For food testing purposes
FOR *IN VITRO* USE ONLY

foodproof® *Enterobacteriaceae* plus *Cronobacter* Detection Kit

- 5'Nuclease -

Version 2, March 2017

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

Order No. R 302 15.1

PCR system for 96 reactions for a maximum of 94 samples

Order No. R 302 15.1L

PCR system for 480 reactions for a maximum of 470 samples

Store the PCR kit at -15 to -25 °C

MICROVAL
European validation and certification organisation

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1. What this Product Does

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.

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:

Kit Contents

Vial / Cap Color	Label	Contents / Function / Storage
1 yellow cap	**food**proof® *Enterobacteriaceae* plus *Cronobacter* Master Mix	• R 302 15: 3 x 600 µl • R 302 15L: 5 x 1.500 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* plus *Cronobacter*-specific sequences. • Store at – 15 to – 25 °C. • Avoid repeated freezing and thawing! • Protect from light!
2 red cap	**food**proof® *Enterobacteriaceae* plus *Cronobacter* Enzyme Solution	• R 302 15: 3 x 32 µl • R 302 15L: 5x 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	**food**proof® *Enterobacteriaceae* plus *Cronobacter* Internal Control	• R 30 15: 3 x 32 µl • R 302 15L: 5x 96 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 °C to +8 °C for up to one month.
4 purple cap	**food**proof® *Enterobacteriaceae* plus *Cronobacter* Control Template	• R 302 15: 1 x 50 µl • R 302 15L: 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 °C to +8 °C for up to one month.
5 colourless cap	H_2O PCR-grade	• R 302 15 and R 302 15L: 1 x 1 ml • Nuclease-free, PCR-grade H_2O. • For use as a PCR run negative control. • Store at -15 to -25 °C.

Additional Equipment and Reagents Required
- real-time PCR cycler suitable for detection of FAM-, VIC/HEX-, and ROX/Texas Red-labeled probes
- real-time PCR compatible tubes, strips or plates with optical cap or foil applicable for the PCR-cycler in use
- **food**proof StarPrep One Kit (Order No. S 400 07)*
- **food**proof Magnetic Preparation Kit IV (Order No. S 400 15)*
- Reagent D (optional, for removal of DNA from dead bacteria; Order No. A 500 02)*
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes
- Sterile reaction tubes for preparing PCR mixes and dilutions

Applicability Statement
The **food**proof® *Enterobacteriaceae* plus *Cronobacter* Detection Kit is intended for the rapid detection of *Enterobacteriaceae* including simultaneous identification of *Cronobacter* spp. DNA isolated from enrichment cultures prepared by valid methods and inoculated with all kinds of foods that are potentially contaminated with *Enterobacteriaceae* or *Cronobacter*, respectively.
The kit is based on the **food**proof® *Enterobacteriaceae* plus *Cronobacter* Detection System - Hybridization Probes (LightCycler® 2.0, 480).

The detection system must not be used in diagnostic procedures.

The performance of the kit described in this Instruction Manual is guaranteed only when it is used with real-time PCR instruments suitable for detection of FAM-, VIC/HEX-, and ROX/Texas Red-labeled probes, e.g.: LightCycler® 480 (Roche Diagnostics), ABI 7500 (Applied Biosystems), iCycler iQ5 (BioRad), Mx3000/Mx3005 (Stratagene) or Rotorgene 6000 (Corbett Life Science).

Note:
A Color Compensation (Color Compensation Set 3; Order No. A 500 10)* is necessary and will be supplied by BIOTECON Diagnostics for users of the LC 480 Systems I and II. Please contact BIOTECON Diagnostics for further information.

* Available from BIOTECON Diagnostics; see Ordering Information

2. How to Use this Product

2.1 Before You Begin

Precautions

Detection of *Enterobacteriaceae* and *Cronobacter* DNA using the **food**proof® *Enterobacteriaceae* plus *Cronobacter* Detection Kit requires DNA amplification by PCR. The detection kit provides all the reagents required for the PCR. However, 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.
- 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.**

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.

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”).

DNA-Extraction

BIOTECON Diagnostics provides sample preparation kits for all kind of foods and raw materials (see “Additional Equipment and Reagents Required”). For more product information please refer to www.bc-diagnostics.com.

Positive Control
Always run a positive control with the samples. To prepare a positive control, replace the template DNA with the provided control DNA [**food**proof® *Enterobacteriaceae* plus *Cronobacter* Control Template (vial 4, purple cap)] or with a positive sample preparation control.

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.

2.2 Procedure

Program Setup

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**	

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 **food**proof® *Enterobacteriaceaea* 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".

* Fluorescence detection in step 2

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. • 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 **food**proof® *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 DNA of *Cronobacter* is analyzed in the fluorescence channel suitable for FAM labeled probes detection. The amplification of DNA of *Enterobacteriaceae* 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.

Channel FAM	Channel VIC/HEX	Channel ROX/Texas Red	sult Interpretation
positive	positive	positive or negative	positive for *Enterobacteriaceae* and *Cronobacter*
negative	positive	positive or negative	positive for *Enterobacteriaceae* (non *Cronobacter*)
negative	negative	positive	negative for *Enterobacteriaceae* or *Cronobacter*
negative	negative	negative	invalid

Note: A prerequisite for the unambignuous discrimination of *Enterobacteriaceae* and *Cronobacter* DNA and Internal Control DNA in this dual-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.

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 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 ROX/Texas Red.	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 **food**proof® *Enterobacteriaceae* plus *Cronobacter* Master Mix (vial 1, yellow cap) at -15 °C 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 **food**proof® *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.
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 seal is dirty (*e.g.,* by direct skin contact).	Always wear gloves when handling the vessel and seal.

4. Additional Information on this Product

How this Product Works

The **food**proof® *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. Whereas 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 **food**proof® *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.

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.

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 **food**proof® *Enterobacteriaceae* plus *Cronobacter* Detection Kit, decontamination can be achieved with the provided reagents.

Product Specifications

Specificity: Inclusivity of the **food**proof® *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 **food**proof® *Enterobacteriaceae* plus *Cronobacter* Detection System detects down to 10^3 - 10^4 cfu/ml of *Enterobacteriaceae/Cronobacter* cultures after enrichment.

References

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

Quality Control

The **food**proof® *Enterobacteriaceae* plus *Cronobacter* Detection Kit is function tested using the LightCycler® 480 System.

5. Supplementary Information

5.1 Ordering Information

In addition to this **food**proof® *Enterobacteriaceae* plus *Cronobacter* Detection Kit BIOTECON Diagnostics is offering a broad range of reagents and services. For a complete overview and for more information, please visit our website at www.bc-diagnostics.com and contact us via email or phone.

5.2 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.

5.3 Trademarks
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Other brand or product names are trademarks of their respective holders.

5.4 Contact and Support
If you have questions or experience problems with this or any other product of BIOTECON Diagnostics, please contact our Technical Support staff (for details see www.bc-diagnostics.com). 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.

6. Change Index
Version 1, April 2015:
Name change from *E. sakazakii* to *Cronobacter.*

Version 2, March 2017
License Notice changed.

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