



**KyIt**<sup>®</sup>



For Feed and Food Testing Applications only.

## **KyIt**<sup>®</sup> *Listeria monocytogenes*

DNA-Extraction and Real-Time PCR Detection Kit for  
*Listeria monocytogenes*

# DIRECTION FOR USE

Art. No. 31013/31014

## Kylt® *Listeria monocytogenes*

### DNA-Extraction and Real-Time PCR Detection Kit for *Listeria monocytogenes*

25 / 100 reactions

*in vitro* Diagnosticum for feed and food testing applications

#### A. Introduction

- Kylt® *Listeria monocytogenes* DNA-Extraction and PCR Detection Kit is for detection of bacterial DNA of *Listeria monocytogenes* from cultured materials originated from feed and food samples as well as from hygienic environmental samples.
- Kylt® *Listeria monocytogenes* comprises all reagents necessary for DNA extraction out of cultured materials as well as all reagents and controls for subsequent detection of bacterial DNA of *Listeria monocytogenes* (*L. monocytogenes*). After bacterial pre-enrichment and DNA extraction the qualitative testing of Kylt® *Listeria monocytogenes* is based on a duplex Real-Time PCR system: In one reaction setting the target genes for *L. monocytogenes* as well as for the included Internal Amplification Control are amplified in parallel by respective primer pairs in the Polymerase Chain Reaction (PCR). Amplified target gene fragments are detected via fluorescently labeled probes during the PCR reaction in real-time (Real-Time PCR). The probes specific for detection of *L. monocytogenes* and Internal Amplification Control target genes are labeled with fluorescent dyes FAM and HEX, respectively, and their emitted fluorescence is separately optically measured by the Real-Time PCR thermal cycler. By means of both individual assays in one reaction vessel per sample and the Negative and Positive Control per run the *L. monocytogenes*-specific status of a sample can be evaluated in the end.
- This kit was developed for use by trained laboratory personnel following standardized procedures. This Direction For Use must be followed strictly.
- It is recommended to follow the general requirements of ISO 22174:2005 (Microbiology of food and feed – Polymerase-chain-reaction (PCR) for detection of pathogenic microorganisms in food – General requirements and terms).

## B. Reagents and Materials

- Kylt® *Listeria monocytogenes* contains the following reagents:

Reagent	Color code of lid	Quantity in kit with 25 / 100 reactions	Storage conditions
DNA Extraction-Mix II	○ white	1 x 20 ml	+2 °C to +8 °C
Lysis Beads	○ transparent	1 x 8 g	+2 °C to +8 °C
Reaction-Mix	● orange	1 x / 4 x 500 µl	+2 °C to +8 °C
Positive Control	● red	2 x / 4 x lyophilisate (final 20 µl each)	+2 °C to +8 °C (lyophilized) -18 °C to -20 °C (rehydrated)
Negative Control	● blue	1 x 1 ml	+2 °C to +8 °C

- After receipt, components of the kit are immediately stored at +2 °C to +8 °C. Alternatively, for long term storage the kit can be stored at -18 °C to -20 °C. Avoid repeated freezing and thawing of the kit or its components. If occasional processing of few samples is expected, you may prepare aliquots of the Reaction-Mix before storage at -18 °C to -20 °C. Reaction-Mix has to be stored dark, do not expose to direct (sun)light! The kit and its components are to be used within the indicated shelf life (see label on the outer packing), if stored properly. The components of different batches may not be mixed.
- Before first use, rehydrate the lyophilized Positive Control: add 20 µl of Negative Control (Nuclease-free water) per vial, incubate briefly at room temperature and mix thoroughly by pulse-vortexing. Generate aliquots of suitable volumes and store them at -18 °C to -20 °C.
- Before first application, reconstitute the final Lysis Buffer by carefully pouring the Lysis Beads into the DNA Extraction-Mix II.
- This kit can be used on all commercially available Real-Time PCR thermal cyclers that detect the emitted fluorescence of the fluorescent dyes FAM and HEX. The following Real-Time PCR thermal cyclers e.g. have been verified for routine diagnostics with this kit: Mastercycler® RealPlex2 (Eppendorf), Rotor-Gene® 3000, 6000 & Q (Corbett / QIAGEN), Chromo4™ & CFX96 Touch™ (BIO-RAD), Applied Biosystems® 7500 & 7500 Fast (Life Technologies), Mx3005P (Stratagene / Agilent Technologies), Eco™ (Illumina®) and LightCycler® 96 & 480 and LightCycler® 2.0 (Roche).
- We recommend to exclusively use certified Nuclease-free materials and powder-free protective gloves. Please wear gloves during the entire experimental procedure. Gloves have to be changed frequently, especially after spillage or other suspected contamination. Pipette tips must be changed between each sample to avoid cross-contamination.

- Apart from the disposables, the following devices are needed (not included in this kit):
  - Stomacher®, masticator or equivalent for homogenizing test samples
  - Incubator, capable of maintaining  $+30\text{ °C} \pm 1\text{ °C}$
  - Dry heating block ( $+100\text{ °C} \pm 3\text{ °C}$ )
  - Table top microcentrifuge
  - Vortex
  - Magnetic stirrer
  - Bench top homogenisator for cell disruption
  - Micropipettes covering volumes of 1  $\mu\text{l}$  to 1000  $\mu\text{l}$
  - Centrifuge for PCR tubes or plates
  - PCR thermal cycler

### C. Control Reactions

- The Negative Control included in the kit allows exclusion of possible contaminations of the reagents. The Positive Control included in this kit provides the ability to monitor the specificity and efficiency of the reagents used, i.e. the performance of amplification itself and of the PCR thermal cycler, respectively. The test is only valid if both, Positive and Negative Controls, are used and verified for validity in every Real-Time PCR run.
- An Internal Amplification Control is included in the Reaction-Mix in defined copy number; it is co-amplified (channel HEX) with every single reaction to detect possible effect of the DNA preparation and to verify true negative results.

### D. Protocol

- The overall protocol of the *L. monocytogenes* analysis consists of the following workflow:
  1. cultural pre-enrichment
  2. DNA preparation
  3. reaction setup and amplification (PCR)
  4. data analysis – validity and qualitative result

#### 1. Cultural Pre-Enrichment

- All samples are homogenized with nine times the sample volume of Half Fraser Broth in a stomacher bag (as a rule e.g. 25 g or 25 ml sample material in 225 ml Half Fraser). To enable sufficient propagation of potentially present *L. monocytogenes* during incubation time, the Half Fraser Broth has to be pre-warmed to the incubation temperature prior to the start of incubation time. The pre-enrichment is incubated at  $+30\text{ °C} \pm 1\text{ °C}$  for 25 h  $\pm$  1 h without agitation.
- Transfer 1.5 ml of the supernatant of the pre-enrichment into a sterile screw cap tube.
- **Attention:** Mixing of the pre-enrichment after incubation by shaking or any other agitation must be avoided. Avoid transferring solid or greasy debris. An aliquot should be taken directly below the surface, avoiding floating particles.
- For samples that are too difficult to separate from the debris after concentration, Stomacher bags with filters can be used for pre-enrichment with Half Fraser Broth.

## 2. DNA Preparation

- Pre-heat heating block to set temperature of +100 °C, the block must have an actual temperature of +100 °C ± 3 °C at first use.
- The final Lysis Buffer (DNA Extraction-Mix II and Lysis Beads) is stirred on a magnetic stirrer, it must be used as a homogenous suspension.  
*Note: Gently shake the final Lysis Buffer by hand first to resuspend the beads. Stir the final Lysis Buffer on a medium moving magnetic stirrer and pipette the reagent during stirring. Use pipettes with a wide tip to allow pipetting of the particles in the homogenized lysis buffer.*
- The aliquoted pre-enrichment in the conical screw cap tube is pelleted via centrifugation at 10.000 g to 12.000 g for 5 min.
- Remove the supernatant using a 1000 µl pipette tip (not by decantation) and discard it.
- The pellet is resuspended by repeated up-and-down pipetting with 250 µl of the final Lysis Buffer. Use a 1000 µl pipette with filtered tips. Avoid formation of bubbles and aerosols.
- Place the screw cap tube in a cell disruptor for 3 min ± 1 min. In case of using instruments with higher efficiency reduce the time according to the manufacturer's instructions.
- Incubate the sample for 10 min to 15 min at +100 °C ± 3 °C.
- Vortex sample thoroughly and centrifuge at 10.000 g to 12.000 g for 5 min; the supernatant is the DNA extract and can be used for Real-Time PCR immediately. Short-term storage (few hours) of the DNA extract at +2 °C to +8 °C is possible. For long-term storage of the DNA extract at -18 °C to -20 °C, take supernatant and transfer to new (screw cap) tube. Before next use in Real-Time PCR incubate DNA extracts that were stored at -18 °C to -20 °C for few minutes at +100 °C ± 3 °C, vortex and spin down.

## 3. Reaction Setup and Amplification

- Before each use, briefly vortex and spin down the Reaction-Mix and the Negative Control (see also chapter B "Reagents and Controls").
- Determine the number of needed PCR-reactions: number of samples + Positive Control and Negative Control.
- Pipette 18 µl of Reaction-Mix to each PCR-reaction tube / each PCR-plate well ("cavity"). Expose the Reaction-Mix as short as possible to (sun)light and return it back to -18 °C to -20 °C right after application. Avoid the formation of bubbles when pipetting the Reaction-Mix, samples and controls.
- Add 2 µl of Negative Control to the corresponding cavity and seal it individually, if possible.
- Add 2 µl of sample DNA (final DNA extract) to the corresponding cavity and seal it individually, if possible. Solely use clear supernatant of DNA extract, **do not transfer particles from the bottom of the tube**.
- To minimize risk of potential cross-contaminations, 2 µl of the Positive Control are added to the corresponding cavity after all previous samples and control reactions are set up. Before each use, briefly vortex and spin down the rehydrated Positive Control (see also chapter B "Reagents and Materials").

- It is recommended to spin the tubes down before start of the PCR run. Place the cavities in the Real-Time PCR thermal cycler and run the test using the following parameters:

Step	Temperature	Duration	
Activation of Polymerase	95 °C	10 min	
Denaturation	95 °C	15 sec	} 42 cycles
Annealing & Extension	60 °C	1 min	
Fluorescence Detection	channels FAM and HEX		

- Please follow the specified instructions of your PCR thermal cycler as recommended by the manufacturer.
- The amplification parameters enable the combination of Kylt® *Listeria monocytogenes* with other Kylt® Real-Time PCR Kits for detection of bacteria. When combining several detection tests, please make sure to detect all relevant channels.

#### 4.Data Analysis – Validity and Qualitative Results

##### General

- The amplification data can be processed automatically using the specific software tool of your Real-Time PCR thermal cycler. Alternatively, the threshold can be set manually considering the following directions: The threshold should be at least a ten-fold standard deviation above the average fluorescence value of cycles 3 to at least 15. The threshold should cross the HEX- and the FAM-curves in the linear area of its slope (log scaling of the y-axis). By setting the thresholds, the crossing points with the HEX- and FAM-curves determine the respective cycle threshold (Ct).
- For the test analysis the validity of the Real-Time PCR run is determined with the Negative and Positive Control. By means of the Internal Amplification Control the validity of each sample reaction and its true test results can be verified according to the Ct-value of the Internal Amplification Control channel (HEX). Finally, the *L. monocytogenes*-specific status of each sample is analyzed (FAM).

##### Test Evaluation

- The Real-Time PCR run is only valid if the FAM-curve of the Negative Control is negative, if the HEX-curve of the Negative Control is positive and if the FAM-curve of the Positive Control is positive. For a valid test the FAM-Ct-value of the Positive Control has to be > 15 and ≤ 35 and the HEX-Ct-value of the Negative Control has to be > 10 and ≤ 40.

HEX-curve is	positive	positive	negative	negative
FAM-curve is	negative	positive	positive	negative
The sample is <i>L. monocytogenes</i>	negative	positive	positive	inhibited

- A **sample** is **negative**, if its FAM-curve is negative and the HEX-curve is positive ( $10 < Ct \leq 40$ ).
- A **sample** is **positive**, if its FAM-curve is positive ( $10 \leq Ct \leq 42$ ), independent of the HEX-curve.
- A **sample** is **inhibited**, if neither the HEX-curve nor the FAM-curve are positive.
- **Recommendation:** In the case of an inhibited sample you may repeat the test by using e.g. a 1:10 dilution of the respective DNA extract. The Negative Control serves as the diluting agent. In this case mix nine volumes of Negative Control with one volume of eluted DNA. Alternatively, repeat the DNA extraction procedure from the start.
- Convenient and reliable sample data input, start of the Real-Time PCR run as well as final qualitative test results analysis and documentation can be managed by the Kylt® Software package. Please feel free to contact us.

Production:

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