foodproof® *Enterobacteriaceae* plus *Cronobacter* Detection Kit

Revision A, April 2024

PCR kit for the qualitative detection of *Enterobacteriaceae* including the simultaneous identification of *Cronobacter* spp. using real-time PCR instruments

Product No. KIT230043

Kit for 96 reactions for a maximum of 94 samples

Store the kit at -15 to -25 °C

For food testing purposes.

FOR *IN VITRO* USE ONLY

MICROVAL® NEN

Table of Contents

1. What This Product Does

1.1 Number of Tests

The detection system is designed for 96 reactions with a final reaction volume of 25 µL each. Up to 94 samples (single sample preparation) plus positive and negative control reactions can be analyzed per run.

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:

Vial / Cap Color	Label	Contents / Function / Storage
1 yellow cap	foodproof *Enterobacteriaceae* plus *Cronobacter* Master Mix	• 3 x 600 µL • Ready-to-use primer and hydrolysis probe mix specific for • *Enterobacteriaceae* and *Cronobacter* DNA and the specific Internal Control • 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	• 3 x 32 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	• 3 x 32 µL • Contains a stabilized solution of plasmid DNA and a yellow dye for better visualization. • For use as an internal amplification control. • 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	• 1 x 50 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	• 1 x 1 mL • Nuclease-free, PCR-grade H_2O. • For use as a PCR run negative control. • Store at -15 to -25 °C.

1.3 Additional Equipment and Reagents Required

- Color Compensation Set 3 (Product No. KIT230006)
- Standard benchtop microcentrifuge containing a rotor for 2.0 mL reaction tubes.
- Real-time PCR cycler suitable for detection of FAM-, VIC/HEX-, and ROX/Texas Red-labeled probes
- Compatible PCR plates/tubes and sealing foil/caps
- foodproof StarPrep® One Kit (Product No. KIT230175 or KIT230176) or
- foodproof Magnetic Preparation Kit IV (Product No. KIT230185) in combination with the KingFisher® Flex System
- Reagent D (KIT230001 or KIT230002)
- foodproof D-Light Instrument (Product No. MCH230039)
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes
- Sterile reaction tubes for preparing PCR mixes and dilutions

1.4 Applicability Statement

The foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit is intended for the rapid detection of *Enterobacteriaceae* and *Cronobacter* spp. isolated by foodproof DNA extraction methods from enrichment cultures of all relevant kinds of foods that are potentially contaminated with *Cronobacter* or other *Enterobacteriaceae*. 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 is 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 (KIT230175) and Reagent D (KIT230001), according to Section 2.3.2 “MicroVal Protocol Including the Manual DNA Extraction with the foodproof StarPrep One Kit”. Semi-automated DNA extraction was performed using the KingFisher Flex System in combination with the foodproof Magnetic Preparation Kit IV (KIT230184) and Reagent D (KIT230001), according to Section 2.3.1 “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* using the foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit requires DNA amplification by PCR. The detection system provides all the 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.,* pipettors, 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.
- Physically separate the workplaces for DNA preparation, PCR set-up, 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 kinds of foods and raw materials (see “Additional Equipment and Reagents required”). For more product information, please refer to www.hygiena.com. Detailed DNA extraction procedures used for the MicroVal validation are described in sections 2.3.1 and 2.3.2.

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 H_2O PCR-grade (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.

How to perform confirmation procedure of the MicroVal Study No. LR08/09/19/20

For *Enterobacteriaceae*:

The first enrichment in BPW was used for isolation onto 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 onto 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 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 3 (KIT230005). 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 of the respective LightCycler Instrument.

2.2 Procedure

Program PCR instrument before preparing the reaction mixes. The amplification is carried out according to the following temperature-time-program (for details on how to program the experimental protocol, see the operation manual of your real-time PCR cycler):

Pre-incubation	**1** cycle
Step 1:	37°C for 4 minutes
Step 2:	95°C for 5 minutes
Amplification	**40** cycles
Step 1:	95°C for 10 seconds
Step 2*:	65°C for 70 seconds, Step down each cycle by 0.1 °C

* Fluorescence detection in step 2

For some real-time PCR instruments, the type of the probe quencher as well as the usage of a passive reference dye has to be specified. The foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit contains probes with a non-fluorescent (“dark”) quencher and no passive reference dye.

Note: For users of the Agilent Mx3005P instrument: Click 'Instrument → Filter Set Gain Settings' to open the Filter Set Gain Settings dialog box in which the gain settings may be viewed and modified. For FAM, the Filter Set Gain Setting has to be modified to 'x1 '.

2.2.1 Preparation of the PCR Mix

Proceed as described below to prepare a 25 µL standard reaction.
Always wear gloves when handling the PCR vessels.

1. Thaw the solutions and, for maximal recovery of contents, briefly spin vials in a microcentrifuge before opening. Mix carefully but thoroughly by pipetting up and down.
2. In a reaction tube (0.5 – 2.0 mL depending on the number of samples), prepare the PCR mix by adding the following components in the order mentioned below.

The volumes indicated below are based on a single 25 µL standard reaction. Prepare the PCR mix by multiplying the amount in the "Volume" column by the number of reactions 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)	18.0 µL
foodproof® *Enterobacteriaceae* plus *Cronobacter* Enzyme Solution, (vial 2, red cap)	1.0 µL
foodproof® *Enterobacteriaceae* plus *Cronobacter* Internal Control (vial 3, white cap)	1.0 µL
Total volume	**20.0 µL**

3. Reaction mixture preparation
   - Mix carefully but thoroughly by pipetting up and down. Do not vortex.
   - Pipet 20 µL PCR mix into each PCR vessel.
   - For the samples of interest, add 5 µL sample DNA.
   - For the negative control, add 5 µL H_2O PCR-grade (vial 5, colorless cap).
   - For the positive control, add 5 µL foodproof® *Enterobacteriaceae* plus *Cronobacter* Control Template (vial 4, purple cap).
4. Seal the PCR vessels accurately with optical caps or foil.
5. Briefly spin the PCR vessels in a suitable centrifuge.
6. Cycle the samples as described above.

2.3 Data Interpretation

The amplification of *Cronobacter* DNA is analyzed in the fluorescence channel suitable for FAM-labeled probe detection. The amplification of *Enterobacteriaceae* DNA is analyzed in the fluorescence channel suitable for VIC/HEX-labeled probes detection. The specific amplification of the Internal Control is analyzed in the fluorescence channel suitable for ROX/Texas Red.

Compare the results from channel FAM (*Cronobacter*), channel VIC/HEX (*Enterobacteriaceae*) and channel ROX/Texas Red (Internal Control) for each sample, and interpret the results as described in the table below:

Cronobacter Channel FAM	*Enterobacteriaceae* Channel VIC/HEX	Internal Control Channel ROX/Texas Red	Result Interpretation
Positive	Positive	Positive OR Negative	Positive for *Enterobacteriaceae* AND *Cronobacter*
Negative	Positive	Positive OR Negative	Positive for *Enterobacteriaceae*, negative for *Cronobacter*
Negative	Negative	Positive	Negative for *Enterobacteriaceae* AND *Cronobacter*
Negative	Negative	Negative	Invalid result

Note: A prerequisite for the unambiguous discrimination of *Enterobacteriaceae* and *Cronobacter* DNA and Internal Control DNA in this tri-color experiment is a suitable calibration of the PCR instrument for channels FAM, VIC/HEX and ROX/Texas Red. Please refer to the operation manual of your real-time PCR cycler for further information.

2.3.1 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

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

Material and Methods

Instruments

- KingFisher Flex System
- foodproof D-Light instrument (for Reagent D treatment); MCH230039
- Real-time PCR instrument (LightCycler 480 II, Mx3005 P, iQ5, ABI 7500)
- Thermoshaker with adapter for 96 DWP (deep well plate, round bottom)

Reagents

- foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit, KIT230043

  (If applicable: foodproof *Salmonella* Detection Kit, KIT230049)
- Reagent D, KIT230001
- foodproof Magnetic Preparation Kit IV, 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 Deep Well plate (Reagent D treatment)

- 96 Round-bottom deep well plates, 2.0 mL
- Breathable viscose foil for biological cultures, sterile
- Reservoirs for reagents

Primary Enrichment

- For primary enrichment, combine sample with pre-warmed (37 °C) BPW according to appropriate sample preparation listed in Tables 1-4. Incubate at 37 °C for 18 ± 2 h.

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

Secondary Enrichment

- Transfer 100 µL of 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* in combination with the foodproof Magnetic Preparation Kit IV, a sub-cultivation of 20-24 h is required for probiotic-containing products.

Reagent D Treatment

- Prefill the Binding Plate with **300 µL Reagent D.**
- Transfer of **100 µL of the subculture** 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.

DNA Extraction

(Please also see kit instructions: KIT230184)

Note: Prepare the required kit components according to the ready reference guide for 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.)
- Prefill 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 **Binding Plate** containing 400 µL of the 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 instrument's display and load the prefilled 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.

Real-time PCR

- Perform real-time PCR 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: KIT230049.

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.

Results Interpretation:

Repetition of Weak Positive Results*, Invalid Results and 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 "reptition"
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 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 completed 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.

How to proceed with samples showing "invalid" results:

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

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

2.3.2 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.

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

Material and Methods

Instruments

- High power halogen light bulk or foodproof D-Light (Product No. MCH230009)
- 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, Mx3005 P, iQ5, ABI 7500)

Reagents

- foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit; KIT230043
  (If applicable: foodproof *Salmonella* Detection Kit; KIT230049)
- Reagent D; KIT230001
- foodproof StarPrep One Kit; KIT230175
- Buffered peptone water (BPW)
- Depending on the matrix: double strength BPW, vancomycin, alpha-amylase

Enrichment

- For primary enrichment, combine sample with pre-warmed (37 °C) BPW according to appropriate sample preparation listed in Tables 1-4. Incubate at 37 °C for 18 ± 2 h.

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: Probiotic-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

Secondary Enrichment

- Transfer 100 µL of 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 of 20-24 h is required for Probiotic-containing samples.

Reagent D Treatment

(For detailed description, see kit instructions: KIT230001)

- Prefill transparent reaction tube with **300 µL Reagent D.**
- Transfer **100 µL of the subculture** into the reaction tube.
- Perform Reagent D treatment with high-power halogen bulb or foodproof D-Light (incubation for 5 min in the dark and for 5 min with light exposure).

DNA Extraction

- Perform DNA extraction with the foodproof StarPrep One Kit (KIT230175) according to the product instructions, Procedure A, starting with 400 µL from the Reagent D treated sample.

Real-time PCR

- Perform 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 instructions in the kit instructions (KIT230049).

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.

Results Interpretation: Repetition of Weak Positive Results*, Invalid Results and 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 "reptition"
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"

   Note: The microproof Diagnostic Interpreter is only available for the *Salmonella* Detection Kit (Kit 230049) in combination with the LightCycler 480. For the *Enterobacteriaceae* plus *Cronobacter* Detection Kit and for other cyclers, amplification curves have to be analyzed manually.

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 completed 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.

How to proceed with samples showing "invalid" results:

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

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

3. Troubleshooting

Observation	Possible Reason	Recommendation
No signal increase is observed, even with positive controls.	Incorrect detection channel has been chosen.	• Set Channel settings to FAM, VIC/HEX or ROX/Texas Red.
	Pipetting errors or omitted reagents.	• Check for correct pipetting scheme and reaction set-up. 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 ROX/Texas Red is observed.	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 H_2O PCR-Grade). • Perform a sub-cultivation of the 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.	• Store the foodproof *Enterobacteriaceae plus Cronobacter Master* Mix (vial 1, yellow cap) at -15 to -25 °C, protected from light. • Avoid repeated freezing and thawing.
	foodproof *Enterobacteriaceae* plus *Cronobacter* Master Mix (vial 1, yellow cap) is not homogeneously mixed.	• Mix the foodproof *Enterobacteriaceae* plus *Cronobacter* Master Mix (vial 1, yellow cap) and 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.
Fluorescence intensity varies.	Insufficient centrifugation of the PCR vessels. Prepared PCR mix is still in the upper part of the vessel.	• Always centrifuge reaction vessels.
	Outer surface of the vessel or the seal is dirty (e.g., by direct skin contact).	• Always wear gloves when handling the vessels and seal.

4. Additional Information on this Product

4.1 How this Product Works

The foodproof® *Enterobacteriaceae* plus *Cronobacter* Detection Kit provides primers and hydrolysis 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, white cap). The IC has to be added to each reaction. A hydrolysis probe was designed to bind specifically to the IC, allowing detection in the ROX/Texas Red channel, whereas the *Cronobacter* spp. DNA is detected in the FAM channel and the *Enterobacteriaceae* DNA is detected in the VIC/HEX channel. 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. In contrast, a negative result for the sample DNA of interest and amplification of the IC clearly indicates the absence of *Enterobacteriaceae* and *Cronobacter* DNA in the sample. The foodproof® *Enterobacteriaceae* plus *Cronobacter* Detection Kit minimizes contamination risk and contains all reagents needed for detection of *Enterobacteriaceae* plus *Cronobacter* spp. DNA. Primers and probes provide specific detection of *Enterobacteriaceae* plus *Cronobacter* DNA in food samples. The described performance of the kit is guaranteed for use on the real-time PCR instruments listed above only.

4.2 Test Principle

1. Using the supplied sequence-specific primers in a polymerase chain reaction (PCR), the PCR instrument and its associated reagents amplify and simultaneously detect fragments of *Enterobacteriaceae* plus *Cronobacter* spp. genomic DNA.
2. The PCR instrument detects these amplified fragments in real time through fluorescence generated by cleavage of the hybridized probe due to the 5´-nuclease activity of the Taq DNA polymerase. The probe is labeled at the 5´-end with a reporter fluorophore and at the 3´-end with a quencher.
3. During the annealing/elongation phase of each PCR cycle, the probe hybridizes to an internal sequence of the amplicon downstream from one of the primer sites and is cleaved by the 5' nuclease activity of the Taq DNA polymerase. This cleavage of the probe separates the reporter dye from the quencher dye, increasing the reporter dye signal.
4. The real-time PCR instrument measures the emitted fluorescence of the reporter dye.

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 of the foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit has been tested with 121 *Cronobacter* strains whereas all of them could be detected. Exclusivity for *Cronobacter* was determined using more than 120 non-*Cronobacter* strains (comprising 61 species) and for *Enterobacteriaceae* using more than 60 non-*Enterobacteriaceae* species (mostly of the closely related genera like *Aeromonas* or *Vibrio*). All *Cronobacter* strains were detected in channel FAM and VIC/HEX, all non-*Cronobacter Enterobacteriaceae* in channel VIC/HEX and none of the non-*Enterobacteriaceae* strains were detected in any channel.

Sensitivity: A relative detection limit of 1 to 10 cells per 25/100 g sample can be achieved with all relevant kinds of foods. The foodproof® *Enterobacteriaceae* plus *Cronobacter* Detection System detects down to 10^3 - 10^4 CFU/mL of *Enterobacteriaceae/Cronobacter* cultures after enrichment.

5. References

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

6. Quality Control

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

7. Supplementary Information

7.1 Ordering Information

In addition to this foodproof *Enterobacteriaceae* plus *Cronobacter* Detection Kit, 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 and contact us via email or phone.

7.2 License

License Notice

The purchase price of this product includes limited, nontransferable rights under U.S. 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. Email: outlicensing@lifetech.com.

7.3 Trademarks

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7.4 Contact and Support

If you have questions or experience problems with this or any other product of Hygiena Diagnostics, please contact our Technical Support staff. Our scientists are committed 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.

7.5 Reference Number

The reference number and original Hygiena Diagnostics GmbH article number: R 302 15.1

8. Change Index

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

Version 2, March 2017
License Notice changed.

Version 3, February 2022
New tables and information regarding the MicroVal validation inserted.

Revision A, April 2024:
Rebranding and new layout. Change of company name and product number. Change document tracking number R 302 15.1 20 → INS-KIT230043-RevA.

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