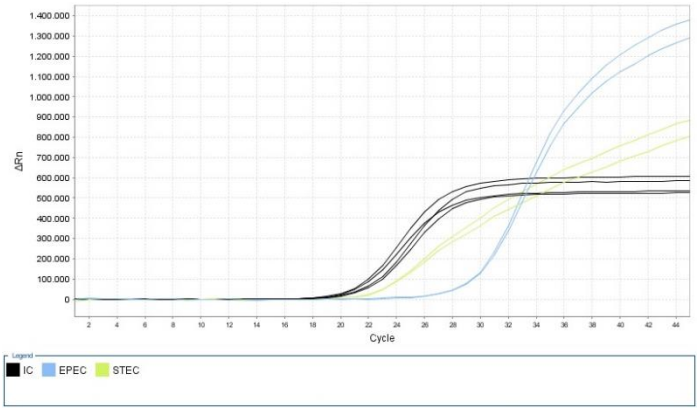
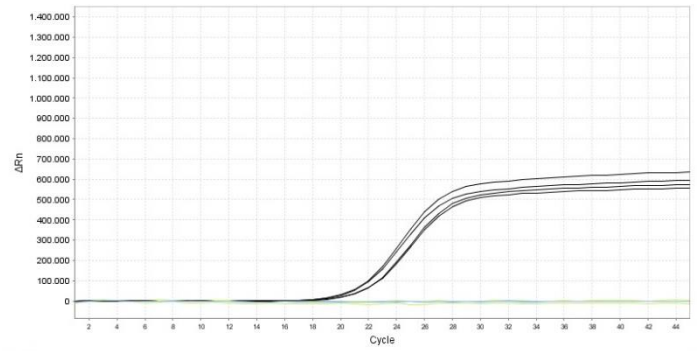
Positive Control –



Negative Control –



11. SPECIFICITY/INCLUSIVITY

a) 100 % Exclusivity, determined using 37 strains of closely related organisms or occurring in the same habitat (Table 1). No statically significant differences were obtained between the performances of small variations in method parameters, which proves that the SUPREME REAL TIME DETECTION KIT E. coli is robust.

b) For inclusivity, 66 *E. coli* strains containing *stx1* and/or *stx2* and *eae* genes (such as O157, O26, O1O3, O111, and O145) of 67 were correctly detected by the corresponding gene target (Table 2). The samples used were mainly culture collections, proficiency tests and local isolates. Each strain were enrich in mTSB+N at 37 ± 1°C for 18–24 h per ISO/TS 1316:20125.

12. PERFORMANCE CHARACTERISTICS

A detection limit of 1 to 10 Cells per 25g of food sample can be achieved after enrichment. In the AOAC validation study, the SUPREME REAL TIME DETECTION KIT *E. coli* showed a comparable detection or performance to the reference method (ISO/TS 13136:20125) for the foods tested, raw ground beef, orange juice, salad (green, purple lettuce and coriander) and cream cheese.

Note: In the context of the AOAC validated method, a positive result is considered presumptive positive and it is recommended further confirmation.

13. CONFIRMATION

Samples producing positive results for SUPREME Real Time Detection Kit *E. coli* can be confirmed according to the ISO/TS 13136: 2012 (or most current ISO reference procedure). EPEC/STEC strains can be isolated from the enrichment by streaking onto tryptone bile x-glucuronide agar as described in Annex F of ISO/TS 13136: 2012. Real-time PCR is conducted directly from isolated colonies using the primers and probes as described in Annex E of ISO/TS 13136:2012.

PERFORMANCE TESTED AOAC RESEARCH INSTITUTE LICENSE NUMBER 081902

AOAC-RI VALIDATION

SUPREME REAL TIME DETECTION KIT *E. coli* has been validated by AOAC-Research Institute under the Performance Tested Method Program for detection of pathogenic *E. coli* associated with pathotypes EPEC, STEC and the sub-group EHEC associated with the combination of the virulence genes *stx1* and/or *stx2* and *eae* from raw ground beef, orange juice, salad (green, purple lettuce and coriander) and cream cheese. A positive result with SUPREME REAL TIME DETECTION KIT *E. coli* should be considered presumptive and it is recommended be confirmed by standard reference methods.

Certificate no. 081902.

BIOPREMIER

SUPREME
REAL TIME DETECTION KIT

Escherichia coli

Detection of Enteropathogenic (EPEC) & Shiga-Toxin (STEC)

Ref: BIOPSFS-0002

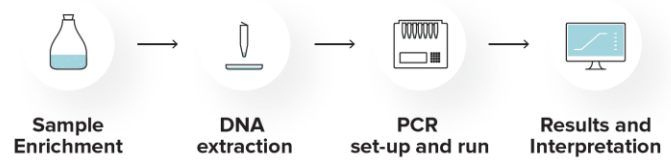
1. PATHOGEN DESCRIPTION

Enteropathogenic *E. coli* (EPEC) was the first recognized pathogenic group, and presently, continues to be a leading cause of diarrhoea among infants from developing countries worldwide. The locus of enterocyte effacement (LEE) is necessary for the pathogenicity and intimin one of the resultant products, coded by *eae* gene. The main pathogenic property of Shiga toxin-producing *E. coli* (STEC) strains is the production of Shiga toxins, coded by *stx* genes. Illnesses associated with STEC are usually watery diarrhoea and are particularly serious in children. Numerous outbreaks have been attributed to STEC strains of serotype O157:H7, but non-O157 serogroups, most commonly O26, O55, O103, O111, O117 O145, O146 and O191 have been shown to cause infections in the EU over recent years. The focus on *E. coli* O157 instead of other serotypes has been further enhanced by the ease of isolation of *E. coli* O157. In contrast, the isolation of other serotypes has been sub-diagnosed by the lack of suitable methods to isolate those strains. EHEC were originally defined as a subset of STEC, that were associated with watery diarrhoea, haemorrhagic colitis, haemolytic-uremic syndrome, and that in addition to the *stx*-encoding genes, usually carry the attaching and effacing gene (*eae*; intimin-coding). EHEC strains are typically isolated from cases of severe disease but are poorly defined because there is no commonly accepted definition of EHEC.

2. INTENDED USE

SUPREME REAL TIME DETECTION KIT *E. coli* is a kit for the detection of pathogenic *E. coli* associated with the pathotypes EPEC, STEC and the subgroup EHEC associated with the combination of the virulence genes *stx1* and/or *stx2* and *eae*. Within Enterobacteriaceae, other species may also contain these virulence genes, therefore, the kit also detects species like *E. albertii*, *Shigella boydii* or *S. sonnei*, when target genes are present. The kit enables a qualitative detection of target genes in food samples by Real Time PCR, after a selective enrichment step, based on ISO/TS 13136:20125. The test may be used with the following matrixes: raw ground beef, orange juice, salad (green, purple lettuce and coriander) and cream cheese. The kit includes an Internal Control (IC). The IC is used for the evaluation of PCR inhibitors in the sample, or for the evaluation of problems that occurred during PCR preparation/amplification. The kit includes Master mix for the target and IC, primers and TaqMan® probes, labelled with non-fluorescent quenchers. The signal for the detection of the target is in the FAM channel, and the one for the IC is in the ROX channel. The kit contains Uracil-DNA Glycosylase (UDG), preventing DNA contamination with PCR products. Included in the kit is also a positive control, allowing an evaluation of the primers and probes used for the detection of the targets and a negative control (nuclease-free water) to confirm the integrity of the kit reagents. The kit was validated in the instruments ABI PRISM® 7500 Fast. The kit is compatible with all thermocyclers working in **FAM** and **ROX** channels. The detection kit must not be used for diagnostic procedures. For Food use only.

The procedure includes the following main steps:



3. TRADEMARKS AND DISCLAIMER

Registered names, trademarks, etc. used in this document, even when not specifically marked as such, are not to be considered unprotected by law. BPMR kit handbooks and user manuals can be requested from BPMR or your local distributor.

4. LIMITED LICENSE AGREEMENT

Use of this product signifies the agreement of the following terms: The kit must be used solely in accordance with the respective Instructions for Use. BPMR grants no license under any of its intellectual property to use or

incorporate the enclosed components of this Kit with any components not included within this kit except as described in the Instructions for Use. This kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.

5. QUALITY CONTROL

In accordance with BPMR's ISO 9001, each lot of the kit is tested against predetermined specifications to ensure consistent product quality.

6. WARNINGS AND PRECAUTIONS

Molecular Biology procedures, such as DNA extractions and PCR amplification, require qualified staff to prevent the risk of erroneous results, especially due to sample contamination or degradation of the nucleic acids contained in the samples. It is strongly recommended to have dedicated areas, materials and equipments for the DNA extraction, preparation of the PCR and post-PCR procedures. Workflow in the laboratory must proceed in a unidirectional manner, beginning in the Extraction Area and moving to the Amplification and Detection Area.

- The user should always pay attention to the following:
- Read all the instructions provided before running the assay.
- Do not mix reagents from different batches.
- Wear proper PPE, including disposable gloves and laboratory coats.
- Store and extract positive material separately from all other reagents.

7. CONTENTS AND STORAGE CONDITIONS

The kit should be stored between -20°C and 5°C and protected from exposure to light. Repeated thawing and freezing (>5) should be avoided, as this may reduce the sensitivity. Considerate to freeze in aliquots to maintain the performance of the assay.
The kit contains reagents for 50 reactions for each target: EPEC and STEC.

CONTENTS	UNITS	COMPOSITION
Master Mix EPEC (blue cap)	1 tube (1 x 683µl)	Buffer, dNTPs, Primers and Probes
Master Mix STEC (green cap)	1 tube (1 x 683µl)	Buffer, dNTPs, Primers and Probes
Enzymes (white cap)	1 tube (1 x 220µl)	DNA polymerase, UDG and storage buffer
Negative Control (clear cap)	1 tube (1 x 130µl)	Nuclease-free water
Positive Control (red cap)	1 tube (1 x 130µl)	Target DNA

8. MATERIALS REQUIRED AND NOT SUPPLIED

- Microcentrifuge
- Laminar Air Flow Cabinets/PCR Cabinets
- Disposable powder-free gloves
- Micropipettes and nuclease-free filter tips
- Real time PCR instrument
- Tubes/Strips/Multiwell plates and acessories specific for each Instrument
- Lysis buffer/DNA extraction kit (expl: BIOPEXT-0400/ BIOPEXT-0609)

9. PROCEDURE

9.1. ENRICHMENT / DNA EXTRACTION

Recommended a pre-enrichment in Tryptone Soy Broth with Novobiocin (mTSB+n) (ISO/TS 13136:2012) or other suitable enrichment.

9.2. DNA EXTRACTION

1. Collect 1 mL of enriched sample and centrifuge at 10,000 - 12,000 g for 5 min.
2. Discard all the supernatants.
3. Wash the pellet: add 1 mL of 0.9% NaCl solution or PBS.
4. Centrifuge at 10,000-12,000 g for 5 min and discard all the supernatant.
5. Use kit for DNA extraction. Such as BIOPREMIER DNA Rapid Extraction Buffer (ref: BIOPEXT-0400) or BIOPREMIER DNA Extraction Kit from Food (ref: BIOPEXT-0609) (not included).

9.3. PCR PREPARATION

A – PCR mix

Always wear gloves for all PCR procedures.

1. Thaw the kit solutions. Mix thoroughly (do not vortex the tube Enzymes) and centrifuge briefly
2. Prepare the reactions, as described below for **EPEC** mix:

CONTENTS	Nº OF SAMPLES	
qPCR reaction	**1**	**10 (10 + 1)**
Master Mix EPEC	13 µl	143 µl
Enzymes	2 µl	22 µl
Total Volume	**15 µl**	**165 µl**

Note: Prepare the PCR reaction for each sample, or in alternative, prepare a Master Mix for the total number of reactions plus 10% (e.g. for 10 samples, prepare a volume for 11). In this case, prepare the Mix in a 1,5mL sterile, nuclease-free tube. Include 2 PCR reactions for the Positive and Negative controls.

3. Prepare the reactions, as described below for **STEC** mix:

CONTENTS	Nº OF SAMPLES	
qPCR reaction	**1**	**10 (10 + 1)**
Master Mix STEC	13 µl	143 µl
Enzymes	2 µl	22 µl
Total Volume	**15 µl**	**165 µl**

Note: Prepare the PCR reaction for each sample, or in alternative, prepare a Master Mix for the total number of reactions plus 10% (e.g. for 10 samples, prepare a volume for 11). In this case, prepare the Mix in a 1,5mL sterile, nuclease-free tube. Include 2 PCR reactions for the Positive and Negative controls.

4. Mix the prepared Mix by inverting the tube and centrifuge briefly
5. Dispense 15 µL aliquots of each prepared Mix into the PCR tubes
6. For the negative control, pipette 5 µL of Negative Control tube (Clear Cap); Pipette 5 µL of DNA sample per well; and for the positive control, pipette 5 µL of Positive Control tube (Red Cap). Each PCR tube / well should have a final PCR volume of 20 µL
7. Centrifuge briefly the plate wells or PCR tubes
8. Place the reactions into the Real Time PCR instrument.

B – Program set up

Prepare the Real-Time PCR instrument according to the following temperature/time program:

PHASE	TEMPERATURE	CYCLES	TIME	ACQUISITION
Incubation	37 ºC	1	15 min	No
UDG Inactivation	95 ºC	1	5 min	No
DNA Amplification	94 ºC	45	15 s	No
	60 ºC		30 s	Collect Data
	72 ºC		15 s	No

Sample Volume: 20 µl Detection format: Hydrolysis Probe
Passive reference dye: None

REACTION	TARGET	CHANNELS
EPEC	Detection of *eae* gene	FAM | Excitation at 465 nm, Emission at 510 nm
	Detection of IC	ROX | Excitation at 533 nm, Emission at 610 nm

REACTION	TARGET	CHANNELS
STEC	Detection of *stx1 / 2* genes	FAM | Excitation at 465 nm, Emission at 510 nm
	Detection of IC	ROX | Excitation at 533 nm, Emission at 610 nm

10. DATA ANALYSIS

For analysis of PCR results, select fluorescence display options. Samples with positive Ct-values are considered positive.
Important: Please, also check amplification curves, not only Ct values. Samples should be inspected both in logarithmic and linear scale view and compared with the negative control. Adjust the Threshold, if necessary. Sample results should be assessed after the positive and negative controls have been examined and determined to be valid. If the results of controls are not valid, the sample results cannot be interpreted.
Interpretation of PCR-data:

a) Controls

To validate the assay, the controls must have the following results:

	E. coli DETECTION FAM	IC DETECTION ROX
Negative Control	Negative	Positive
Positive Control	Positive	Positive

Note that if the controls do not match these results, the experiment must be repeated.

b) Samples

Interpretation of sample results is summarized in the following table:

EPEC DETECTION FAM	STEC DETECTION FAM	IC DETECTION ROX	INTERPRETATION
Positive	Positive	Positive/Negative **	DNA detected, sample Positive for *E. coli* STEC and EPEC
Negative	Negative	Positive	No DNA detected, sample Negative for *E. coli* STEC and EPEC
Positive	Negative	Positive/Negative **	DNA detected, sample Positive for *E. coli* EPEC
Negative	Positive	Positive/Negative **	DNA detected, sample Positive for *E. coli* STEC
Negative	Negative	Negative	Invalid Result*

*When both *E. coli* and IC detection are Negative, means the presence of PCR inhibitors in the sample. Dilute the sample or perform another DNA extraction.
** High DNA concentration of the target in the sample can lead to a reduced or absent fluorescence signal of the IC.

Table 1: Exclusivity of the SUPREME Real Time Detection Kit *E. coli*

ID	Strain	SUPREME Kit Result	
		stx1/2	eae
NCTCa 11351	Campylobacter jejuni subsp jejuni	NEG	NEG
DSMb 20171	Brochothrix thermosphacta	NEG	NEG
DSM 756	Clostridium perfringens	NEG	NEG
CECTc 4022	Lactobacillus paracasei subsp paracasei	NEG	NEG
NCTC 11994	Listeria monocytogenes	NEG	NEG
NCTC 6571	Staphylococcus aureus	NEG	NEG
DSM 16636	Citrobacter rodentium	NEG	NEG
MB 198	Citrobacter sp.	NEG	NEG
MB 118	Cronobacter sakazakii	NEG	NEG
MB 490	Enterobacter cloacae	NEG	NEG
MB 20	Klebsiella oxytoca	NEG	NEG
MB 9	Pseudomonas aeruginosa	NEG	NEG
DSM 13772	Salmonella bongori	NEG	NEG
MB 164	S. enterica subsp. enterica serovar Enteritidis	NEG	NEG
NCTC 74	S. enterica subsp. enterica serovar Typhimurium	NEG	NEG
MB 168	Shigella flexneri	NEG	NEG
DSM 7532	Shigella boydii	NEG	NEG
DSM 5570	Shigella sonnei	NEG	NEG
534-1715343	Shigella sonnei	NEG	NEG
MB 249	Yersinia enterocolitica	NEG	NEG
MF 22	Aspergillus flavus	NEG	NEG
MF 101	S. cerevisiae	NEG	NEG
534-1715344	Escherichia coli K12	NEG	NEG
NCTC 9007	Escherichia coli O7	NEG	NEG
DSM 1103	Escherichia coli O6	NEG	NEG
MB 189	Escherichia coli	NEG	NEG
MB 190	Escherichia coli	NEG	NEG
MB 239	Escherichia coli - Hemolítica	NEG	NEG
MB 607	Escherichia coli - Hemolítica	NEG	NEG
MB 264	Escherichia coli BLSE+	NEG	NEG
NCTC 7464	Bacillus cereus	NEG	NEG
NCTC 11366	Campylobacter coli	NEG	NEG
NCTC 11352	Campylobacter lari	NEG	NEG
MB 282	Legionella pneumophila	NEG	NEG
MB 116	Listeria innocua	NEG	NEG
NCTC 11348	Vibrio cholerae	NEG	NEG
MB 135	Vibrio parahaemolyticus	NEG	NEG

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BPMR is certified ISO 9001:2015

MKT-0131 Last revision: November 2023

Table 2: Inclusivity of the SUPREME Real Time Detection Kit *E. coli*

ID	Strain	stx1 gene	stx2 gene	eae gene	SUPREME Kit Result		Result comparison
					stx1/2	eae	
LMVa_E_2	E. coli O26	+	+	+	POS	POS	Matched
LMV_E_3	E. coli O111	+	+	+	POS	POS	Matched
LMV_E_4	E. coli O145	+	-	+	POS	POS	Matched
LMV_E_5	E. coli O157	+	+	+	POS	POS	Matched
LMV_E_6	E. coli O157	+	-	+	POS	POS	Matched
LMV_E_7	E. coli O103	-	+	+	POS	POS	Matched
MB E111	E. coli O103	-	+	+	POS	POS	Matched
MB LM21	E. coli O157	+	+	+	POS	POS	Matched
NTCCd 12079	E. coli O157:H7	+	+	+	POS	POS	Matched
CECTe 4267	E. coli O157:H7	+	+	+	POS	POS	Matched
CECT 4783	E. coli O157:H7	+	+	+	POS	POS	Matched
CECT 4782	E. coli O157:H7	+	+	+	POS	POS	Matched
CCCf-1-12	E. coli O157	-	+	+	POS	POS	Matched
CCC-3-12	E. coli O26	+	+	+	POS	POS	Matched
CCC-5-12	E. coli O157	-	+	+	POS	POS	Matched
CCC-7-12	E. coli O157	-	+	+	POS	POS	Matched
CCC-10-12	E. coli O157	-	+	+	POS	POS	Matched
CCC-11-12	E. coli O157	+	-	+	NEG	POS	Not Matched
CCC-12-12	E. coli O157	-	+	+	POS	POS	Matched
CCC-13-12	E. coli O157	-	+	+	POS	POS	Matched
CCC-14-12	E. coli O157	-	+	+	POS	POS	Matched
CCC-15-12	E. coli O157	-	+	+	POS	POS	Matched
CCC-16-12	E. coli O157	-	+	+	POS	POS	Matched
CCC-18-12	E. coli O157	-	+	+	POS	POS	Matched
CCC-20-12	E. coli O26	+	-	+	POS	POS	Matched
CCC-21-12	E. coli O26	+	-	+	POS	POS	Matched
CCC-22-12	E. coli O26	+	-	+	POS	POS	Matched
00760EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
00960EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
01068EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
01076EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
01144EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
01320EO2A6H	E. coli O157:H7	-	+	+	POS	POS	Matched
01511EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
01863EO1A6H	E. coli O157:H7	-	+	+	POS	POS	Matched
02171EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
02172EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
02264EO1A6H	E. coli O157:H7	-	+	+	POS	POS	Matched
02269EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
02270EO1A24H	E. coli O157:H7	-	+	+	POS	POS	Matched
02306EO1A6H	E. coli O157:H7	-	+	+	POS	POS	Matched
02309EO1A6H	E. coli O157:H7	-	+	+	POS	POS	Matched
02450EO1A24H	E. coli O157:H7	-	+	+	POS	POS	Matched
02871EO1A6H	E. coli O157:H7	-	+	+	POS	POS	Matched
02922EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
02942EO1A6H	E. coli O157:H7	-	+	+	POS	POS	Matched
03084EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
03085EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
03220EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
03222EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
03321EO1C6H	E. coli O157:H7	+	+	+	POS	POS	Matched
03322EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
03650EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
03804EO1A6H	E. coli O157:H7	+	+	+	POS	POS	Matched
03878EO1A6H	E. coli O157:H7	-	+	+	POS	POS	Matched
MSUh TW14960	E. coli O111	+	+	+	POS	POS	Matched
MSU DEC8D	E. coli O111	+	+	+	POS	POS	Matched
PSUj 5.0959	E. coli O111	+	+	+	POS	POS	Matched
PSU 7.1686	E. coli O111	+	+	+	POS	POS	Matched
PSU 7.1711	E. coli O145	+	+	+	POS	POS	Matched
PSU 10.0707	E. coli O145	+	+	+	POS	POS	Matched
MSU TW09153	E. coli O145	+	+	+	POS	POS	Matched
MSU TW07596	E. coli O145	+	+	+	POS	POS	Matched
MSU TW11239	E. coli O103	+	+	+	POS	POS	Matched
MSU TW07697	E. coli O103	+	+	+	POS	POS	Matched
PSU 5.1658	E. coli O103	+	+	+	POS	POS	Matched
PSU 7.1691	E. coli O103	+	+	+	POS	POS	Matched