# Report of the 43rd inter-laboratory study on the detection of Shiga toxin-producing *E. coli* (STEC) in sprouts (PT43) - 2025

**Edited by:**

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## 1. OBJECTIVES OF THE STUDY

The study consisted in the detection and isolation of STEC in sprout samples and the **objectives** were:

- to improve the preparedness of the NRLs towards testing sprouts in compliance with Regulation (EU) No 209/2013;
- to improve the preparedness of the NRLs towards the detection and isolation of STEC strains not belonging to the O157 serogroup;
- to give further support to the NRLs for the accreditation of the ISO TS 13136:2012.

This document represents the full evaluation report of the study.

## 2. PARTICIPANTS

Thirty-one NRLs from the following 24 EU Member States and two EFTA Countries participated in the study.

- Austria, Institut für Medizinische Mikrobiologie und Hygiene, AGES
- Belgium, SCIENSANO – Foodborne Pathogens
- Bulgaria, National Diagnostic and Research Veterinary Institute
- Croatia, Laboratory for food microbiology, Croatian Veterinary Institute
- Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO), Cyprus Veterinary Services
- Czech Republic,State Veterinary Institute Prague
- Estonia, National Centre for Laboratory Research and Risk Assessment (LABRIS)
- Finland, Finnish Food Safety Authority Evira, Research and Laboratory Department, Food and Feed Microbiology Research Unit
- France, Vetagro Sup
- Germany, Federal Institute for Risk Assessment,
- Hungary, National Food Chain Safety Office, Food Chain Safety Directorate
- Ireland, Department of Agriculture, Food and the Marine
- Italy, Istituto Superiore di Sanità
- Latvia, Institute of Food Safety, Animal Health and Environment (BIOR)
- Lithuania, National Food and Veterinary Risk Assessment Institute
- Luxembourg, Laboratoire National de Santé
- The Netherlands, National Institute for Public Health and the Environment - RIVM
- The Netherlands, Wageningen Food Safety Research WFSR
- Norway, Norwegian Veterinary Institute

- Poland, National Institute of Public Health (NIH)
- Poland, National Veterinary Research Institute (NVRI)
- Portugal, Instituto Nacional de Investigação Agrária e Veterinária
- Romania, Institute for Hygiene and Veterinary Public Health
- Slovakia, Veterrinary and Food Institute SVFI Dolny Kubin
- Slovenia, University of Ljubljana Veterinary Faculty, National Veterinary Institute
- Spain, National Plant Health and Hygiene Laboratory
- Spain, Laboratorio Central de Veterinaria de Algete (MAPA)
- Spain, Centro Nacional de Alimentación-AESAN
- Sweden, Swedish Food Agency
- Sweden, National Veterinary Institute
- Switzerland, Agroscope

After the deadline for submitting the results, each NRL received its own individual participation report indicating the expected and the reported results.

## 3. MATERIALS AND METHODS

### 3.1. Sample preparation

Three test samples (1, 2 and 3), each consisting of 25 g of Alfalfa sprouts potentially contaminated with STEC, were sent in the blind to the NRLs.

The sprouts used have been acquired as a single batch from a local producer and contained a natural background microflora of $6x10^6$ bacterial CFU per gram of sprouts ($3x10^6$ CFU of Enterobacteria per gram of sprouts). The sprouts were portioned in 25 g samples in sterile stomacher bags and placed at + 4 °C until the artificial contamination was carried out. Two 25 g portions of sprouts of the same batch were initially assayed for the presence of STEC by applying the ISO TS 13136:2012 method. Both samples were negative for all the target genes at the screening.

The artificial contamination of the samples was carried out on the 31st of March 2025, using appropriate dilutions of an exponential liquid culture (0.5 OD read at 600 nm) of the STEC strain C1178-04 (O145:H28) possessing the *stx1* gene and positive for the presence of the *eae* gene. The characteristics of the samples are reported in Table 1.

An uncertainty of measurement of 0.37 log cfu/ml was associated with the standardized inoculum, using the procedure described in the ISO TS 19036:2006. The samples were spiked with three different levels of contamination: zero, low and high level (Table 1). Serial

dilutions of the inoculum suspensions of strain C1178-04 added to the samples were plated onto MacConkey agar plates to confirm the titer.

The test samples were labeled with randomly generated numerical codes different for each participant laboratory and stored at +4°C until shipped refrigerated on 31st of March 2025 by courier. The NRLs were requested to record the date of receipt and sample temperature and to start the analyses immediately upon receipt.

**Table 1: Characteristics of the red radish sprout samples assessed in the study**

| Contaminant (*Genotype*) | Contamination level in: | | |
|---|---|---|---|
| | **Sample 1** | **Sample 2** | **Sample 3** |
| Strain C1178-04, STEC O145 *(stx1+, stx2-, eae+ ihp1$_{O145}$+)* | - | Low: 50 CFU/g | High: 200 CFU/g |

### 3.2. Assessment of the stability and homogeneity of the samples

The stability and homogeneity of the samples were evaluated according to the requirements of ISO 17043:2010.

The stability was assessed using samples spiked on the 6th of February 2025 and tested by ISO TS 13136:2012 after 0, 2, 4, and 7 days since the initial contamination. The Real Time PCR screening was positive for the STEC target genes after 4 days from the spiking. Isolation was successful for all the samples spiked with the low and high level of contamination up to two days from spiking.

When the test samples were prepared, six bags for each of the three levels of contamination were randomly selected for homogeneity testing, enriched at 37°C and analyzed by Real Time PCR for the presence of the contaminating STEC on the 31st of March 2025. The Real Time PCR screening carried out for the homogeneity tests were positive for the STEC target genes in contaminated samples only, as expected.

### 3.3. Laboratory methods

Laboratories were requested to identify the presence of STEC using the ISO TS 13136:2012 method, taking into account the adaptation provided by the EU Reference Laboratory for *E. coli* (EURL-VTEC) for the specific detection of STEC O104:H4

(EURL VTEC_Method_04_Rev 1: "*Detection and identification of Verocytotoxin-producing Escherichia coli (VTEC) O104:H4 in food by Real Time PCR*").

**4.4. Collection and elaboration of the NRL results**

The results were submitted through a dedicated website developed by the EURL for *E. coli* and the deadline was set on 22 April 2025.

**4.5. Analysis of the NRL results**

***4.5.1. Evaluation of the NRL performance in the Real Time PCR screening step***

The performance of each NRL in identifying STEC target genes in the enrichment cultures was evaluated by assigning four penalty points to each incorrect or missing result concerning the identification of *stx1* and *stx2*, and two penalty points for the incorrect or missing identification of *eae* gene as well as the top-5 and O104 serogroups.

***4.5.2. Evaluation of the NRL performance in the isolation of STEC strains from the PCR-positive enrichment cultures***

The performance of each NRL in the isolation and characterization of the isolated STEC strain was also assessed. In detail, two penalty points were assigned in case of lack of isolation of STEC from samples 2 or 3. As for strain characterization, four penalties have been assigned to incorrect detection of Stx-coding genes and two for incorrect detection of *eae* gene. Two penalties were assigned to the laboratories for not reporting the information on the serogroup of the STEC strain isolated or reporting an incorrect serogroup.

***4.5.3. Evaluation of the NRL performance in the overall procedure***

The sum of the penalty points obtained in the different steps of the procedure originated a total score, used to evaluate the overall performance of the NRLs in the PT. The performance of laboratories that obtained a score higher than eight was considered unsatisfactory.

**4.6. Evaluation of the performance of the method**

Sensitivity (*Se*) and Specificity (*Sp*) were calculated for the various STEC characters considered in the study and for the different steps of the ISO TS 13136:2012. Therefore, Se and *Sp* were calculated for the PCR screening for *stx1*, and *eae* genes, and for the isolation of the STEC strain. *Se* was evaluated also for $ihp1_{O145}$ gene determination and *Sp* could be calculated for the *stx2* gene target as well. The sensitivity and specificity were calculated according to the following formulas:

Sensitivity: *Se* = [true positives / (true positives + false negatives)] x 100

Specificity: *Sp* = [True negatives / (true negatives + false positives)] x 100

## 5. RESULTS

All the 31 Laboratories received the samples in good condition.

As for the delivery time, all but seven NRLs received the samples within 24 hours. The remaining seven Laboratories received the test materials after 48 h.

The results submitted by the participating laboratories are summarized in **Figures 1 – 3**.

**Figure 1. Proportion of Laboratories reporting the correct screening results (a) and isolating the STEC strain (b) (green: correct result; red: incorrect result).**

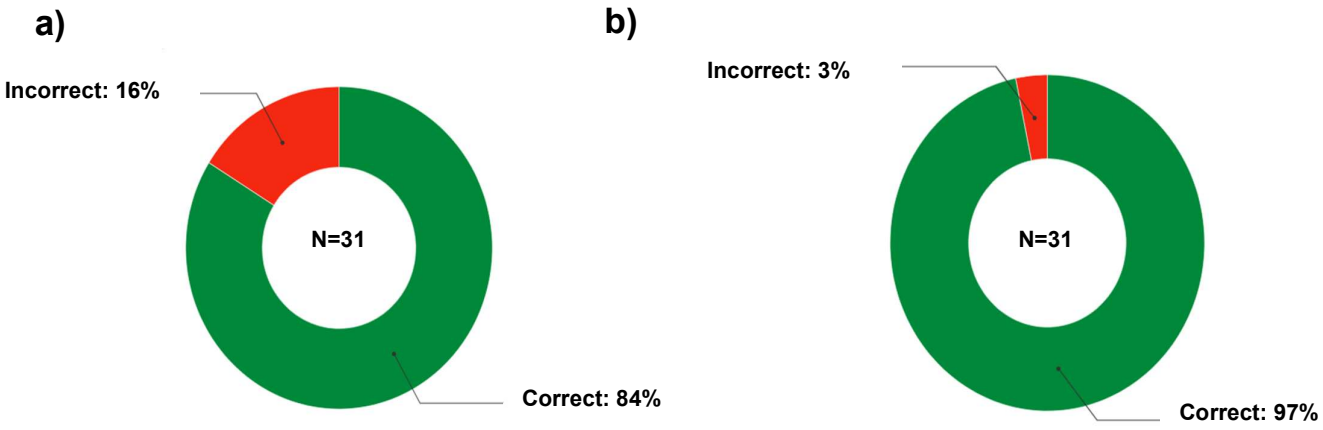


**Figure 2. Real-time PCR detection of virulence and serogroup-associated genes in the enrichment cultures (yellow boxes represent the gold standards; green boxes: correct results and red boxes: incorrect results).**

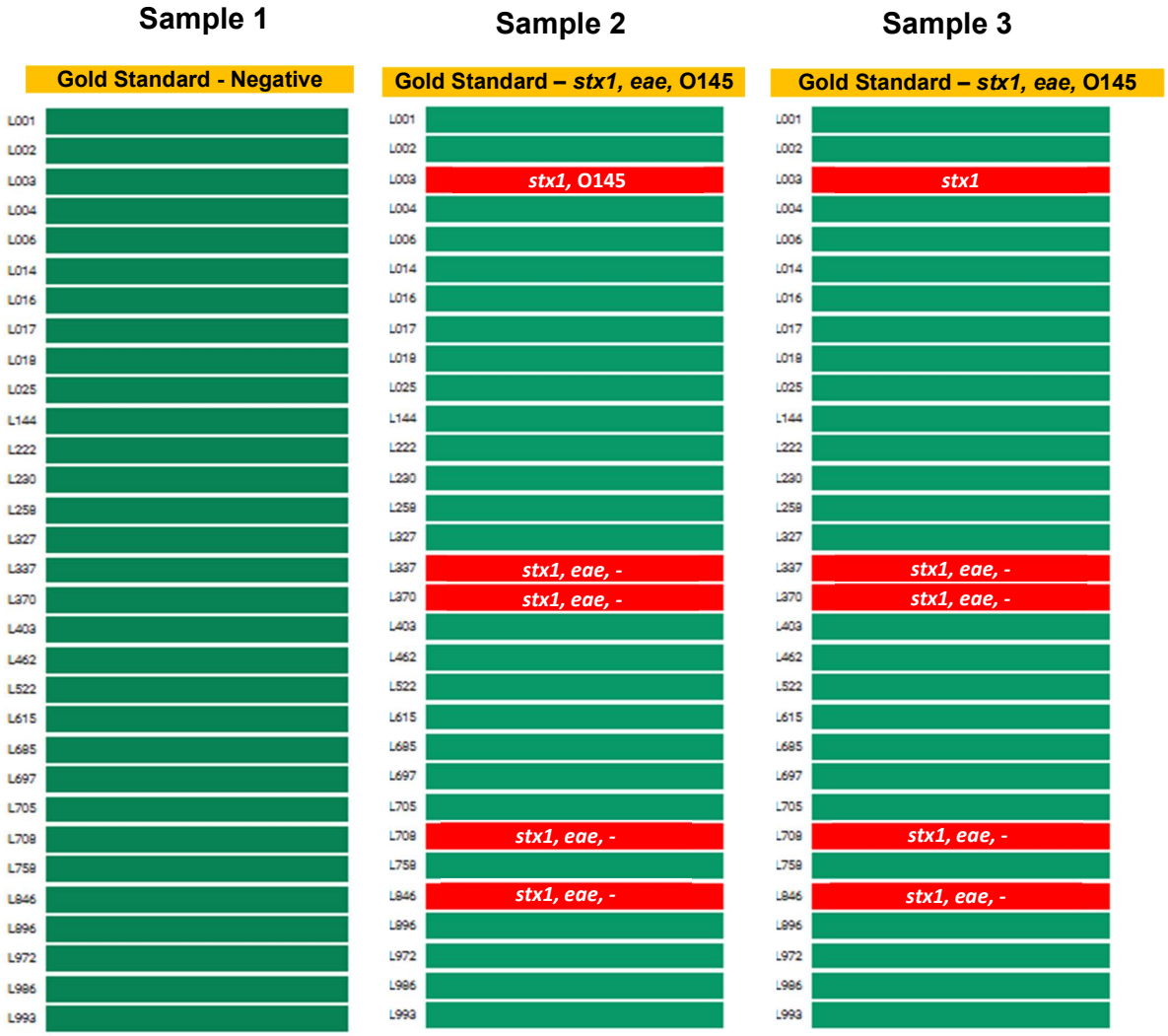
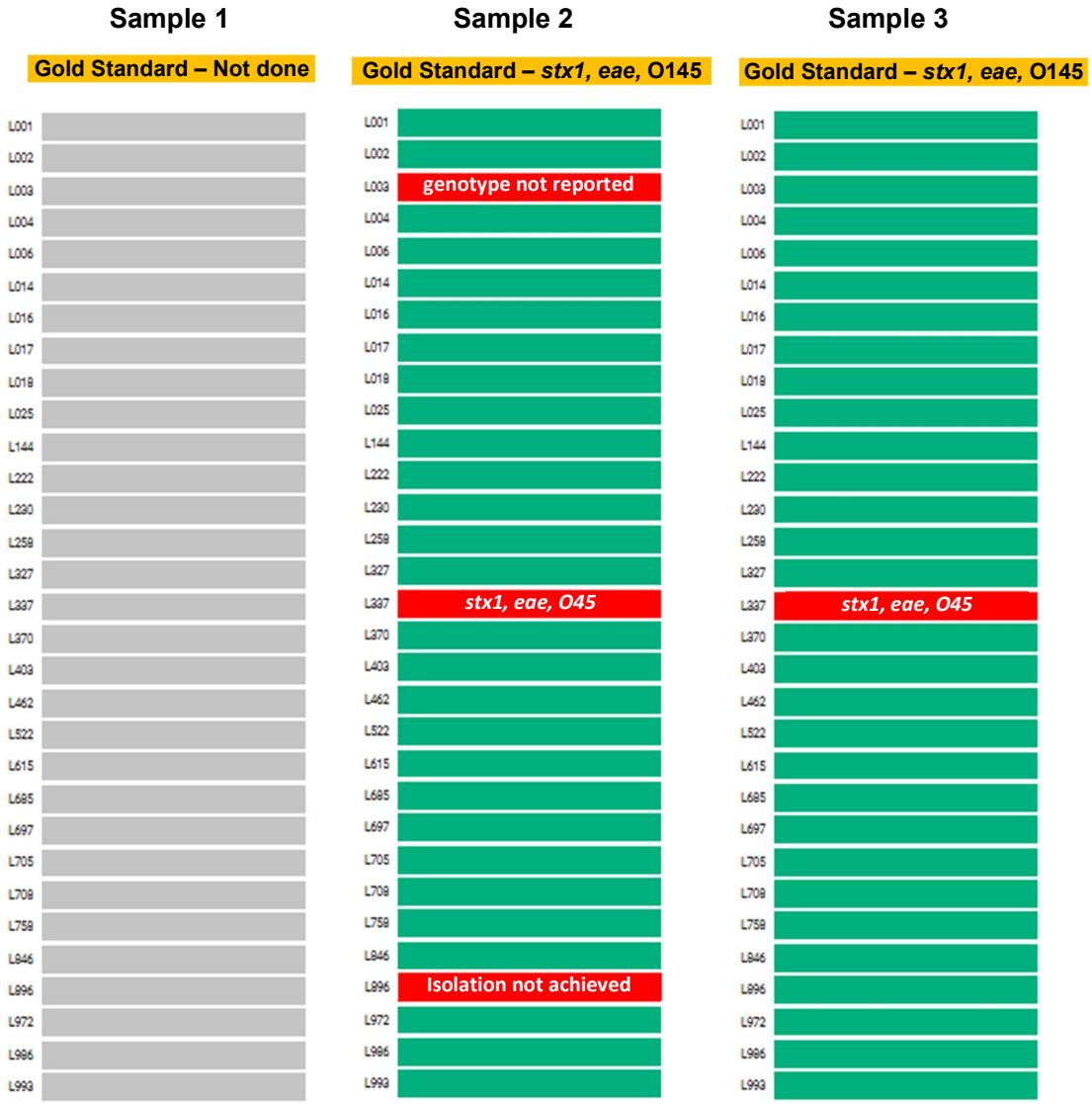


**Figure 3. Isolation and genotyping of STEC strains from the sprouts samples (Yellow boxes represent the gold standards; green boxes: correct results and red boxes: incorrect results).**

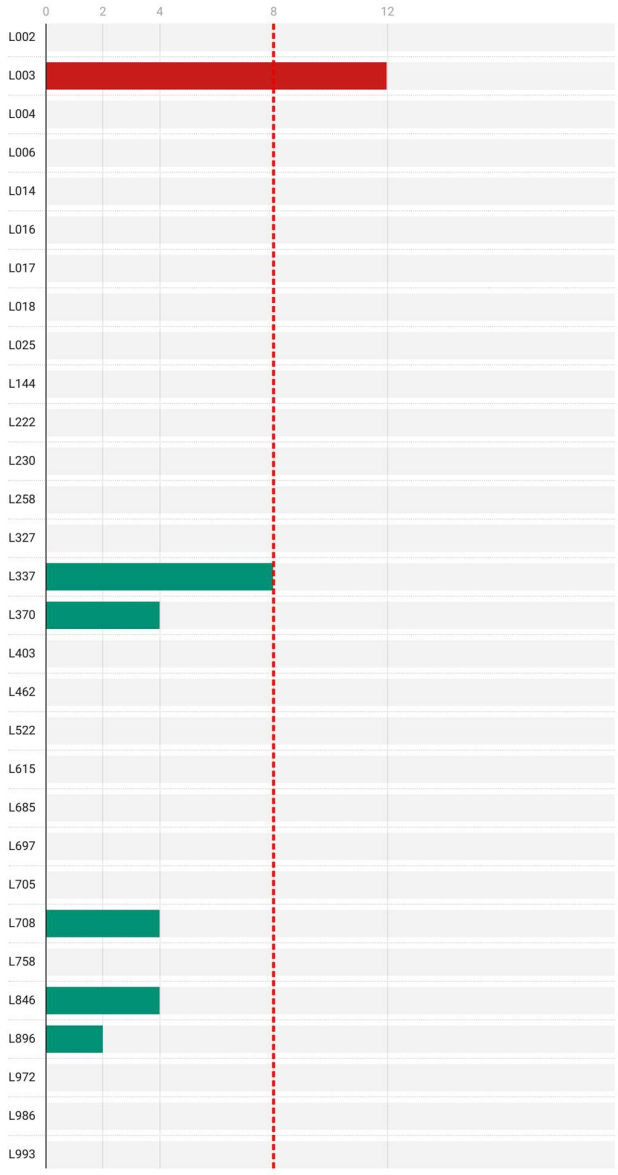



A participant (L003) reported to have isolated STEC in sample 2, but didn't provide any information about its characterization. One Laboratory reported that isolation was achieved by applying the acid treatment to the enrichment cultures, whereas another one reported to have applied IMS. L846 informed in the note field not to perform the detection of serogroup-associated genes in the screening, except for O104: as this is part of the ISO TS 13136:2012 procedure, this laboratory was assigned two penalty points per sample in the screening for missing the detection of O145-associated gene *ihp1*$_{O145}$.

Two laboratories reported to have tested and detected the presence of serogroup O45 in the screening, and another one incorrectly typed the STEC isolated strain as O45.

For each NRL, the number of penalty points was determined using the criteria described in section 4.5. **Figure 4** shows the score achieved by each NRL. Only one laboratory did not comply with the definition of satisfactory proficiency.

**Figure 4. Evaluation of the NRLs performance in the PT procedures (screening and isolation steps). A score higher than 8 (red bars) was considered unsatisfactory.**



The calculation of ***Se* and *Sp* in the screening step** was performed based on the results provided by all 31 participating NRLs.

**Table 1. Sensitivity and Specificity of the screening step.**

| | ***Se*** ***(low level)*** | ***Se*** ***(high level)*** | ***Sp*** |
|---|---|---|---|
| ***stx1*** | 100% | 100% | NA |
| ***stx2*** | NA | NA | 100% |
| ***eae*** | 96.8% | 96.8% | NA |
| ***Ihp1$_{O145}$*** | 87.1% | 87.1% | NA |

The ***Se* in the isolation step** was 96.8% in the low-level contamination sample and 100% in the high-level contamination sample. The Limit of detection ($LOD_{50}$) of the isolation step was not evaluated.

## 6. CONCLUDING REMARKS

PT43 concerned the application of the ISO TS 13136:2012 on sprout samples for the benefit of the network of NRLs, as this is the only matrix for which a micro-criterion has been established according to Regulation (EU) No 209/2013.

The analysis of the results provided by 31 Laboratories participating in PT43 induces the following conclusions:

1. A high participation rate was observed, confirming the consolidation of the network of National Reference Laboratories for *E. coli*;
2. The virulence genes of the contaminating STEC strain were identified with high sensitivity in the spiked samples.
3. Almost all the laboratories could isolate the STEC from both the samples with low level and high level of contamination.
4. Only one participating Laboratory presented a non-satisfactory performance and will be contacted for follow up.
5. The $LOD_{50}$ of the isolation step was not calculated as almost all Laboratories could isolate the contaminating STEC strain in both samples with high and low levels of contamination.
6. The performance parameters calculated in PT43 will be added to those already determined for other couples matrix/STEC strain and made available through publication

in the EURL-VTEC website with the aim to support the NRLs and Official Laboratories, which can refer to such parameters to have the method correctly accredited.