Detection and identification of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 in food by Real Time PCR

1. Aims and field of application:

The large outbreak of STEC infections in Germany was caused by a STEC strain belonging to serotype O104:H4, a serotype (and serogroup), not comprised among those usually associated with severe infections in Europe and worldwide.

The characteristics of the outbreak strain are the following:

1. It produces Stx2, and harbors the *stx2a* gene subtype.
2. It lacks the gene coding for the adherence factor intimin (*eae* gene), which is considered as a hallmark of the pathogenic STEC.
3. It possesses the genetic markers of typical of Enteroaggregative *Escherichia coli* (EAggEC): the *aggR, aatA*, *aaiC* and *aap* genes.

Therefore, the specific genetic markers to be considered for the molecular screening of *stx*-positive enrichment cultures are the O104 antigen-associated gene wzx_{O104} and the gene encoding the H4 flagellar antigen, $fliC_{H4}$.

The markers associated with the enteroaggregative adhesion should be considered for confirmation and characterization of the isolated strains. A laboratory method for such tests is available at the EURL-VTEC website.

The proposed method aims at the identification of the presence of O104 antigen-associated gene (wzx_{O104}) in *stx*-positive enrichment cultures. The molecular design of this Real Time PCR has been described in the literature

(Bugarel *et al*., 2010). The same assay can be used to identify and confirm the O104:H4 serotype of isolated strains in combination with the *fliC*$_{H4}$ RT-PCR that has been deployed at the EURL-VTEC on the *fliC* gene sequence of the *E. coli* strain U9-41 present in GenBank under the accession number AY249989.

2. Screening of food samples

Enrichment cultures are performed by adding a 25 g test portion of food sample or 25 ml of liquid sample to 225 ml of enrichment broth, as it is specified in the ISO/TS 13136:2012, and incubating for 18-24 h at 37 ± 1 °C. One ml aliquot of such a culture is used for DNA extraction and purification. For testing of seeds used for production of sprouts to be consumed raw, refer to **Annex 2**.

This Real Time PCR protocol is used to test all the samples that give positive results for the presence of *vtx* genes by using the first step of the ISO/TS 13136:2012 – Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and determination of O157, O111, O26, O103 and O145 serogroups - Qualitative Real-time polymerase chain reaction (PCR)-based Method.

The Real-time PCRs for *wzx*$_{O104}$ and *fliC*$_{H4}$ are performed using the primers and probes described in **Annex 1**. Amplification conditions to be applied will depend on the system used, and will refer to the instructions supplied with the instrument and the reagents' kit of choice.

A standard two-step thermal profile used at EURL-VTEC for both the reactions is the following:

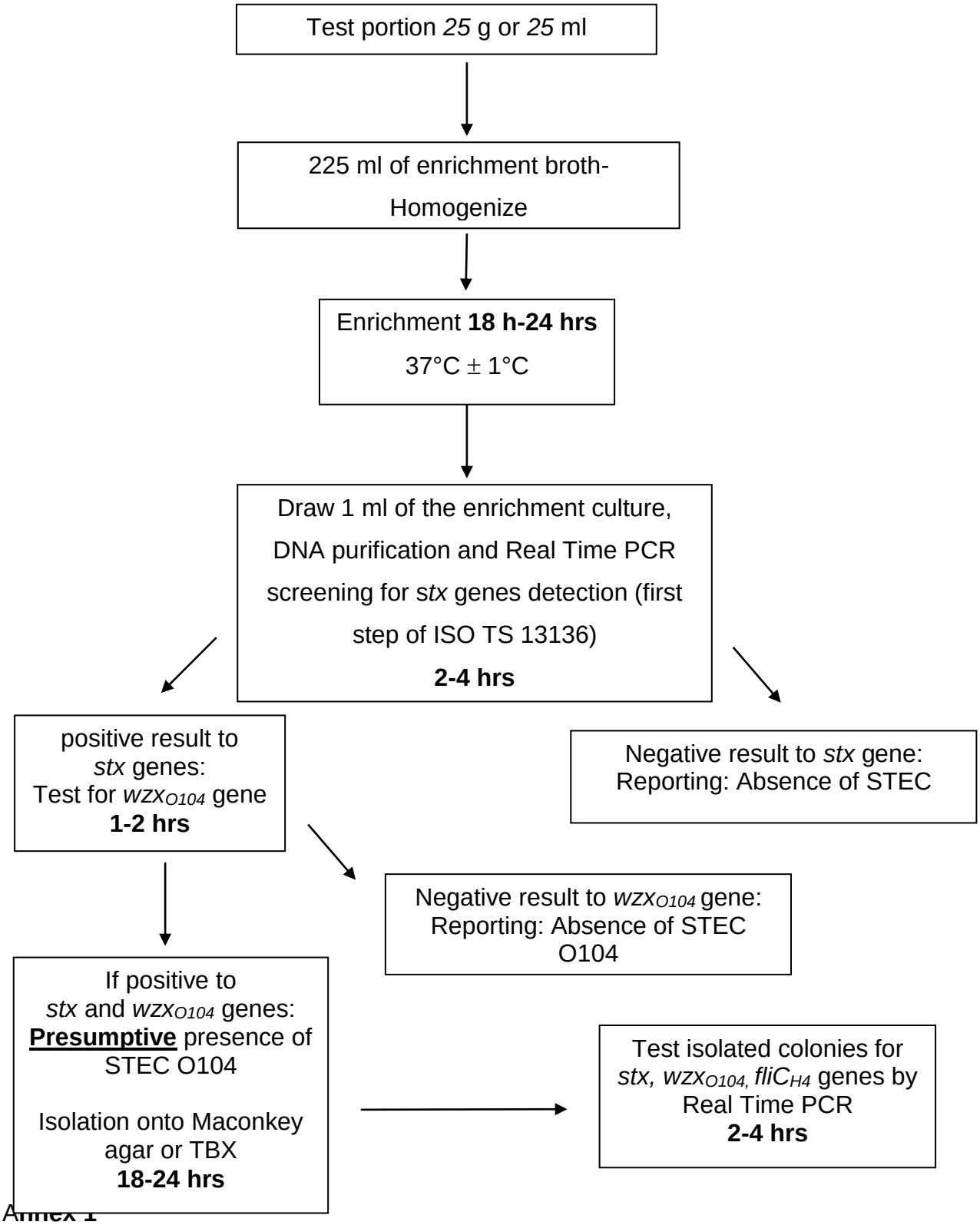
95 °C X 10'
35 cycles of
95 °C X 15''
60 °C X 1'

Enrichment cultures positive for presence of wzx_{O104} gene are streaked onto MacConkey agar or TBX plates or any other media suitable for *E. coli* isolation.

Up to 50 isolated colonies with typical *E. coli* morphology are collected and point-inoculated on Nutrient Agar (NA) (single colonies) and H_2O (5 pools by 10 colonies each). The *stx* gene detection is performed on the isolated colonies or pools by Real Time or conventional PCR (reference methods can be found at the EURL website). Colonies positive for *stx* genes will be tested for the O104 antigen-associated gene wzx_{O104} and the gene encoding the H4 flagellar antigen, $fliC_{H4}$. Colonies can be also tested for the presence of the markers associated with the enteroaggregative adhesion using the laboratory method available at the EURL-VTEC website.

For strain characterization, the Real-time PCRs for wzx_{O104} and $fliC_{H4}$ can be run as duplex PCR, labeling the two probes with compatible phluorophors (*e.g.* FAM and HEX).

The whole laboratory procedure for the detection of STEC O104:H4 in food samples is summarized in the flow diagram below.


Test portion *25* g or *25* ml
225 ml of enrichment broth- Homogenize
Enrichment **18 h-24 hrs** 37°C ± 1°C
Draw 1 ml of the enrichment culture, DNA purification and Real Time PCR screening for s*tx* genes detection (first step of ISO TS 13136) **2-4 hrs**
positive result to *stx* genes: Test for wzx_{O104} gene **1-2 hrs**
Negative result to *stx* gene: Reporting: Absence of STEC
Negative result to wzx_{O104} gene: Reporting: Absence of STEC O104
If positive to *stx* and wzx_{O104} genes: **Presumptive** presence of STEC O104
Isolation onto Maconkey agar or TBX **18-24 hrs**
Test isolated colonies for *stx*, wzx_{O104}, $fliC_{H4}$ genes by Real Time PCR **2-4 hrs**

Annex 1

Real-time PCR for the detection of wzx_{O104} and $fliC_{H4}$ genes

1. Principle of the method

This Real-time PCR protocol aims at the detection of the O104 serogroup-associated gene, wzx_{O104}, coding for the O-antigen flippase Wzx and the gene encoding the flagellar antigen H4, $fliC_{H4}$.

2. Primer and probes

The primers and probes to be used are listed in the **Table** below. A typical Real Time PCR reaction is described below. The chemistry of the reporter and quencher phluorophores are not indicated being largely dependent on the Real-time PCR systems available in each laboratory.

Table 1. Primers and probes used in 5' nuclease PCR assays

Target gene (Ref.)	Forward primer, reverse primer and probe sequences (5'-3') [a]	Amplicon size (bp)	Location within sequence	GenBank accession number
wzx_{O104}(1)	TGTCGCGCAAAGAATTTCAAC AAAATCCTTTAAACTATACGCCC Probe- TTGGTTTTTTTGTATTAGCAATAAGTGGTGTC	100	2,333,750–2,333,730 2,333,673–2,333,651 2,333,724–2,333,693	CU928145
$fliC_{H4}$(2)	GCTGGGGGTAAACAAGTCAA CCAGTGCTTTTAACGGATCG Probe- TCTTACACTGACACCGCGTC	192	604-623 796-777 631-650	AY249989

(1) Bugarel M. et al. Int J Food Microbiol 2010 142:318-329
(2) EURL-VTEC

For <u>isolated strains</u> characterization the Real-time PCR for wzx_{O104} and $fliC_{H4}$ can be run as duplex PCR, labeling the two probes with compatible phluorophors (e.g. FAM and HEX).

RT PCR reaction assembly:

Buffer 10X	to 1X ($MgCl_2$ 3mM)
Primer Fwd	500 nM
Primer Rev	500 nM
Probe	200nM
DNA	2 µl of DNA purified from 1 ml of culture and diluted 1:10 can be sufficient
Water	to final volume

3. Controls

A STEC strain belonging to serotype O104:H4 should be used as positive control.

The Real-time PCR procedure requires an **inhibition/extraction control**. Details on the possible systems to be used as inhibition/extraction control are given below. In particular, two different internal amplification controls (IACs) can alternatively be used:

- A commercially available TaqMan® Exogenous Internal Positive Control (Applied Biosystems, Foster City, CA, USA). The kit includes all reagents necessary (primers, a Vic™probe, IAC target DNA and blocking solution). The IAC target DNA must be diluted 10 times to achieve a copy number of approximately 100 per PCR reaction. The PCR product length is not declared to the customer.
- The open formula pUC 19 based internal amplification control IAC developed by Fricker et al. (2007. Appl Environ Microbiol 73**,** 1892-1898). Approximately 100 copies of target DNA (pUC 19) should be used per PCR reaction. The size of the IAC is 119 bp.

The latter may be also used as an extraction control by adding 100 copies of the pUC 19 plasmid to the sample aliquot prior to the DNA purification step.

Annex 2

Testing of seeds intended for the production of sprouts

When the matrix to be analyzed is constituted by seeds the following considerations have to be made:

1. The seeds are generally contaminated at very low levels (0.1 to 1.8 cfu/gr, as has been assessed for Salmonella. See reference below). Nonetheless, the sprouting process is characterized by conditions (humidity, heat) favoring the pathogen's enrichment.
2. The seeds are generally dried. Therefore, the contaminating pathogens are supposed to be stressed.
3. The contamination may occur on the surface of the seed as well as being internal. The latter case occurs when the seeds are contaminated during the growth of the plants used for their production (primary contamination of crops). Contamination of the seeds' surface can occur during all the phases of preparation, storage and general handling of the seeds (secondary contamination).
4. The enrichment cultures of seeds may contain inhibitors of the DNA polymerase used for the PCR screening of the samples

In order to increase the analytical power of the proposed method, the following steps are carried out when dealing with seeds:

1. 50 gr of seeds are analyzed instead of the usual 25 gr of food items in order to increase the sensitivity of the assay.
2. The seeds are smashed in a sterile container (e.g. a stomacher bag) using a mortar with pestle or other similar tools before adding the enrichment broth.
3. The smashed seeds are transferred to a sterile container (flask or a new stomacher bag) added with 450 ml BPW and incubated for 24 hrs at 37°C (static or in agitation).

4. A 5 ml aliquot of the enrichment culture is taken, mixed by a vortex (in order to detach any possible Enteroaggregative bacteria adhering to seeds), centrifuged at 500 X g 1 min to sediment the seeds' debris. One ml aliquot of the supernatant is taken at this stage and used for DNA preparation.
5. The DNA is diluted 1:10 before using it. In case of absence of amplification in the reactions containing the IAC, the DNA template is used at the dilution of 1:30.

Reference

Ching-Hsing Liao, William F. Fett. 2003. Isolation of Salmonella from alfalfa seed and demonstration of impaired growth of heat-injured cells in seed homogenates. International Journal of Food Microbiology 82: 245– 253