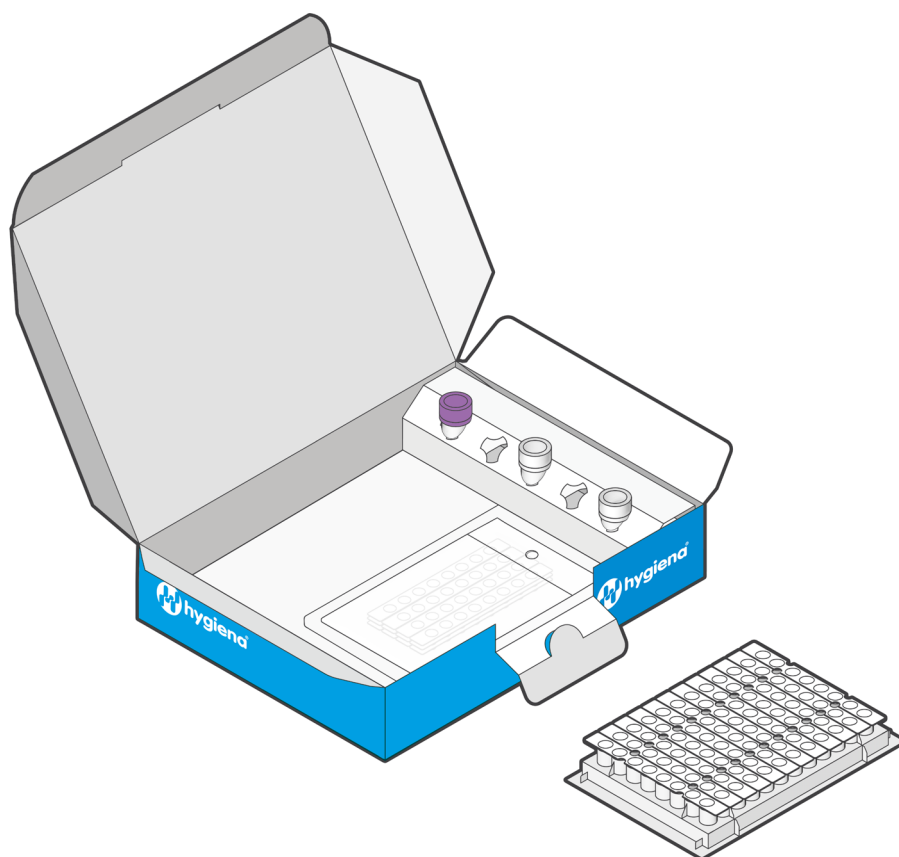




foodproof®

STEC

Identification LyoKit

PRODUCT INSTRUCTIONS

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

Product No. KIT230079 / KIT230080

foodproof®
STEC
Identification LyoKit

Product No.

LP: KIT230079

RP: KIT230080

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

Store kit at 2 to 8 °C

For testing of food
and environmental samples

Approval:



PRODUCT INSTRUCTIONS

Revision A, May 2024

1. OVERVIEW 4

1.1 General Information 4

1.2 Applicability..... 4

1.3 Kit Contents 5

2. INSTRUCTIONS 6

2.1 Required Material 6

2.2 Precautions and Preparations..... 7

2.3 Enrichment and DNA Extraction 8

 2.3.1 Certified Methods 8

2.4 Procedure 9

 2.4.1 Workflow 9

 2.4.2 Program Setup..... 10

 2.4.3 Data Interpretation 10

2.5 Troubleshooting 11

2.6 Support..... 12

3. ADDITIONAL INFORMATION 13

3.1 Testing Principle 13

3.2 Trademarks 14

3.3 License 14

3.4 Reference Number 14

3.5 Change Index 14

ANNEX A..... 15

ANNEX B..... 17

OVERVIEW

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 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 with silica gel pads. Protect from light and moisture.

LyoKit Tube Profiles

The LyoKit is available in two different tube profiles: white low profile tubes (LP) and clear regular profile tubes (RP).

The majority of real-time PCR cyclers use low profile tubes, 0.1 mL (LP). However, the Dualo 32® R2 and a few other cyclers require clear low profile tubes, 0.1 mL (DP) or clear regular profile tubes, 0.2 mL (RP).

Please refer to the cycler and tube profile compatibility chart for LyoKits in the Hygiena® document library at www.Hygiena.com/documents.

1.2 Applicability

The foodproof® 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 foodproof STEC Screening LyoKit KIT230077 /78) isolated from enrichment cultures prepared by valid methods with all relevant kinds of samples that are potentially contaminated with these microorganisms, e.g., meat or sprouts.

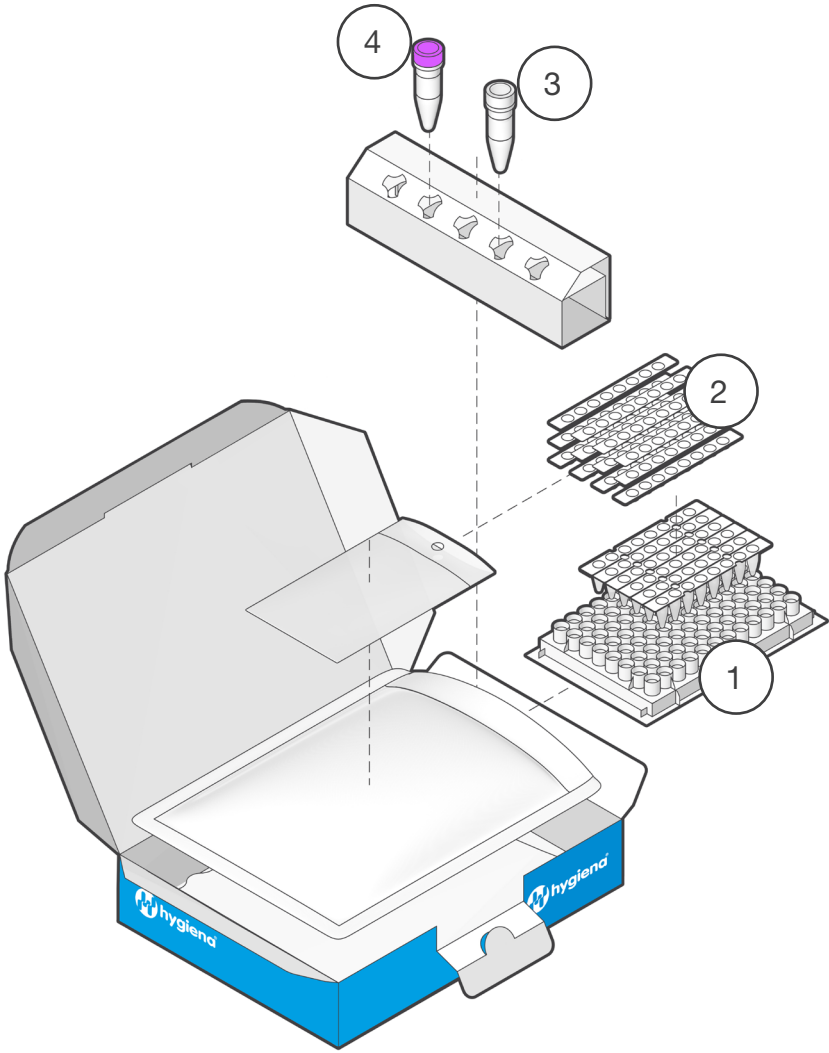
The kit has been developed for real-time PCR instruments with FAM, HEX, ROX and Cy5 detection channels, and capable of performing a melting curve analysis. The performance of the kit was tested with the following real-time PCR instruments: LightCycler® 480, LightCycler® 96 (Roche Diagnostics), AriaMx, Mx3005P® (Agilent Technologies), PikoReal24, Applied Biosystems™ 7500 Fast (Thermo Scientific), and CFX96™ (Bio-Rad).

The kit must not be used in diagnostic procedures.

1.3 Kit Contents

A schematic representation of the foodproof STEC Identification LyoKit with all its components.

LP: KIT230079
RP: KIT230080



	Component	Details
1	Microplate	6 x 8-tube strips, prefilled with lyophilized ready-to-use PCR mix. Different tube profiles are available: 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 ₂ O 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

INSTRUCTIONS

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 in the instrument, the samples should be transferred to appropriate PCR vessels after resuspension of the lyophilized PCR mix.

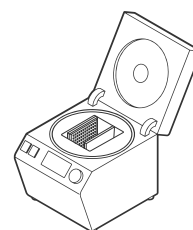
Material

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



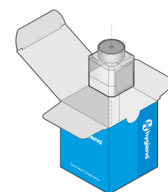
- ☐ **PCR strip or plate centrifuge**

- *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*



- ☐ **DNA Extraction Kits**

- *foodproof StarPrep Three Kit (KIT230187) or*
- *foodproof Magnetic Preparation Kit I (KIT230180)*



INSTRUCTIONS

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 nucleases, 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 and other 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.hygiena.com/sds.

INSTRUCTIONS

2.3 Enrichment and DNA Extraction

The foodproof STEC Identification LyoKit is intended for the rapid detection of Shiga toxin-producing *E. coli* DNA isolated from enrichment cultures. For DNA extraction, please use the kits mentioned in Section 2.1. Required Material.

2.3.1 Certified Methods

The foodproof STEC Identification LyoKit was validated according to the AOAC RI *Performance Tested Methods*SM program (License No. 102004) for the category raw meat for identification of eight (8) serogroups of *E. coli* STEC strains.

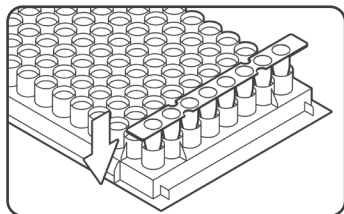
The validation includes 375 g test portions enriched in Modified Tryptone Soy Broth (mTSB) (diluted 1:4) at 42 ± 1 °C for 12 to 24 h and 25 g test portions enriched in mTSB (1:10) for 8 to 24 h. DNA isolation was done according to the package insert of the foodproof StarPrep® Three Kit – Extraction Procedure 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 foodproof STEC Screening LyoKit was validated in combination with the foodproof STEC Identification LyoKit and positive samples were confirmed via the method described in Annex A in the package insert of the foodproof STEC Identification LyoKit and via the reference method described in the USDA/FSIS-MLG 5C.00.

INSTRUCTIONS

2.4 Procedure

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

2.4.1 Workflow

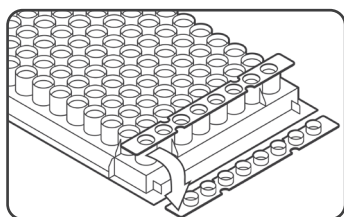


1. PLACE STRIPS IN RACK

Take needed number of PCR tube strips out of aluminum bag.

Important: close bag tightly afterwards. Place strips in a suitable PCR tube rack.

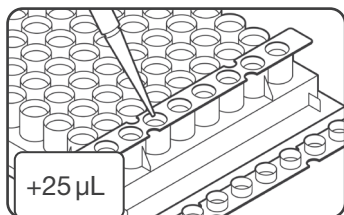
If needed, gently tap the tubes to move the lyophilized pellets to the bottom of all tubes.



2. DECAP

Open strips carefully immediately before filling and discard caps.

Important: Recap immediately after hydration. Do not leave open longer than necessary.

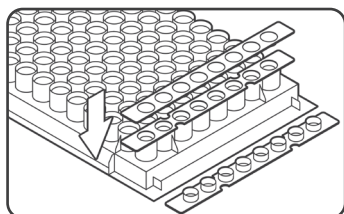


3. ADD SAMPLES AND CONTROLS

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

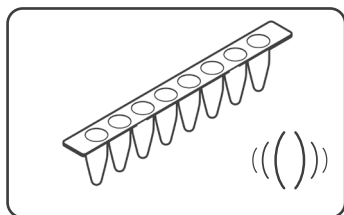
If using less volume, add PCR-grade H₂O to reach 25 µL.

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.

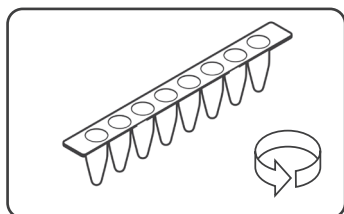


5. MIX

Resuspend pellet after sealing by mixing thoroughly.

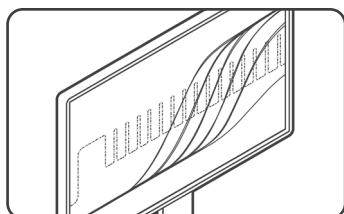
Alternatively, resuspend pellet by pipetting up and down multiple times in step 3.

Note: Hygiena recommends to use vortex centrifuges for steps 5,6: Multispin MSC-6000 (for tube strips) or CVP-2 (for plates).



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).

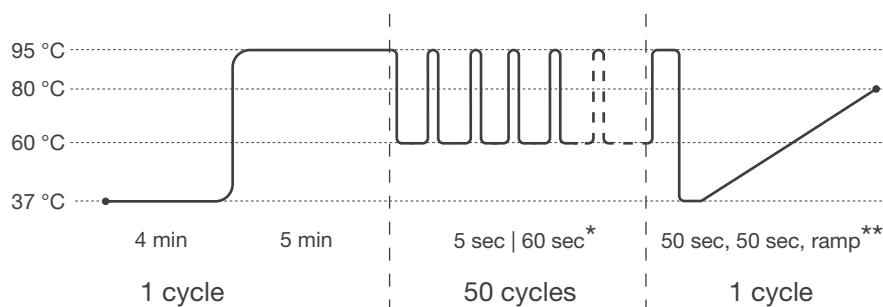
Place tubes in a vertical, balanced order into the cycler, e.g., two strips can be placed in the first and last column.

INSTRUCTIONS

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 (Product No. KIT230005).

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.

Applied Biosystems 7500 Fast: Please reach out to Hygiena for detailed instructions.

2.4.3 Data Interpretation

Verify results of positive (Control Template) and negative control (H₂O), before interpreting sample results. Always compare samples to positive and negative controls. Review data from each channel and interpret results as described in the table.

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

Melting Curve	Channel	Melting Temperature (T _m) Range for LC480, LC96, AriaMx®, PikoReal24™		
	HEX	O26: 43 to 48 °C	O104: 48.5 to 54 °C	O103: 59 to 64 °C
	ROX	O157: 45 to 50 °C	O111: 51 to 56 °C	O145: 59 to 64 °C
	Cy5	O121: 50 to 55 °C	O45: 58 to 63 °C	IC: > 65 °C

Melting Curve	Channel	Melting Temperature (T _m) Range for iQ5, Mx3005P®, ABI 7500 Fast, CFX96		
	HEX	O26: 41 to 45.5 °C	O104: 46 to 51 °C	O103: 58 to 63 °C
	ROX	O157: 42 to 47 °C	O111: 48 to 53 °C	O145: 57 to 62 °C
	Cy5	O121: 48 to 53 °C	O45: 56 to 61 °C	IC: > 65 °C

INSTRUCTIONS**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. 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 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

INSTRUCTIONS

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/support

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 foodproof 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 amplification inhibition of 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 in Section 1.2: Applicability. For other instruments, please contact us.

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 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 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.

ADDITIONAL INFORMATION

3.2 Trademarks

foodproof®, **microproof®**, **vetproof®**, **ShortPrep®**, **StarPrep®**, **RoboPrep®** and **LyoKit®** are registered trademarks of Hygiena Diagnostics GmbH.

Hygiena® is a registered trademark of Hygiena.

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

3.3 License

License Notice

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

3.4 Reference Number

The reference number and original Hygiena Diagnostics GmbH article numbers:
R 602 12-1 (KIT230079), and R 602 12-2 (KIT230080).

3.5 Change Index

Version 1, January 2015:

First version.

Version 2, September 2016:

New table and texts.

Version 3, March 2017:

License Notice changed.

Introduction of vortex centrifuges into the PCR Setup Procedure.

Version 4, October 2020:

Addition of AOAC certification information.

Version 5, February 2022:

Rebranding, new document layout and content, new order number.

Revision A, August 2023

Rebranding, new document layout, images and content.

R 602 12 20 -> INS-KIT230079-80-REVA

ANNEX**ANNEX A**

Alternative confirmation of presumptive positive PCR results (from the enrichment culture). In case of positive PCR results with the foodproof STEC Screening LyoKit and foodproof STEC Identification LyoKit, continue with the confirmation procedure as described below (See 2.3.1 Certified Method for additional information).

General Preparation:

- Prewarm E Buffer to at least 18 °C.
- Remove mRBA plates or equivalent from 2 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 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 a culture or control has drained through, wash the column by applying 1.0 mL of E Buffer to each column and allow to drain. Repeat 3 more times for a total of 4 washes.
- (j) 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.
- (k) 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.
- (l) 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.

ANNEX

- (m) 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.
- (n) Place the microcentrifuge tubes containing the acid treated suspension on a rotating tube agitator and rotate tubes for 1 h at 18 to 30 °C.
- (o) Following incubation, dilute the suspension by adding 475 µL of E buffer.
- (p) 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.
- (q) 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.
- (r) 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.
- (s) 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.
- (t) Extract DNA from agglutination positive colonies using the foodproof StarPrep Three Kit according to the "Extraction procedure: Colonies" described in the package insert.
- (u) Confirm agglutination positive colonies with both the foodproof STEC Screening LyoKit and foodproof STEC Identification LyoKit following the procedure described in the respective package inserts.
- (v) 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* or 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 are 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".

ANNEX**ANNEX B****Colony Confirmation Procedure**

The following procedure has been optimized for the rapid confirmation and identification of presumptive STEC of serogroups O26, O45, O103, O104, O111, O121, O145 and O157.

Prepare single colonies with the microproof Suspension Buffer (Product No. KIT2301178) or with the foodproof StarPrep Three Kit (Product No. KIT230187) according to the “Extraction procedure: Colonies”. Safety precautions and protocols are described in the respective package inserts.

Note: Colonies prepared with microproof Suspension Buffer are not inactivated before the preincubation step of PCR and are still considered hazardous.

Program Setup (Colony confirmation):

The following procedure has been developed for a real-time PCR instrument with a FAM (amplification of the target serotypes), HEX (melting curve differentiation), ROX (melting curve differentiation) and Cy5 (amplification of the Internal Control and melting curve differentiation) detection channels. Program the PCR instrument before preparing the PCR samples. Use the following real-time PCR protocol for the foodproof STEC Identification LyoKit. For details on how to program the experimental protocol, see the Instrument Operator’s Manual of your real-time PCR cycler.

Alternative Confirmation Procedure:

<u>Preincubation</u>	1 cycle
Step 1:	37 °C for 4 minutes
Step 2:	95 °C for 5 minutes
<u>Amplification</u>	30 cycles
Step 1:	95 °C for 5 seconds
Step 2*:	60 °C for 60 seconds

* Fluorescence detection in step 2

<u>Melting Curve</u>	1 cycle
Step 1:	95 °C for 50 seconds
Step 2:	37 °C for 50 seconds
Step 3*:	Ramp up to 80 °C

* Fluorescence detection during 37 to 80 °C ramp with 1 – 2 measurements/°C

Notes:

- 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 STEC Identification LyoKit contains probes with a non-fluorescent (“dark”) quencher and no passive reference dye.
- For users of the 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.

ANNEX

- For users of the CFX96™ system, please modify melting curve protocol:
Step 1: 95 °C for 45 seconds
Step 2: Melt Curve 37 to 80 °C, increments of 0.5 °C, for 0.02 + Plate Read
- Please contact Hygiena Diagnostics if you have questions on how to program your cycler.

Preparation of the PCR Mix (Colony confirmation):

Proceed as described below to prepare a 25 µL standard reaction. Always wear gloves when handling strips or caps.

Note: The PCR strips must be stored in the provided aluminum bag with the silica gel pads to avoid liquid absorption.

1. Take the needed number of PCR tube strips out of the aluminum bag. Use scissors or scalpel to cut the strips apart. Tightly seal the bag afterward and store under the recommended conditions.
2. Place the PCR tube strips containing the lyophilized reagents in a suitable PCR tube rack. Check that the reagent pellets are at the bottom of the tubes. If not, briefly centrifuge or flick the pellets to the bottom before proceeding.
3. Decap the tube strips cautiously and discard the cap strips.
Note: Do not leave strips open for extended periods of time. To avoid unwanted liquid absorption, open strips only shortly before filling.
4. Pipetting of samples:
 - Pipet 20 µL Internal Amplification Control (Product No. KIT230015) into each PCR vessel.
 - Add 5 µL of suspended colony material to each well.**Note:** To reduce the risk of cross-contamination, it is recommended to prepare only one PCR tube strip at a time.
5. Pipetting of controls:
 - For the negative control, pipet 20 µL Internal Amplification Control into a PCR vessel and add 5 µL PCR-grade H₂O (vial 3, colorless cap).
 - For the positive control, add 25 µL foodproof STEC Identification LyoKit Control Template (vial 2, purple cap).
6. Seal the vessels accurately and tightly with the colorless cap strips.
7. Mix thoroughly using a vortex centrifuge.
Note: Hygiena Diagnostics recommends vortex centrifuge Multispin MSC-6000 for PCR strips or vortex centrifuge CVP-2 for PCR plates. Dedicated protocols are available for these centrifuges. Alternatively, resuspend the pellet by manual mixing. This may be achieved by cautiously pipetting the sample up and down multiple times during step 4 or flipping the tube strips after sealing while pressing down the cap strip.
8. Spin the PCR tube strips for 30 seconds at 150 – 200 x g in a suitable centrifuge.
Note: If your centrifuge exceeds 200 x g, do not centrifuge for more than 5 seconds. Avoid centrifugation forces exceeding 1000 x g!
9. Place the samples in your PCR cycler and run the program as described above.
Note: For using any LightCycler 480 instrument, a special adapter is necessary. For some PCR instruments, the PCR strips should be placed in a balanced order into the cycler block. For example, two strips can be placed in columns 1 and 12.

Hygiena®

Camarillo, CA 93012
USA

diagnostics.support@hygiena.com

Manufactured by

Hygiena Diagnostics GmbH

Hermannswerder 17
14473 Potsdam
Germany

www.hygiena.com