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# Listeria monocytogenes FLUO kit

**REF MBK0056–50 Reactions**

**REF MBK0090–100 Reactions**

**REF MBK0077 PL–384 Reactions (96 x 4)**

**INTENDED USE**

The kit could be used for the identification of *Listeria monocytogenes* by multiplex Real-Time PCR.

**INTRODUCTION**

Intoxications and infections caused by food-borne pathogens represent an increasing public health problem, with nearly a quarter of the population at higher risk for illness today (Oliver et al., 2005). Several outbreaks of food-borne illnesses following consumption of several food caused by *L. monocytogenes* have been reported in recent years, indicating the importance of this problem in safeguarding public health.
The fast and accurate identification of *L. monocytogenes* from food samples by Public health agencies and diagnostic laboratories insures not only a better quality of products, but also the possibility to adopt timely precautionary measures to limit the spread of infection in case of an outbreak. Conventional assays in common use can take up to days or more.
The *Listeria monocytogenes FLUO kit* represents an alternative PCR-based approach for the qualitative detection of this pathogen. The kit uses DNA primers and fluorescent probe specific for the target organism. If pathogen is present, DNA is amplified and the increased fluorescence signals are recorded in real time. The internal control, present in the amplification mix, assesses the efficiency of amplification reaction by checking the presence of inhibitory factors and ensuring reliability of negative results. A multiplex assay with two dyes is used: probes for the target DNA and the internal control, each labelled with different fluorophores are in the same tube. Results are obtained within a few hours following an enrichment step and subsequent DNA extraction.

Specificity: 100% (tested on a panel of 90 bacterial strains target and non target).
Sensitivity: 1 cfu after enrichment.

**PRODUCT DESCRIPTION**

The *Listeria monocytogenes FLUO kit* provides an easy-to-use Master mix dedicated for the use with the instruments with two-plex capability. The kit contains reagent, enzyme and positive control for the successful amplification and detection of DNA from *L. monocytogenes* in multiplex Real-Time PCR using dual-labelled probes. Up to 2 genes (1 control gene and 1 target gene) can be detected simultaneously in the same reaction.

**KIT CONTENTS**

| Component | MBK0056 | MBK0090 | MBK0077 PL |
|---|---|---|---|
| 1X Master mix | 2 x 0.5 ml | 4 x 0.5 ml | 8 x 1.100 µl |
| DNA polymerase (5U/µl) | 1 x 15 µl | 2 x 15 µl | 1 x 90 µl |
| Positive Control ($10^5$ cells/5 µl) | 1 x 0.1 ml | 2 x 0.1 ml | 1 x 0.1 ml |
| PCR grade water | 1 x 1 ml | 2 x 1 ml | 1 x 1 ml |
| ROX | 2 x 5 µl | 4 x 5 µl | 4 x 10 µl |

**ADDITIONAL EQUIPMENT**

- Gloves
- Pipette and pipette tips with aerosol preventive filter
- 1.5 ml microcentrifuge tubes
- 96 well PCR plate
- Vortex
- Microcentrifuge

**STORAGE**

The product should be stored immediately upon arrival at -20 °C, protect from light.
If properly stored, see the expiration date for the stability of the kit.

**PRECAUTIONS**

The user should always pay attention to:

- use pipette tips with aerosol-preventive filters, deionised DNA-free water and gloves;

- store positive material (specimens, controls and amplicons) separately from all other reagents and if possible, add it to the reaction mix in a separated space;
- do not use the same precision pipettes for reaction mix and DNA;
- thaw all components and samples at room temperature before starting an assay, when thawed, mix the components and centrifuge briefly;
- do not use reagents after their expiration date
- verify the accuracy and precision of pipettes, as well the correct functioning of instruments;
- clean works space periodically with at least 10% bleach or other decontaminant agent.

## 1. PROCEDURE

### 1.1 DNA ISOLATION

*Food and environmental samples*

For sample preparation please refer to manual of Bacterial DNA Isolation Single Step (Diatheva cod. MBK0063-MBK0076).

*Colony*

1. Dispense 100 µl of DNase free water in a 1.5 ml tube and dissolve the colony.
2. Boil the sample for 10 minutes.
3. Centrifuge at 14 000 rpm for 10 minutes.
4. Collect the supernatant in a 1.5 ml tube taking care to do not disrupt the pellet.
5. Mix and use 2 µl of the sample in the Real-Time PCR reaction.

### 1.2 PCR SET-UP

All detection experiments should include an NTC (No Template Control), containing all the components of the reaction except for the template. This enables detection of potential contamination. Moreover include at least one positive control. Total volume per reaction is 25 µl.

- Thaw the components protect from light. Vortex 1X Master Mix and ROX for 10" and centrifuge briefly.

- Upon first use of 1X Master Mix, for instruments that require a passive reference dye (see below), it is necessary to add the ROX:
  -Low ROX: ABI Prism® 7500, 7500 Fast, QuantStudio3. QuantStudio5
  -High ROX: ABI Prism® 7000, 7300, 7700, 7900, 7900HT, StepOne, StepOne Plus.

- The 1X Master Mix should be completed immediately before the use according to the instructions below:
- For Low Rox instruments → add 15µl of the PCR grade water to the vial containing the 10µl ROX and vortex for 30". Proceed by completing 1X Master Mix with the addition of ROX according to table below.

| | MBK0056 or MBK0090 (1 vial-500 µl) | MBK0077 PL (1 vial-1100 µl) |
|---|---|---|
| Low Rox | 0.35 µl | 0.78 µl |

- -For High Rox instruments → the ROX provided in the kit is ready to-use (no dilution is required). Proceed by completing 1X Master Mix with the addition of ROX according to table below.

| | MBK0056 or MBK0090 (1 vial-500 µl) | MBK0077 PL (1 vial-1100 µl) |
|---|---|---|
| High Rox | 1.56 µl | 3.5 µl |

- Note: It is recommended to complete the ROX and add to the 1X Master Mix only before the use.
- Vortex for 10'.

In one sterile tube prepare the amplification reaction mix needed for each sample to be tested plus one NTC and one Positive Control following the pipette scheme below:

| | 1 reaction* | 96 well PCR Plate** |
|---|---|---|
| 1X Master Mix | 19.8 µl | 2098.8 µl |
| DNA Polymerase (5U/µl) | 0.2 µl | 21.2 µl |
| Total volume | 20 µl | 2120 µl |

*For the analysis of more than one sample, multiply the volumes of 1X Master Mix and DNA Polymerase for the number of samples to be tested plus one or two additional reactions to cover pipetting losses.
**For 96 well PCR plate 10 additional reactions are considered.

- Vortex for 10" the vial containing the prepared master mix and centrifuge briefly;
- Aliquot 20 µl of 1X Master Mix in the plate prepared for the experiment;
- Add 5 µl of PCR Grade water into NTC;
- In a separate area, add 5 µl of DNA samples to be tested into the corresponding well containing amplification mixes;
- Add 5 µl of Positive Control;

If possible after pipetting the negative control and the samples, the tubes must be sealed in order to avoid cross-contamination during the addition of Positive Control.

**1.3 THERMAL PROFILE**

Optimal instrument and fluorescence analysis settings are a prerequisite for accurate results. For details, please refer to the manual provided with your Real-Time PCR instrument. The kit has been optimized to be used with ABI 7500 (Applied Biosystems), StepOne and StepOne Plus instrument (Applied Biosystems). Program the Real-Time PCR instrument according to the operator's manual:

| | | | |
|---|---|---|---|
| Denaturation | 95°C | 1 min | 1 X |
| Denaturation | 95°C | 15 sec | 50 X |
| Annealing | 60°C | 30 sec | |
| Extension | 72°C | 30 sec | |
| Acquire on the GREEN (FAM) and YELLOW (VIC) channels **during the annealing step.** Set the gain optimization on NTC sample before 1st acquisition (ROTOR GENE ISTRUMENTS) | | | |

**Please note:** if your instrument requires ROX as passive reference, select ROX as passive reference dye. Verify that fluorophores available for the acquisition channels listed above are calibrated. Select non fluorescent quencher.

| **Target species** | **Acquisition channel** |
|---|---|
| *L. monocytogenes* | Green, FAM (Ex 495-Em 520 nm) |
| Internal Amplification Control | Yellow, VIC/HEX/JOE (Ex 538-Em 554 nm) |

**1.4 ANALYSIS SETUP**

The analysis of the results should be done with the program included in the recommendations provided by the manufacturer of the instrument. In some cases, it is possible that the program will go automatically setting the baseline. In this case it is advisable to check these settings. For a correct definition of the threshold, it is necessary to select a value distinction from the background after the linear phase growth. Analyze each sample in the two acquisition channels.

**1.5 INTERPRETATION OF RESULTS**

**Controls:** Before interpreting sample results, it is necessary to verify the positive and negative controls. For the experiment to be valid, the controls must have the following results:

| | **Target** | **Internal Amplification Control** |
|---|---|---|
| **Negative control** | No amplification | 20≤Ct≤32 |
| **Positive control** | 15≤Ct≤25 | Not significant* |

*The amplification in this channel may also not be present.

**Samples:** check that the curves are typical amplification curves. If the Ct value in the Green channel is ≤10, verify in the raw data that the curve is a regular amplification curve. If correct the sample could be considered positive for *L. monocytogenes*.

| **Target** | **Internal Amplification Control** | **Interpretation** |
|---|---|---|
| No amplification | 20≤Ct≤32 | Sample negative for *L. monocytogenes* |
| No amplification | No amplification | *Inhibition |
| Ct≥10 | Not significant | Sample positive *for L. monocytogenes* |

*In case of inhibition is necessary dilute sample and repeat a further PCR.

**1.6 CONFIRMATION OF POSITIVE RESULTS**

All positive PCR results need to be confirmed according to the reference method ISO 11290 or following the scheme below.

*FLOW DIAGRAM OF CONFIRMATION STEP*
*of PCR positive results*

| Procedure for foods and environmental sample |
|---|
| Streak 100 µl onto<br>OAA formulation<br>↓<br>Incubate for 24 h ± 3 h<br>at 37°C ± 1°C<br>↓<br>Observe characteristic colonies<br>of *L. monocytogenes* |
| OAA-Ottaviani and Agosti (O&A) formulation agar |

**TROUBLESHOOTING**

| | | |
|---|---|---|
| No signal, poor Rn value (PCR) or signal detected late in PCR | Pipetting error or missing reagent | Check the storage conditions of the reagents, repeat the assay. |
| | Problems with starting template DNA | Check the concentration, storage conditions, and quality of the template and control DNA.<br><br>Efficient removal of PCR inhibitors is essential for optimal results. Purify nucleic acids from your sample using an appropriate purification method.<br><br>Insufficient or degraded template DNA, increase the amount of template DNA if possible. |

**REFERENCES**

**Oliver, S. P., Jayarao, B. M. and Almeida, R. A.**, 2005. Foodborne pathogens in milk and the dairy farm environment: Food safety and public health implications. Foodborne Pathogen Disease 2, 115-129.

**IMAGES**

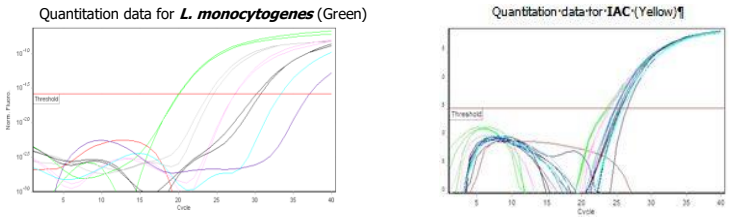


**Fig. 1:** Representative amplification plots are shown. Serial dilutions of *L. monocytogenes* genomic DNA, equivalent to $10^6$ down to 10 target molecules per reaction were used. Each graph displays amplification plots generated with the indicated concentrations.