Via Sant Anna 131-135
61030 Cartoceto PU (IT)
Telephone + 39 (0)721830605
FAX +39 (0)721837154
e-mail:info@diatheva.com
www.diatheva.com

MultipathogenFLUO kit

REF MBK0019 - 50 Reactions

INTRODUCTION AND PRODUCT DESCRIPTION

Foodborne diseases are a serious global health problem. Microbial food safety is an increasing public health concern worldwide. Each year, as many as 600 million, or almost one in 10 people in the world, fall ill after consuming some sort of contaminated food. Several outbreaks of food-borne illnesses following consumption of several food caused by Shiga toxin-producing E. coli, Salmonella spp. and L. monocytogenes have been reported in recent years, indicating the importance of this problem in safeguarding public health. (CDC, 2015). Conventional bacteriological method are often long and tedious.
The MultiphatogenFLUO kit is intended for the simultaneous molecular detection of *Salmonella* spp., *L. monocytogenes* and *E. coli* O157, by multiplex Real-time PCR. The kit uses DNA primers and fluorescent probes specific for the target organisms. If pathogens are present, DNAs are amplified and the increased fluorescence signals are recordered in Real-Time. The internal amplification control, presents in the amplification mix, assesses the efficiency of each amplification reaction by checking the presence of inhibitory factors and ensuring reliability of negative results. Results are obtained within few hours following an enrichment step and subsequent DNA extraction.

The MultipathogenFLUO kit provides a ready-to-use Master Mix dedicated for the use with the instruments with four-plex capability, and positive control.

KIT CONTENTS

Component	MBK0019
1X Master Mix	2 x 0.5 ml
Positive Control (10^5 cells for each target species/5 µl)	1 x 0.2 ml
PCR Grade Water	1 x 1 ml

STORAGE

Store the kit at -20°C upon arrival, protect from light. If properly stored, see the expiration date for the stability of the kit.

ADDITIONAL EQUIPMENT

- Gloves
- Pipette and pipette tips with aereosol preventive filter
- 1.5 ml microcentrifuge tubes
- 0.2 ml PCR tubes
- Vortexer
- Microcentrifuge

PRECAUTIONS AND RECOMMENDATIONS

- The test must be performed by specialised, trained and authorised staff,
- Do not use reagents after the expiry date printed on the label,
- Use gloves as well as sterile pipet tips with filters. Change gloves often, especially if you suspect a possible contamination of them,
- It is suggested to provide separate and dedicated spaces, material and equipment for preand post-PCR amplification stages,
- Clean working space periodically with at least 5% sodium hypochlorite or another decontaminant agent.

1. PROCEDURE

1.1 DNA ISOLATION

Food 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 MIX PREPARATION

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 PCR reaction is 25 µl.

- Thaw the components (1X Master Mix, PCR Grade Water and Positive Control), protect from light, vortex 15" and centrifuge briefly.
- Aliquot 20µl of 1X Master Mix in 0.2 ml tubes or alternatively in the plate prepared for the experiment;
- Add 5 µl of PCR Grade Water into NTC;
- In a separate area, add 5µl of DNA samples to be tested into the corresponding PCR tubes or wells containing amplification mix;
- Add 5 µl of Positive control into the corresponding PCR tubes or wells containing amplification mix;
- Seal hermetically the PCR tubes or plate and load into the real-time PCR instrument, following the manufacturer's instructions.

Note: for PCR instrument with 96 wells block check that the reagents are at the bottom of each well, if not, briefly centrifuge at 800 x g for 1 minute.

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 Rotor-Gene Q (Qiagen), Rotor-Gene 6000 (Corbett) instruments. Otherwise it could be used also with other instruments provided with acquisition channels listed below.

1.3 PROGRAM SETUP

Program the Real-Time PCR instrument according to the operator's manual and the table below:

STEP	*TEMPERATURE*	*TIME*	*CYCLES*
Initial denaturation	95°C	3 min	1X
Denaturation	95°C	20 sec	45 X
Annealing/Extension	60°C	1 min	
Acquire on the GREEN (FAM), YELLOW (VIC), RED (QUASAR 670), ORANGE (CAL Fluor Red 610) channels during Annealing/Extension; if possible, set the gain optimization on NTC sample before 1st acquisition.			

Please note: The kit is formulated for Real-Time PCR instruments that not required ROX as passive reference (e.g. Rotor-Gene 6000 Corbett; CFX96 Biorad; MX3000P, Stratagene).
PLEASE NOTE: Verify that fluorophores available for the acquisition channel listed in the table above are calibrated. Select non fluorescent quencher.

Target species	**Acquisition channel**
Salmonella spp.	Green, FAM (Ex 495-Em 520 nm)
L. monocytogenes	Red, QUASAR 670 (Ex 647-Em 670 nm)
E. coli O157	Orange, CAL FLUOR RED 610 (Ex 590-Em 610nm)
Internal Amplification Control	Yellow, VIC (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. 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 four 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:

	***Salmonella* spp. (Green)**	***L.monocytogenes* (Red)**	***E. coli* O157 (Orange)**	**Internal Amplification Control (Yellow)**
Negative Control	No amplification	No amplification	No amplification	$20 \leq Ct \leq 30$
Positive Control	$15 \leq Ct \leq 25$	$15 \leq Ct \leq 25$	$15 \leq Ct \leq 25$	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, Red or Orange 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 *Salmonella* spp., *L. monocytogenes* or *E. coli* O157, respectively.

Target*	**Internal Amplification Control**	**Interpretation**
No amplification	$20 \leq Ct \leq 32$	Negative
No amplification	No amplification	Inhibition**
$Ct \geq 10$	Not significant	Positive

**Salmonella* spp., *L. monocytogenes* and *E. coli* O157
**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 (for *L monocytogenes*), ISO 6579 (for *Salmonella* spp.) and ISO 16654 (for *E. coli* O157)

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. Efficient removal of PCR inhibitors is essential for optimal results. Purify nucleic acids from your sample using an appropriate purification method. Insufficient or degraded template DNA, increase the amount of template DNA if possible.

REFERENCES

Centres for Disease Control (CDC). 2015. Surveillance of foodborne diseases outbreaks in the United States, 2013: annual report. Atlanta, Georgia: US Department of Health and Human Services, CDC