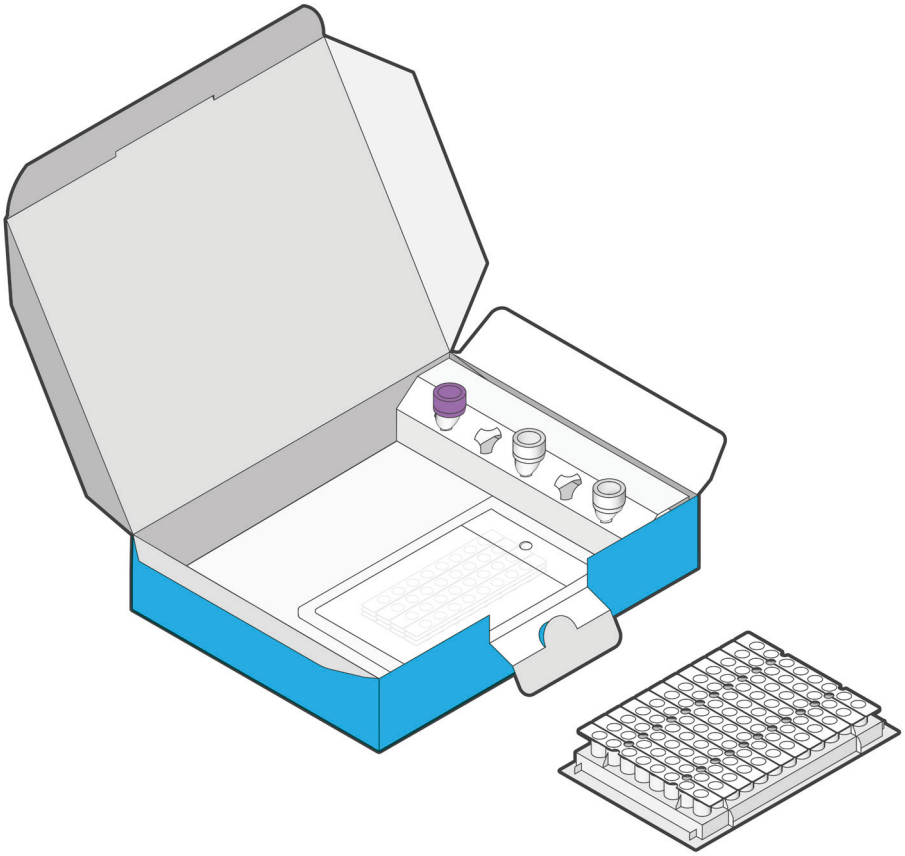


foodproof®

STEC Identification LyoKit

MANUAL

Documentation for the qualitative detection and simultaneous identification of Shiga toxin-producing *Escherichia coli* (STEC) of the serogroups O26, O45, O103, O104, O111, O121, O145 and O157

Order No. KIT 2300 79 / KIT 2300 80

foodproof®

STEC
Identification LyoKit

Order No.

LP: KIT 2300 79

RP: KIT 2300 80

Kit for 48 reactions (lyophilized)
for a maximum of 46 samples

Store kit at 2 °C to 8 °C

For testing of food
and environmental samples

Approval:

PERFORMANCE TESTED
AOAC
RESEARCH INSTITUTE
LICENSE NUMBER 102004

Manual:

Version 5, February 2022

TABLE OF CONTENTS

1. OVERVIEW

1.1 General Information

Number of Reactions

The kit is designed for 48 reactions with a final reaction volume of 25 µl each. Up to 46 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

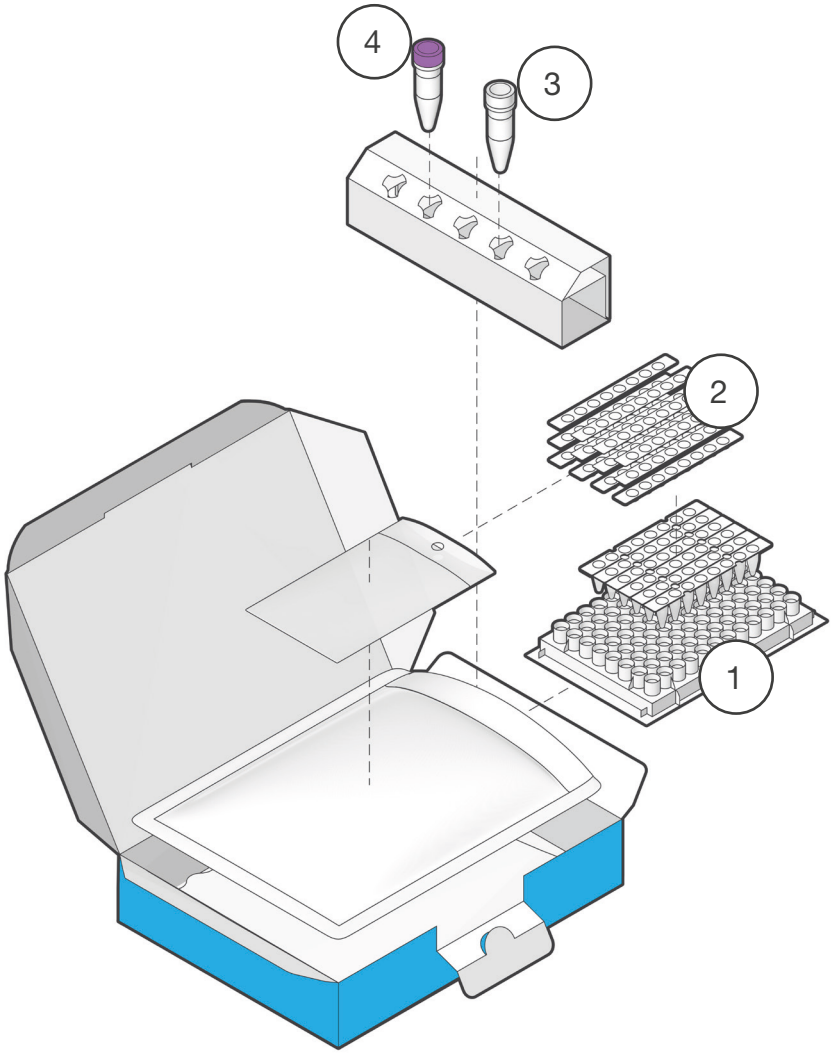
The **food**proof® STEC Identification LyoKit is intended for the rapid analysis of samples previously tested positive for Shiga toxin-producing *E. coli* DNA (e.g., with the **food**proof® STEC Screening LyoKit KIT 2300 77 / 78) isolated from enrichment cultures prepared by valid methods and inoculated with all relevant kinds of samples that are potentially contaminated with Shiga toxin-producing *E. coli*.

The kit 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), AriaMx, Mx3005P® (Agilent Technologies), Applied Biosystems™ 7500 Fast (Thermo Scientific), CFX96 (Bio-Rad), and others.

1.3 Kit Contents

A schematic representation of the **food**proof® STEC Identification LyoKit with all its components.

LP: KIT 2300 79
RP: KIT 2300 80



	Component	Details
1	Microplate	6 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	1 x H_2O PCR-grade (colorless cap)	1 ml nuclease-free, for use as a PCR run negative control.
4	Control Template (purple cap)	250 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

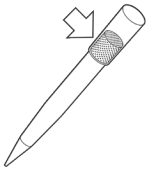
2.1 Required Material

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.

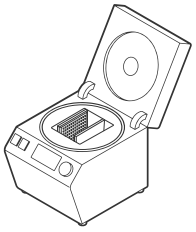
Material

☐ 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 **food**proof® STEC Identification LyoKit is intended for the rapid detection of Shiga toxin-producing *E. coli* DNA isolated from enrichment cultures prepared by valid methods and inoculated with all relevant kinds of foods and primary production stage (PPS) samples.

2.3.1 Certified Methods

The **food**proof® STEC Identification LyoKit was validated according to the AOAC RI *Performance Tested Methods*℠ program (license number 102004) for the category raw meat for identification of 8 serogroups of *E. coli* STEC strains.

The validation includes 375 g test portions enriched in Modified Tryptone Soy Broth (mTSB) (1:4) at 42 ± 1 °C for 12 to 24 h and 25 g test portions enriched in mTSB (1:10) for 8 to 24 h. DNA isolation was done according to the package insert of the **food**proof® StarPrep Three Kit – Extraction Procedure A. For 25 g meat samples with 8 to 20 h enrichment time and for 375 g meat samples with 12 to 20 h enrichment time, 500 µl of the enrichment culture were used for DNA extraction. For enrichment times between 20 h and 24 h 100 µl of the enrichment culture were used for DNA extraction. The **food**proof® STEC Screening LyoKit was validated in combination with the **food**proof® STEC Identification LyoKit and positive samples were confirmed via the method described in Annex A in the package insert of the **food**proof® STEC Identification LyoKit and via the reference method described in the USDA/FSIS-MLG 5C.00.

2.4 Procedure

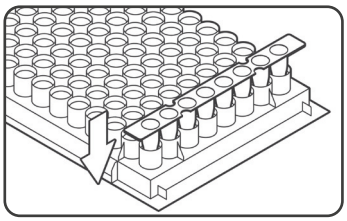
This protocol describes how to perform the analysis of DNA extracts by real-time PCR.

2.4.1 Workflow

1. PLACE STRIPS IN RACK

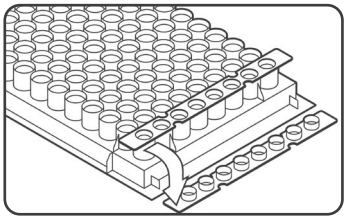
Take needed number of PCR tube strips out of aluminum bag.
Important: close bag tightly afterwards. Place strips in a suitable PCR tube rack.

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



2. DECAP

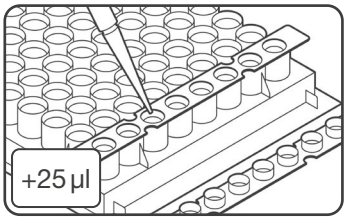
Open strips carefully direct before filling and discard caps.
Important: do not leave open longer than necessary.



3. ADD SAMPLES AND CONTROLS

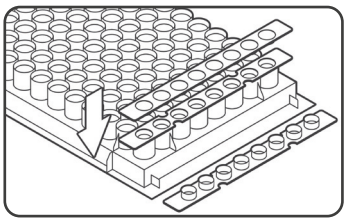
Pipette 25 µl of samples, negative control (colorless cap) or Control Template (purple cap) into respective wells.

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

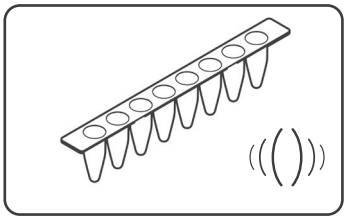
Seal the tubes with the provided 8-cap strips tightly.



5. MIX

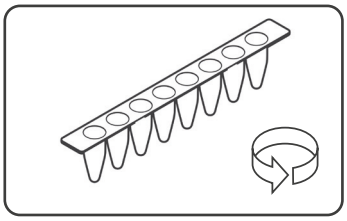
Resuspend pellet after sealing by mixing thoroughly.

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



6. CENTRIFUGE

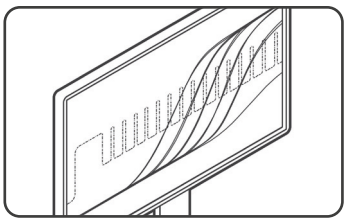
Briefly spin strips, e.g., 5 sec at 500 - 1,000 x g, in a suitable centrifuge.



7. START REAL-TIME PCR RUN

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

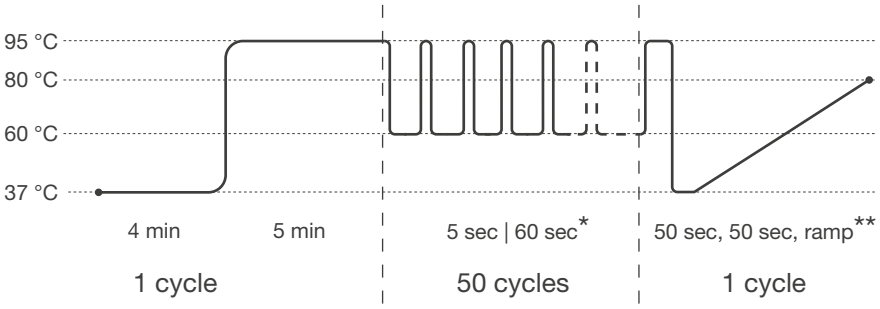
Place tubes in a vertical, balanced order into the cycler, e.g., two strips can be placed in the first and last column.



2.4.2 Program Setup

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

- FAM (all 8 serotypes), HEX (O26, O103, O104), ROX (O111, O145, O157), and Cy5 (O45, O121, 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 80 °C

* Fluorescence detection ** Fluorescence detection during 37 - 80 °C ramp with 1 - 2 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 (Order No. KIT 2300 05).
Agilent Mx3005P™ instrument: Choose Experiment Type “SYBR® Green (with Dissociation Curve)” and add HEX, ROX, and CY5 channels for data collection in the setup section.
CFX96™ system melting curve protocol: Step 1: 95 °C for 45 seconds; Step 2: Melt Curve 37 °C to 80 °C, increment 0.5 °C, for 0.02 + Plate Read

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 table.

	FAM	Cy5	Result Interpretation
Amplification Curve	+	+/-	Positive for one or more: O26, O45, O103, O104, O111, O121, O145, O157
	-	+	Negative for serogroups: O26, O45, O103, O104, O111,O121, O145, O157
	-	-	Invalid

	Channel	Tm Range for LC480, LC96, AriaMx		
Melting Curve	HEX	O26: 43 - 48 °C	O104: 48.5 - 54 °C	O103: 59 - 64 °C
	ROX	O157: 45 - 50 °C	O111: 51 - 56 °C	O145: 59 - 64 °C
	Cy5	O121: 50 - 55 °C	O45: 58 - 63 °C	IC: > 65 °C

	Channel	Tm Range for Mx3005P, 7500 Fast, CFX96		
Melting Curve	HEX	O26: 41 - 45.5 °C	O104: 46 - 51 °C	O103: 58 - 63 °C
	ROX	O157: 42 - 47 °C	O111: 48 - 53 °C	O145: 57 - 62 °C
	Cy5	O121: 48 - 53 °C	O45: 56 - 61 °C	IC: > 65 °C

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 12 -1, and R 602 12 -2.

3.4 Change Index

Version 5, February 2022:
Rebranding, new document layout and content, new order number.

ANNEX A

Alternative confirmation of presumptive positive PCR results (from the enrichment culture)
In case of positive PCR results with the **food**proof® STEC Screening LyoKit and **food**proof® STEC Identification LyoKit continue with the confirmation procedure as described below.

General Preparation:

- Prewarm E Buffer to at least 18 °C.
- Remove mRBA plates or equivalent from 2 °C to 8 °C.
- Prepare and label reaction tubes.

Alternative Confirmation Procedure:

(a) Following incubation for 15-24 h shake enrichment culture gently.

(b) Place a 40 µm cell strainer on a labeled 50 ml conical centrifuge tube. Pipette 5 ± 1 ml of enrichment culture into the respective cell strainer and collect at least 1.0 ml of filtrate.

(c) Transfer appropriate immunomagnetic capture beads (volume recommended by the manufacturer) determined by the serogroup PCR screen results to a sterile, labeled microcentrifuge tube.

(d) Transfer 1.0 ml of a filtrate to the corresponding microcentrifuge tube containing the immunomagnetic bead suspension and place in the clips of the rotating tube agitator. Rotate the tubes for 10 15 mins at 18 °C to 30 °C.

(e) Attach the OctoMACS® magnet to the multistand.

(f) Label and place the appropriate number of large cell separation columns on the OctoMACS® Magnet. Leave the plungers in the bags at this time to maintain sterility.

(g) Place a rack with tubes below the columns for collecting liquid waste. Inactivate and discard flow-through following step (i).

(h) Transfer at least 0.5 ml E Buffer to the top of each column and let the buffer run through, then transfer each culture and control to its corresponding column.

(i) After the last washing step, remove the column from the OctoMACS® magnet and insert the tip into an empty labeled 12 x 75 mm tube. Apply 1.0 ml of E Buffer to the column, and using the plunger supplied with the column, immediately flush out the beads into the tube. Use a smooth, steady motion to avoid splattering. Cap the tubes.

(j) Make a 1:10 dilution of each treated bead suspension by adding 100 µl of the bead suspension to a 12 x 75 mm labeled tube containing 900 µl E Buffer. Make a 1:100 dilution by adding 0.1 ml of the 1:10 dilution to a 12 x 75 mm labeled tube containing 900 µl E Buffer.

(k) Vortex briefly to maintain beads in suspension and plate 100 µl from each suspension (undiluted column eluate, 1:10 dilution and 1:100 dilution) onto a labeled mRBA plate. Use a hockey stick or spreader to spread plate the beads.

(l) For acid treatment transfer 450 µl of the undiluted column eluate to an empty labeled microcentrifuge tube. Add 25 µl of 1N hydrochloric acid (HCl) to this bead suspension and vortex briefly.

(m) Place the microcentrifuge tubes containing the acid treated suspension on a rotating tube agitator and rotate tubes for 1 h at 18 °C to 30 °C.

(n) Following incubation, dilute the suspension by adding 475 µl of E buffer.

(o) Vortex briefly to maintain beads in suspension and plate 100 µl of the diluted suspension onto a labeled mRBA plate. Use a hockey stick or spreader to spread plate the beads.

(p) Add 100 µl of the suspension to a labeled tube containing 900 µl E buffer and vortex briefly, representing a 1:10 dilution of the acid-treated cell suspension. Plate 100 µl of the diluted suspension onto a labeled mRBA plate.

(q) As soon as there is no visible moisture on the agar surface, invert plates and incubate for 20 to 24 h at 35 ± 2 °C.

(r) After incubation of mRBA, plates are to be examined for colonies that agglutinate with latex agglutination reagents specific for the serogroup of interest. Perform serological agglutination assays for STECs following manufacturer's instructions. Samples that have no growth on mRBA or colonies that are agglutination negative for the O group of interest can be reported as negative for STEC.

(s) Extract DNA from agglutination positive colonies using the **food**proof® StarPrep Three Kit according to the "Extraction procedure Alternative 2" described in the package insert.

(t) Confirm agglutination positive colonies with both the **food**proof® STEC Screening LyoKit and **food**proof® STEC Identification LyoKit following the procedure described in the respective package insert.

(u) The sample is considered positive for non-O157 STEC if the isolate is agglutination positive for one or more of the seven non-O157 STEC serogroups (O26, O45, O103, O104, O111, O121, O145) positive for *stx* and *eae*, positive for one or more of the seven non-O157 serogroup genes. If the isolate and any additional colony picks from mRBA are ultimately determined to be negative for either *stx*, *eae*, or serogroup genes, the sample is negative for non-O157 STEC.

Isolates that serologically and genetically determined to be "*E. coli* O157" are additionally considered as positive if they are positive for Shiga toxin production, positive for *stx* gene(s) or genetically determined to be "H7".

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www.hygiena.com/technical-support-request

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