Detection of *Escherichia coli* serogroups DNA using real-time PCR

VALIDATION REPORT

ASSAY

Escherichia coli O157, O26, O111, O103, and O145 detection by 5' nuclease real time-PCR assay.

VALIDATED EQUIPMENT

Applied Biosystems, 7500 model; Thermo Scientific, model PikoReal.

SAMPLES (N)

Enrichments prepared from suitable food samples.

VALIDATION ASSAYS

Start – 01-10-2013
Finish – 01-05-2014

SCOPE OF THE METHOD

The test is designed for the detection the genes associated with the serogroups O157, O111, O26, O103, and O145, based on a 5' nuclease real-time PCR reactions. The method's performance was tested according ISO 22118:2011 and ISO/TS 13136:2012 and fulfils the requirements set in this International Standard.

Conventional serotyping is a method based in antibody-antigen agglutination reactions. These is a very complex and time-consuming method. Recently, rapid and sensitive methods have been found to be attractive alternatives to the *E. coli* conventional serotyping method. These methods are PCR-based methods, in particular real-time-PCR-based, that allows the rapid detection of different antigen genes, which in turn allow the determination of *E. coli* serogroups.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 5 food samples enriched according the method described in ISO 16654:2001 and 45 *Escherichia coli* strains. After enrichment, STEC colonies suspected were isolated, resuspended in the lysis solution and DNA extracted. These food samples included 5 different matrices, aleatory taken from commercial shops:

- Cheese
- Milk
- Meat
- Processed meat
- Soil

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing mustard DNA

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

	Target detection Chanels 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

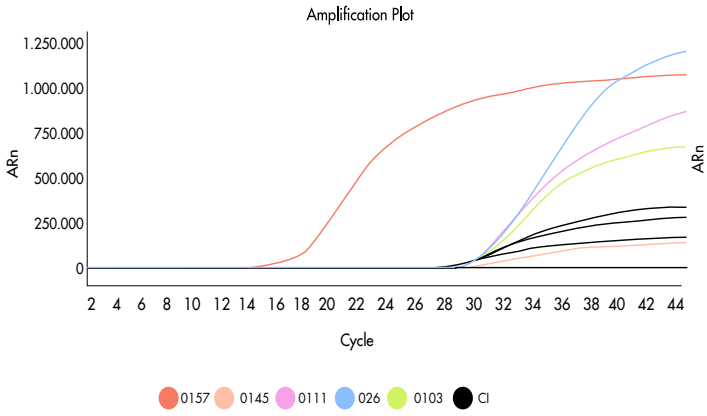
If the controls do not match these results, the experiment must be repeated.

The results obtained by the present method shall be interpreted as follows:

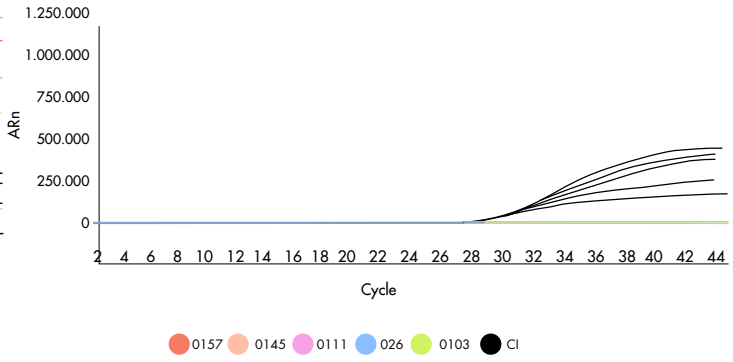
Target detection Chanels 1	Target detection Chanels 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

**When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

Positive control



Negative control



2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific).

3. SPECIFICITY

For this propose we used pre- enriched positive samples for O157, O26, O111, O103, and O145 of Escherichia coli that were identified by an accredited method. A total of 5 pre-enriched samples were tested (Table 3.1).

Code	Strain identification	Other informations
E 2	*Enriched cheese*	
E 112	*Enriched milk*	
E 218	*Enriched processed meat*	
E 600	*Enriched soil*	
E 662	*Enriched meat*	

Table 3.1 – List of Escherichia coli strains used for inclusivity test

Result:

All strains were detected with the present method, corresponding to 100% inclusivity.

3.2. Exclusivity test

The strains for exclusivity test were chosen following the recommendations of International Standard ISO 22118:2011. A total of 30 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.2). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations
MB 385	*Listeria monocytogenes*	CECT 4031
MB 388	*Listeria monocytogenes*	Serotype 1/2a ou 3a
MB 381	*Listeria innocua*	CECT 910
MB 357	*Escherichia coli*	NCTC 9001
MB 372	*Campylobacter jejuni subsp jejuni*	NCTC 11351
MB 373	*Campylobacter coli*	NCTC 11366
MB 399	*Salmonella Typhimurium*	CECT 443
MB 22	*Vibrio cholerae*	Local isolate
MB 356	*Vibrio parahaemolyticus*	NCTC 10885
MB 363	*Bacillus cereus*	IFM1600
MB 115	*Citrobacter freundii*	Local isolate
MB 451	*Cronobacter muytjensii*	ATCC 51329
MB 141	*Lactobacillus plantarum*	CECT 748 T
MB 144	*Lactobacillus paracasei subsp paracasei*	CECT 4022T
MB 150	*Staphylococcus aureus*	NCTC 6571
MB 302	*Pseudomonas aeruginosa*	Local isolate
MB 13	*Serratia marcescens*	Local isolate
MB 163	*Proteus vulgaris*	Local isolate
MB 365	*Enterococcus faecalis*	Local isolate
MB 306	*Enterococcus hirae*	Local isolate
MB 147	*Staphylococcus epidermidis*	Local isolate
MB 267	*Shigella flexneri*	Local isolate
MB 249	*Yersinia enterocolitica*	Local isolate
MB 178	*Klebsiella pneumoniae*	Local isolate
MB 449	*Brochothrix thermosphacta*	ATCC 11509
MB 274	*Legionella pneumophila*	Local isolate
MB 278	*Legionella micdadei*	Local isolate
MB 362	*Clostridium perfringens*	Local isolate
MF 128	*Saccharomyces cerevisae*	CECT
MF 129	*Aspergillus niger*	Local isolate

Table 3.2 – List of strains used for exclusivity test

Result:
The method presented 100% exclusivity as none of the strains showed a positive results and positive result.

4. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 4.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer		Not applicable
Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 4.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

5. TRUENESS

Trueness of the method was evaluated using 6 positive and 11 negative food samples. All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%.

Performance declaration

Accordingly with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.

The present test allows the "Detection of *E. coli* serogroups O157, O26, O111, O103, and O145 using real-time PCR".

Condalab

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