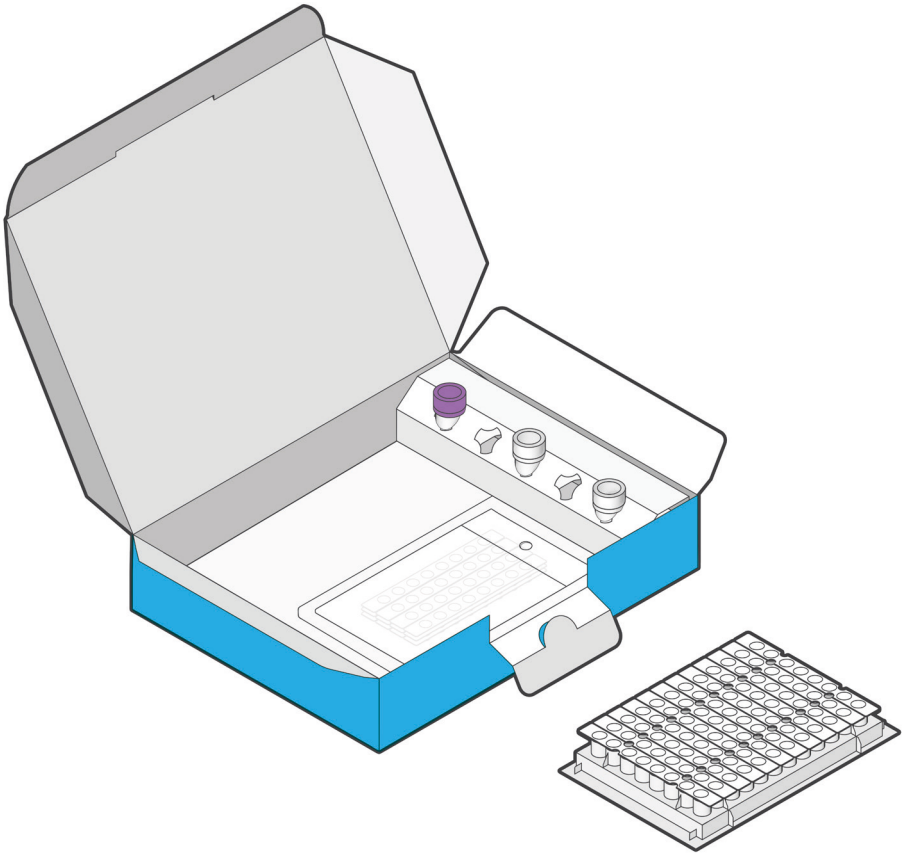

foodproof®

Vibrio Detection LyoKit

MANUAL

Documentation for the qualitative detection of *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio cholerae* and their associated toxin genes *tdh*, *trh1*, *trh2* and *ctx*

Order No. KIT 2301 17 / KIT 2301 18

foodproof®

Vibrio
Screening LyoKit

Order No.

LP: KIT 2301 17

RP: KIT 2301 18

Kit for 96 reactions (lyophilized)
for a maximum of 94 samples

Store kit at 2 °C to 8 °C

For testing of food
and environmental samples

Approval:

PERFORMANCE TESTED
AOAC
RESEARCH INSTITUTE
LICENSE NUMBER 061901

Manual:

Version 3, February 2022

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1. OVERVIEW

1.1 General Information

Number of Reactions

The kit is designed for 96 reactions with a final reaction volume of 25 µl each. Up to 94 samples plus positive and negative control can be analyzed per run.

Storage and Stability

Store all components at 2 °C to 8 °C. They are guaranteed to be stable through the expiration date printed on the label. Opening of the kit does not shorten the expiration date.
The PCR strips must be stored in the provided aluminum bag. Protect from light and moisture.

LyoKit Tube Profiles

The LyoKit is available in three different tube profiles: white low profile tubes (LP), clear regular profile tubes (RP), and clear low profile tubes (DP).
The majority of real-time PCR cyclers use low profile tubes (LP). For the Dualo 32® R² and a few other cyclers, please use clear low profile tubes (DP). For a detailed overview, please have a look at our compatibility chart.

1.2 Applicability

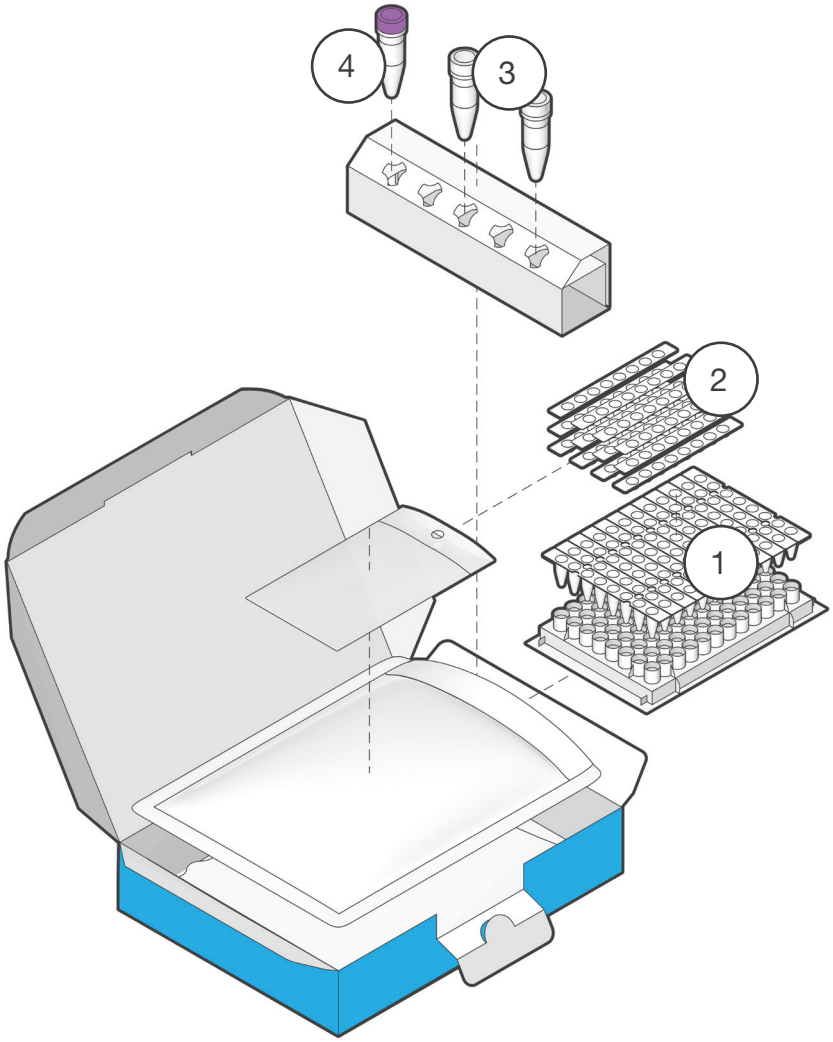
The **food**proof® *Vibrio* Detection LyoKit is intended for the qualitative detection of the three major human pathogenic *Vibrio* species *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio cholerae*, isolated from enrichment cultures prepared by valid methods with all relevant kinds of samples that are potentially contaminated with these microorganisms, e.g. seafood. In addition, presence of the pathogenicity associated toxin genes *thermostable direct hemolysin* (*tdh*), *tdh-related hemolysin* (*trh1* and *trh2*) and *cholera toxin* (*ctx*) in isolates may be qualitatively assessed by melting curve analysis. To be used in the analytical laboratory for food, bioburden and environmental testing applications. The kit must not be used in diagnostic procedures. The kit described in this instruction manual has been developed for real-time PCR instruments with a FAM, a HEX, a ROX and a Cy5 detection channel, and capable of performing a melting curve analysis.

The performance of the kit was tested with the following real-time PCR instruments: LightCycler® 480, LightCycler® 96 (Roche Diagnostics), Mx3005P®, AriaMx (Agilent Technologies), Applied Biosystems™ 7500 Fast (Thermo Scientific), CFX96™ (Bio-Rad), and others.

1.3 Kit Contents

A schematic representation of the **food**proof® *Vibrio* Detection LyoKit with all its components.

LP: KIT 2301 17
RP: KIT 2301 18



	Component	Details
1	Microplate	12 x 8-tube strips, prefilled with lyophilized ready-to-use PCR mix. Available are different tube profiles: white low profile tubes (LP), and clear regular profile tubes (RP).*
2	12 x 8-cap strips	For use in real-time PCR after addition of samples.
3	2 x H_2O PCR-grade (colorless cap)	1 ml nuclease-free, for use as a PCR run negative control.
4	Control Template (purple cap)	350 µl, contains a stabilized solution of DNA for use as a PCR run positive control.

* Tube profile and instrument compatibility chart is available online: www.bc-diagnostics.com/foodproof-compatibility-chart

2. INSTRUCTIONS

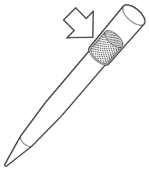
2.1 Required Material

Most of the required equipment and reagents are available through Hygiena™.
Please contact us for further information.

Use a real-time PCR cycler suitable for detection of respective probes as well as for using low or regular profile strip tubes.
In case the strip tubes don´t fit for the instrument, the samples should be transferred to appropriate PCR vessels after resuspension of the lyophilized PCR mix.

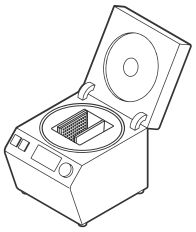
Material

☐ Nuclease-free, aerosol-resistant pipette **filter tips**.



☐ **PCR strip / plate centrifuges**

- *Without vortex: Mini microcentrifuge for 4 x 8-strips*
- *With vortex: Multispin MSC-6000 for 4 x 8-strips*
- *With vortex: CVP-2 for 12 x 8-strips and plates*



2.2 Precautions and Preparations

The kit provides all 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:

- ☐ Keep the kit components 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 barrier 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.
- ☐ **Sample Material**: Use any sample material suitable for PCR in terms of purity, concentration, and absence of inhibitors.
- ☐ **DNA Extraction**: We provide sample preparation kits suitable for all kind of food samples and primary production stage samples.
- ☐ **Positive Control**: Always run a positive control with the samples. Use the provided control DNA (Control Template) or 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 PCR-grade water. 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.
- ☐ **Confirmation**: If required, positive results may be confirmed by appropriate methods (e.g., reference method).
- ☐ **Waste Disposal:** All contaminated and potentially infectious material, like enrichment cultures or food samples, should be autoclaved before disposal and eliminated according to local rules and regulations. For more information, e.g., proper disposal of unused chemicals, please refer to the appropriate safety data sheet (SDS).

Keep the PCR mix away from light and moisture.

For more information, please refer to the appropriate safety data sheet (SDS). The SDS is available online at www.bc-diagnostics.com.

2.3 Enrichment and DNA extraction

The enrichment can be done for 16 to 18 hours in media according to e.g. ISO/TS 21872-1:2017 (Alkaline Saline Peptone Water, ASPW) or FDA BAM Chapter 9 (Alkaline Peptone Water, APW).

2.3.1 Certified Methods

The **food**proof® *Vibrio* Detection LyoKit was validated according the AOAC RI *Performance Tested Methods*℠ program (license number 061901). 25 g samples were enriched in APW for 16 to 18 hours at 36 ± 1 °C. For raw shrimps, 6 to 8 hours can also be used. DNA extraction was performed using **food**proof® StarPrep Three Kit and Reagent D, according to the respective manual.

For the AOAC RI validation the performance of the kit was tested with the following real-time PCR instruments: LightCycler® 480, LightCycler® 96 (Roche Diagnostics), AriaMx (Agilent Technologies), Applied Biosystems™ 7500 Fast (Thermo Scientific) and CFX96™ (Bio-Rad).

2.4 Procedure

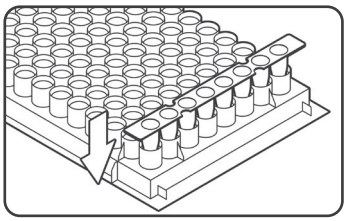
This protocol describes how to perform the analysis of DNA extracts by real-time PCR.

2.4.1 Workflow

1. PLACE STRIPS IN RACK

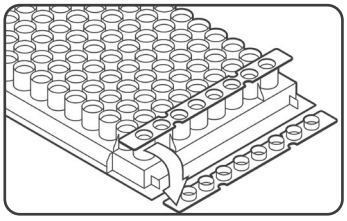
Take needed number of PCR tube strips out of aluminum bag.
Important: close bag tightly afterwards. Place strips in a suitable PCR tube rack.

If needed, gently tap the tubes to move the lyophilized pellets to the bottom of all tubes.



2. DECAP

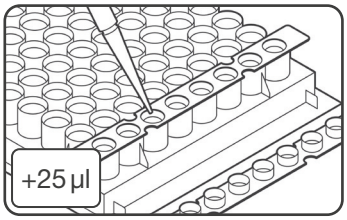
Open strips carefully direct before filling and discard caps.
Important: do not leave open longer than necessary.



3. ADD SAMPLES AND CONTROLS

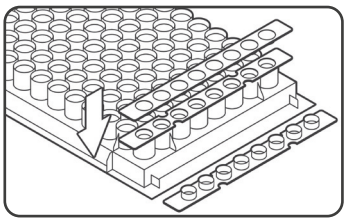
Pipette 25 µl of samples, negative control (colorless cap) or Control Template (purple cap) into respective wells.

If using less volume, add PCR-grade H_2O to reach 25 µl.
To reduce the risk of cross-contamination, prepare only one strip at a time.



4. SEAL

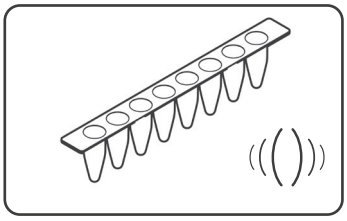
Seal the tubes with the provided 8-cap strips tightly.



5. MIX

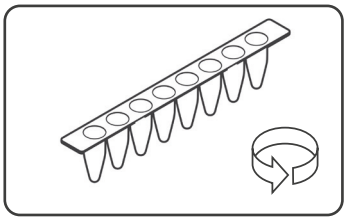
Resuspend pellet after sealing by mixing thoroughly.

Alternatively, resuspend pellet by pipetting up and down multiple times in step 3.



6. CENTRIFUGE

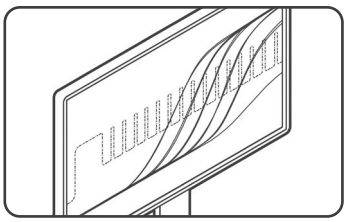
Briefly spin strips, e.g., 5 sec at 500 - 1,000 x g, in a suitable centrifuge.



7. START REAL-TIME PCR RUN

Cycle samples as described in the program setup (2.4.2).

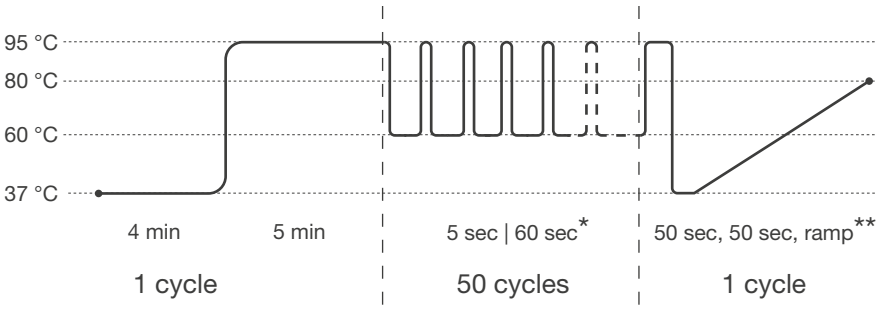
Place tubes in a vertical, balanced order into the cycler, e.g., two strips can be placed in the first and last column.



2.4.2 Program Setup

Program your real-time PCR instrument before setting up the PCR reactions. Select the following channels:

▶ FAM (*V. parahaemolyticus, tdh*), HEX (*V. vulnificus, trh1* and *trh2*), ROX (*V. cholerae, ctx*), and Cy5 (Internal Control).



Pre-incubation: 1 cycle
Step 1: 37 °C for 4 min
Step 2: 95 °C for 5 min

Amplification: 50 cycles
Step 1 : 95 °C for 5 sec
Step 2*: 60 °C for 60 sec

Melting Curve: 1 cycle
Step 1 : 95 °C for 50 sec
Step 2 : 37 °C for 50 sec
Step 3**: ramp up to 65 °C

* Fluorescence detection ** Fluorescence detection during 37 - 65 °C ramp with 2 - 4 measurements/°C

For some real-time PCR instruments the probe quencher as well as the usage of a passive reference dye has to be specified. This kit contains probes with a non-fluorescent “dark” quencher and no passive reference dye.
A Color Compensation is necessary for users of the LightCycler® 480 System: Color Compensation Set 3 (LC 480 I) or 5 (LC 480 II).
Mx3005P instrument: Choose Experiment Type “SYBR® Green (with Dissociation Curve)” and add HEX, ROX, and CY5 channels for data collection in the setup section. Click “Instrument” and “Filter Set Gain Settings” to open the Filter Set Gain Settings dialog box. For FAM and HEX the Filter Set Gain Setting must be modified to “x4”. For ROX and Cy5 the Filter Set Gain Setting must be modified to “x1”.

2.4.3 Data Interpretation

Verify results of positive (Control Template) and negative controls (H_2O), before interpreting sample results. Always compare samples to positive and negative control. Review data from each channel and interpret results as described in the tables.

Amplification curves

FAM	HEX	ROX	Cy5	Result Interpretation
+	+	+	+ or -	Positive for *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*
-	+	+	+ or -	Positive for *V. vulnificus* and *V. cholerae*
+	-	+	+ or -	Positive for *V. parahaemolyticus* and *V. cholerae*
+	+	-	+ or -	Positive for *V. parahaemolyticus* and *V. vulnificus*
-	+	-	+ or -	Positive for *V. vulnificus*
+	-	-	+ or -	Positive for *V. parahaemolyticus*
-	-	+	+ or -	Positive for *V. cholerae*
-	-	-	+	Negative for *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*
-	-	-	-	Invalid

The Control Template contains all target sequences and usually generates higher fluorescent values than positive samples. This can affect positive/negative calls in automatic analysis of amplification curves by the respective instrument software. Always check results visually for plausibility.

Melting curves

Samples that show a positive amplification signal in the FAM or ROX detection channel can be further differentiated using a melting curve analysis. A prerequisite for the unambiguous detection and discrimination of the *Vibrio* toxin types in this multi-color experiment is a suitable calibration of the PCR instrument for channels FAM, HEX and ROX. Please refer to the Operator's Manual of your real-time PCR cycler for further information.

The following table lists the detectable *Vibrio* toxin types in the respective channels and their expected melting peak temperatures (± 2 °C dependent on the real-time PCR instrument):

***Vibrio* toxin**	**FAM channel – melting peak temperature**	**HEX channel – melting peak temperature**	**ROX channel – melting peak temperature**
tdh	49 °C ± 2 °C	none	none
trh1	none	53.5 °C ± 2 °C	none
trh2	none	47 °C ± 2 °C	none
ctx	none	none	49 °C ± 2 °C
Control Template	49 °C ± 2 °C	53.5 °C ± 2 °C	49 °C ± 2 °C

The melting peak temperature ranges given in the above table mainly reflect the variability between instruments and their respective analysis software. The Control Template contains a mixture of all target sequences except *trh2*. Functionality of *trh2* detection is provided by the *trh1* melting curve. Mind that melt curves in channel HEX are associated with *Vibrio parahaemolyticus* (detected in channel FAM). *tdh*, *trh1* and *trh2* are associated with pathogenic *Vibrio parahaemolyticus*. *ctx* is associated with pathogenic *Vibrio cholerae*. *V. vulnificus* does not contain any of the toxin genes detected with this kit.

Melting peaks can occur at a temperature greater than 60 °C. These are not attributed to one of the target toxin types and can safely be ignored.

The peak height of positive samples may vary according to the initial cell concentration. Melting peaks of positive samples may be absent, if target is detected with an amplification cycle later as 30. If the melting curve is absent at an amplification cycle later as 30, please subcultivate the sample 1:10 in APW at 37 °C for 12 to 18 hours, and repeat DNA extraction and PCR. Note that the presence or absence of specific melting peaks should be checked manually for all positive samples as the peak finding algorithms of the respective PCR instrument software may not detect all relevant maxima of the melting curve. A guarantee for the identification via melting curves cannot be given.

2.5 Troubleshooting

Problem	Possible Cause	Recommendation
Squashed or crooked tubes, or open / dislodged tube lids after run, or the cycler does not open or close properly.	Wrong tube format.	Choose the correct tube format for your cycler. Tube profile and instrument compatibility chart is available online: www.bc-diagnostics.com/compatibility-chart If necessary, the samples can be transferred to appropriate PCR vessels after resuspension of the lyophilized PCR mix.
	Wrong placement of tubes.	Place tubes into the cycler in a vertical and balanced order, as described in the instructions for the PCR instrument.
No signal increase is observed, even with positive controls.	Incorrect detection channel has been chosen.	Set channel settings for respective dyes accordingly.
	Pipetting errors.	Check for correct reaction setup and repeat the PCR run. Always run a positive control along with your samples.
	No data acquisition programmed.	Check the cycle programs.
A sample shows no signals, including the internal control. Positive and negative control have proper signals.	Inhibitory effects of the sample material (e.g., caused by insufficient purification).	Use the recommended DNA extraction kit. Dilute samples or pipette a lower amount of sample DNA (e.g., 20 µl PCR-grade water and 5 µl sample instead of 25 µl sample).
Negative control samples are positive.	Carry-over contamination.	Exchange all critical solutions and reagents for DNA/RNA extraction. Repeat the complete experiment with fresh batches 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 is too low.	Inappropriate storage of kit components.	Store lyophilized PCR mix at 2 °C to 8 °C, protected from light and moisture.
	Low initial amount of target DNA.	If possible, increase the amount of sample DNA. Depending on the chosen DNA isolation method, inhibitory effects may occur.

Troubleshooting continues on the next page

Problem	Possible Cause	Recommendation
Strong decrease of fluorescence baseline.	Resuspension of lyophilized PCR mix not complete.	Always resuspend lyophilized PCR mix thoroughly. Use the recommended vortex centrifuge with the correct settings.
Fluorescence intensity varies or changes abruptly during the run.	Insufficient centrifugation of the PCR strips, e.g., resuspended PCR mix is still in the upper part of the vessel or bubbles trapped in the mix.	Always centrifuge PCR strips. Use the centrifuge models and settings recommended in this manual. Avoid the introduction of air bubbles during pipetting.
	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. Do not mark vessels on the outside of the tubes or directly on top of the reaction mix.
Pellets are difficult to dissolve.	The lyophilized PCR mix started to rehydrate.	Store the lyophilized PCR mix always in the aluminum bag with the silica gel pads. Make sure that the lids are tightly closed. Remove strips from the aluminum bag only shortly before PCR setup. Open strip shortly before filling.

2.6 Support

If you have questions or experience any problems with our products, please contact us:

www.hygiena.com/technical-support-request

Our aim is to provide you with a solution as quickly and effectively as possible. We would also like you to contact us if you have any suggestions for improving the product or in case you would like to use our product for a different application. We highly value your feedback.

3. ADDITIONAL INFORMATION

3.1 Testing Principle

The **food**proof® kit provides all necessary reagents and a control template for reliable interpretations of results. To ensure maximum reliability of the kit and to prevent misinterpretation of negative results due to inhibition of the amplification, an Internal Control (IC) is included. A hydrolysis probe was designed to bind specifically the IC, allowing detection in the respective channel, whereas the target DNA is detected in another channel. In case of a negative result due to inhibition of the 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 parameter in the sample. The real-time PCR kit minimizes contamination risk and contains all reagents (except for template DNA) needed for the detection of target DNA. Primers and probes provide specific detection of target DNA in food and environmental samples, including primary production stage samples. The described performance of the kit is guaranteed for use only on the real-time PCR instruments listed above.

Step-by-Step Procedure

1. Using the kit‘s sequence-specific primers in a polymerase chain reaction (PCR), the PCR instrument and the supplied reagents amplify fragments of specific sequences for target 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 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 PCR instrument measures the emitted fluorescence of the reporter dye.

Prevention of Carry-Over Contamination

The heat-labile Uracil-DNA N-Glycosylase (UNG) is suitable for preventing carry-over contamination between PCR’s. 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 target 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 this kit, decontamination can be achieved with the provided reagents.

3.2 Trademarks

foodproof®, **micro**proof®, **vet**proof®, ShortPrep®, RoboPrep®, and LyoKit® are trademarks of BIOTECON Diagnostics GmbH.
Hygiena™ is a registered trademark of Hygiena.
Other brand or product names are trademarks of their respective holders.

3.3 Reference Number

The reference number and original BIOTECON Diagnostics article numbers:
R 602 44 -1, and R 602 44 -2.

3.4 Change Index

Version 3, February 2022:
Rebranding, new document layout and content, new order number.

Hygiena LLC

CA 93012 Camarillo
USA

www.hygiena.com/technical-support-request

Manufactured by

BIOTECON
Diagnostics GmbH

Hermannswerder 17
14473 Potsdam
Germany

bcd@bc-diagnostics.com
www.bc-diagnostics.com