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CHAPTER

## 17.9.41 AOAC Official Method 2016.01 *Salmonella* spp. in Select Foods and Environmental Surfaces: Neogen® Molecular Detection Assay (MDA) 2–*Salmonella* Method

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First Action 2016

Final Action 2019

[Applicable to detection of *Salmonella* spp. in raw ground beef (73% lean), raw ground chicken, chicken carcass rinse, chicken carcass sponge, pasteurized liquid whole egg, cooked breaded chicken, instant nonfat dry milk, black pepper, cocoa powder, raw whole shrimp, raw bagged spinach, creamy peanut butter, dry dog food, pasteurized processed American cheese, spent sprout irrigation water, and sealed concrete, stainless steel, and sealed ceramic tile environmental surfaces.]

**Caution:** The Molecular Detection Assay (MDA) 2–*Salmonella* 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, cosmetics, clinical, or veterinary samples. The MDA 2–*Salmonella* 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–*Salmonella* has only been evaluated for use with the enrichment media specified in the manufacturers instructions for use.

The MDS 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–*Salmonella*. Retain the safety instructions for future reference.

Periodically decontaminate laboratory benches and equipment (pipets, cap/decap tools, etc.) with a 1–5% (v/v in water) household bleach solution 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 exposure to chemicals and biohazards, (1) perform pathogen testing in a properly equipped laboratory under the control of trained personnel; (2) always follow standard laboratory safety practices, including wearing appropriate protective apparel and eye protection while handling reagents and contaminated samples; (3) avoid contact with the contents of the enrichment media and reagent tubes after amplification; and (4) dispose of enriched samples according to current industry standards.

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

See Tables 2016.01A and B for a summary of results of the interlaboratory study supporting acceptance of the method.

## A Principle

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The MDA 2–*Salmonella* method is used with the MDS for the rapid and specific detection of *Salmonella* in enriched food, feed, and food-process environmental samples. The MDA 2–*Salmonella* 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 preenriched in ISO BPW.

## B Apparatus and Reagents

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Items (a)–(m), (x), and (y) are available from Neogen Corp. (Lansing, MI, USA, [www.neogen.com](http://www.neogen.com)). Items (b)–(e) are available as the MDA 2–*Salmonella* kit from Neogen Corp. (Cat. No. MDA2SAL96; Product SKU 700002157).

- (a) *MDS*.—Model MDS100; Product SKU 700002195.
- (b) *MDA 2–Salmonella reagent tubes*.—Twelve strips of eight tubes.
- (c) *Lysis solution (LS) tubes*.—Twelve strips of eight tubes.
- (d) *Extra caps*.—Twelve strips of eight caps.
- (e) *Reagent control*.—Eight reagent tubes.
- (f) *Molecular Detection speed loader tray*.
- (g) *Molecular Detection chill block insert*.
- (h) *Molecular Detection heat block insert*.
- p. C17-256 (i) *Molecular Detection cap/decap tool for reagent tubes*.
- (j) *Molecular Detection cap/decap tool for lysis tubes*.
- (k) *Empty lysis tube rack*.
- (l) *Empty reagent tube rack*.
- (m) *ISO BPW*.—Formulation equivalent to ISO 6579:2002 Annex B or Neogen equivalent.
- (n) *Disposable pipet*.—Capable of 20  $\mu$ L.
- (o) *Multichannel (eight-channel) pipet*.—Capable of 20  $\mu$ L.
- (p) *Sterile filter-tip pipet tips*.—Capable of 20  $\mu$ L.
- (q) *Filter Stomacher<sup>®</sup> bags*.—Seward or equivalent.
- (r) *Stomacher*.—Seward or equivalent.
- (s) *Thermometer*.—Calibrated range to include  $100 \pm 1^\circ\text{C}$ .
- (t) *Dry block heater unit*.—Capable of maintaining  $100 \pm 1^\circ\text{C}$ .
- (u) *Incubators*.—Capable of maintaining  $37 \pm 1^\circ\text{C}$  or  $41.5 \pm 1^\circ\text{C}$ .
- (v) *Refrigerator*.—Capable of maintaining  $2\text{--}8^\circ\text{C}$ , for storing the MDA components.
- (w) *Computer*.—Compatible with the MDS instrument.
- (x) *Neogen Enviroswab*.—Hydrated with Lethen Broth.
- (y) *Neogen hydrated sponge stick with 10 mL D/E*.

## C General Instructions

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- (1) Store the *MDA 2–Salmonella* at  $2\text{--}8^\circ\text{C}$ . 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  $2\text{--}8^\circ\text{C}$  for no longer than 1 month. Do not use *MDA 2–Salmonella* past the expiration date.



**Table 2016.01A** Summary of results for the detection of *Salmonella* in raw ground beef (325 g)<sup>a</sup>

Neogen MDA 2- <i>Salmonella</i> results	Inoculation level		
	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	4/156	83/156	155/156
POD <sub>CP</sub>	0.03 (0.01–0.06)	0.53 (0.44–0.62)	0.99 (0.96–1.00)
s <sub>r</sub>	0.15 (0.14–0.17)	0.49 (0.44–0.52)	0.08 (0.07–0.15)
s <sub>L</sub>	0.04 (0.00–0.09)	0.09 (0.00–0.24)	0.00 (0.00–0.03)
s <sub>R</sub>	0.16 (0.14–0.19)	0.50 (0.45–0.52)	0.08 (0.07–0.09)
P <sub>T</sub>	0.0315	0.1725	0.4395
Candidate confirmed positive/total No. of samples analyzed	3/156	83/156	155/156
POD <sub>CC</sub>	0.02 (0.01–0.06)	0.53 (0.43–0.63)	0.99 (0.96–1.00)
s <sub>r</sub>	0.14 (0.12–0.15)	0.49 (0.44–0.52)	0.08 (0.07–0.15)
s <sub>L</sub>	0.03 (0.00–0.07)	0.12 (0.00–0.27)	0.00 (0.00–0.03)
s <sub>R</sub>	0.14 (0.12–0.15)	0.50 (0.45–0.52)	0.08 (0.07–0.09)
P <sub>T</sub>	0.0877	0.0715	0.4395
Candidate confirmed positive/total No. of samples analyzed	2/156	82/156	155/156

POD <sub>C</sub>	0.01 (0.01–0.05)	0.53 (0.43–0.62)	0.99 (0.96–1.00)
S <sub>r</sub>	0.11 (0.10–0.15)	0.49 (0.44–0.52)	0.08 (0.07–0.15)
S <sub>L</sub>	0.03 (0.01–0.07)	0.10 (0.00–0.25)	0.00 (0.00–0.03)
S <sub>R</sub>	0.11 (0.10–0.13)	0.50 (0.45–0.52)	0.08 (0.07–0.09)
P <sub>T</sub>	0.0184	0.1272	0.4395
Positive reference samples/total No. of samples analyzed	2/156	77/156	156/156
POD <sub>R</sub>	0.01 (0.00–0.05)	0.49 (0.41–0.58)	1.00 (0.98–1.00)
S <sub>r</sub>	0.11 (0.10–0.15)	0.49 (0.45–0.52)	0.00 (0.00–0.15)
S <sub>L</sub>	0.00 (0.00–0.04)	0.06 (0.00–0.22)	0.00 (0.00–0.15)
S <sub>R</sub>	0.11 (0.10–0.13)	0.50 (0.45–0.52)	0.00 (0.00–0.21)
P <sub>T</sub>	0.5167	0.2813	1.0000
dLPOD <sub>C</sub> (candidate versus reference) <sub>b</sub>	0.00 (–0.03 to 0.03)	0.03 (–0.10 to 0.16)	–0.01 (–0.04 to 0.02)
dLPOD <sub>CP</sub> (candidate presumptive versus candidate confirmed) <sub>b</sub>	0.01 (–0.03 to 0.05)	0.00 (–0.14 to 0.14)	0.00 (–0.03 to 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.

- (2) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

## D Sample Enrichment

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- (a) *Foods*.—(1) Allow ISO BPW enrichment medium to equilibrate to ambient laboratory temperature (20–25°C) or prewarm to  $41.5 \pm 1^\circ\text{C}$  depending on matrixes tested. See Table 2016.01C for matrix-specific enrichment protocols.
- (2) Aseptically combine the enrichment medium and sample. For all meat and highly particulate samples, the use of filter bags is recommended.
- (3) Homogenize thoroughly for  $2 \pm 0.2$  min. Incubate matrixes according to the instructions provided in Table 2016.01C.

- p. C17-257 (b) *Environmental samples (not analyzed for this collaborative study)*.—(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. Neutralizing solution can be D/E neutralizing broth (NB) or Lethen Broth. It is recommended to sanitize the area after sampling.

*Caution:* If NB that contains aryl sulfonate complex as the hydrating solution for the sponge is used, it is required to perform a 1:2 dilution (one part sample into one part sterile enrichment broth) of the enriched environmental sample before testing in order to reduce the risks associated with a false-negative result leading to the release of contaminated product. Another option is to transfer 10  $\mu\text{L}$  NB enrichment into the LS tubes.

- (2) The recommended size of the sampling area for verifying the presence or absence of the pathogen on the surface is at least 100  $\text{cm}^2$  (10  $\times$  10 cm or 4  $\times$  4 in.). When sampling with a sponge, cover the entire area going in two directions (left to right and then

**Table 2016.01B** Summary of results for the detection of *Salmonella* in creamy peanut butter (25 g)

Neogen MDA 2- <i>Salmonella</i> results <sup>a</sup>	Inoculation level		
	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	3/144	83/144	144/144
POD <sub>CP</sub>	0.02 (0.01–0.06)	0.58 (0.49–0.66)	1.00 (0.97–1.00)
s <sub>r</sub>	0.14 (0.12–0.16)	0.50 (0.45–0.52)	0.00 (0.00–0.16)
s <sub>L</sub>	0.03 (0.00–0.08)	0.00 (0.00–0.16)	0.00 (0.00–0.16)
s <sub>R</sub>	0.14 (0.13–0.17)	0.50 (0.45–0.52)	0.00 (0.00–0.22)
P <sub>T</sub>	0.0976	0.7877	1.0000
Candidate confirmed positive/total No. of samples analyzed	2/144	82/144	144/144
POD <sub>CC</sub>	0.01 (0.00–0.05)	0.57 (0.49–0.65)	1.00 (0.97–1.00)
s <sub>r</sub>	0.11 (0.10–0.16)	0.51 (0.46–0.52)	0.00 (0.00–0.16)
s <sub>L</sub>	0.04 (0.01–0.07)	0.00 (0.00–0.12)	0.00 (0.00–0.16)
s <sub>R</sub>	0.12 (0.11–0.14)	0.51 (0.46–0.52)	0.00 (0.00–0.22)
P <sub>T</sub>	0.0221	0.9709	1.0000
Candidate confirmed positive/total No. of samples analyzed	1/144	81/144	144/144

POD <sub>C</sub>	0.01 (0.00–0.04)	0.56 (0.48–0.65)	1.00 (0.97–1.00)
S <sub>r</sub>	0.08 (0.07–0.16)	0.51 (0.45–0.52)	0.00 (0.00–0.16)
S <sub>L</sub>	0.00 (0.00–0.03)	0.00 (0.00–0.13)	0.00 (0.00–0.16)
S <sub>R</sub>	0.08 (0.07–0.10)	0.51 (0.46–0.52)	0.00 (0.00–0.22)
P <sub>T</sub>	0.4368	0.9393	1.0000
Positive reference samples/total No. of samples analyzed	0/144	73/144	144/144
POD <sub>R</sub>	0.00 (0.00–0.03)	0.51 (0.42–0.59)	1.00 (0.97–1.00)
S <sub>r</sub>	0.00 (0.00–0.16)	0.51 (0.46–0.52)	0.00 (0.00–0.16)
S <sub>L</sub>	0.00 (0.00–0.16)	0.00 (0.00–0.14)	0.00 (0.00–0.16)
S <sub>R</sub>	0.00 (0.00–0.22)	0.51 (0.46–0.52)	0.00 (0.00–0.22)
P <sub>T</sub>	1.0000	0.8963	1.0000
dLPOD <sub>C</sub> (candidate versus reference) <sup>b</sup>	0.01 (–0.02 to 0.04)	0.06 (–0.06 to 0.17)	0.00 (–0.03 to 0.03)
dLPOD <sub>CP</sub> (candidate presumptive versus candidate confirmed) <sup>b</sup>	0.01 (–0.03 to 0.05)	0.01 (–0.11 to 0.13)	0.00 (–0.03 to 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.

up and down) or collect environmental samples after your current sampling protocol or according to the FDA BAM, USDA–FSIS MLG, or ISO 18593 (1) guidelines.

- (a) Prewarm ISO BPW enrichment medium to  $41.5 \pm 1^\circ\text{C}$  depending on matrixes tested. See Table 2016.01C.
- (b) Aseptically combine the enrichment medium and sample.
- (c) Homogenize thoroughly by blending, stomaching, or hand-mixing for  $2 \pm 0.2$  min. Incubate at  $41.5 \pm 1^\circ\text{C}$  for 18–24 h.

## E Preparation of the Molecular Detection Speed Loader Tray

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- (1) Wet a cloth or paper towel with a 1–5% (v/v in water) household bleach solution and wipe the Molecular Detection speed loader tray.
- (2) Rinse the Molecular Detection speed loader tray with water.
- (3) Use a disposable towel to wipe the Molecular Detection speed loader tray dry.
- (4) Ensure that 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  $100 \pm 1^\circ\text{C}$ .

p. C17-23 **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  $100 \pm 1^\circ\text{C}$ .

## G Preparation of the Molecular Detection Instrument

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- (1) Launch the Molecular Detection software and log in.
- (2) Turn on the Molecular Detection instrument.
- (3) Create or edit a run with data for each sample. Refer to the MDS User Manual for details.

**Note:** The MDS instrument must reach and maintain a temperature of  $60^\circ\text{C}$  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.

**Table 2016.01C** Neogen MDA 2–*Salmonella* enrichment protocols

Sample matrix	Sample size	Enrichment broth volume	Enrichment temperature, $\pm 1^{\circ}\text{C}$	Enrichment time, h
Raw ground beef	25 g	225 mL ISO BPW (prewarmed)	41.5	10–24
	325 g	975 mL ISO BPW (prewarmed)		
Raw ground chicken	25 g	225 mL ISO BPW (prewarmed)	41.5	10–24
	325 g	975 mL ISO BPW (prewarmed)		14–24
Cooked breaded chicken	325 g	2925 mL ISO BPW	37	18–24
Dry dog food	25 g	225 mL ISO BPW	37	18–24
	375 g	1500 mL ISO BPW		
Black pepper, raw whole shrimp, raw bagged spinach, pasteurized processed American cheese	25 g	225 mL ISO BPW	37	18–24
Chicken carcass rinse	30 mL	30 mL ISO BPW (prewarmed)	41.5	18–24
Chicken carcass sponge	1 sponge	50 mL ISO BPW (prewarmed)	41.5	18–24
Instant nonfat dry milk	25 g	225 mL ISO BPW	37	20–24

Cocoa powder	25 g	225 mL ISO BPW	37	24–28
Pasteurized liquid whole egg	100 mL	900 mL ISO BPW	37	18–24
Spent sprout irrigation water	375 mL	3375 mL ISO BPW	37	18–24
Creamy peanut butter	25 g	225 mL ISO BPW	37	18–24
	375 g	3375 mL ISO BPW		
Environmental				
Sealed concrete	1 sponge	225 mL ISO BPW (prewarmed)	41.5	18–24
Stainless steel	1 swab	10 mL ISO BPW (prewarmed)	41.5	18–24
Sealed ceramic tile	1 sponge	50 mL ISO BPW (prewarmed)	41.5	18–24



## H Lysis

- (a) Allow the LS tubes to warm up to room temperature by setting the rack on the laboratory bench for at least 2 h. Invert room-temperature capped lysis tubes to mix. Sample aliquots must be transferred to lysis tubes within 4 h of mixing.
- (b) Remove the enrichment broth from the incubator and gently agitate the contents.
- (c) One LS tube is required for each sample and the negative control (NC) sample.
  - (1) LS tube strips can be cut to the desired LS tube numbers. 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 tubes strip at a time and use a new pipet tip for each transfer step.
- (d) Transfer enriched sample to LS tubes as described below:

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

- (1) Use the Molecular Detection cap/decap tool for lysis tubes to decap one LS tube strip, one strip at a time.
- (2) Discard the LS tube cap. If lysate will be retained for retest, place the caps into a clean container for reapplication after lysis.
- (3) Transfer 20  $\mu\text{L}$  sample into an LS tube.
- (4) Repeat step H(d) (2) until each individual sample has been added to a corresponding LS tube in the strip. See Figure 2016.01A.
- (5) Repeat steps H(d)(1)–(4), as needed, for the number of samples to be tested. When all samples have been transferred, transfer 20  $\mu\text{L}$  NC into an LS tube. Do not recap tubes.
- (6) Verify that the temperature of the Molecular Detection heat block insert is at  $100 \pm 1^\circ\text{C}$ . Place the rack of LS tubes in the Molecular Detection heat block insert and heat for  $15 \pm 1$  min. During heating, the LS solution will change from pink (cool) to yellow (hot).
- (e) Remove the uncovered rack of LS tubes from the heating block and allow to cool in the Molecular Detection chill block insert at least 5 min and for a maximum of 10 min. The Molecular Detection chill block insert, used at ambient temperature ( $20\text{--}25^\circ\text{C}$ ) without the Molecular Detection chill block tray, should sit directly on the laboratory bench. When cool, the LS will revert to a pink color.

- p. C17-259 (f)  $\hookrightarrow$  Remove the rack of LS tubes from the Molecular Detection chill block insert.



**Figure 2016.01A**

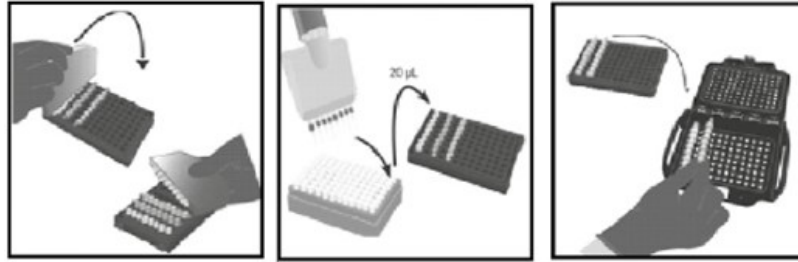


Figure 2016.01B

## I Amplification

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- (a) One reagent tube is required for each sample and the NC.
  - (1) Reagent tubes strips can be cut to the 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 for reagent tubes to decap one reagent tube strip, one strip at a time. Discard cap.
- (2) Transfer 20  $\mu$ L sample lysate from the upper one-half of the liquid (avoid precipitate) in the LS tube into the corresponding reagent tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down five times.
- (3) Repeat step I(d) (2) until individual sample lysate has been added to a corresponding reagent tube in the strip.
- (4) Cover the reagent tubes with the provided extra cap and use the rounded side of the Molecular Detection cap/decap tool-reagent to apply pressure in a back and forth motion ensuring that the cap is tightly applied.
- (5) Repeat steps I(d) (1)–(4), as needed, for the number of samples to be tested.
- (6) When all sample lysates have been transferred, repeat I(d) (1)–(4) to transfer 20  $\mu$ L NC lysate into a reagent tube.
- (7) Transfer 20  $\mu$ 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. Close and latch the Molecular Detection speed loader tray lid. See Figure 2016.01B.
- (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 MDS instrument and close the lid to start the assay. Results are provided within 60 min, although positives may be detected sooner.
- (i) After the assay is complete, remove the Molecular Detection speed loader tray from the MDS instrument and dispose of the tubes by soaking in a 1–5% (v/v in water) household bleach solution 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 tubes. Always dispose of sealed reagent tubes by soaking in a 1–5% (v/v in water) household bleach solution for 1 h and away from the assay preparation area.

## J Results and Interpretation

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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 results should be confirmed using your preferred method or as specified by the FDA BAM, USDA-FSIS MLG, or ISO 6579 reference method, starting from the Neogen primary enrichment, followed by transfer to a secondary enrichment or direct plating onto media through 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–*Salmonella* amplification reagents have a “background” relative light unit reading.

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

## References:

(1) ISO 18593 (2004) *Microbiology of food and animal feeding stuffs–Horizontal methods for sampling techniques from surfaces using contact plates and swabs*, International Organization for Standardization, Geneva, Switzerland

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