Assurance® GDS *Cronobacter* Tq II

AOAC® *Performance Tested Method*℠ 121903

AOAC® *Official Method of Analysis*℠ 2021.08

Part No: 71038-100 (100 tests)
71038-576ATM (576 tests)

General Description

Assurance® GDS for *Cronobacter* Tq II is an automated nucleic acid amplification system for the detection of *Cronobacter* in infant nutritional formula, ingredients, and environmental samples. Assurance® GDS assays are designed for use by qualified lab personnel who follow appropriate microbiology laboratory practices.

Kit Components

Each Assurance® GDS for *Cronobacter* Tq II kit (**100** tests) contains the following:

Amplification Tubes Tq
Concentration Reagent
Resuspension Buffer Tq
Wash Solution

Each Assurance® GDS for *Cronobacter* Tq II **576ATM** kit contains the following:

Amplification Tubes Tq
Concentration Reagent

The following are also necessary for **576ATM** kit but sold separately:

61031-100	Wash Solution Kit
34724-100C	Resuspension Buffer Tq

Equipment / Materials Required

Other necessary materials not provided include:

Media per **Appendix A**

Assurance® GDS Rotor-Gene® thermocycler
GDS rotor and locking ring

Laptop computer and software v2.3.103

PickPen® device and PickPen® tips

Vortex mixer (IKA® MS 3, or equivalent)

Adhesive film strips

GDS sample wells and sample wells base

Resuspension plate

Gel cooling block

Stomacher® paddle homogenizer, or equivalent

Stomacher®-type bags with filter, or equivalent

8-channel micropipette capable of accurately dispensing 30 µL

Repeat pipette

Repeat pipette tips (0.5 mL and 10 mL)

Adjustable micropipette capable of accurately dispensing 20 µL, 45 µL, 0.3 mL, 0.5 mL, 0.7 mL, and 1.0 mL

Filter-barrier micropipette tips (50 µL and 1.0 mL)

Incubator capable of maintaining 36 ± 2 °C

Incubator capable of maintaining 41.5 ± 1 °C

Incubator capable of maintaining 44 ± 1 °C

Freezer capable of maintaining -20 ± 5 °C

Refrigerator capable of maintaining 5 ± 3 °C

TEST PORTION PREPARATION & ENRICHMENT

See **Appendix A** for selection of appropriate enrichment media for sample type. See **Appendix B** for enrichment media preparation guidelines.

Note: For preparations of initial suspensions, follow instructions of EN ISO 22964 and EN ISO 6887 standards, unless stated.

Note: For this method, when a temperature of 36 °C is specified, the acceptable temperature range is 36 ± 2 °C.

A. Sample Preparation & Enrichment – <u>Infant formula, infant cereals, non-fat dry milk (NFDM) and milk powders, and other infant nutritional ingredients</u>

Note: The media must be prewarmed to 36 °C prior to sample addition.

1. **Samples <u>without</u> subculture in brain heart infusion (BHI) broth**
   a. **Infant formula without probiotics,** most **non-probiotic ingredients** (except **NFDM and milk powders**): For 10 – 375 g of sample, maintain a 1:10 dilution in Buffered Peptone Water (BPW, Appendix B). For example, to 10 g, add 90 mL BPW; to 25 g, add 225 mL BPW; to 375 g, add 3375 mL BPW.

      Infant formula with probiotics: For 10 – 375 g of sample, maintain a 1:10 dilution in BPW with vancomycin (BPW+v, Appendix B). For example, to 10 g, add 90 mL BPW+v; to 25 g, add 225 mL BPW+v; to 375 g, add 3375 mL BPW+v.

      Infant cereals without probiotics: For 10 – 375 g of sample, maintain a 1:10 dilution in BPW with amylase (BPW+a, Appendix B). For example, to 10 g, add 90 mL BPW+a; to 25 g, add 225 mL BPW+a; to 375 g, add 3375 mL BPW+a.

      Infant cereal with probiotics: For 10 – 375 g of sample, maintain a 1:10 dilution in BPW with vancomycin and amylase (BPW+v+a, Appendix B). For example, to 10 g, add 90 mL BPW+v+a; to 25 g, add 225 mL BPW+v+a; to 375 g, add 3375 mL BPW+v+a.

   b. For powdered or dry products, do not shake; instead, allow media and sample to stand undisturbed at 18 – 27 °C (laboratory temperature) for 30 min – 60 min. If after this time the powder is not homogenized, mix the content till dissolved.

   c. Incubate 10 – 25 g samples for 20 – 28 h at 36 °C. Incubate 26 – 375 g samples for 24 – 32 h at 36 °C.

   d. Continue to SAMPLE EXTRACTION PROTOCOL.

2. **Samples <u>with</u> subculture in BHI**
   a. **NFDM and milk powders**: For 10 – 375 g of sample, maintain a 1:10 dilution in Brilliant Green Water (BGW, Appendix B). For example, to 10 g, add 90 mL BGW; to 25 g, add 225 mL BGW; to 375 g, add 3375 mL BGW.

   b. Do not shake; instead, allow media and sample to stand undisturbed at 18 – 27 °C (laboratory temperature) for 30 min – 60 min. If after this time the powder is not homogenized, mix the content till dissolved.

c. Incubate 10 – 25 g samples for 20 – 28 h at 36 °C. Incubate 26 – 375 g samples for 24 – 32 h at 36 °C.

d. Transfer enriched samples to BHI (Appendix B) broth for 2 – 4 h at 36 °C as described in C. Sample Extraction Protocol, Step 10.

e. Continue to SAMPLE EXTRACTION PROTOCOL.

B. Sample Preparation & Enrichment – <u>Environmental Samples</u>

1. **Samples without subculture in BHI**

a. **Environmental monitoring** (stick swabs and sponges): Pre-moisten sterile dehydrated sponges with 10 mL BPW. Hydrate sterile swab by soaking in BPW. If neutralization is needed, substitute D/E (Dey/Engley) Broth or Letheen Broth for the BPW to pre-moisten. After collecting sample from surface, add sponge or swab to 100 mL or 10 mL of BPW, respectively. Mix well. Incubate samples for 20 – 28 h at 36 °C.

Areas of sampling:

- Food (and non-food) product contact surfaces, work surfaces and adjacent areas (e.g., blenders, worktables, drip shields, housing)
- Non-food contact surfaces not close to food product work surfaces (e.g., drains, floors, walls, cart wheels)

b. **Dusts and process water**: Aseptically weigh 25 g sweepings into 225 mL BPW. Aseptically add 25 mL process water to 225 mL of BPW. Mix well. Incubate samples for 20 – 28 h at 36 °C.

C. Sample Extraction Protocol

Change gloves prior to handling reagents.

1. Vortex **Concentration Reagent**. Immediately transfer 20 µL to each of the required number of GDS sample wells (1 well/sample) using a repeat pipette and a 0.5 mL pipette tip. Add 0.7 mL of **Wash Solution** to sample wells containing Concentration Reagent using a repeat pipette and a 10 mL pipette tip. Cover sample wells with adhesive film strips.

2. Dispense 1.0 mL of **Wash Solution** to the required number of GDS sample wells (1 well/sample) using a repeat pipette and a 10 mL pipette tip. Cover sample wells with adhesive film strips.

   For **NFDM and milk powders** samples, dispense 0.5 mL of sterile BHI broth to sample wells (1 well/sample) <u>instead of</u> Wash Solution using a repeat pipette and a 10 mL pipette tip. Cover sample wells with adhesive film strips.

3. Dispense 45 µL of **Resuspension Buffer Tq** to the sample wells in the resuspension plate (1 well/sample) using a repeat pipette and a 0.5 mL pipette tip. Cover resuspension plate with adhesive film strips.

4. Carefully remove adhesive film from 1 strip of sample wells containing Concentration Reagent and Wash Solution. Mix incubated sample gently to ensure homogeneity. Add 0.3 mL of incubated sample to each sample well using adjustable micropipette and 1.0 mL filter barrier tips. Avoid transferring food particles. A new pipette tip must be used for each sample. Cover each strip of sample wells with a new adhesive film prior to adding samples to a new strip of wells. **Immediately return samples to incubator for confirmation, if necessary**.

5. Place sealed GDS sample wells containing Concentration Reagent and samples on the vortex mixer and vortex at 900 rpm for 10 – 20 min. If necessary, adjust rpm to be certain that liquid does not contact adhesive film.

6. Carefully remove and discard adhesive film from 1 strip of samples. Remove corresponding adhesive film from a strip of sample wells containing Wash Solution or BHI.

7. Load tips onto the PickPen® device, ensuring that the tips are firmly in place on the PickPen® tool. Extend the PickPen® magnets and insert tips into the first strip of sample wells. Stir gently for 30 s while continually moving up and down from the surface to the bottom of the well. Gently tap the PickPen® tips against the side of the sample wells to remove excess media droplets.

8. For all samples **except NFDM and milk powders**, transfer PickPen® tips to corresponding sample wells containing Wash Solution. With tips submerged, gently stir the PickPen® tips from side to side for 10 s (do

not release particles into solution). Tap the PickPen® tips against the side of the wells to remove excess Wash Solution droplets.

9. Remove adhesive film strip from resuspension plate. Transfer PickPen® tips to the corresponding column of the prepared resuspension plate. With tips submerged, retract the PickPen® magnets and tap tips gently to release particles into the Resuspension Buffer Tq. Cover the resuspension plate column with an adhesive film strip and continue with step 11.

10. For **NFDM and milk powders** samples:

    a. Remove adhesive film strip from sample wells containing BHI. Transfer PickPen® tips to corresponding sample wells containing BHI. With tips submerged, retract the PickPen® magnets and tap tips gently to release particles into the BHI. Cover each strip of BHI containing Concentration Reagent with a new adhesive film strip prior to adding samples to a new strip. Incubate sample wells containing BHI and particles for 2 – 4 h at 36 °C.

    b. Remove adhesive film strip from resuspension plate. Following incubation, transfer the particles from the BHI sample wells to the corresponding column of the prepared resuspension plate using the PickPen® device, as indicated in steps C7 – C9. With tips submerged, retract the PickPen® magnets and tap tips gently to release particles into the Resuspension Buffer Tq. Cover the resuspension plate column with an adhesive film strip and continue with step 11.

11. Repeat steps C6 through C10 for all samples using new tips for each strip of samples.

PROCEED TO TEST PROCEDURE SECTION

Test Procedure (Amplification & Detection)

Change gloves prior to handling reagents.

A. Preparation of Gel Cooling Block

1. Prior to initial use, the gel cooling block must be stored in the freezer (-20 ± 5 °C) for minimum 6 h. When frozen, the gel cooling block will change color from pink to purple. When not in use, the gel cooling block should continue to be stored upside-down at -20 ± 5 °C.

2. Between each use, the gel cooling block should be returned to the freezer and stored upside-down until it has turned completely purple, indicating it is ready for use. This may take up to 2 h.

B. Preparation of Amplification Tubes Tq

1. The Assurance® GDS Rotor-Gene® set-up and data entry should be completed prior to transferring samples from the resuspension plate into the **Amplification Tubes Tq**.

2. Remove Amplification Tubes from foil pouch and place them in the frozen gel cooling block. Reseal pouch.

3. Open Amplification Tubes. Briefly pipette up and down resuspension solution to resuspend beads. Transfer 30 µL of sample from resuspension plate well into each Amplification Tube using a multi-channel pipette and filter barrier tips. Firmly press down on each Amplification Tube cap to close. Visually inspect each tube to ensure that the cap is securely sealed.

4. Place Amplification Tubes into the Assurance® GDS Rotor-Gene® in sequential order, beginning with position #1.

5. Start Rotor-Gene® cycle. Refer to Assurance® GDS user manual (No. 55342 / 20516474) for detailed instructions on operating the Rotor-Gene® thermocycler.

Note: The Assurance® GDS Rotor-Gene® must be started within 20 min after addition of the samples to the Amplification Tubes.

Note: Enriched samples can be stored at 5 ± 3 °C for up to 72 h prior to testing with GDS *Cronobacter*.

Note: Cover resuspension plate with adhesive film and store at refrigeration temperature if cultural confirmation according to Protocol C is performed.

Results

Upon completion of the run, the Assurance® GDS thermocycler software will provide a results table. Each sample will be identified as **Positive**, **Negative**, or **No Amp**.

Positive: Samples are potentially positive for *Cronobacter* spp.

Negative: Samples are negative for *Cronobacter* spp.

No Amp: Amplification did not occur. Repeat the test beginning from **Sample Extraction Protocol**. If the No Amp result repeats, contact Technical Services (BioMTS@milliporesigma.com).

No.	Color	Name	Result	Description	Kit Lot Number
1		Sample 1	Positive	*Cronobacter*	1234567
2		Sample 2	Negative	*Cronobacter*	1234567
3		Sample 3	No Amp	*Cronobacter*	1234567

Cultural Confirmation

Enriched samples can be stored at 5 ± 3 °C for up to 72 h prior to confirmation. For **NFDM and milk powder** samples, store BGW (and not BHI subculture) enrichment at 5 ± 3 °C.

A. Confirm typical colonies via ISO 22964 (2017): Horizontal method for the detection of *Cronobacter* spp. Transfer 0.1 mL enrichment broth to 10 mL *Cronobacter* Selective broth (CSB). Incubate CSB for 24 ± 2 h at 41.5 ± 1 °C. Streak 10 µL CSB onto Chromogenic *Cronobacter* Isolation (CCI) agar (Merck 1205960500, or equivalent). Incubate plates for 24 ± 2 h at 41.5 ± 1 °C.

B. *Cronobacter* may be isolated from GDS–positive samples [22 – 28 h / 26 – 32 h, based on sample size] by directly streaking enrichment to a choice of chromogenic agars: CCI agar (Merck 1205960500, or equivalent), Brilliance *Cronobacter sakazakii* agar (DFI, ThermoFisher CM1055, or equivalent), RAPID'*Sakazakii* agar (RSA, Bio-Rad 3564976, or equivalent). Streak plates for isolation. Incubate CCI plates for 24 ± 2 h at 41.5 ± 1 °C. Incubate DFI plates for 24 ± 2 h at 36 ± 2 °C. Incubate RSA plates for 24 ± 2 h at 44 ± 1 °C. Refer to manufacturer's instructions on identification of typical colonies from chromogenic agars.

   For direct streak of **NFDM or milk powder** samples, isolate only after same-day analysis by GDS and only onto CCI plates.

   Confirm colonies using rapid biochemical gallery (e.g., bioMérieux API® 20E or VITEK 2 kit), or appropriate ISO 16140-6:2019 validated confirmation method (e.g., Bruker MALDI-ToF Biotyper®).

   Note: If *Cronobacter* is not isolated using the direct streak method (above), *Cronobacter* may then be isolated using the resuspension plate containing remaining IMS particles targeting *Cronobacter* in resuspension buffer from the GDS-positive samples. See option C, below.

   Note: There is a possibility of weak color development of typical colonies on CCI or DFI agar. Also, weak color development of non-targets may occur on RSA. Restreak from enrichment broth to a different chromogenic agar if unable to confirm positive result of GDS.

C. *Cronobacter* may be isolated from GDS-positive samples by plating the GDS concentration reagent which remains in resuspension plate after analysis.

   1. From the sample resuspension plate previously used for GDS *Cronobacter* analysis (Test Procedure, Step **B**), briefly pipette up and down remaining liquid contained in the well. This will resuspend the IMS beads contained in Resuspension Buffer Tq (approximately 10-15 µL volume remains).

   2. Transfer all volume (10-15 µL) of suspended IMS beads to first quadrant of choice of chromogenic agar: CCI or DFI plates. Streak plates for isolation. Incubate CCI plates for 24 ± 2 h at 41.5 ± 1 °C. Incubate DFI plates for 24 ± 2 h at 36 ± 2 °C.

3. Confirm colonies using rapid biochemical gallery (e.g., bioMérieux API® 20E or VITEK 2 kit), or appropriate ISO 16140-6:2019 validated confirmation method (e.g., Bruker MALDI ToF Biotyper®).

Note: The original GDS *Cronobacter* sample resuspension plate can be stored at 5 ± 3 °C (refrigeration) for up to 48 h prior to confirmation.

Storage

Store Assurance® GDS for *Cronobacter* Tq II kit components at 5 ± 3 °C.

Precautions

Comply with Good Laboratory Practice (refer to EN ISO 7218 standard).

Assurance® GDS for *Cronobacter* Tq II must be used as described herein. Do not use test kit beyond expiration date on the product box label.

Safety

Assurance® GDS for *Cronobacter* Tq II kit.—This product is not intended for human or veterinary use. Assurance® GDS for *Cronobacter* Tq II must be used as described in the package insert. The user should read, understand, and follow all safety information in the instructions for the Assurance® GDS for *Cronobacter* Tq II kit. Retain the safety instructions for future reference.

Contents of the test may be harmful if swallowed or taken internally.

Do not open or autoclave used Amplification Tubes. After run is complete, place used Amplification Tubes into a sealed container with sufficient volume of a 10% bleach solution to cover tubes for a minimum of 15 min or double bag Amplification Tubes and dispose outside of the lab. Decontaminate and dispose of materials in accordance with good laboratory practices and in accordance with local, state, and federal regulations. If contamination is suspected, moisten paper towel with 10% bleach solution and wipe all lab benches and equipment surfaces. Avoid spraying bleach solution directly onto surfaces. Allow bleach solution to remain on surfaces for a minimum of 15 min before wiping clean with 70% isopropyl alcohol solution.

To prepare 10% bleach solution, add 10 mL of commercially available bleach containing at least 5% sodium hypochlorite to 90 mL of deionized water. The minimum final concentration of sodium hypochlorite in the bleach solution should be 0.5%. The bleach solution is stable for 7 days from preparation. To prepare 70% isopropyl alcohol solution, add 70 mL of pure isopropyl alcohol to 30 mL of deionized water or buy commercially available 70% isopropyl alcohol.

Assurance® GDS Rotor-Gene®.—Improper use of the Assurance® GDS Rotor-Gene® may cause personal injuries or damage to the instrument. Some components may pose a risk of personal injury due to excessive heat if improperly handled. For safe use, the instrument must only be operated by qualified laboratory personnel who have been appropriately trained. Servicing of the instrument must only be performed by MilliporeSigma/Merck KGaA Service Engineers.

Sample Enrichment.— 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 personal 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. Decontaminate and dispose of materials in accordance with good laboratory practices and in accordance with local, state/provincial, and federal regulations.

Cronobacter Precautions.— *Cronobacter* is a biosafety level-2 organism. Biological samples, such as enrichments, have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations on disposal of biological wastes. Wear appropriate protective equipment which includes, but is not limited to: protective eyewear, face shield, clothing/laboratory coat, and gloves. All work should be conducted in properly equipped facilities utilizing the appropriate safety equipment (e.g., physical containment devices). Individuals should be trained in accordance with applicable regulatory and company/institutional requirements before working with potentially infectious materials. All enrichment broths should be sterilized following any culture-based confirmatory steps. Clean the work stations and laboratory equipment with a disinfectant of choice before and after lab activities (e.g., sodium hypochlorite solution, phenol solution, quaternary ammonium solution, etc.).

APPENDIX A – *Cronobacter* Enrichment Methods

Table A: Sample Type and Enrichment Method for *Cronobacter* (Infant Formula, Infant Cereals, and Ingredients)

Food Type	Media	Sample Size	Dilution Factor	Enrichment Time	Incubation Temperature
No BHI subculture					
Infant Formula without Probiotics	BPW	10 – 25 g	1:10	20 – 28 h	36 ± 2°C
		26 – 375 g	1:10	24 – 32 h	36 ± 2°C
Infant Cereals without Probiotics	BPW+a	10 – 25 g	1:10	20 – 28 h	36 ± 2°C
		26 – 375 g	1:10	24 – 32 h	36 ± 2°C
Non-probiotic Ingredients (except NFDM)	BPW	10 – 25 g	1:10	20 – 28 h	36 ± 2°C
		26 – 375 g	1:10	24 – 32 h	36 ± 2°C
Infant Formula with Probiotics	BPW+v	10 – 25 g	1:10	20 – 28 h	36 ± 2°C
		26 – 375 g	1:10	24 – 32 h	36 ± 2°C
Infant Cereals with Probiotics	BPW+v+a	10 – 25 g	1:10	20 – 28 h	36 ± 2°C
		26 – 375 g	1:10	24 – 32 h	36 ± 2°C
With BHI subculture					
NFDM and milk powders	BGW	10 – 25 g	1:10	20 – 28 h	36 ± 2°C
		26 – 375 g	1:10	24 – 32 h	36 ± 2°C

Table B: Sample Type and Enrichment Method for *Cronobacter* (Environmental Monitoring)

Environmental Sample	Media	Sample Size	Dilution Factor	Enrichment Time	Incubation Temperature
Environmental samples (Nearby food contact surfaces*) (Distant non-food contact surfaces**)	BPW	Swab Sponge	10 mL 100 mL	20 – 28 h	36 ± 2°C
Process water	BPW	25 mL	1:10	20 – 28 h	36 ± 2°C
Dust, sweepings	BPW	25 g	1:10	20 – 28 h	36 ± 2°C

* Food (and non-food) product contact surfaces, work surfaces and adjacent areas (e.g., blenders, worktables, drip shields, housing)

**Non-food contact surfaces not close to work surfaces (e.g., drains, floors, walls, cart wheels)

APPENDIX B – Enrichment Media Preparation

Buffered Peptone Water (BPW)

Follow the manufacturer's instructions for preparation of media.

Buffered Peptone Water w/ Vancomycin (BPW+v)

Prepare BPW as described above.

For 1:10 enrichments, on day of use, add 0.675 mL of 0.2% Vancomycin solution to 225 mL BPW (10.1 mL of Vancomycin solution to 3375 mL BPW).

Buffered Peptone Water w/ Amylase (BPW+a)

Prepare BPW as described above.

For 1:10 enrichments, on day of use, add 2.25 mL of 1% Amylase solution to 225 mL BPW (33.8 mL of Amylase solution to 3375 mL BPW).

Buffered Peptone Water w/ Vancomycin and Amylase (BPW+v+a)

Prepare BPW as described above.

For 1:10 enrichments, on day of use, add 0.675 mL of 0.2% Vancomycin solution and 2.25 mL of 1% Amylase solution to 225 mL BPW (10.1 mL of Vancomycin solution and 33.8 mL of Amylase solution to 3375 mL BPW).

0.2% Vancomycin Solution

Dissolve 0.2 g of Vancomycin (hydrochloride) in 100 mL of sterile deionized water. Store in dark at 5 ± 3 °C.

1% Amylase Solution

Dissolve 1.0 g of alpha-Amylase (~50 U/mg) in 100 mL of sterile deionized water. Store in dark at 5 ± 3 °C.

Note: Verify amylase source does not also contain dextrin and is from bacterial origin. We recommend Sigma #10070 or MP Biomedicals #0210044725.

Brain Heart Infusion (BHI) Broth

Suspend 37 g of BHI in 1 L of deionized water. Mix thoroughly and dispense into desired aliquots. Autoclave at 121 °C for 15 min.

1% Brilliant Green Dye Solution

Dissolve 1 g of Brilliant Green dye in 100 mL of sterile deionized water (do not autoclave). Store in dark at 5 ± 3 °C.

Brilliant Green Water (BGW)

For 1:10 enrichments, on day of use, add 0.45 mL of 1% Brilliant Green Dye solution to 225 mL of sterile deionized water (6.75 mL of 1% Brilliant Green Dye solution to 3375 mL of sterile deionized water).

Manufacturing Entity

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Lit. No. MK_DS5776EN v2.0
01/2025
20516488 v4.0
55403