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CHAPTER

17.17.01 AOAC Official Method 2018.01 *Cronobacter* species in Select Foods and Environmental Surfaces: Neogen® Molecular Detection Assay (MDA) 2– *Cronobacter* Method

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[Applicable to detection of *Cronobacter* species in powdered infant formula with probiotics (10 and 300 g), powdered infant cereal without probiotics (10 and 300 g), lactose powder (10 g), and environmental surface sponges (stainless steel).]

See Table 2018.01 for a summary of results of the collaborative study. *See* additional table in *J. AOAC Int.* **102**, 108(2019) for detailed results of the collaborative study.

A Principle

The Molecular Detection Method (MDA) 2 – *Cronobacter* method is used with the Neogen Molecular Detection System (MDS) for the rapid and specific detection of *Cronobacter* species in select enriched food and food process environmental samples. The MDA 2 – *Cronobacter* uses loop-mediated isothermal amplification (LAMP) 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, and negative results are displayed after the assay is completed. Samples are pre-enriched in buffered peptone water (BPW)-ISO formulation.

B Apparatus and Reagents

Items **B(a)** –(**m**) and (**x**) are available from Neogen Corp. (Lansing, MI, USA, www.neogen.com). Items **B(b)**–(**e**) are available as the MDA 2 – *Cronobacter* kit from Neogen Corp. (Cat. No. MDA2CRO96; Product SKU 700002257).

(a) *Molecular Detection System Model MDS100; Product SKU 700002195.*

(b) *Molecular Detection Assay 2 – Cronobacter 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 (RC).*—Eight reagent tubes.

(f) *Molecular Detection Speed Loader Tray.*

(g) *Molecular Detection Chill Block Insert.*

(h) *Molecular Detection Heat Block Insert.*

(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.*

p. C17-274 **(m)** ↳ *BPW-ISO formulation.*

(n) *Micropipet.*—Capable of 20 μL.

(o) *Multichannel (8-channel) pipet.*—Capable of 20 μL.

(p) *Sterile filter pipet tips*—Capable of 20 μL.

(q) *Filter Stomacher® bags.*—Seward or equivalent.

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

(s) *Thermometer.*—Calibrated range to include 100 ± 1°C.

(t) *Dry block heater unit.*—Capable of maintaining 100 ± 1°C.

(u) *Incubators.*—Capable of maintaining 37 ± 1°C.

(v) *Refrigerator.*—Capable of maintaining 2–8°C for storing the MDA components.

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

(x) *Neogen Hydrated Sponge Stick.*

C General Instructions

(a) Store the MDA 2 – *Cronobacter* at 2–8°C. Do not freeze. Keep kit away from light during storage. After opening kit, check that foil pouch is undamaged. If 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 lyophilized reagents. Store resealed pouches at 2–8°C for no longer than 60 days. Do not use MDA 2 – *Cronobacter* past expiration date.

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

(c) The MDA 2 – *Cronobacter* 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 – *Cronobacter* has not been evaluated with all possible food products, food processes, testing protocols, or with all possible strains of bacteria.

(d) As with all test methods, the source of enrichment medium can influence results. The MDA 2 – *Cronobacter* has only been evaluated for use with the enrichment media specified in the *Instructions for Use* section.

D Safety Precautions

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 not exceed the recommended temperature setting on the heater or the recommended heating time.

Use an appropriate, calibrated thermometer to verify the Molecular Detection Heat Block Insert temperature (e.g., a partial immersion thermometer or digital thermocouple thermometer, not a total immersion thermometer). The thermometer must be placed in the designated location in the Molecular Detection Heat Block Insert.

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

To reduce the risks associated with exposure to chemicals and biohazards, 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.

After use, the enrichment medium and the MDA 2 – *Cronobacter* tubes can potentially contain pathogenic materials. Periodically decontaminate laboratory benches and equipment (pipets, cap/decap tools, etc.) with 1–5% (v/v in water) household bleach solution (5250–6500 ppm) or DNA removal solution. When testing is complete, follow current industry standards for disposal of contaminated waste. Consult Safety Data Sheet for additional information and local regulations for disposal.

Table 2018.01 Summary of results for detection of *Cronobacter* in powdered infant formula with probiotics

Method[a]		**Inoculation level**		
Neogen MDA 2 – *Cronobacter*	**Uninoculated**		**Low**	**High**
Candidate presumptive positive/total No. of samples analyzed	2/168		88/168	168/168
$LPOD_{CP}$	0.01 (0.01, 0.04)	0.52	(0.45, 0.60)	1.00 (0.98, 1.00)
s_r	0.11 (0.10, 0.15)	0.51	(0.46, 0.52)	0.00 (0.00, 0.15)
s_L	0.00 (0.00, 0.04)	0.00	(0.00, 0.15)	0.00 (0.00, 0.15)
s_R	0.11 (0.10, 0.12)	0.51	(0.46, 0.52)	0.00 (0.00, 0.21)
P value[b] Candidate confirmed positive/total No. of samples analyzed	0.5158 0/168		0.7750 86/168	1.0000 168/168
$LPOD_{CP}$	0.00 (0.00, 0.02)	0.51	(0.43, 0.59)	1.00 (0.98, 1.00)
s_r	0.00 (0.00, 0.15)	0.51	(0.46, 0.52)	0.00 (0.00, 0.15)
s_L	0.00 (0.00, 0.15)	0.00	(0.00, 0.11)	0.00 (0.00, 0.15)
s_R	0.00 (0.00, 0.21)	0.51	(0.47, 0.52)	0.00 (0.00, 0.21)
P value Candidate confirmed positive/total No. of samples analyzed	1.0000 0/168		0.9494 84/168	1.0000 168/168
Candidate presumptive positive that confirmed $LPOD_C$	0.00 (0.00, 0.02)	0.50	(0.42, 0.58)	1.00 (0.98, 1.00)
s_r	0.00 (0.00, 0.15)	0.51	(0.46, 0.52)	0.00 (0.00, 0.15)

s_L	0.00 (0.00, 0.15)	0.00	(0.00, 0.13)	0.00 (0.00, 0.15)
s_R	0.00 (0.00, 0.21)	0.51	(0.47, 0.52)	0.00 (0.00, 0.21)
P value Positive ref. samples/total No. of samples analyzed	1.0000 0/168		0.9462 86/168	1.0000 168/168
Ref. LPOD	0.00 (0.00, 0.02)	0.51	(0.43, 0.59)	1.00 (0.98, 1.00)
s_r	0.00 (0.00, 0.15)	0.51	(0.46, 0.52)	0.00 (0.00, 0.15)
s_L	0.00 (0.00, 0.15)	0.00	(0.00, 0.11)	0.00 (0.00, 0.15)
s_R	0.00 (0.00, 0.21)	0.51	(0.47, 0.52)	0.00 (0.00, 0.21)
P value	1.0000		0.9494	1.0000
dLPOD (candidate vs. ref.)[c]	0.00 (−0.02, 0.02)	−0.01	(−0.12, 0.10)	0.00 (−0.02, 0.02)
dLPOD (candidate presumptive vs. candidate confirmed)[c]	0.01 (−0.01, 0.04)	0.01	(−0.10, 0.12)	0.00 (−0.02, 0.02)

a Results include 95% confidence intervals.

b *P* value = Homogeneity test of laboratory PODs.

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

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

E Sample Enrichment

Food matrixes.—

(a) Allow BPW-ISO to equilibrate to ambient laboratory temperature (20–25°C) for 10 g test portions or environmental samples or to 37°C for 300 g test portions.

(b) Enrich samples following 1:9 enrichment ratio.

(1) For example, to 10 g test portions, 90 mL volume of BPW-ISO is added.

(2) For 300 g powdered infant formula and powdered infant cereal with probiotics, 10 mg/L Vancomycin is required to be supplemented into 2700 mL BPW-ISO.

(c) Homogenize thoroughly by blending, stomaching, vortex mixing, or hand mixing for 2 ± 0.2 min, or until all lumps are completely dissolved and the enrichment suspension is homogeneous.

(d) *Incubation.*—(1) Incubate powdered infant formula and powdered infant cereal (10 g) for 18–20 h at 37 ±1°C.

(2) Incubate powdered infant formula nonprobiotic (300 g) for 18–24 h at 37 ± 1°C.

(3) Incubate powdered infant formula and powdered infant cereal with probiotics (300 g) for 22–24 h at 37 ± 1°C.

(4) Incubate lactose (10 g) for 18–24 h at 37 ± 1°C.

Environmental samples.—

(a) Sample collection devices should be a sponge-hydrated with Dey-Engley Neutralizing Broth. It is recommended to sanitize the area after sampling.

(b) The recommended size of the sampling area to verify the presence or absence of the pathogen on the surface is at least 100 cm^2 (10 × 10 cm or 4 × 4 in.). When sampling with a sponge, cover entire area going in two directions (left to right then up and down) or collect environmental samples following current sampling protocol or ISO 18593:2004 guidelines.

(c) Allow BPW-ISO to equilibrate to ambient laboratory temperature (20–25°C).

(d) Enrich samples by adding 90 mL volume BPW-ISO to sampling sponge.

(e) Homogenize thoroughly by stomaching or hand mixing for 2 ± 0.2 min. Incubate at 37 ± 1°C for 18–24 h.

Figure 2018.01A

F Preparation of the Molecular Detection Speed Loader Tray

(a) Wet a cloth or paper towel with a 1–5% (v/v in water) household bleach (5250–6500 ppm) solution 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.

G Preparation of the Molecular Detection Chill Block Insert

Place the Molecular Detection Chill Block Insert directly on the laboratory bench; the Molecular Detection Chill Block Tray is not used. Use the block at ambient laboratory temperature (20–25°C).

H Preparation of the Molecular Detection Heat Block Insert

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, 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.

I Preparation of the Molecular Detection Instrument

(a) Launch the Molecular Detection Software and log in. Contact your Neogen representative to ensure you have the most updated version of the software.

(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.

J Lysis

(a) Allow the LS tubes to warm up by setting the rack at room temperature (20–25°C) overnight (16–18 h). Alternatives to equilibrate the LS tubes to room temperature are to set the LS tubes on the laboratory bench for at least 2 h, incubate the LS tubes in a 37 ± 1°C incubator for 1 h, or place 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 after inverting.

(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 desired LS tube number.*—Select the number of individual LS tubes or 8-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. 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. If lysate will be retained for retest, place the caps into a clean container for reapplication after lysis.

(6) Agitate the enrichment bag before collecting the sample from the filtered side when working with viscous samples.

(7) Transfer 20 μL sample into a LS tube (*see* Figure 2018.01A).

(e) Repeat steps (*1*)–(4) as needed for the number of samples to be tested. When all samples have been transferred, then transfer 20 μL NC into a LS tube. Do not recap tubes.

(f) 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). 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 Molecular Detection Instrument.

(g) 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 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 lysis solution will revert to a pink color.

(h) Remove the rack of LS tubes from the Molecular Detection Chill Block Insert.

K Amplification

(a) One MDA 2 – *Cronobacter* reagent tube is required for each sample and the NC.

(1) Reagent tubes strips can be cut to desired tube number. Select the number of individual reagent tubes or 8-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 RC tube and place in rack.

(c) To avoid cross-contamination, decap one reagent tubes strip at a time and use a new pipet tip for each transfer step.

(d) Transfer lysate to reagent tubes and RC tube. Transfer each sample lysate into individual reagent tubes first followed by the NC. Hydrate the RC tube last.

(e) Use the Molecular Detection Cap/Decap Tool-Reagent to decap the reagent tubes one reagent tubes strip at a time. Discard cap.

(1) Transfer 20 µL 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.

p. C17-276 (2) Repeat step **I(e)**(*1*) until each individual sample lysate has been added to a corresponding reagent tube in the strip.

(3) 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.

(4) Repeat **I(e)**(*1*)–(*3*) as needed for the number of samples to be tested.

(5) When all sample lysates have been transferred, repeat **I(e)**(*1*)–(*3*) to transfer 20 µL NC lysate into a reagent tube.

(6) Transfer 20 µL NC lysate into an RC tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down five times.

(f) Load capped tubes into a clean and decontaminated Molecular Detection Speed Loader Tray. *See* Figure 2018.01B. Close and latch the Molecular Detection Speed Loader Tray lid.

(g) Review and confirm the configured run in the Molecular Detection Software.

Figure 2018.01B

(h) Click the "Start" button in the software and select instrument for use. The selected instrument's lid automatically opens.

(i) 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.

(j) 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 1–5% (v/v in water) household bleach (5250–6500 ppm) solution for 1 h and away from the assay preparation area.

Note: To minimize the risk of false positives from 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 1–5% (v/v in water) household bleach (5250–6500 ppm) solution for 1 h and away from the assay preparation area.

Reference:

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