foodproof® *Listeria* plus *L. monocytogenes* Detection LyoKit

Revision A, April 2024

PCR kit for the qualitative detection of the *Listeria* sensu stricto species (*L. monocytogenes, L. seeligeri, L. ivanovii, L. welshimeri, L. innocua and L. marthii*), including the simultaneous identification of *Listeria monocytogenes*, using real-time PCR instruments.

Product No. KIT230129 (LP), KIT230130 (RP), KIT230369 (DP)

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

Store the kit at 2 to 8 °C

FOR *IN VITRO* USE ONLY

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1. What This Product Does

1.1 Number of Tests

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

1.2 Storage and Stability

- Store the kit at 2 to 8 °C through the expiration date printed on the label.
- Once the kit is opened, store the components as described in the following contents table:

1.3 Kit Contents

Component	Label	Contents / Function / Storage
foodproof® *Listeria* plus *L. monocytogenes* Detection LyoKit Microplate, prefilled with 96 reactions (lyophilized)	Aluminum bag containing a 8-tube strip mat • KIT230129 with white low profile (LP) tubes* • KIT230130 with clear regular profile (RP) tubes* • KIT230369 with clear low profile (DP) tubes*	• 96 prefilled reactions (lyophilized). • Ready-to-use PCR mix containing primer and hydrolysis probes specific for *Listeria*-DNA and the Internal Control (IC) as well as Taq DNA Polymerase and Uracil-DNA Glycosylase (UNG, heat-labile) for prevention of carry-over contamination. • For amplification and detection of *Listeria* sensu stricto and *Listeria monocytogenes* specific sequences. • Store at 2 to 8 °C in the aluminum bag (sealed and containing silica gel pads). • **Protect from light and moisture!**
Control Template	Vial 2 (purple cap)	• 1 x 250 µL • Contains a stabilized solution of DNA. • For use as a PCR run positive control. • Store at 2 to 8 °C.
H_2O PCR-grade	Vial 3 (colorless cap)	• 2 x 1 mL • Nuclease-free, PCR-grade H_2O. • For use as a PCR run negative control.
Cap strips	Plastic bag containing 8-cap strips	• 12 x 8-cap strip • For use in real-time PCR after addition of samples.

*****Tube profile and instrument compatibility chart is available online: www.hygiena.com/documents

1.4 Additional Equipment and Reagents Required

Real-time PCR cycler suitable for detection of FAM-, HEX- and ROX-labeled 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.

- Sample Preparation Kit
    - foodproof StarPrep Two Kit (Product No. KIT230177)
    - foodproof StarPrep Two 8-Strip Kit (Product No. KIT230186)
    - foodproof ShortPrep II Kit (Product No. KIT230171) **or**
    - foodproof Magnetic Preparation Kit II (Product No. KIT230181)

- Nuclease-free, aerosol-resistant pipette tips
- Pipettes

and optionally

- Vortex centrifuge Multispin MSC-6000 for PCR strips **with**
- SR-32, Rotor for MSC-3000/6000 **or**
- Vortex centrifuge CVP-2 for PCR plates
  For users of the Agilent AriaMx:
- 0.1 mL thin-wall 8-tube strip (low profile)

1.5 Applicability Statement

The foodproof *Listeria* plus *L. monocytogenes* Detection LyoKit is intended for the rapid detection of *Listeria* sensu stricto species isolated by foodproof DNA extraction methods from enrichment cultures of all relevant kinds of food and environmental samples that are potentially contaminated with *Listeria*. 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 VIC/Yakima Yellow or HEX, and a ROX or Texas Red detection channel. The performance of the kit was tested with the following real-time PCR instruments: LightCycler® 480, LightCycler 96 (Roche Diagnostics), Mx3005P®, AriaMx (Agilent Technologies), ABI 7500 fast (Thermo Fisher), CFX96 (Bio-Rad) and PikoReal® 24 (Thermo Fisher).

Note: When testing the samples on a LightCycler 480 instrument, it is recommended to carry out the DNA extraction with a wash step (foodproof StarPrep Two Kit, Product No. KIT230177, Procedure A: STANDARD protocol).

Note*:* A color compensation (Color Compensation Set 3; Product No. KIT230005) is necessary and will be supplied by Hygiena Diagnostics for users of the LC 480 Systems I and II. Please contact Hygiena Diagnostics for further information.

Note: For users of the Agilent AriaMx. it is recommended that transparent low-profile tubes be used.

The performance of the foodproof *Listeria* plus *L. monocytogenes* Detection LyoKit in combination with different foodproof DNA extraction procedures [foodproof StarPrep Two Kit (procedure A and B) and the foodproof StarPrep Two 8-Strip Kit (procedure A.1)] has been approved in a NordVal International method validation (Certificate No. 054). For this validation study, the following categories were tested: composite foods/ready-to-eat and ready-to-reheat, meat products, milk and dairy products, vegetables, seafood and fishery products, and environmental samples. For further information about the enrichment protocols and the DNA extraction procedures, please refer to ANNEX 1 at the end of the product instructions.

2. How to Use this Product

2.1 Before You Begin

2.1.1 Precautions

Detection of *Listeria* DNA using the foodproof *Listeria* plus *L. monocytogenes* Detection LyoKit requires DNA amplification by PCR. 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-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 *Listeria* plus *L. monocytogenes* Detection lyophilized PCR mix away from light and moisture.

2.1.2 Sample Material

Use any sample material suitable for PCR in terms of purity, concentration, and absence of inhibitors. For preparation of genomic DNA from various sample enrichments, refer to the corresponding product package inserts of a suitable sample preparation kit (see *"1.4 Additional Equipment and Reagents Required"*).

2.1.3 DNA Extraction

Hygiena Diagnostics provides sample preparation kits suitable for all kinds of food and environmental samples (see *"1.4 Additional Equipment and Reagents Required"*). For more product information, please refer to www.hygiena.com.

2.1.4 Positive Control

Always run a positive control with the samples. To prepare a positive control, replace the template DNA with the provided control DNA [foodproof *Listeria* plus *L. monocytogenes* Detection Control Template (vial 2, purple cap)] or with a positive sample preparation control.

2.1.5 Negative Control

Always run a negative control with the samples. To prepare a negative control, replace the template DNA with PCR-grade H_2O (vial 3, 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

2.2.1 Program Setup

The following procedure is optimized for a real-time PCR instrument with a FAM (for *Listeria monocytogenes*), HEX (for *Listeria* sensu stricto) and ROX (for the Internal Amplification Control) detection channel. Program the PCR instrument before preparing the samples. Use the following real-time PCR protocol for the foodproof *Listeria* plus *L. monocytogenes* Detection LyoKit. For details on how to program the experimental protocol, see the Instrument Operator's Manual for your real-time PCR cycler:

<u>Pre-incubation</u> **1** cycle

Step 1: 37 °C for 4 minutes
Step 2: 95 °C for 5 minutes

<u>Amplification</u> **50** cycles

Step 1: 95 °C for 5 seconds
Step 2*: 60 °C for 60 seconds

* Fluorescence detection in step 2

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 *Listeria* plus *L. monocytogenes* Detection LyoKit contains probes with a non-fluorescent ("dark") quencher and no passive reference dye.
- 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 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'.

2.2.2 Preparation of the PCR Mix

Proceed as described below to prepare a 25 µL standard reaction. Always wear gloves when handling strips or caps. Use any sample material suitable for PCR in terms of purity, concentration, and absence of inhibitors.

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

1. Take the needed number of PCR tube strips out of the aluminum bag. Use scissors or a 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: To avoid unwanted liquid absorption, open strips only shortly before filling.

4. Pipet 25 µL sample into each PCR vessel:
    - For the samples of interest, add 25 µL sample DNA (for less volume, add PCR-grade water to achieve a total volume of 25 µL).

Note: For DNA samples prepared from acriflavine-containing enrichment broth (e.g., Fraser broth) with the foodproof StarPrep Two or foodproof ShortPrep II Kit, it is recommended to use 5 µL sample instead of 25 µL sample to avoid the negative influence of residual acriflavine residues on the fluorescence detection.

- For the negative control, add 25 µL PCR-grade H_2O (vial 3, colorless cap).
- For the positive control, add 25 µL *Listeria* plus *L. monocytogenes* Control Template (vial 2, purple cap).

Note: To reduce the risk of cross-contamination, it is recommended that only one PCR tube strip be prepared at a time.

5. Seal the vessels accurately and tightly with the colorless cap strips.
6. Mix thoroughly using a vortex centrifuge.

Note: Hygiena Diagnostics recommends vortex centrifuges Multispin MSC-3000 for PCR strips or vortex centrifuge CVP-2 for PCR strips and plates. Dedicated protocols are available for this centrifuge.

Note: Alternatively, resuspend the pellet by manually mixing by cautiously pipetting the sample up and down multiple times during step 4 or by flipping the tube strips after sealing while pressing down the cap strip.

7. 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 at forces exceeding 1,000 x g!

8. Place the samples in your PCR cycler and run the program as described above.

Note: When 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.

2.3 Data Interpretation

The details of data analysis depend on the real-time PCR instrument that you used; refer to the appropriate user guide for further instructions on how to analyze your data.

If a threshold value has to be set manually, ensure that the threshold lies within the exponential phase of the fluorescent curves but above any background signal and background noise.

The procedure chosen for setting the threshold should be used consistently.

- Ensure that any positive result has an exponential amplification curve.
- Control template and negative control must be clearly positive and negative, respectively.

The amplification of the *Listeria monocytogenes*-specific DNA region is analyzed in the fluorescence channel suitable for FAM-labeled probe detection. The amplification of the *Listeria* sensu stricto-specific DNA region is analyzed in the fluorescence channel suitable for HEX-labeled probe detection, and the specific amplification of the Internal Control is analyzed in the fluorescence channel suitable for ROX-labeled probes.

Compare the results from channels FAM (*Listeria monocytogenes*), HEX (*Listeria* sensu stricto) and ROX (Internal Amplification Control) for each sample, and interpret the results as described in the table below.

Channel FAM	Channel HEX	Channel ROX	Result Interpretation
Positive	Positive	Positive or Negative	Positive for *Listeria monocytogenes* and potentially for *L. seeligeri, L. ivanovii, L. welshimeri, L. innocua, L. marthii*
Negative	Positive	Positive or Negative	Positive for one or more of the following species*: L. seeligeri, L. ivanovii, L. welshimeri, L. innocua, L. marthii* Negative for *L. monocytogenes*[1]
Negative	Negative	Positive	Negative for *Listeria* sensu stricto (including *L. monocytogenes*)
Negative	Negative	Negative	Invalid

[1] If the amplification in channel HEX is very weak (Cq > 36), the result in FAM may be negative due to slight differences in the limit of detection of the assays in this multiplex-PCR system. In this case, prolonging the enrichment or using a larger amount of sample DNA might be used to increase the sensitivity.

Note: A prerequisite for the unambiguous discrimination of the targets in this multi-color experiment is a suitable calibration of the PCR instrument for channels FAM, HEX and ROX. 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, HEX or ROX.
	Pipetting errors.	• Check for correct 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 from sample, including Internal Control. Positive and Negative Controls have proper signals (No increase in channel ROX).	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., 20 µL PCR-grade water + 5 µL sample DNA instead of 25 µL sample DNA).
Fluorescence intensity is too low.	Inappropriate storage of kit components.	• Store the foodproof *Listeria* plus *L. monocytogenes* Detection Lyophilized PCR Mix at 2 to 8 °C, protected from light and moisture.
	Low initial amount of target DNA.	• Increase the amount of sample DNA. Depending on the chosen DNA isolation method, inhibitory effects may occur.
Strong decrease of fluorescence baseline.	Resuspension of lyophilized PCR mix not complete.	• Resuspend lyophilized PCR mix thoroughly.
Negative control samples are positive.	Carry-over contamination.	• Exchange all critical solutions. • Repeat the complete experiment with fresh aliquots of all reagents. • 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 strips. Resuspended PCR mix is still in the upper part of the vessel.	• Centrifuge PCR strips.
	Outer surface of the vessel or the seal is dirty (e.g., by direct skin contact).	• Wear gloves when handling the vessels and seal.
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 pad. • Open strip shortly before filling.

4. Additional Information on this Product

4.1 How this Product Works

The foodproof *Listeria* plus *L. monocytogenes* Detection LyoKit 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 ROX channel, whereas the *Listeria* DNA is detected in the FAM and the HEX 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 *Listeria* sensu stricto in the sample. The foodproof *Listeria* plus *L. monocytogens* Detection LyoKit minimizes contamination risk and contains all reagents (except for template DNA) needed for the detection of *Listeria* DNA. Primers and probes provide specific detection of *Listeria* sensu stricto DNA in food and environmental samples. The described performance of the kit is guaranteed for use on the real-time PCR instruments listed above only.

4.2 Test Principle

1. Using the kit's sequence-specific primers in a polymerase chain reaction (PCR), the PCR instrument and the supplied reagents amplify fragments of specific sequences for the target *Listeria* species.
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 amplicon sequence 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.

4.3 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 *Listeria* genomic DNA) does not contain uracil and is therefore not degraded by this procedure. Since dTTP is replaced with dUTP and UNG is included in the foodproof *Listeria* plus *L. monocytogenes* Detection LyoKit, decontamination can be achieved with the provided reagents.

4.4 Background Information

Listeria monocytogenes is considered to be one of the most important food-borne pathogens. It can cause severe disease, including meningoencephalitis, septicemia, and abortion, with mortality rates up to 33%. The CDC estimates that *Listeria* is the third leading cause of death from food poisoning in the United States [1]. Infections have been traced to the consumption of contaminated foods that often have relatively short shelf lives, emphasizing the need for rapid detection methods. *L. monocytogenes* is often found in samples that contain other *Listeria* spp. Therefore, the detection of *Listeria* species is often used as an indicator for the presence of *L. monocytogenes* and general process hygiene.

The number of known species belonging to the genus *Listeria* has increased from six to currently 20 in less than ten years. Most of the newly described species, however, differ substantially from the originally described *Listeria*, rendering them unsuitable as indicator organisms for the pathogenic *L. monocytogenes*. Based on phenotypic and genomic characteristics, a subdivision into new genera and *Listeria* “sensu stricto”, which includes the species *L. monocytogenes*, *L. seeligeri*, *L. ivanovii*, *L. welshimeri*, *L. marthii* and *L. innocua*, has been proposed [2].

4.5 References

1. Centers for Disease Control and Prevention – Listeriosis http://www.cdc.gov.
2. Orsi RH and Wiedmann M. 2016. Characteristics and distribution of *Listeria* spp., including *Listeria* species newly described since 2009. *Appl Microbiol Biotechnol* 100, 5273–5287

4.6 Quality Control

The foodproof *Listeria* plus *L. monocytogenes* Detection LyoKit is function tested using the LightCycler 480 System (KIT230129), the Mx3005P (KIT230130) and the Dualo 32 R2 (KIT230369).

5. Supplementary Information

5.1 Ordering Information

Hygiena Diagnostics is offering a broad range of reagents and services. For a complete overview and for more information, please visit our website at www.hygiena.com.

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

foodproof® is a registered trademark of Hygiena Diagnostics GmbH.
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 Hygiena Diagnostics GmbH, please contact our Technical Support staff (www.hygiena.com/support). 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.

5.5 Reference Number

The reference numbers and original Hygiena Diagnostics GmbH article numbers:
R 602 51-1 (KIT230129, LP) and R 602 51-2 (KIT230130, RP), R 602 51-3 (KIT230369, DP).

6. Change Index

Version 1, April 2019
First version of the package insert.

Version 2, September 2021
Introduction of NordVal International logo.
Introduction of ANNEX 1: NordVal #054 Validation Table and additional information in the Applicability Statement.

Revision A, April 2024
Rebranding and new layout.
R 602 51 20 -> INS-KIT-230129-130-369-RevA

ANNEX 1

NordVal #054 Validation Table for the foodproof® *Listeria* plus *L. monocytogenes* Detection LyoKit

The following table shows the recommended enrichment time for the tested food categories and environmental samples in Actero *Listeria* Enrichment Media (ALEM) and Half Fraser broth in combination with different foodproof DNA extraction procedures that have been analyzed for the NordVal International method validation of the foodproof *Listeria* plus *L. monocytogenes* Detection LyoKit (Certificate No. 054).

For further information regarding the DNA extraction procedures below, please refer to the appropriate Hygiena Diagnostics package inserts on www.hygiena.com.

DNA Extraction	Enrichment time in ALEM at 36 °C	Enrichment time in Half Fraser Broth at 30 °C	DNA extract for PCR
foodproof StarPrep Two Kit Extraction Procedure A STANDARD	22 h ± 2 h	25 h ± 1 h	5 µL + 20 µL PCR-grade water
foodproof StarPrep Two Kit Extraction Procedure B RAPID	---	48 h ± 2 h	5 µL + 20 µL PCR-grade water
foodproof StarPrep Two 8-Strip Kit Extraction Procedure A.1 STANDARD	22 h ± 2 h	25 h ± 1 h	5 µL + 20 µL PCR-grade water

Tested categories:

- Composite foods/ready-to-eat and ready-to-reheat
- Meat products
- Milk and dairy products
- Vegetables
- Seafood and fishery products
- Environmental samples

Sample size:

- 25 g + **150 mL** Actero *Listeria* Enrichment Media or **225 mL** Half Fraser broth
- 1 swab + 10 mL Actero *Listeria* Enrichment Media or 10 mL Half Fraser broth
- 1 sponge + 100 mL Actero *Listeria* Enrichment Media or 100 mL Half Fraser broth
- 1 wipe + 225 mL Actero *Listeria* Enrichment Media or 225 mL Half Fraser broth

- For sampling after cleaning process, premoisten:
    - 1 swab + 1 mL broth universal neutralizing (+ 9 mL ALEM or Half Fraser)
    - 1 sponge + 10 mL broth universal neutralizing (+ 90 mL ALEM or Half Fraser)
    - 1 wipe + BPW + 10 % neutralizing agent (+ 225 mL ALEM or Half Fraser)

Reference method: ISO 11290-1/A1 (May 2017)

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