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E. coli O157 FLUO kit

REF MBK0071 – 50 Reactions

INTENDED USE

The kit could be used for the identification of *E. coli* O157:H7 by 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). The fast and accurate identification of *E.coli* O157:H7 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.
The *E. coli O157 FLUO kit* represents an alternative PCR-based approach for the qualitative detection of this pathogen from enriched samples or bacterial colony. 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. Results are obtained within a few hours following an enrichment step and subsequent DNA extraction.

SENSIBILITY

Sensibility: 1 cfu after enrichment.
Limit of detection: 10 Genome Units (GU).

SPECIFICITY

Specificity: 100% (tested on a panel of 90 bacterial strain target and non target).

KIT CONTENTS

2 x 0.5 ml 1X Master mix
1 x 10 µl 5U/µl Hot rescue DNA polymerase
1 x 0.2 ml Positive Control (10^5 cells of E. coli O157/5µl)
1 x 1 ml DNAse free water
2 x 5 µl ROX
1 x 2 ml Dilution Buffer

ADDITIONAL EQUIPMENT

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

STORAGE

The product should be stored immediately upon arrival at -20°C, protect from light. Avoid repeated freeze-thawing cycles. For discontinued use, storage of mixes in working aliquots is recommended.

PRECAUTIONS

The user should always pay attention to:

- use pipette tips with aerosol-preventive filters, deionized 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.

PROCEDURE

1.1 DNA ISOLATION

Food and environmental samples

Enrichment media must be warmed at room temperature before using it. It is strongly recommended the use of stomacher bags with filter.

Prepare the sample placing x g of sample into a stomacher bag with filter and add y ml of the enrichment culture medium. The sample must be diluted 1:10 in the enrichment medium (for example 25 g in 225 ml, 10 g in 90 ml). Homogenize the sample and incubate it at temperature and times described in the ISO reference methods.

Isolate the bacterial DNA using the Fast DNA Extraction kit (Diatheva, Cod. MBK0061) or Bacterial DNA Isolation single step (Diatheva cod. MBK0063) according to the procedure.

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

- Thaw the components protect from light. Vortex Multiplex PCR Master Mix for 10" and centrifuge briefly.
- Upon first use of Multiplex PCR Master Mix, for instruments that require a passive reference dye (ex. Applied Biosystems), it is necessary to add the ROX.
  Dilute the ROX (5 µl) by the addition of Dilution Buffer according to the type of Real-Time PCR thermal cycler:

Instrument	ABI Prism® 7300 7500	ABI Prism® 7000- 7700-7900HT SDS GeneAMP5700
Volume of Dilution Buffer to add to ROX	195	45

Note: it is recommended to dilute the ROX just before use, and directly into the vial of ROX provided. The kit provides 2 vials of ROX, one for each vials of Multiplex PCR Master Mix provided in the kit.

- Vortex for 20" and centrifuge briefly.
- Proceed by completing Multiplex PCR Master Mix (directly in the vial in which is provided) with the addition of ROX or Dilution Buffer according to the following scheme.

	For 1 vial	For 1 vial	For 1 vial
Real-Time PCR Instrument	ABI Prism® 7300, 7500	ABI Prism® 7000, 7700, 7900HT SDS GeneAMP5700	Bio-Rad, Rotor-Gene Q, Rotor-Gene 6000 Stratagene 3005/3000p
Multiplex PCR Master Mix	500 µl	500 µl	500 µl
ROX	4.5 µl	4.5 µl	-
Dilution Buffer	-	-	4.5 µl

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.

In one sterile 1.5 ml 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	6 samples + 1 NTC + 1 Positive control *
Multiplex PCR Master Mix	19.8 µl	158.4 µl
DNA Polymerase (5U/µl)	0.2 µl	1.6 µl
Total volume	20 µl	160 µl

*For the analysis of more than one sample, simply multiply the volumes of Multiplex PCR Master Mix and DNA Polymerase for the number of samples to be tested considering the NTC and Positive control.

- Vortex for 15" the vial containing the prepared master mix and centrifuge briefly;
- Aliquot 20 µl of master mix in 0.2 ml tubes or alternatively in the plate prepared for the experiment
- Add 5 µl of DNAse free water into NTC;
- In a separate area, add 5 µl of DNA samples to be tested into the corresponding PCR tubes or wells containing amplification mixes;
- Add 5 µl of Positive Control;

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 Rotor-Gene Q (Qiagen), Rotor-Gene 6000 (Corbett) and ABI 7500 (Applied Biosystems) instrument. Otherwise it could be used also with other instruments provided with acquisition channel listed below.
Program the Real-Time PCR instrument according to the operator's manual considering a total volume per reaction of 25 µl.

Denaturation	95°C	10 min	1 X
Denaturation	95°C	20 sec	40 X*
Annealing/Extension	63°C	1 min	
Acquire on the GREEN (FAM) and YELLOW (VIC) channels during Annealing/Extension; if possible, set the gain optimization on NTC sample before 1st acquisition.			

*45 cycles for instrument based on Peltier block technology (ex. 7500 Applied Biosystem, CFX96 Biorad, MX3000P Stratagene etc.)

Please note: if your instrument require 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**
E. coli O 157	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 Green	**Controllo interno di amplificazione Yellow**
Negative control	No amplification	20≤Ct≤36
Positive control	15≤Ct≤25	Not significant*

*The amplification in this signal 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 *E. coli* O 157.

Target	**IAC**	**Interpretation**
No amplification	20≤Ct≤36	Sample negative
No amplification	No amplification	Inhibition
Ct≥10	Positive/negative	Sample positive

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

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