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Microbiology of food and animal feed — Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens — Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups

Microbiologie des aliments — Méthode basée sur la réaction de polymérisation en chaîne (PCR) en temps réel pour la détection des micro-organismes pathogènes dans les aliments — Méthode horizontale pour la détection des Escherichia coli producteurs de Shigatoxines (STEC) et la détermination des sérogroupes O157, O111, O26, O103 et O145

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Contents

Page

Foreword ... iv

Introduction ... v

1 **Scope** ... 1

2 **Normative references** ... 1

3 **Terms and definitions** ... 2

4 **Principle** ... 2
4.1 **General** ... 2
4.2 **Microbial enrichment** ... 2
4.3 **Nucleic acid extraction** ... 3
4.4 **Target genes** ... 3
4.5 **Detection** ... 3
4.6 **Isolation** ... 3

5 **Diluents, culture media and reagents** ... 3
5.1 **Culture media** ... 4
5.2 **Reagents for nucleic acid extraction** ... 5
5.3 **Reagents for PCR** ... 5

6 **Equipment** ... 5

7 **Sampling** ... 6

8 **Preparation of test sample** ... 6

9 **Procedure** ... 6
9.1 **Test portion and initial suspension** ... 6
9.2 **Enrichment** ... 7
9.3 **Nucleic acid extraction** ... 7
9.4 **PCR amplification (for real-time PCR)** ... 7
9.5 **Strain isolation** ... 8

10 **Expression of results** ... 8

11 **Performance data** ... 8

Annex A (normative) **Flow diagram of the screening procedure** ... 12

Annex B (normative) **Flow diagram of the isolation and confirmation procedure** ... 13

Annex C (informative) **Identification of Shiga toxin-producing *Escherichia coli* (STEC) by multiplex PCR amplification of virulence genes and detection of PCR products by agarose gel electrophoresis** ... 14

Annex D (informative) **Internal amplification control** ... 18

Annex E (informative) **Primers and probes for the PCR assays** ... 19

Annex F (normative) **Isolation of STEC strains** ... 21

Bibliography ... 22

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Foreword

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are pathogenic *E. coli*, which can cause diarrhoea as well as more severe diseases in humans such as haemorrhagic colitis and haemolytic uremic syndrome (HUS). Although STEC may belong to a large number of serogroups, those that have been firmly associated with the most severe forms of the disease, in particular HUS, belong to O157, O26, O111, O103, and O145 (Reference [1]).

The following nomenclature has been adopted in this Technical Specification:

— *stx*: Shiga toxin genes (synonymous with *vtx)*;

— Stx: Shiga toxin (synonymous with Vtx: Verocytotoxin);

— STEC: Shiga toxin-producing *Escherichia coli (*synonymous with VTEC: Verocytotoxin-producing *Escherichia coli).*

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IMPORTANT — It is necessary to consider any STEC as pathogenic to humans and potentially to cause severe disease depending on both the risk profile of the food commodity (ready-to-eat foods vs. foods intended to be consumed after technological treatment such as pasteurization, cooking etc. used to reduce any bacteria present in the food) and the health status of the subject ingesting the food.

Moreover, given the high genomic plasticity of this bacterial species, it is possible that novel arrangements of virulence features can give rise to novel sero-pathogroups such as the Shiga toxin-producing enteroaggregative *E. coli* O104 that caused the HUS outbreaks in Germany and France in 2011-05/06. Novel atypical *E. coli* sero-pathogroups can arise from the acquisition of an *stx*-converting bacteriophage by an *E. coli* strain belonging to pathogroups different from STEC.

Such atypical strains fall in the scope of this method and can be efficiently detected as they are positive for the presence of the *stx* genes.

1 Scope

This Technical Specification describes the identification of Shiga toxin-producing *Escherichia coli* (STEC) by means of the detection of the following genes:

a) the major virulence genes of STEC, *stx* and *eae* (References [2][3]);

b) the genes associated with the serogroups O157, O111, O26, O103, and O145 (References [3][4]).

In any case, when one or both of the *stx* genes is/are detected, the isolation of the strain is attempted.

The isolation of STEC from samples positive for the presence of the genes specifying the serogroups in the scope of this method can be facilitated by using serogroup-specific enrichment techniques (e.g. immunomagnetic separation, IMS).

The protocol uses real-time PCR as the reference technology for detection of the virulence and serogroup-associated genes.

This Technical Specification is applicable to:

1) products intended for human consumption and the feeding of animals;

2) environmental samples in the area of food production and food handling;

3) environmental samples in the area of primary production.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

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ISO 20838, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for amplification and detection for qualitative methods*

ISO 22174, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

Definitions 3.1 to 3.3 have been compiled from the epidemiological data on disease caused by STEC managed by organizations such as the US Centers for Disease Control and, in the EU, by the European Centre for Disease Prevention and Control and the European Food Safety Authority.

3.1
Shiga toxin-producing *Escherichia coli*
STEC
E. coli strains possessing the Stx-coding genes

3.2
Shiga toxin-producing *Escherichia coli* causing the attaching and effacing lesion
STEC causing the attaching and effacing lesion
E. coli strains possessing the Stx-coding genes and the intimin-coding gene *eae*

NOTE This combination of virulence genes is often associated with the most severe forms of STEC-induced disease.

3.3
Shiga toxin-producing *Escherichia coli* belonging to highly pathogenic serogroups
STEC belonging to highly pathogenic serogroups
E. coli strains possessing the Stx-coding genes, the intimin-coding gene *eae* and belonging to one of the serogroups O157, O111, O26, O103, and O145

4 Principle

4.1 General

The method specified comprises the following sequential steps:

a) microbial enrichment;

b) nucleic acid extraction;

c) detection of virulence genes;

d) detection of serogroup-associated genes;

e) isolation from positive samples.

Figure A.1 is a flow diagram of the screening procedure.

4.2 Microbial enrichment

The number of STEC cells to be detected is increased by incubating the test portion in a non-selective liquid nutrient medium chosen from:

a) modified tryptone-soy broth (tryptone soy broth supplemented with 1,5 g/l bile salts No.3, mTSB) supplemented with 16 mg/l of novobiocin (mTSB+N);

b) buffered peptone water (BPW);

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c) modified tryptone-soy broth (tryptone-soy broth supplemented with 1,5 g/l bile salts No.3, mTSB) supplemented with 12 mg/l of acriflavin (mTSB+A) for analysis of milk and dairy products.

The mTSB shall be used when analysing matrices suspected to contain high levels of contaminating microflora. Novobiocin and acriflavin inhibit the growth of Gram-positive bacteria and promote the growth of Gram-negative cells, including STEC. The BPW shall be used to analyse samples which are assumed to contain stressed target bacteria (such as frozen products), to resuscitate stressed STEC cells, and expected lower levels of contaminating microflora than in fresh samples.

NOTE The addition of novobiocin is controversial and has been investigated by several authors. It has been observed that the minimum inhibitory concentration of the antibiotic for non-O157 STEC is lower than for O157 strains (Reference [5]). The addition of novobiocin in the enrichment mTSB at the usual concentration of 20 mg/l, as specified in ISO 16654,[19] seems to inhibit the growth of about one-third of non-O157 strains (Reference [6]) increasing the risk of false-negative results.

4.3 Nucleic acid extraction

The nucleic acid is extracted according to the requirements of the adopted detection system.

4.4 Target genes

The purified nucleic acid is used for the detection of the following target genes:

— the main virulence genes for STEC: *stx* genes, encoding the Shiga toxins and the *eae* gene, encoding a 90 kDa protein, the intimin, involved in the attaching and effacing mechanism of adhesion, a typical feature of the STEC strains causing severe disease. The *stx* genes encode a family of toxins including two main types: *stx*1 and *stx*2. The latter comprises seven recognized variants (from *stx*2*a* to *stx*2*g*) (Reference [22]). Only the variants *stx*2*a*, *stx*2*b*, and *stx*2c have been found to be produced by the STEC strains included in Clause 1, and therefore constitute the target Stx-coding genes of this Technical Specification. The GenBank accession numbers corresponding to the *stx*2 variants-coding genes are:

  — *stx*2a: X07865

  — *stx*2*b*: L11078

  — *stx*2*c*: M59432

— the intimin-coding *eae* gene

— the *rfbE(O157), wbdl(O111), wzx(O26), ihp1(O145)* and *wzx(O103)* genes, to identify the corresponding serogroups.

4.5 Detection

The detection of the target genes is performed according to the adopted detection system.

4.6 Isolation

If the presence of a STEC is suspected, the isolation is attempted. If one of the serogroups specified in the scope of this Technical Specification is detected, a serogroup-specific enrichment (e.g. IMS) can be performed followed by plating on to tryptone–bile–glucuronic agar (TBX) or a specific selective medium if available (see Annex F, Notes 2 and 3) in order to facilitate the isolation of the STEC from the background flora.

5 Diluents, culture media and reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and sterile distilled or demineralized water or water of equivalent purity.

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5.1 Culture media

5.1.1 Modified tryptone- soy broth (mTSB)

5.1.1.1 Basic medium

Composition and pH

Enzymatic digest of casein	17 g
Enzymatic digest of soy	3 g
D(+)-Glucose	2,5 g
Sodium chloride	5 g
Dipotassium hydrogenphosphate (K_2HPO_4)	4 g
Bile salts No. 3	1,5 g
Water	to 1 000 ml

pH 7,4 ± 0,2

Preparation

Dissolve the components or the dehydrated medium in water. Adjust pH with a pH-meter to pH 7,4 ± 0,2 at 25 °C and sterilize by autoclaving at 121 °C for 15 min.

5.1.1.2 Novobiocin solution

Composition

Novobiocin	0,16 g
Water	10 ml

Preparation

Dissolve the novobiocin in the water and sterilize by membrane filtration using 0,22 µm or 0,45 µm filters.

Prepare on the day of use.

5.1.1.3 Acriflavin solution

Composition

Acriflavin	0,12 g
Water	10 ml

Preparation

Dissolve the acriflavin in the water and sterilize by membrane filtration using 0,22 µm or 0,45 µm filters.

Prepare on the day of use.

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5.1.1.4 Preparation of the complete medium

Immediately before use, add 1 ml of novobiocin (5.1.1.2) or acriflavin solution (5.1.1.3) to 1 000 ml of cooled mTSB (5.1.1.1).

The final concentration of novobiocin shall be 16 mg/l of mTSB.

The final concentration of acriflavin shall be 12 mg/l of mTSB.

5.1.2 Buffered peptone water (BPW)

Composition and pH

Peptone	10 g
Sodium chloride	5,0 g
Disodium phosphate (Na_2HPO_4)	3,5 g
Potassium dihydrogenphosphate (KH_2PO_4)	1,5 g
Water	to 1 000 ml

pH 7,0 ± 0,2

Preparation

Dissolve the components or the dehydrated powder in the water. Adjust pH with a pH-meter to pH 7,0 ± 0,2 at 25 °C and sterilize by autoclaving at 121 °C for 15 min.

5.2 Reagents for nucleic acid extraction

The reagents to be used for nucleic acid extraction are not listed, being dependent on the method adopted (9.3).

5.3 Reagents for PCR

See ISO 20838.

5.3.1 Oligonucleotides (primers) and detection probes

Primers and probes for specific detection of the target gene sequences by standard and real-time PCR are listed in Annexes C and E.

6 Equipment

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

6.1 **Water bath** or **heating block** capable of being maintained at temperatures up to 100 °C.

6.2 **Incubator** according to ISO 7218, capable of being maintained at 37 °C± 1 °C.

6.3 **Nucleic acid extraction apparatus**.

Appropriate equipment according to the method adopted (if needed).

6.4 **Pipettes** of capacities between 1 µl and 100 µl, ISO 7550.[16]

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6.5 Thin walled real-time PCR microtubes (0,2 ml/0,5 ml reaction tubes), multi-well PCR microplates or other suitable light transparent disposable plasticware.

6.6 Thermal cycler. Several brands of apparatus are available and can be chosen according to the laboratory policies.

6.7 PCR product detection apparatus.

Light emission following 5’ nuclease PCR assay is detected by the real-time PCR apparatus.

6.8 Peristaltic blender with sterile bags, possibly with a device for adjusting speed and time.

7 Sampling

Sampling is not part of the method specified in this Technical Specification. See the specific International Standard dealing with the product concerned or specific regulations. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

It is important that the laboratory receive a truly representative sample which has not been damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion and initial suspension

9.1.1 General

Use the quantity of enrichment medium necessary to give a final dilution of 10^{-1} of the original test portion.

9.1.2 For matrix samples assumed to contain a high level of background flora

For solid matrices, aseptically transfer a test portion (x g) of sample to a peristaltic blender bag containing 9x ml of mTSB to which novobiocin or acriflavin has been added (5.1.1.4). Bags with filters are preferred.

Homogenize in a peristaltic blender (see ISO 7218) (6.8).

For liquid matrices, transfer the test portion (x ml) of liquid sample, using a sterile pipette, into the tube or bottle containing 9x ml of the enrichment mTSB to which novobiocin or acriflavin has been added (5.1.1.4).

9.1.3 For matrix sample assumed to contain stressed target bacteria

Allow frozen products to thaw at room temperature, and then transfer the test portion (x g or x ml) to a peristaltic blender bag or tube containing 9x ml of BPW (5.1.2) and proceed as above.

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9.2 Enrichment

9.2.1 Incubation

Incubate the peristaltic blender bag, tube or bottle (9.1.2) at 37 °C ± 1 °C for 18 h to 24 h.

9.2.2 Process control (for real-time PCR)

Perform process control according to ISO 22174.

Guidance on internal amplification control (IAC) and process control are given in Annex D and C.3.8.

9.3 Nucleic acid extraction

Use an appropriate nucleic acid extraction procedure for Gram-negative bacteria. A comprehensive collection of methods can be found in Reference [10]. Alternatively, commercial kits may be used according to the manufacturer's instructions.

9.4 PCR amplification (for real-time PCR)

9.4.1 General

The PCR amplification approach described is based on real-time PCR.

Follow all requirements for the PCR amplification as specified in ISO 20838.

Primers and detection probes for conducting real-time PCR are described in Annex E.

9.4.2 Detection of PCR products

Light emission is captured by the apparatus once generated during the amplification.

9.4.3 Interpretation of PCR results

The PCR results obtained, including the controls specified in ISO 22174 and in Annex D, are interpreted by the software linked to the apparatus. During amplification, the software monitors 5' nuclease PCR amplification by analysing fluorescence emissions of the reporter dye for each sample, R_n. ΔR_n is R_n minus the baseline reporter dye intensity established in the first few cycles. At the end of the PCR cycles, a reaction is considered positive if its ΔR_n curve exceeds the threshold, defined as 10 times the standard deviation of the mean baseline emission calculated between the first few cycles. The cycle threshold, C_t, is defined as the cycle number at which the ΔR_n fluorescence of a sample crosses the determined threshold value.

If the results are ambiguous, check the emission curves. Positive samples give a curve with a clear increase in fluorescence, starting from a number of cycles corresponding to the C_t.

If the controls yield unexpected results, repeat the procedure.

The method is sequential (see the flowchart in Figure A.1):

— step 1: detection of the Stx-coding genes and the *eae* gene (PCR A in Annex E — this can also be done in duplex PCR);

— step 2 a): samples positive for *stx* and the *eae* genes are tested for the molecular serogrouping (PCR B in Annex E);

— step 2 b): samples positive for the Stx-coding genes are subjected to strain isolation — a serogroup-specific enrichment method (e.g. IMS) can be used to improve the isolation of STEC from samples positive for one of the serogroups within the scope of this Technical Specification (see 9.5 and Figure B.1).

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9.5 Strain isolation

The isolation of the STEC strains is required to confirm that the positive PCR signals are generated from genes present in the same live bacterial cell.

In case of positivity to one of the genes associated with the serogroups in the scope of this Technical Specification, a serogroup-specific enrichment may be used to facilitate the isolation step followed by direct plating on to suitable solid media and screening of the colonies for the presence of the virulence genes.

The standard PCR and the real-time PCR protocols in Annexes C or E or any other equivalent PCR protocol shall be used in order to confirm the presence of the virulence genes in the isolated colonies.

A STEC isolation flowchart appears in Figure B.1 and a procedure in Annex F.

10 Expression of results

a) **Samples negative for *stx* gene**: STEC not detected in the test portion of x g or x ml (see ISO 7218).

In the absence of *stx* genes, the procedure is stopped without proceeding to the determination of the intimin-coding *eae* gene or the genes associated with the serogroups in the scope of this method.

If isolation is *not* acheived from samples positive to the *stx* screening:

b) **Samples positive for *stx* gene**: Presumptive detection of STEC in the test portion of x g or x ml.

c) **Samples positive for *stx* and *eae* genes**: Presumptive detection of STEC causing the attaching and effacing lesion in the test portion of x g or x ml.

d) **Samples positive for *stx* and *eae* genes as well as to genes associated to one of the serogroups in the scope of this Technical Specification**: Presumptive detection of STEC of XX[1)] serogroup in the test portion of x g or x ml

If isolation and confirmation are achieved from samples positive to the *stx* screening:

e) **Isolated *E. coli* strains positive for *stx* gene**: Presence of STEC in the test portion of x g or x ml.

f) **Isolated *E. coli* strains positive for *stx* and *eae* genes**: Presence of STEC causing the attaching and effacing lesion in the test portion of x g or x ml.

g) **Isolated *E. coli* strains positive for *stx* and *eae* genes as well as for genes associated with one of the serogroups in the scope of this Technical Specification**: Presence of STEC of XX[2)] serogroup in the test portion of x g or x ml.

11 Performance data

11.1 The real-time PCR approach described in this Technical Specification has been the subject of a validation study carried out according to ISO 16140:2003[17] rules. Since the only International Standard available as a reference method at the time was ISO 16654[19] for the detection of *E. coli* O157 in foodstuffs, the method has only been validated and certified by an NF validation (Reference [7]) for the detection of STEC belonging to the O157 serogroup only.

Following this study, the part of the method regarding STEC O157 has been certified by AFNOR as equivalent to ISO 16140:2003[17] (certificate number GEN 25/04-11/08). The complete validation dossier is available in Reference [7].

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1) XX indicates the serogroup specified by the presence of the genes assessed.

2) XX indicates the serogroup the isolated strain belongs to as assessed by the presence of the corresponding genes or by phenotypic determination.

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11.2 Some performance characteristics of the real-time PCR screening section of the method have been determined in published studies. In particular, the sensitivity and the limit of detection of the real-time PCR have been determined by using dilutions of plasmids containing the cloned genes encoding the different targets (Reference [8]). The results of this study are summarized in Table 1.

Table 1 — Sensitivity and limit of detection of the 5′ nuclease PCR assays for some of the genes targeted in this Technical Specification (adapted from Reference [8])

Target gene	**Limit of detection** No. copies/reaction	**Efficiency** %
*stx*1	5	94,7
*stx*2	5	94
rfbE	1	94,2
wbdI	5	94
wzx	5	97,7
ihp1	5	99,6

Another study (Reference [17]) considered the performances of the proposed real-time PCR approaches in screening mixed cultures of STEC belonging to the serogroups in the field of application of this Technical Specification, with a K-12 laboratory strain (C600). The results are summarized in Table 2.

11.3 This Technical Specification was used in the third collaborative study organized in 2009 by the European Union Reference Laboratory (EURL) for *E. coli* including STEC. The study consisted of the examination of a set of five simulated carcass swabs (moistened sponges) containing the organisms of interest, including both STEC O157 and O26, together with background microbial flora (Table 3). The choice of using simulated carcass swabs as the matrix to be analysed in the proficiency test was driven by the principles included in the European Food Safety Authority guidelines for the forthcoming monitoring plans for STEC (ISO 16654[19]).

There were 14 national reference laboratories (NRLs) for *E. coli* that participated in the study. The evaluation of the performance of the method for non-O157 STEC (O26 STEC) returned the following values:

Sensitivity (*Se*): 100 % [95 % confidence interval (CI) 96,97 % to 100 %]

Specificity (*Sp*): 99,62 % (95 % CI 97,5 % to 100 %)

The analytical results per NRL are summarized in Table 4.

The NRLs were also asked to isolate the non-O157 STEC contaminating the samples. The outcome of the isolation step is showed in Table 5.

The report with the complete analysis of the results of the 3rd EU-RL-STEC collaborative study is publicly available.[9]

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Table 2 — Detection of low numbers of STEC in mixed cultures (adapted from Reference [15])

STEC serotype	CFU/ml	C_t values							
		*stx*1/*stx*2	*eae*	*rfbE* (*O157*)	*wbd1* (*O111*)	*wzx* (*O103*)	*ihp1* (*O145*)	*fliC* (*H7*)	*wzx* (*O26*)
O26:H11	2 to 3	28 to 31	31 to 32	—	—	—	—	—	31 to 33
	10 to 20	25 to 27	28 to 29	—	—	—	—	—	29
O103:H2	2 to 3	29 to 30	31 to 32	—	—	32 to 33	—	—	—
	10 to 20	26 to 27	29 to 30	—	—	29 to 31	—	–	—
O111:[H8]	2 to 3	26 to 27	30 to 31	—	30 to 31	—	—	–	—
	10 to 20	24 to 25	29 to 30	—	27 to 28	—	—	–	—
O145:[H28]	2 to 3	32 to 33	31 to 32	—	—	—	31 to 34	–	—
	10 to 20	30 to 32	29 to 31	—	—	—	30	–	—
O157:H7	2 to 3	29 to 30	29 to 31	31 to 32	—	—	—	33 to 38	—
	10 to 20	26 to 28	26 to 29	29 to 31	—	—	—	31 to 33	—

Table 3 — Composition of samples for the third EURL-STEC collaborative study

Values in colony-forming units per millilitre

Sample/contaminant	Sample A	Sample B	Sample C	Sample D	Sample E
STEC O157 *stx*1, *stx*2, *eae*	2	2×10^3	20	0	0
STEC O26 *stx*1, *eae*	0	40	4×10^3	40	0
E. coli	10^2	10^2	10^2	10^2	10^2
K. pneumoniae	2×10^2	2×10^2	2×10^2	2×10^2	2×10^2
S. faecalis	5×10^2	5×10^2	5×10^2	5×10^2	5×10^2

The expanded uncertainty, U, associated with the level of inoculum was 0,22 $\log_{10}$(CFU/ml) for *E. coli* strains as determined according to ISO/TS 19036:2006.[20]

Table 4 — Detection of virulence and serogroup-associated genes in the enrichment cultures by real-time PCR (screening section of the method; third EURL-STEC collaborative study)

Target gene	Sample		Participating laboratories													
			L_1	L_2	L_4	L_7	L_8	L_9	L_{12}	L_{14}	L_{15}	L_{17}	L_{21}	L_{22}	L_{25}	L_{30}
stx[a]	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

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Table 4 *(continued)*

Target gene	Sample		Participating laboratories													
			L_1	L_2	L_4	L_7	L_8	L_9	L_{12}	L_{14}	L_{15}	L_{17}	L_{21}	L_{22}	L_{25}	L_{30}
eae	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
O26	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
O111	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
O103	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
O145	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] This includes either *stx*1 or *stx*2.

Table 5 — Isolation of STEC O26 from the RT PCR-positive enrichment cultures (Confirmation of positive cultures by isolation of the infecting strain section; third EURL-STEC collaborative study)

Test	Sample	True value	Laboratories													
			L_1	L_2	L_4	L_7	L_8	L_9	L_{12}	L_{14}	L_{15}	L_{16}	L_{17}	L_{21}	L_{25}	L_{30}
Isolation of O26	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

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Annex A
(normative)

Flow diagram of the screening procedure

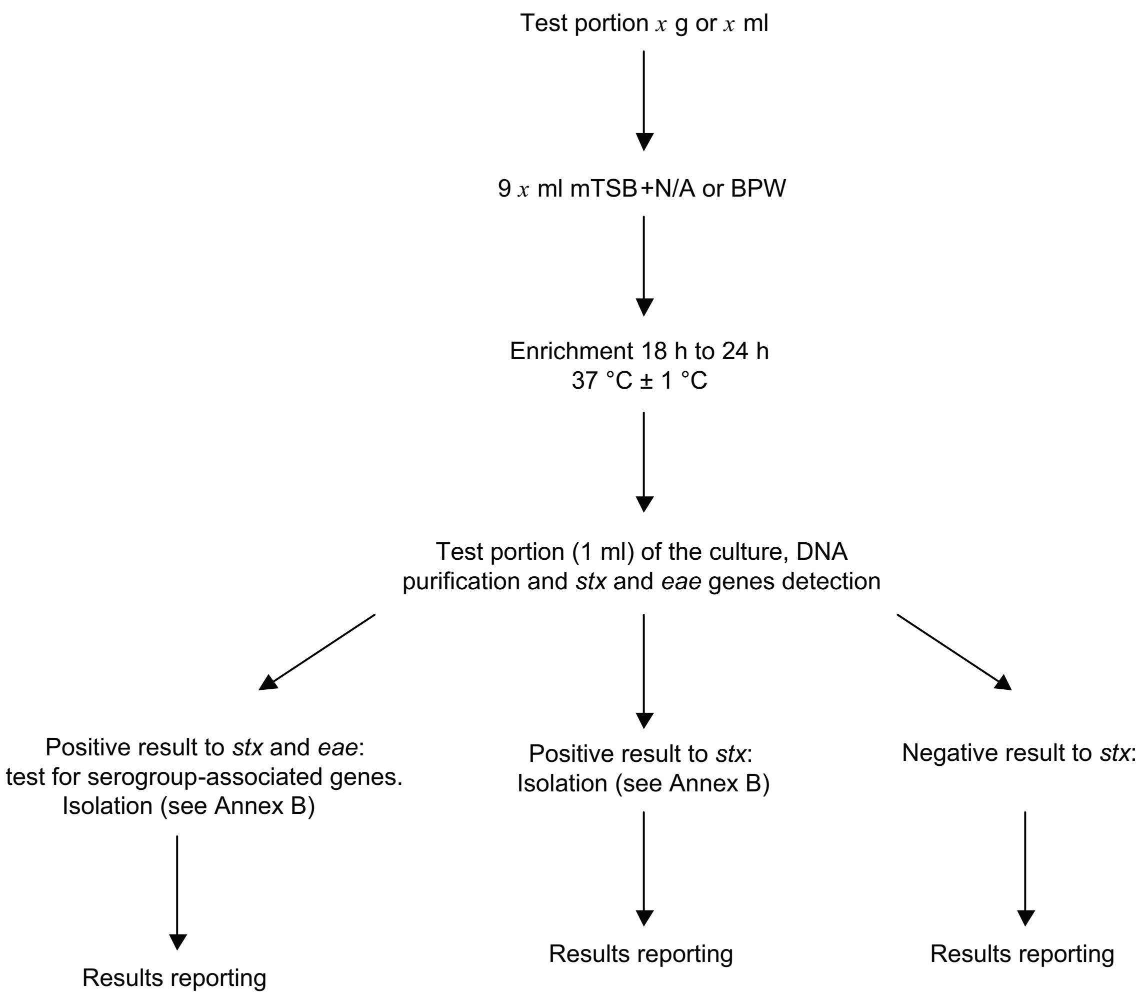

Figure A.1 — Flow diagram of the screening procedure

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Annex B
(normative)

Flow diagram of the isolation and confirmation procedure[3)]

If the sample was positive for one of the serogroup-associated genes in the scope of the method, a serogroup-specific enrichment (SSE) may be performed in order to facilitate the isolation.

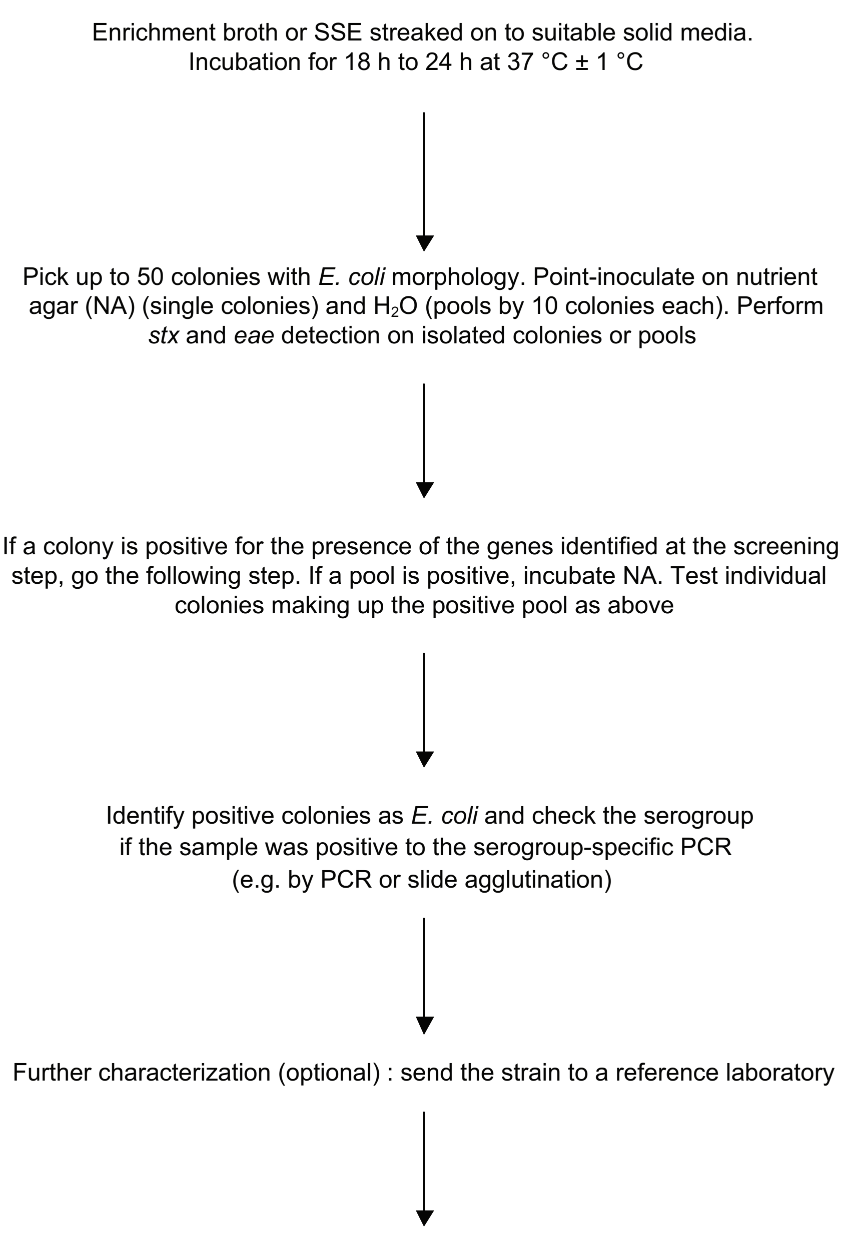

Figure B.1 — Flow diagram of the isolation and confirmation procedure

3) See Annex F.

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Annex C
(informative)

Identification of Shiga toxin-producing *Escherichia coli* (STEC) by multiplex PCR amplification of virulence genes and detection of PCR products by agarose gel electrophoresis

C.1 General

STEC are *Escherichia coli* strains harbouring lysogenic bacteriophages carrying genes encoding the production of Shiga toxins (Reference [12]). The method described in this annex is performed in order to detect by multiplex PCR the presence of Stx-coding genes in *E. coli* cultures, for their identification as STEC. The determination of the presence of the intimin-coding gene *eae* is also included, since it is associated with STEC strains causing sever disease in to humans.

The primer pairs used, *stx*1*F*/*stx*1*R* and *stx*2*F*/*stx*2*R* (Reference [11]), are able to detect the genes s*tx*1 and s*tx*2, respectively. The latter recognize all the variants of s*tx*2 except s*tx*2*f*. However, this variant is not considered to be of major importance in terms of public health, having been mainly observed in STEC isolated from birds (ISO/TS 19036[20]). The primers used for the detection of *eae* (Reference [11]) recognize all the reported polymorphic variants of this gene.

WARNING — The method described in this annex is intended for use with pure bacterial cultures only for confirmation of the strains isolated.

C.2 Abbreviations

S.U.: Sample unit

C.3 Procedure

C.3.1 Principle of the method

The method is based on PCR amplification of specific DNA regions from a template DNA, with oligonucleotides triggering the start of the PCR reaction.

Detection of s*tx*1, s*tx*2, and *eae* genes is performed by a multiplex PCR reaction using specific primers (Table C.1). The method is composed of the following steps:

— template preparation;

— setting-up the PCR reaction;

— determination of the PCR results by horizontal agarose gel electrophoresis.

C.3.2 Template preparation

Cultures streaked on to solid media, e.g. tryptone-soy agar (TSA), are processed as follows:

— pick up a single bacterial colony with a sterile 1 µl loop;

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— prepare the template by suspending the bacteria in 100 µl of 0,22 µm filter-sterilized milliQ[4] water and boil for 10 min.

C.3.3 Setting up the PCR reaction

For each sample, set up a 50 µl reaction [reaction buffer 1×, $MgCl_2$ 1,2 mmol/l, deoxynucleotidetriphosphates (dNTPs) 0,2 mmol/l each, 50 pmol of each primer, 2 units of *Taq* polymerase and 10 µl of DNA template]. Define the volume of the reagents according to the final volume of reaction. Use milliQ water for PCR reactions.

In each PCR assay, include a positive and two negative controls. The positive control is a DNA template obtained from an *E. coli* strain possessing the virulence genes tested, while a negative control is the DNA from a non-pathogenic *E. coli* isolate (no virulence genes harboured) and the other is constituted by a sample without template added.

Incubate the reactions in a thermal cycler programmed with the thermal profile described in Reference [11] (Table C.1).

C.3.4 Agarose gel electrophoresis

Prepare a 20 g/l agarose gel in 1× tris/borate/EDTA (TBE) or tris/acetate/EDTA (TAE). Load each well of the gel with 15 µl of each PCR reagent mixed with loading dye at 1× final concentration. Run the samples in 1× running buffer (TBE or TAE) at constant voltage (100 V). Use a molecular weight marker suitable for assignment of the correct molecular weights to the amplicons produced (refer to Table C.1).

WARNING — Consider that a correct band assignment is a crucial point in the assessment of the presence of the virulence genes. Make sure that the bands produced by the reference strains match exactly the expected molecular weight.

Ethidium bromide should be added to agarose gels to allow the visualization of DNA. This reagent is a DNA intercalating agent commonly used as a nucleic acid stain in molecular biology laboratories. When exposed to ultraviolet light, it fluoresces with a red-orange colour.The final concentration of ethidium bromide shall be of 0,5 µg/ml when added before pouring the agarose gel into the electrophoresis gel cast. Alternatively, the agarose gel can be stained after electrophoresis in a 0,5 µg/ml ethidium bromide aqueous solution.

DNA gel stains other than ethidium bromide are commercially available and may be used following the manufacturer’s instructions.

C.3.5 Apparatus

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

C.3.5.1 Laminar flow hood for PCR.

C.3.5.2 Pipette, capacity 1 ml, sterile.

C.3.5.3 Sterile loops for bacteriology.

C.3.5.4 Borosilicate glass bottles, capacity 500 ml.

C.3.5.5 Borosilicate glass cylinders, capacity 500 ml.

C.3.5.6 Borosilicate glass cylinders, capacity 1 l.

C.3.5.7 Incubator, capable of being maintained at 37 °C ± 1 °C.

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4) milliQ is the trade name of a product supplied by Millipore Corporation. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

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C.3.5.8 Technical bench scales.

C.3.5.9 Autoclave.

C.3.5.10 Pipette aid.

C.3.5.11 Micropipettes.

C.3.5.12 Micropipette tips, sterile.

C.3.5.13 Microcentrifuge tubes, capacity 1,5 ml.

C.3.5.14 PCR tubes, capacity 0,2 ml or 0,5 ml.

C.3.5.15 Thermal cycler.

C.3.5.16 Magnetic plate stirrer.

C.3.5.17 Magnetis stirring bars.

C.3.5.18 milliQ deionizer.

C.3.5.19 Electrophoresis apparatus.

C.3.5.20 UV transilluminator.

C.3.5.21 Ice machine.

C.3.5.22 Microwave oven.

C.3.6 Reagents and media

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and sterile distilled or demineralized water or water of equivalent purity.

C.3.6.1 Agar plates.

C.3.6.2 dNTPs stock solution.

C.3.6.3 Synthetic oligonucleotides solution.

C.3.6.4 *Taq* DNA polymerase and **reaction buffer** 10× with or without $MgCl_2$.

C.3.6.5 Electrophoresis running buffer.

C.3.6.6 Molecular weight DNA marker.

C.3.6.7 Loading dye.

C.3.6.8 Agarose.

C.3.6.9 Ethidium bromide solution or other DNA gel stains.

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C.3.7 Safety and protection devices

Some STEC strains can infect human beings at a very low infectious dose and can cause severe disease. Laboratory-acquired infections have been reported. Therefore, working with STEC requires good laboratory practices and the use of protection devices. Ethidium bromide is a mutagen and toxic agent; therefore it should be used in compliance with the safety sheet provided and with protection devices (lab-coats and latex gloves). UV light can cause damage to eyes, so the use of polymethylmethacrylate shields and protective glasses is mandatory.

C.3.8 Reference strains and process control

Use a STEC strain harbouring s*tx*1, s*tx*2, and *eae* genes as positive control for all these genes. An example is the *E. coli* O157 EDL933 reference strain (WDCM 00188) (References [12][13]).

Use any *E. coli* K12 strain, such as MG1655, as a negative control.

PCR controls are prepared as specified in C.3.2. Control templates can be prepared in advance and stored in 10 µl ready to use aliquots at –20 °C for 8 months.

C.3.9 Interpretation of the results

Samples showing amplification fragments of the expected size (see C.3.4 and Table C.1) are considered as positive for related target genes.

Include positive and negative controls in each reaction, giving positive and negative results, respectively. Should the controls give ambiguous results, repeat the entire procedure.

The validation of the amplicon by means of direct sequencing or other suitable methods can be considered redundant, since this method is intended for confirmation of strains isolated and already subjected to real-time PCR for the same characteristics.

The confirmation of the amplicons (e.g. by direct sequencing or by restriction endonuclease assay) should be performed when ambiguous results are obtained.

Table C.1 — Amplification fragments of the expected size

Target gene	Primer name[11]	Primer sequence	Amplicon size bp
eae	*eaeAF*	GAC CCG GCA CAA GCA TAA GC	384
	eaeAR	CCA CCT GCA GCA ACA AGA GG	
*stx*1	*stx*1*F*	ATA AAT CGC CAT TCG TTG ACT AC	180
	stx1R	AGA ACG CCC ACT GAG ATC ATC	
*stx*2 (group)	*stx*2F	GGC ACT GTC TGA AAC TGC TCC	255
	*stx*2*R*	TCG CCA GTT ATC TGA CAT TCTG	
Thermal profile[11]: 35 PCR cycles, each consisting of 1 min of denaturation at 95 °C; 2 min of annealing at 65 °C for the first 10 cycles, decrementing to 60 °C from cycles 11 to 15 (1 °C per cycle); and 1,5 min of elongation at 72 °C, incrementing to 2,5 min from cycles 25 to 35.			

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Annex D
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Internal amplification control

Three different internal amplification controls (IACs) can be used in real-time PCR:

— TaqMan®[5] is a exogenous internal positive control. The reagent kit includes all reagents necessary (primers, a Vic™[6] probe, IAC target DNA and blocking solution). The IAC target DNA requires dilution by 10 times to achieve a copy number of approximately 100 per PCR reaction. The PCR product length is not declared to the customer.

— For the open formula pUC 19 based internal amplification control IAC, see Reference [27]. Approximately 100 copies of target DNA (pUC 19) should be used per PCR reaction. The size of the IAC was 119 bp.

— A recombinant plasmid (named pIAC-STEC) can be used in the *stx*-specific real-time PCR assay.[7] This IAC contains the following DNA fragment cloned into the *Eco*RI site of pUC19:

  5′-ATT**TTTGTTACTGTGACAGCTGAAGCTTTACG**TGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATA**GATGTTGATCTTACATTGAACTGGGG**AATT-3’ (bold letters: *stx*1/*stx*2 forward and reverse primers binding sites sequences; underlined sequence: IAC-probe binding site).

  The IAC is co-amplified with *stx* genes using the same primers as *stx* (Annex E), under the same conditions and in the same PCR tube.[14] It is detected by a specific DNA probe (5’ [Red640]-CAAGGCGACAAGGTGCTGATGCCG-[BHQ2] 3’) included in the PCR mix at a concentration identical to that of the *stx*1 and *stx*2 DNA probes (both labelled with [fluorescein] and [BHQ1] at their 5’ and 3’ ends, respectively). A total of 64 copies of the IAC should be used per PCR reaction. The IAC PCR product is 96 bp long. The performance of the resulting *stx*-IAC real-time PCR assay has been shown using artificially and naturally contaminated food samples (Reference [5]).

The second and third systems may also be used as an extraction control by adding 100 copies of the pUC 19 plasmid or of the pIAC-STEC to the sample aliquot prior to the DNA purification step.

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5) TaqMan is the trade name of a product supplied by Applied Biosystems. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

6) Vic is the trade name of a product supplied by Applied Biosystems. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

7) Auvray et al. personal communication.

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Annex E
(informative)

Primers and probes for the PCR assays

The real-time PCR protocol described is based on the use of the following primers and probes, which should be considered as reference reagents. However, other methods involving the use of different primers and probes may be used provided that they have been recognized equivalent to those indicated in the Tables E.1 and E.2 according to ISO 16140:2003[17] rules.

E.1 Primers and probes

Tables E.1 and E.2 provide the primers and probes sequences, respectively, for:

— the detection of *stx* and *eae* genes by real-time-PCR (PCR A);

— the detection of serogroup-related genes using real-time-PCR (PCR B).

In Tables E.1 and E.2, the chemistry of the reporter and quencher fluorophores is not indicated, as this is largely dependent on the real-time PCR instruments available in each laboratory.

Table E.1 — Degenerate primers and probes used for 5'-nuclease PCR assays

Target gene	Forward primer, reverse primer and probe sequences (5'-3')[a]	Amplicon size bp	Location within sequence	GenBank accession No.
*stx*1 Reference [3]	TTT GTY ACT GTS ACA GCW GAA GCY TTA CG CCC CAG TTC ARW GTR AGR TCM ACR TC Probe-CTG GAT GAT CTC AGT GGG CGT TCT TAT GTAA	131	878 to 906 983 to 1008 941 to 971	M16625
*stx*2[b] Reference [3]	TTT GTY ACT GTS ACA GCW GAA GCY TTA CG CCC CAG TTC ARW GTR AGR TCM ACR TC Probe-TCG TCA GGC ACT GTC TGA AAC TGC TCC	128	785 to 813 887 to 912 838 to 864	X07865
eae Reference [2]	CAT TGA TCA GGA TTT TTC TGG TGA TA CTC ATG CGG AAA TAG CCG TTA Probe-ATA GTC TCG CCA GTA TTC GCC ACC AAT ACC	102	899 to 924 1000 to 979 966 to 936	Z11541

[a] In the sequence Y is (C, T), S is (C, G), W is (A, T), R is (A, G), M is (A, C).

[b] This combination of primer/probe recognizes all the *stx*2 variants except the *stx*2*f*.

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Table E.2 — Primers and probes used for amplification of O antigen specific genes in 5'-nuclease PCR assays

Target gene (serogroup)	Forward primer, reverse primer and probe sequences (5'-3')	Amplicon size bp	Location within sequence	GenBank accession number
rfbE (O157) Reference [3]	TTT CAC ACT TAT TGG ATG GTC TCAA CGA TGA GTT TAT CTG CAA GGT GAT Probe-AGG ACC GCA GAG GAA AGA GAG GAA TTA AGG	88	348 to 372 412 to 435 381 to 410	AF163329
wbdI (O111) Reference [3]	CGA GGC AAC ACA TTA TAT AGT GCT TT TTT TTG AAT AGT TAT GAA CAT CTT GTT TAGC Probe-TTG AAT CTC CCA GAT GAT CAA CAT CGT GAA	146	3464 to 3489 3579 to 3609 3519 to 3548	AF078736
wzx (O26) Reference [3]	CGC GAC GGC AGA GAA AATT AGC AGG CTT TTA TAT TCT CCA ACT TT Probe-CCC CGT TAA ATC AAT ACT ATT TCA CGA GGT TGA	135	5648 to 5666 5757 to 5782 5692 to 5724	AF529080
ihp1 (O145) Reference [3]	CGA TAA TAT TTA CCC CAC CAG TAC AG GCC GCC GCA ATG CTT Probe-CCG CCA TTC AGA ATG CAC ACA ATA TCG	132	1383 to 1408 1500 to 1514 1472 to 1498	AF531429
wzx (O103) Reference [4]	CAA GGT GAT TAC GAA AAT GCA TGT GAA AAA AGC ACC CCC GTA CTT AT Probe-CAT AGC CTG TTG TTT TAT	99	4299 to 4323 4397 to 4375 4356 to 4373	AY532664

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Annex F
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Isolation of STEC strains

Adopt the procedure specified to isolate STEC strains from real-time PCR positive samples.

a) In case of positivity to one of the genes associated with the serogroups within the scope of this Technical Specification, a serogroup-specific enrichment (SSE) on the remaining enrichment culture may be performed in order to facilitate the isolation of the STEC (see Note 1).

b) Streak the enrichment culture or the SSE on to TBX or other suitable medium (see Note 2). Incubate for 18 h to 24 h at 37 °C ± 1 °C.

c) Pick up 50 colonies with *E. coli* morphology or with characteristic aspect (see Note 5) and point-inoculate on nutrient agar (NA) (see Note 3) and H_2O (the colonies may be pooled in water to a total of 10 per pool).

d) Perform the detection of the Stx-coding genes on the isolated colonies or the H_2O pools (see Note 4).

e) If a pool is positive, go back to NA and assay the individual colonies forming the positive pool in order to select one single positive colony.

f) Identify the colonies as *E. coli* and confirm the presence of the *eae* gene and serogroup if identified in the screening step (e.g. by PCR B in Annex E), see Note 5.

g) Isolates may be sent to a reference laboratory for further characterization.

NOTE 1 Serogroup-specific enrichment can be achieved by using immunocapture systems such as IMS or equivalent. Generally, refer to the instructions supplied by the manufacturer.

NOTE 2 For O157 positive samples; ISO 16654[19] or alternative methods validated according to ISO 16140-2[18] are appropriate. Sorbitol-fermenting *E. coli* O157 are susceptible to tellurite contained in the CT SMAC medium indicated in ISO 16654.[19] Therefore the use of a second SMAC isolation plate without antibiotics is appropriate. In the absence of sorbitol-negative colonies on the plates, the screening of sorbitol-positive colonies is indicated.

For STEC O26 isolation, a differential solid medium (MacConkey) containing rhamnose instead of lactose is commercially available (RMAC). It is very effective in distinguishing STEC O26 strains, which do not ferment rhamnose, from other *E. coli*.

NOTE 3 There are several types of nutrient agar media commercially available either ready to use plates or prepared in house from dehydrated powders. Every type of non-selective nutrient agar media (e.g. TSA) is suitable for the purpose of maintaining the colonies for further characterization. Enterohaemolysin agar can also be used. It has the advantage of detecting enterohaemolysin production, which is a common feature of STEC pathogenic to humans.

NOTE 4 The real-time PCR described in this protocol can be adopted to confirm the presence of the *stx* and *eae* in the isolated strains. Conventional PCR can be used as an alternative (Annex C).

NOTE 5 Colony confirmation as *E. coli* can be achieved by using any commercial biochemical multi-assay or by assessing the production of indole. Confirmation of the serogroup can be achieved either by PCR or by agglutination with commercial antisera.

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