of the Molecular Detection Cap/Decap \hookrightarrow Tool– Reagent to apply pressure in a back-and-forth motion, ensuring that the cap is tightly applied.
- (5) Repeat steps (d)(1)–(4), as needed, for the number of samples to be tested.
- (6) When all sample lysates have been transferred, repeat steps (d)(1)–(4) to transfer 20 μ L NC lysate into a reagent tube.
- (7) Transfer 20 μ L NC lysate into an RC tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down five times.
- (e) Load capped tubes into a clean and decontaminated Molecular Detection Speed Loader Tray. See Figure 2016.08B. Close and latch the Molecular Detection Speed Loader Tray lid.
- (f) Review and confirm the configured run in the Molecular Detection Software.
- (g) Click the Start button in the software and select instrument for use. The selected instrument's lid automatically opens.
- (h) Place the Molecular Detection Speed Loader Tray into the Molecular Detection Instrument and close the lid to start the assay. Results are provided within 75 min, although positives may be detected sooner.
- (i) After the assay is complete, remove the Molecular Detection Speed Loader Tray from the Molecular Detection Instrument and dispose of the tubes by soaking in a household bleach solution (1–5%, v/v in water; 5250–6500 ppm) for 1 h and away from the assay preparation area.

Note: To minimize the risk of false positives due to cross-contamination, never open reagent tubes containing amplified DNA. This includes RC, reagent, and matrix control tubes. Always dispose of sealed reagent tubes by soaking in a household bleach solution (1–5%, v/v in water; 5250–6500 ppm) for 1 h and away from the assay preparation area.

J Results and Interpretation

An algorithm interprets the light output curve resulting from the detection of the nucleic acid amplification. Results are analyzed automatically by the software and are color-coded based on the result. A positive or negative result is determined by analysis of a number of unique curve parameters. Presumptive positive results are reported in real time, whereas negative and “inspect” results will be displayed after the run is completed.

Presumptive positive samples should be confirmed as per the laboratory's standard operating procedures or by using the current version of the appropriate reference method confirmation (FDA/BAM or USDA/FSIS-MLG), beginning with transfer from the primary enrichment to the secondary enrichment broth (if applicable), followed by subsequent plating and confirmation of isolates using appropriate biochemical and serological methods.

Note: Even a negative sample will not give a zero reading because the system and MDA 2–*Listeria monocytogenes* amplification reagents have a “background” relative light unit reading.

In the rare event of any unusual light output, the algorithm labels this as “inspect.” Neogen recommends the user to repeat the assay for any “inspect” samples. If the result continues to be “inspect,” proceed to confirmation testing using your preferred method or as specified by local regulations.

Reference:

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