foodproof® *Enterobacteriaceae* plus *Cronobacter* Detection Kit

Hybridization Probes (LC 2.0, 480)

Revision A, January 2024

PCR kit for the qualitative detection of *Enterobacteriaceae* DNA including the simultaneous identification of *Cronobacter* spp. using the LightCycler® 2.0 or 480 Systems.

Product No. KIT230068, KIT230069

PCR kit for 96 reactions for a maximum of 90 or 94 samples / 450 or 470 samples

Store the kit at -15 °C to -25 °C

For food testing purposes.

FOR *IN VITRO* USE ONLY

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1. What this Product Does

1.1 Number of Tests

The detection kit is designed for 96 reactions or 480 reactions with a final reaction volume of 20 µL each. Up to 30 samples (single sample preparation) plus positive and negative control reactions can be analyzed per LightCycler® Carousel-Based System run (i.e., the complete detection kit allows analysis of a maximum of 90 or 450 samples). Using the LightCycler® 480 System up to 94 samples or 470 samples can be analyzed.

1.2 Storage and Stability

- Store the kit at -15 to -25 °C through the expiration date printed on the label.
- Once the kit is opened, store the components as described in the following Contents table:

1.3 Kit Contents

Vial / Cap Color	Label	Contents / Function / Storage
1 yellow cap	foodproof *Enterobacteriaceae* plus *Cronobacter* Master Mix	• KIT230068: 3 x 420 µL • KIT230069: 5 x 1.260 µL • Ready-to-use primer and Hybridization Probe mix specific for *Enterobacteriaceae* or *Cronobacter* DNA respectively as well as the specific Internal Control (IC). • For amplification and detection of *Enterobacteriaceae* and *Cronobacter* specific sequences. • Store at -15 to -25 °C. • **Avoid repeated freezing and thawing!** • **Protect from light!**
2 red cap	foodproof *Enterobacteriaceae* plus *Cronobacter* Enzyme Solution	• KIT230068: 3 x 32 µL • KIT230069: 5 x 96 µL • Contains DNA-free Taq DNA Polymerase and Uracil-DNA Glycosylase (heat labile) for prevention of carry-over contamination. • Store at -15 to -25 °C.
3 white cap	foodproof *Enterobacteriaceae* plus *Cronobacter* Internal Control -LC 2.0	• KIT230068: 3 x 32 µL • KIT230069: 5 x 96 µL • Contains a stabilized solution of plasmid DNA. • For use as an internal amplification control using LightCycler 2.0. • Store at -15 to -25 °C. • After first thawing store at 2 to 8 °C for up to one month.
4 purple cap	foodproof *Enterobacteriaceae* plus *Cronobacter* Control Template	• KIT230068: 1 x 50 µL • KIT230069: 1 x 100 µL • Contains a stabilized solution of plasmid DNA. • For use as a PCR run positive control. • Store at -15 to -25 °C. • After first thawing store at 2 to 8 °C for up to one month.

5 colorless cap	H_2O PCR-grade	• KIT230068 and KIT230069: 1 x 1 mL • Nuclease-free, PCR-grade H_2O. • For use as a PCR run negative control. • Store at -15 to -25 °C.
6 black cap	foodproof *Enterobacteriaceae* plus *Cronobacter* Internal Control -LC 480	• KIT230068: 3 x 32 µL • KIT230069: 5 x 96 µL • Contains a stabilized solution of plasmid DNA and a yellow dye for better visualization. • For use as an internal amplification control using LightCycler 480. • Store at -15 to -25 °C. • After first thawing store at 2 °C to 8 °C for up to one month.

1.4 Additional Equipment and Reagents Required

- LightCycler Carousel-Based System 2.0 Instrument
- LightCycler 20 µL - Capillaries
- Color Compensation Set 1 (Product No. KIT230004)
- Standard benchtop microcentrifuge containing a rotor for 2.0 ml reaction tubes.
- The LightCycler (LC) Carousel-Based System provides adapters that allow LightCycler Capillaries to be centrifuged in a standard microcentrifuge rotor.
  or
- LC Carousel Centrifuge 2.0 for use with the LightCycler 2.0 Sample Carousel (optional).
  or
- LightCycler 480 I or II System
- LightCycler 480 compatible PCR plate and sealing foil
- foodproof StarPrep One Kit (Product No. KIT230175 Product No. KIT230176) or
- foodproof Magnetic Preparation Kit IV (Product No. KIT230184) in combination with the KingFisher™ Flex System
- Reagent D (Product No. KIT230001 or Product No. KIT230002)
- foodproof D-Light Instrument (Product No. MCH230039)
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes
- Sterile reaction tubes for preparing PCR mixes and dilutions

1.5 Applicability Statement

The foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit – Hybridization Probes (LC 2.0, 480) is intended for the rapid detection of DNA of *Enterobacteriaceae* isolated from enrichment cultures prepared by valid methods and inoculated with all kinds of foods that are potentially contaminated with these microorganisms. In addition, the detection kit allows the specific identification of *Cronobacter* spp. The *Enterobacteriaceae* plus *Cronobacter* Detection Kit can also be used for pre-screening of *Salmonella* followed by confirmation with the foodproof *Salmonella* Detection Kit.

The detection kit must not be used in diagnostic procedures.

The foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit (Product No. KIT230068) was validated according to ISO 16140-2:2016, MicroVal certificate number LR08/09/19/20. The validation was performed in comparison to the ISO methods for *Cronobacter* (ISO 22964:2017) and *Enterobacteriaceae* (ISO 21528-1:2017).

For the validation, the LightCycler 480 II (software version 1.5.1) from Roche Diagnostics was used. Manual DNA extraction was performed with the foodproof StarPrep One Kit (Product No. KIT230175) and Reagent D (Product No. KIT230001), according to section 2.5 "MicroVal Protocol including the manual DNA extraction with the foodproof StarPrep One Kit". Semi-automated DNA extraction was performed with the KingFisher Flex System in combination with the foodproof Magnetic Preparation Kit IV (Product No. KIT230184) and Reagent D (Product No. KIT230001), according to section 2.4 "MicroVal Protocol including the semi-automated DNA extraction". The alternative methods for *Enterobacteriaceae* and *Cronobacter* detection were validated to be applicable to the scope: infant formula and infant cereals, probiotics containing products, ingredients and environmental samples.

2. How to Use this Product

2.1 Before You Begin

2.1.1 Precautions

Detection of *Enterobacteriaceae* DNA using the foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit requires DNA amplification by PCR. The detection system provides all reagents required for PCR. In order to achieve reliable results, the entire assay procedure must be performed under nuclease-free conditions. Follow the instructions below to avoid nuclease-, carry-over-, or cross- contamination:

- Prepare appropriate aliquots of the solutions and keep them separate from other reagents in the laboratory.
- Use nuclease-free labware (e.g., pipettes, pipette tips, reaction vials).
- Wear gloves when performing the assay.
- To avoid cross-contamination of samples and reagents, use fresh aerosol-preventive pipette tips.
- To avoid carry-over contamination, transfer the required solutions for one experiment into a fresh tube, rather than directly pipetting from stock solutions.
- Do not touch the surface of the capillaries. Always wear gloves when handling the capillaries.
- Physically separate the workplaces for DNA preparation, PCR setup, and PCR to minimize the risk of carry-over contamination. Use a PCR hood for all pipetting steps.
- Keep the foodproof *Enterobacteriaceae* plus *Cronobacter* Master Mix (vial 1, yellow cap) away from light.

2.1.2 Waste Disposal

Place any waste and biohazard material potentially contaminated with pathogenic bacteria in an appropriate plastic contaminated waste bag and label as follows: CONTAMINATED waste, room number, date and initials. The bag should be autoclaved and then disposed of according to local regulations.

2.1.3 Sample Material

Use any sample material suitable for PCR in terms of purity, concentration, and absence of inhibitors. For preparation of genomic DNA from raw material or from food enrichments, refer to the corresponding product package inserts of a suitable sample preparation kit (see "Additional Equipment and Reagents required").

2.1.4 DNA Extraction

Hygiena Diagnostics provides sample preparation kits for all kind of foods and raw materials (see "Additional Equipment and Reagents required"). For more information, please contact us at www.hygiena.com/support. Detailed DNA extraction procedures used for the MicroVal validation are described in sections 2.4 and 2.5.

2.1.5 Positive Control

Always run a positive control with the samples. To prepare a positive control, replace the template DNA with the provided control DNA (Control Template; vial 4, purple cap) or with a positive sample preparation control.

2.1.6 Negative Control

Always run a negative control with the samples. To prepare a negative control, replace the template DNA with PCR-grade water (vial 5, colorless cap). Include a negative control during sample preparation to monitor reaction purity and cross-contamination. This extraction control can be used as an additional negative control reaction.

2.1.7 Cultural Confirmation

Positive PCR results should be confirmed with cultural confirmation methods, e.g., recommended by the reference methods for *Cronobacter* (ISO 22964:2017) and *Enterobacteriaceae* (ISO 21528-1:2017). For further information, please visit the following web address: www.iso.org.

Detailed confirmation procedure of the MicroVal study No. LR08/09/19/20

For *Enterobacteriaceae*:
The first enrichment in BPW was used for isolation on VRBG agar (incubation at 37 ± 1°C for 18 ± 2 h). Confirmation was done according to ISO 21528-1 by Oxidase reaction and Fermentation test in Glucose OF medium.

For *Cronobacter*:
0.1 ml of the first enrichment in BPW was transferred to 10 mL CBS and incubated at 41.5° C for 24 ± 2 h. Isolation was done on CCL agar. Confirmation was done according to ISO 22964 by using the ID 32E identification test kit.

2.1.8 Color Compensation

The use of a previously generated color compensation (CC) object is a prerequisite for the unambiguous discrimination of *Enterobacteriaceae* DNA*, Cronobacter* DNA and internal control (IC) DNA amplification in this multi-color experiment. A suitable color compensation object can be generated using dedicated reagents available as Color Compensation Set 1 (Product No. KIT230004). As color compensation is instrument-specific, it is necessary to generate a CC object for every LightCycler Instrument. A new object has to be created after the optical system has been repaired.

For additional information on color compensation, please refer to the manual for the respective LightCycler instrument being used.

2.2 PCR Procedure

The following procedures are optimized for the LightCycler 480 System and the LightCycler 2.0 Carousel-Based System. Program the LightCycler Systems before preparing the reaction mixes. The protocols contain the following programs:

- Pre-incubation to prevent carry-over contamination (UNG), to activate Taq polymerase and for DNA denaturation
- Amplification of the target DNA
- Cooling of the LightCycler System

2.2.1 LightCycler 480 System Protocol

The following procedure is optimized for use with the LightCycler 480 System. Program the LightCycler before preparing the reaction mixes. Use the following LightCycler 480 System PCR program for the foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit (for details on how to program the experimental protocol, see the LightCycler 480 System Operator's Manual):

Setup		
Detection Format	**Block Type**	**Reaction Volume**
Multi Color HybProbe	96	20 µL
Filter Setting	Dynamic mode, **LC 480 I:** Fluos (483-533), Red 610 (483-610), Red 640 (483-640) and Cy 5 (483-670) **LC480 II:** Fluos (465-510, Red 610 (498-610), Red 640 (498-640) and Cy 5/ Cy 5.5 (498-660)	
Programs		
Program Name	**Cycles**	**Analysis Mode**
Pre-Incubation	1	None
Amplification	38	Quantification
Cooling	1	None

Temperature Targets							
Program/ Segment	**Target [°C]**	**Acquisition Mode**	**Hold [hh:mm:ss]**	**Ramp Rate [°C/s]**	**Secondary Target Temperature [°C]**	**Step Size [°C]**	**Step Delay [Cycles]**
Pre-Incubation							
Segment 1	37	None	00:04:00	4.4	0	0.0	0
Segment 2	95	None	00:05:00	4.4	0	0.0	0
Amplification							
Segment 1	95	None	00:00:10	4.4	0	0.0	0
Segment 2	65	Single	00:00:40	2.2	61	0.2	8
Segment 3	72	None	00:00:25	4.4	0	0.0	0
Cooling							
	40	None	00:00:30	2.2	0	0.0	0

2.2.2 LightCycler 2.0 Carousel-Based System Protocol

The following procedure is optimized for use with the LightCycler 2.0 Carousel-Based System. Program the LightCycler before preparing the reaction mixes. Use the following LightCycler 2.0 PCR program for the foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit (for details on how to program the experimental protocol, see the LightCycler 2.0 Instrument Operator's Manual):

Pre-incubation (Prevention of Carry-over Contamination, Activation of Taq DNA Polymerase, Denaturation of Template DNA)		
Programs/Cycle Program Data	***Value***	
Cycles	1	
Analysis Mode	None	
Temperature Targets	***Segment 1***	***Segment 2***
Target/Target Temperature [°C]	37	95
Hold/Incubation Time [h:min:s]	00:04:00	00:02:00
Ramp Rate/Temperature Transition Rate [°C/s]	20	20
Sec Target/Secondary Target Temperature [°C]	0	0
Step Size [°C]	0.0	0.0
Step Delay [Cycles]	0	0
Acquisition Mode	None	None

Amplification (of the target DNA)			
Programs/Cycle program data	***Value***		
Cycles	34		
Analysis Mode	Quantification		
Temperature Targets	***Segment 1***	***Segment 2***	***Segment 3***
Target/Target Temperature [°C]	95	64	72
Hold/Incubation Time [h:min:s]	00:00:02	00:00:35	00:00:20
Ramp Rate/Temperature Transition Rate [°C/s]	20	20	20
Sec Target/Secondary Target Temperature [°C]	0	60	0
Step Size [°C]	0.0	0.2	0.0
Step Delay [Cycles]	0	8	0
Acquisition Mode	None	Single	None

Cooling (of the Rotor and Thermal Chamber)	
Programs/Cycle program data	***Value***
Cycles	1
Analysis Mode	None
Temperature Targets	***Segment 1***
Target/Target Temperature [°C]	40
Hold/Incubation Time [h:min:s]	00:00:30
Ramp Rate/Temperature Transition Rate [°C/s]	20
Secondary Target/Secondary Target Temperature [°C]	0
Step Size [°C]	0.0
Step Delay [Cycles]	0
Acquisition Mode	None

2.2.3 LightCycler 2.0 Fluorescence and Run Setup Parameters

Parameter	Setting
All LightCycler Software Versions	
Seek Temperature	30 °C
LightCycler Software Version 4.x	
Default channel • during run • for analysis	 • Fluorescence channel 640, 670 or 610 • 640/Back 530, 670/Back 530 or 610/Back 530
Fluorescence Gains	• not required
“Max. Seek Pos”	Enter the number of samples including controls.
“Instrument Type”	’6 Ch.’: for LightCycler 2.0 Instrument (selected by default).
“Capillary Size”	Select ’20 µL’ as the capillary size for the experiment.

2.2.4 Preparation of the PCR Mix

Proceed as described below to prepare a 20 µL standard reaction.

Do not touch the surface of the capillaries. Always wear gloves when handling the capillaries. For LightCycler 480 users, do not touch the upper surface of the PCR multiwell plate.

1. Depending on the total number of reactions, place the required number of LightCycler capillaries in centrifuge adapters or in a LightCycler Sample Carousel in a LC Carousel Centrifuge Bucket. For LightCycler 480 instruments, use a suitable multiwell plate.
2. Thaw the solutions and, for maximal recovery of contents, briefly spin vials in a microcentrifuge before opening. Mix carefully by pipetting up and down.

3. Prepare the PCR Mix for one 20 µL reaction in a 1.5 mL reaction tube by adding the following components in the order mentioned below, then mix gently by pipetting up and down.

The volumes indicated below are based on a single 20 µL standard reaction. Prepare the PCR mix by multiplying the amount in the "Volume" column by the number of reactions plus one positive and on negative control to be cycled plus one or two additional reactions to cover pipetting losses.

Component	Volume
foodproof *Enterobacteriaceae* plus *Cronobacter* Master Mix, (vial 1, yellow cap)	13 µL
foodproof *Enterobacteriaceae* plus *Cronobacter* Enzyme Solution, (vial 2, red cap)	1 µL
foodproof *Enterobacteriaceae* plus *Cronobacter* Internal Control - LC 2.0, (vial 3, white cap) or foodproof *Enterobacteriaceae* plus *Cronobacter* Internal Control - LC 480, (vial 6, black cap)	1 µL
Total volume	**15 µL**

4. Mix carefully by pipetting up and down. Do not vortex.
   - Pipette 15 µL PCR mix into each LightCycler capillary or plate well, respectively.
   - For the samples of interest, add 5 µL sample DNA to a capillary or a well (LC 480); seal the capillary with a stopper.
   - For the negative control, add 5 µL PCR-grade water (vial 5, colorless cap); seal the capillary with a stopper.
   - For the positive control, add 5 µL foodproof *Enterobacteriaceae* plus *Cronobacter* Control Template (vial 4, purple cap); seal the capillary with a stopper.
5. For LightCycler Carousel Based System:
   - Place the adapters (containing the capillaries) in a standard benchtop microcentrifuge. (Place the centrifuge adapters in a balanced arrangement within the centrifuge.)
   - Centrifuge at 700 x g for 5 s (3,000 rpm in a standard benchtop microcentrifuge). Alternatively, use the LC Carousel Centrifuge for spinning the capillaries.
   - Transfer the capillaries to the LightCycler.
6. For LightCycler 480 System:
   - Seal the plate accurately with an optical sealing foil. Place the plate in a swing bucket centrifuge and centrifuge at 1,500 x *g* for 30 s.
7. Cycle the samples as described above.

2.3 Data Interpretation

Analyze real-time PCR results in channels 640/Back-530 (LC 480 I: 483-640, LC 480 II: 498-640), 670/Back-530 (LC 480 I: 483-670, LC 480 II: 498-660) and 610/Back-530 (LC 480 I: 483-610, LC 480 II: 498-610) using the Qualitative Detection module of the LightCycler Analysis Software for LightCycler 2.0 and the Absolute Quantification module for the LightCycler 480 Systems. The instrument- and assay-specific color compensation object (see section 2.1) must be activated for all channels in order to compensate for crosstalk between the detection channels. Check for a positive result of the Internal Control (visible signal in the channel for 610 nm detection) for each sample that is negative for *Enterobacteriaceae* and *Cronobacter* DNA (no signal in the channels for 640 and 660/670 nm detection). Compare the results for each sample, and interpret as described in the table below:

Enterobacteriaceae **Channel 640**	***Cronobacter*** **Channel 660/670**	**Internal Control Channel 610**	**Result Interpretation**
Positive	Positive	Positive OR Negative	Positive for *Enterobacteriaceae* AND *Cronobacter**
Positive	Negative	Positive OR Negative	Positive for *Enterobacteriaceae*; Negative for *Cronobacter*
Negative	Negative	Positive	Negative for *Enterobacteriaceae* AND *Cronobacter*
Negative	Negative	Negative	Invalid result

*In case of very small amounts of *Cronobacter* DNA, it is possible that the *Cronobacter* channel (660/670) gives a positive signal, whereas the channel for *Enterobacteriaceae* (640) is negative. This indicates a positive result for both *Enterobacteriaceae* and *Cronobacter* due to a slightly higher sensitivity of the detection system for *Cronobacter.*

Notes:
For LightCycler 480 System: Use the "High Sensitivity" setting of the LightCycler Software to calculate results.
For LightCycler 2.0 and LightCycler 480 System: Always verify the software results ("positive", "negative", "uncertain") for plausibility by inspection of the amplification curves.

Data interpretation of PCR results can be done with the microproof Diagnostic Interpreter, according to the user manual for the Diagnostic Interpreter.

2.4 MicroVal Protocol Including the Semi-Automated DNA Extraction

Enterobacteriaceae / Cronobacter: Protocol for the semi-automated DNA isolation and real-time PCR detection in infant formula and infant cereals, probiotic-containing products, ingredients and environmental samples with the foodproof Magnetic Preparation Kit IV in combination with the KingFisher Flex System.

Salmonella spp.: Protocol for the semi-automated DNA isolation and real-time PCR detection in infant formula and infant cereals and probiotic-containing products with the foodproof Magnetic Preparation Kit IV in combination with the KingFisher Flex System.

2.4.1 Introduction

The procedure for the detection of *Enterobacteriaceae*/*Cronobacter* consists of five consecutive steps:

1. Primary enrichment
2. Secondary enrichment
3. Reagent D treatment
4. DNA extraction
5. Real-time PCR

2.4.2 Material and Methods

Instruments

- KingFisher Flex System
- foodproof D-Light instrument (for Reagent D treatment); Product No. MCH230039
- Real-time PCR instrument (LightCycler 480 II, LightCycler 2.0)
- Thermoshaker with adapter for 96 DWP (round bottom)

Reagents

- foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit, Product No. KIT230068
- If applicable: foodproof *Salmonella* Detection Kit, Product No. KIT230070
- Reagent D; Product No. KIT230001
- foodproof Magnetic Preparation Kit IV, Product No. KIT230184
- Buffered peptone water (BPW)
- Depending on the matrix: double strength BPW, vancomycin, alpha-amylase

Consumables

- Consumables for KingFisher Flex:
    - Riplate® 96 tip comb; 60 pieces
    - Riplate® 96 SRW magnetic, 2.0 mL; 60 pieces (binding plate, washing plates)
    - Riplate® 96 SRW magnetic, 0.2 mL; 60 pieces (elution plate, tip plate)
    - Seal for microplates; 100 pieces
- Plate cover for KF DeepWell plate (Reagent D treatment)
- 96 Round-bottom deep-well plates, 2.0 mL (4titude)
- Breathable viscose foil for biological cultures, sterile
- Reservoirs for reagents

2.4.3 Primary Enrichment

- Enrichment in pre-warmed (37 °C) BPW for **18 ± 2h at 37 °C**

Table 1 Category: Infant Formula and Infant Cereals

Sample Type	Sample Preparation
Infant formula (intended for infants < 1 year)	100 g in 900 mL BPW
Infant formula (intended for infants > 1 year)	100 g in 900 mL BPW
Infant cereals	100 g in 900 mL BPW plus alpha-amylase (alpha-amylase at 50 mg per 100 g sample in 900 mL BPW for products with high starch content)

Table 2 Category: Probiotics Containing Products

Sample Type	Sample Preparation
Probiotic infant formula (*L. paracasei, L. rhamnosis, L. reuteri*) at a level < 10^8 CFU/g (consumer products)	100 g in 900 mL BPW
Probiotic infant formula (*L. johnsonii, S. thermophilus, B. lactis, B. longum*) at a level < 10^8 CFU/g (consumer products)	100 g in 900 mL BPW plus vancomycin (vancomycin at 10 mg/L)
Probiotic infant cereals (*Bifidus* bacteria) at a level < 10^8 CFU/g (consumer products)	100 g in 900 mL BPW plus vancomycin (vancomycin at 10 mg/L) alpha-amylase at 50 mg per 100 g sample in 900 mL BPW for products with high starch content
Probiotic ingredients containing *L. reuteri* at ~10^{10} CFU/g	100 g in 900 mL double-strength BPW
Probiotic ingredients containing *L. rhamnosis* and/or *B. longum* at ~10^{10} CFU/g	100 g in 900 mL double-strength BPW plus vancomycin (vancomycin at 10 mg/L)

Table 3 Category: Ingredients

Sample Type	Sample Preparation
Infant formula ingredients (e.g., milk cow powder, whey cow powder, lactose, maltodextrin)	100 g in 900 mL BPW
Infant cereals ingredients (e.g. starch, oatmeal, rye meal, wheat(flour), buckwheat)	100 g in 900 mL BPW plus alpha-amylase (alpha-amylase at 50 mg per 100 g sample in 900 mL BPW for products with high starch content)
Premix, Duomix (containing minerals, vitamins)	12.5 g in 900 BPW

Table 4 Category: Environmental Samples

Sample Type	Sample Preparation
Sweep samples/equipment swabs	Submerge swab/sponge in 90 mL BPW
Traject samples (in-line factory)	100 g in 900 mL BPW
Vacuum cleaner residues	100 g in 900 mL BPW

2.4.4 Secondary Enrichment

- Transfer 100 µL of primary enrichment broth in 900 µL of fresh BPW in a deep well plate (round bottom).
- Cover the plate with a breathable foil.
- Incubate for 3-4 h at 37 °C with shaking on a thermoshaker (at maximum speed, 900 U/min).

Notes:
Probiotic-containing samples may also be sub-cultivated for 20-24 h.

For *Salmonella* detection in combination with the foodproof Magnetic Preparation Kit IV, a secondary enrichment of 20-24 h is required for probiotic-containing products.

2.4.5 Reagent D Treatment

- Pre-filling of the Binding Plate with **300 µL Reagent D.**
- Transfer of **100 µL of the secondary enrichment culture** into the Binding Plate (for the KingFisher Flex instrument).
- Add a sterile plate cover.
- Reagent D treatment with the D-Light instrument (incubation for 5 min in the dark and for 5 min with light exposure).
- After Reagent D treatment, the Binding Plate can be directly used for DNA extraction with the foodproof Magnetic Preparation Kit IV in combination with KingFisher Flex System.

2.4.6 DNA Extraction

(Please also see product instructions for KIT230184)

Note: Prepare the required kit components according to the quick reference procedure of the foodproof Magnetic Preparation Kit IV.

The following protocol describes the automated DNA isolation from 400 µL sample material with the KingFisher Flex System:

- Switch on the KingFisher Flex System.

Note: Before starting the purification process with the KingFisher Flex System, please read the user manual carefully!

Resuspend/Vortex the Magnetic Beads thoroughly directly before use!

- **Tip Plate:** Place the Tip Comb 96 DWH on a Tip Plate (Use one Elution Plate as Tip Plate.)
- Pre-fill the Washing Plates and the Elution Plate as described below:

- **Washing Plate I:** Add 1000 µL **Wash Buffer I**
- **Washing Plate II:** Add 1000 µL **Wash Buffer II**
- **Washing Plate III:** Add 1000 µL **Wash Buffer III**
- **Elution Plate**: Add 50 µL **Elution Buffer**
- Add reagents to the **Binding Plate** containing 400 µL of sample (100 µL sample plus 300 µL Reagent D):

  Binding Plate: Add 500 µL **Lysis Buffer,** 20 µL **Lysozyme** and 30 µL **Magnetic Beads**
- Choose assay file 'foodproof_MPK_IV' on instrument and press 'START'.
- Follow instructions on the instruments display and load the pre-filled buffer plates in the right position. Confirm with 'START' after each loading step; the instrument will then provide the next free loading position automatically.
- When all plates are loaded, press 'START' again to initialize the program.

2.4.7 Real-time PCR

- Run real-time PCR assay according to the instructions of section 2.2 in this kit insert.
- In case of a positive result for *Enterobacteriaceae*, also optionally test for *Salmonella* according to the instructions in the kit insert KIT230070 (foodproof *Salmonella* Detection Kit).
- Interpretation of PCR results using the microproof® Diagnostic Interpreter for the kits KIT230069 (foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit) and KIT230070, according to the user manual for the Diagnostic Interpreter.

2.4.8 Confirmation

Positive PCR results have to be culture-confirmed using ISO reference methods for *Cronobacter* (ISO 22964:2017) and/or *Enterobacteriaceae* (ISO 21528-1:2017) and/or *Salmonella* (ISO 6579-1:2017), as applicable.

2.4.9 Repetition of Weak Positive Results, of Invalid Results and of Results with PCR Inhibition*

Weak positive results, samples that show a PCR inhibition and invalid results should be repeated.

1. A weak positive result means:
   - for LightCycler 480 in combination with the microproof Diagnostic Interpreter: a result that indicates "repetition"

2. PCR inhibition means:
   - for LightCycler 480 in combination with the microproof Diagnostic Interpreter: a result that indicates "inhibition"

3. An invalid result means:
   - for LightCycler 480 in combination with the microproof Diagnostic Interpreter: a result that indicates "invalid"

*weak positive result = high Cp/Ct-value due to low amount of initial target DNA.

How to proceed with samples showing “repetition”/”inhibition”:

- Transfer 50 µL of the second BPW enrichment broth into 450 µL of pre-warmed BPW to perform a third BPW enrichment.
  - or- Transfer 100 µL of the second BPW enrichment broth into 900 µL of pre-warmed BPW to perform a third BPW enrichment at 900 U/min.
- Incubate at 37 ± 1°C for 3 h minimum and 16-18 h maximum.
- Prepare a new Reagent D treatment and DNA extraction from the third BPW enrichment (preferably with fresh reagents) and perform a new real-time PCR.

How to proceed with samples showing “repetition” twice:

- If not already done, incubate the third BPW enrichment for a total of 16-18 h at 37 ± 1°C
- Prepare a new DNA extraction from this third BPW enrichment and perform a new real-time PCR.

If this sample also shows a “repetition”, the sample has to be considered as slightly positive.

How to proceed with samples showing “invalid” results:

- Repeat the real-time PCR with the DNA extract obtained from the second BPW enrichment broth.

2.5 MicroVal Protocol including the Manual DNA Extraction with the foodproof StarPrep One Kit

Protocol for the manual DNA isolation and real-time PCR detection of *Enterobacteriaceae / Cronobacter* and *Salmonella* spp. in infant formula and infant cereals, probiotic containing products, ingredients and environmental samples with the foodproof StarPrep One Kit.

2.5.1 Introduction

The procedure for the detection of *Enterobacteriaceae*, *Cronobacter* and *Salmonella* spp. consists of five consecutive steps:

1. Primary enrichment
2. Secondary enrichment
3. Reagent D treatment
4. DNA extraction
5. Real-time PCR

2.5.2 Material and Methods

Instruments

- High-power halogen light bulk or foodproof D-Light (Product No. MCH230039)
- Cooling block for 1.5 mL/ 2.0 mL tubes
- Centrifuge for 1.5 mL/ 2.0 mL tubes
- Real-time PCR instrument (LightCycler 480-II, LightCycler 2.0)

Reagents

- foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit (Product No. KIT230068)
- If applicable: foodproof *Salmonella* Detection Kit (Product No. KIT230070)
- Reagent D (Product No. KIT230001)
- foodproof StarPrep One Kit (Product No. KIT230175)
- Buffered peptone water (BPW)
- Depending on the matrix: double strength BPW, vancomycin, alpha-amylase

2.5.3 Primary Enrichment

- Enrichment in pre-warmed (37 °C) BPW for **18 ± 2 h at 37 °C**

Table 1 Category: Infant Formula and Infant Cereals.

Sample Type	Sample Preparation
Infant formula (intended for infants < 1 year)	100 g in 900 mL BPW
Infant formula (intended for infants > 1 year)	100 g in 900 mL BPW
Infant cereals	100 g in 900 mL BPW plus alpha-amylase (alpha-amylase at 50 mg per 100 g sample in 900 mL BPW for products with high starch content)

Table 2 Category: Probiotics Containing Products.

Sample Type	Sample Preparation
Probiotic infant formula (*L. paracasei, L. rhamnosis, L. reuteri*) at a level < 10^8 CFU/g (consumer products)	100 g in 900 mL BPW
Probiotic infant formula (*L. johnsonii, S. thermophilus, B. lactis, B. longum*) at a level < 10^8 CFU/g (consumer products)	100 g in 900 mL BPW plus vancomycin (vancomycin at 10 mg/L)
Probiotic infant cereals (*Bifidus* bacteria) at a level < 10^8 CFU/g (consumer products)	100 g in 900 mL BPW plus vancomycin (vancomycin at 10 mg/L) alpha-amylase at 50 mg per 100 g sample in 900 mL BPW for products with high starch content
Probiotic ingredients containing *L. reuteri* at ~10^{10} CFU/g	100 g in 900 mL double strength BPW
Probiotic ingredients containing *L. rhamnosis* and/or *B. longum* at ~10^{10} CFU/g	100 g in 900 mL double strength BPW plus vancomycin (vancomycin at 10 mg/L)

Table 3 Category: Ingredients.

Sample Type	Sample Preparation
Infant formula ingredients (e.g., milk cow powder, whey cow powder, lactose, maltodextrin)	100 g in 900 mL BPW
Infant cereals ingredients (e.g., starch, oatmeal, rye meal, wheat(flour), buckwheat)	100 g in 900 mL BPW plus alpha-amylase (alpha-amylase at 50 mg per 100 g sample in 900 mL BPW for products with high starch content)
Premix, Duomix (containing minerals, vitamins)	12.5 g in 900 BPW

Table 4 Category: Environmental Samples.

Sample Type	Sample preparation
Sweep samples/equipment swabs	Submerge swab/sponge in 90 mL BPW
Trajectory samples (in-line factory)	100 g in 900 mL BPW
Vacuum cleaner residues	100 g in 900 mL BPW

2.5.4 Secondary Enrichment

- Transfer 100 µL of primary enrichment broth in 900 µL of fresh BPW in a deep well plate (round bottom)
- Cover the plate with a breathable foil
- Incubate for 3-4 h at 37 °C with shaking on a thermoshaker (at maximum speed, 900 U/min)

Notes:
Probiotic-containing samples may also be sub-cultivated for 20-24 h.
Alternatively, 50 µL of BPW culture in 450 µL fresh BPW (pre-warmed at 37 °C in Eppendorf tube) can be used for *Salmonella* detection without shaking. In this case, a secondary enrichment for 20-24 h is required for probiotic-containing samples.

2.5.5 Reagent D Treatment

(For detailed description, see Product Instructions for KIT230001)

- Pre-filling of transparent reaction tube with **300 µL Reagent D**
- Transfer of **100 µL of the secondary enrichment culture** into the reaction tube
- Reagent D treatment with a high-power halogen bulb or foodproof D-Light (incubation for 5 min in the dark and for 5 min with light exposure)

2.5.6 DNA Extraction

- Use the foodproof StarPrep One Kit
- According to product instructions for KIT230175-76, follow procedure A starting with 400 µL from the Reagent D treated sample

2.5.7 Real-time PCR

- Real-time PCR according to the instructions in section 2.2 of this kit insert.
- In case of a positive result for *Enterobacteriaceae*, optionally also test for *Salmonella* according to the product instructions for KIT230070.
- Interpretation of PCR results using the microproof Diagnostic Interpreter for kits, KIT230070 and KIT230068, according to the product instructions for the Diagnostic Interpreter.

2.5.8 Confirmation

Positive PCR results have to be culture-confirmed using the ISO reference methods for *Cronobacter* (ISO 22964:2017) and/or *Enterobacteriaceae* (ISO 21528-1:2017) and/or *Salmonella* (ISO 6579-1:2017), as applicable.

2.5.9 Repetition of weak positive results, of invalid results and of results with PCR Inhibition*

Weak positive results, samples that show a PCR inhibition and invalid results should be repeated.

1. Weak positive result means:

- for LightCycler 480 in combination with the microproof Diagnostic Interpreter result that indicates "repetition"

2. PCR inhibition means:

- for LightCycler 480 in combination with the microproof Diagnostic Interpreter: result that indicates "inhibition"

3. Invalid result means:

- for LightCycler 480 in combination with the microproof Diagnostic Interpreter: result that indicates "invalid"

How to proceed with samples showing "repetition"/"inhibition":

- Transfer 50 µL of the second BPW enrichment broth into 450 µL of pre-warmed BPW to perform a third BPW enrichment.
  -or- Transfer 100 µL of the second BPW enrichment broth into 900 µL of pre-warmed BPW to perform a third BPW enrichment at 900 U/min.
- Incubate at 37 ± 1°C for 3 h minimum and 16-18 h maximum.
- Prepare a new DNA extraction from the third BPW enrichment (preferably with fresh reagents) and perform a new real-time PCR.

How to proceed with samples showing "repetition" twice:

- If not done so far incubate the third BPW enrichment for a total of 16-18 h at 37 +/- 1°C
- Prepare a new DNA extraction from this third BPW enrichment and perform a new real-time PCR.

If this sample also shows a "repetition", the sample has to be considered as slightly positive.

*weak positive result = high CP/Ct-value due to low amount of initial target DNA.

How to proceed with samples showing “invalid” results:

- Repeat the real-time PCR with the DNA extract obtained from the second BPW enrichment broth.

3. Troubleshooting

Observation	Possible Reason	Recommendation
No signal increase is observed, even with positive controls.	Incorrect detection channel has been chosen.	• For Carousel-based LightCycler: Set Channel settings to 610, 640 or 670. Fluorescence data is acquired for all channels during the run, regardless of the channel settings. If the incorrect channel is selected, there is NO need to abort and redo the run. • For LC 480: Set channel settings to 610, 640 or 660/670.
	Pipetting errors or omitted reagents.	• Check for correct pipetting scheme and reaction setup. Repeat the PCR run. • Always run a positive control along with your samples.
	No data acquisition programmed.	• Check the cycle programs.
No signal increase in channel 610.	Inhibitory effects of the sample material (e.g., caused by insufficient purification).	• Use the recommended DNA sample preparation kit to purify template DNA. • Dilute samples or pipet a lower amount of sample DNA (e.g., 2.5 µL instead of 5 µL, substitute with H2O PCR-Grade). • Perform a secondary enrichment of the primary enrichment culture (e.g., 1:10 in Buffered Peptone Water) to dilute the portion of food matrix in the sample.
Fluorescence intensity is too low.	Inappropriate storage of kit components.	• Low initial amount of target DNA.
	foodproof *Enterobacteriaceae* plus *Cronobacter* Master Mix (vial 1, yellow cap) or the complete PCR mix is not homogeneously mixed.	• Mix the foodproof *Enterobacteriaceae* plus *Cronobacter* Master Mix (vial 1, yellow cap) and also the entire PCR mix thoroughly before pipetting.
	Low initial amount of target DNA.	• Increase the amount of sample DNA. Depending on the chosen DNA isolation method, inhibitory effects may occur.

Negative control samples are positive.	Carry-over contamination.	• Exchange all critical solutions. • Repeat the complete experiment with fresh aliquots of all reagents. • Always handle samples, kit components and consumables in accordance with commonly accepted practices to prevent carry-over contamination. • Add positive controls after sample and negative control reaction vessels have been sealed. • For Carousel-based LightCycler: Add positive controls after sample capillaries and negative control capillaries have been sealed with stoppers.
Fluorescence intensity varies.	Insufficient centrifugation of the capillaries or plate. For Carousel-based LightCycler: • Prepared PCR mix is still in the upper vessel of the capillary. • Air bubble is trapped in the capillary tip.	• Always centrifuge capillaries or plates (loaded with the reaction mix) as described.
	For Carousel-based LightCycler: • Outer surface of the capillary tip is dirty (e.g., by direct skin contact). For LightCycler 480: • Surface of the sealing foil is dirty (e.g., by direct skin contact).	• Always wear gloves when handling the capillaries or plates.

4. Additional Information on this Product

4.1 How this Product Works

The foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit provides primers and Hybridization Probes (for sequence-specific detection), convenient premixed reagents, and a control template for reliable interpretations of results. To ensure maximum reliability of the detection system and to prevent misinterpretation of negative results due to inhibition of the amplification, an Internal Control (IC) is supplied (vial 3 and vial 6, respectively). The IC has to be added to each reaction. Hybridization Probes were designed to bind specifically the IC, allowing detection in channel 610, whereas the *Enterobacteriaceae* DNA is detected in channel 640 and *Cronobacter* DNA in channel 670 or 660 for LC 480 II, respectively. In case of a negative result due to inhibition of amplification by the sample DNA of interest, the amplification of the IC is suppressed as well. Whereas a negative result for the sample DNA of interest and amplification of the IC clearly indicates the absence of *Enterobacteriaceae/Cronobacter* DNA in the sample. The foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit minimizes contamination risk and contains all reagents (except for template DNA) needed for detection. The detection kit described in this Manual Instruction has been developed for the LightCycler 2.0 Carousel-Based System and the LightCycler 480 Systems.

4.2 Test Principle

1. Using the product's supplied sequence-specific primers in a polymerase chain reaction (PCR), the LightCycler and its associated reagents amplify and simultaneously detect specifically DNA of *Enterobacteriaceae/Cronobacter*.
2. The LightCycler System detects these amplified fragments in real time through fluorescence generated by their corresponding pair of sequence-specific probes. For each amplicon, one probe is labeled at the 5´-end with an acceptor fluorophore and, to avoid extension, is modified at the 3´-end by phosphorylation. The other oligonucleotide probe is labeled at the 3´-end with a donor fluorophore.
3. During the annealing phase of each PCR cycle, these probes hybridize to an internal sequence of the amplicon. Only while hybridized in close proximity to each other do these probes result in fluorescence resonance energy transfer (FRET) between the two fluorophores. During FRET, the light source of the LightCycler excites the donor fluorophore and part of the excitation energy is transferred to the acceptor fluorophore.
4. The LightCycler System measures the emitted fluorescence of the acceptor fluorophore.

4.3 Prevention of Carry-Over Contamination

The heat-labile Uracil-DNA Glycosylase (UNG) is suitable for preventing carry-over contamination between PCRs. This technique relies on the incorporation of deoxyuridine triphosphate (dUTP) during all amplification reactions and the pretreatment of all successive PCR mixtures with the heat-labile UNG. The UNG cleaves DNA at any site where a deoxyuridine residue has been incorporated. The resulting abasic sites are hydrolyzed due to the high temperatures during the initial denaturation step and can no longer serve as PCR templates. The heat-labile UNG is inactivated during the initial denaturation step. Native DNA (e.g., the isolated *Enterobacteriaceae* or *Cronobacter* genomic DNA) does not contain uracil and is therefore not degraded by this procedure. Since dTTP is replaced with dUTP and UNG is included in the foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit, decontamination can be achieved with the provided reagents.

4.4 Product Specifications

Specificity: Inclusivity and Exclusivity of the foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit has been tested with 160 *Cronobacter* spp. strains comprising strains of all genogroups (*Cronobacter sakazakii*, *C. malonaticus, C. turicensis, C. muytjensii, C. dublinensis* and *C.* genomospecies 1) and all 16 biogroups, more than 120 non-*Cronobacter* strains of the family *Enterobacteriaceae* comprising 61 species (including the most closely related *Enterobacter helveticus* and *E. pulveris*) as well as more than 60 non-*Enterobacteriaceae* species (mostly of the closely related genera like *Aeromonas* or *Vibrio)*.

All *Cronobacter* spp. strains were detected in channel 640 and 660/670, all non-*Cronobacter Enterobacteriaceae* in channel 640 and none of the non-*Enterobacteriaceae* strains were detected in any channel.

4.5 References

1. C. Grönewald, M. Kiehne, K. Berghof-Jäger, Hygiene Report 1-2006, 22.

4.6 Quality Control

The foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit is function-tested using the LightCycler Carousel-Based System and the LightCycler 480 System.

5. Supplementary Information

5.1 Ordering Information

Hygiena Diagnostics offers a broad range of reagents and services. For a complete overview and for more information, please visit our website at www.hygiena.com.

5.2 License Notice

The purchase price of this product includes limited, nontransferable rights under US Patent No. 7,687,247 owned by Life Technologies Corporation to use only this amount of the product to practice the claims in said patent solely for activities of the purchaser for bioburden testing, environmental testing, food testing, or testing for genetically modified organisms (GMO) in accordance with the instructions for use accompanying this product. No other rights are conveyed, including no right to use this product for *in vitro* diagnostic, therapeutic, or prophylactic purposes. Further information on purchasing licenses under the above patent may be obtained by contacting the Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, CA 92008.

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5.3 Trademarks

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5.4 Contact and Support

If you have questions or experience problems with this or any other product of Hygiena Diagnostics GmbH, please contact our Technical Support staff (www.hygiena.com/support). Our scientists commit themselves to providing rapid and effective help. We also want you to contact us if you have suggestions for enhancing our product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to us and the worldwide research community.

5.5 Reference Number

The reference number and original Hygiena Diagnostics GmbH article number: R 310 15.1

6. Change Index

Version 1, April 2015
Name change from *Enterobacter sakazakii* to *Cronobacter.*

Version 2, April 2016
Information about large version of the kit with 480 reactions has been added.

Version 3, March 2017
License Notice changed.

Version 4, September 2017
License Notice changed.

Version 5, February 2022
New tables and information regarding the MicroVal validation inserted.

Revision A, January 2024
Rebranding and new layout.
R 310 15.1 20 -> INS-KIT-230068-69-RevA

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