



ISO TS 13136:2012

Report on the primary validation of the PCR screening reactions and the determination of the performance parameters, based on the results of the analytical tests carried out within the EURL-VTEC proficiency testing program (2009-2022)

1. Primary validation of the real-time PCR reactions for the detection of the *stx1*, *stx2*, and *eae* genes as well as of the O26, O103, O111, O145, and O157 serogroup-associated genes according to CEN ISO/TS

13136:2012 (from: Kagkli DM *et al.*, *Appl. Environ. Microbiol.* October 2011 vol. 77 no. 19 6954-6963)

1.1. Dynamic Range.

The dynamic range is defined as the range of gene target concentrations over which the method performs in a linear manner with acceptable levels of accuracy and precision. It was determined based on six repetitions of DNA serial dilutions. The amplification reactions for all the targets performed satisfactorily over a range of 1 to 10⁴ copies per reaction (fig 1).

1.2. PCR efficiency.

The efficiency (*E*) of every single PCR reaction was calculated according to the following formula: $E = [10^{(-1/\text{slope})}] - 1$. The slope was that of the curves obtained for the dynamic range determination (fig. 1).

The PCR efficiency values ranged between 80 % for O103 and 95 % for O26-specific PCR. With the exception of the O103 and O145 specific PCR, whose efficiency was below 90 %, the remaining six methods had an efficiency above that value.

1.3. Limit of Detection (LOD).

Reactions containing the DNA targets with a concentration falling in the interval between 0.1 and 20 copies were used to determine the LOD_{100%} of each PCR module. Sixty reactions, each containing a theoretical copy number of 15, 10, 5, and 0.1 target copies, were performed for each of the targets. In some cases (e.g., serogroups), no clear decision could be made regarding the LOD_{100%} determination in that range, and therefore, a similar analysis at 20 copies was performed.

The LOD_{100%} of each PCR was set at 1 dilution above the level in which only 59 out of 60 reactions gave a positive result.

The LOD_{100%} for the *stx*₁ and *stx*₂ was between 10 and 15 copies, while for the *eae* gene it was between 5 and 10 copies.

The PCR for the serogroups O26, O111 and O103 gave a LOD_{100%} result placed between 5 and 15 copies.

The PCR for O145 had a LOD_{100%} between 10 and 15 copies.

The PCR for O157 had a LOD_{100%} between 10 to 20 copies.

A synopsis of the results is reported in table 1.

1.4. Linearity with food matrices.

Twenty-five grams or ml of three different matrices, namely, minced meat, ready-to eat salad, and pasteurized skimmed milk, were artificially inoculated with 2 to 10 CFU g⁻¹ (or ml) of each STEC serogroup and incubated overnight at 37 °C. The total DNA was extracted and serial dilutions were prepared, from 20 ng down to 0.05 ng per reaction when possible. 1:4 serial dilutions of the DNA were prepared and analyzed by real-time PCR. All PCRs performed well, as demonstrated by the linearity of the curve and the efficiencies obtained (Fig 2).

Note: The terms VTEC and STEC are synonyms and will be both used in this document. The same consideration applies to the terms *stx* and *vtx*, indicating the Shiga toxins (verocytotoxins)-coding genes

Fig. 1. Dynamic Range of CEN ISO/TS 13136:2012 PCR (from: Kagkli DM *et al.*, *Appl. Environ. Microbiol.* October 2011 vol. 77 no. 19 6954-6963).

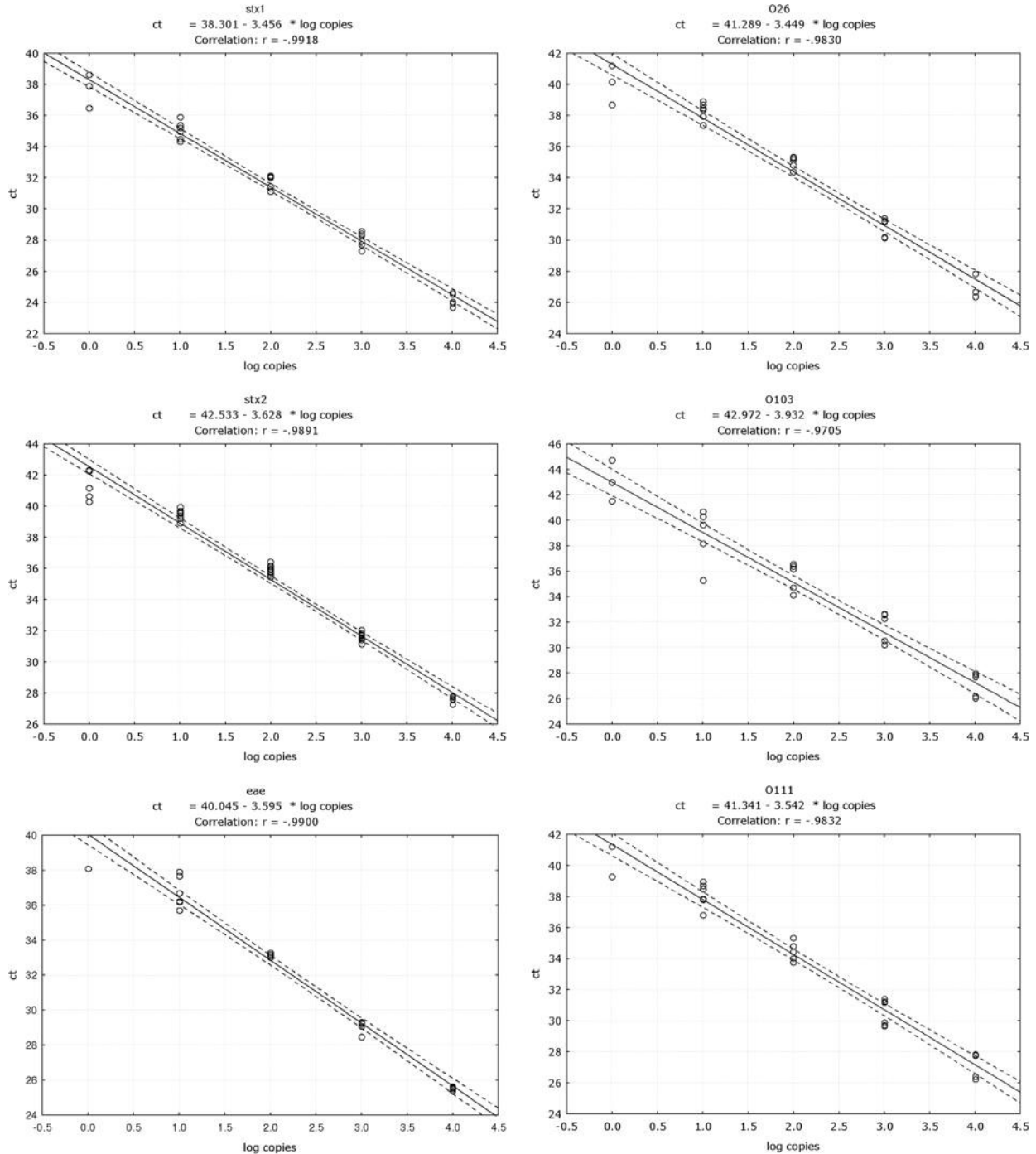


Fig. 1. Continued

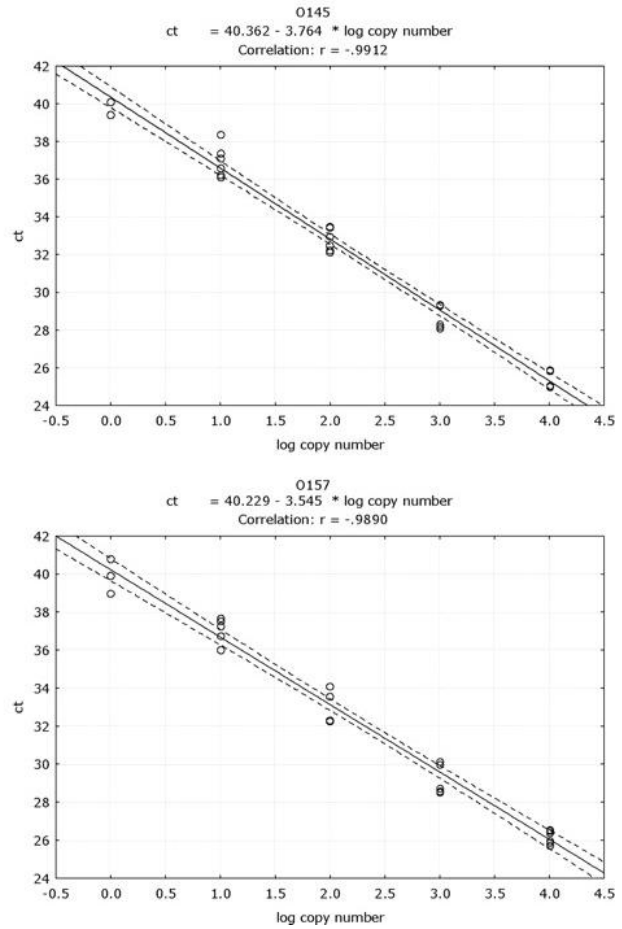


Fig. 2. Linearity of CEN ISO/TS 13136:2012 PCR with food matrices (from: Kagkli DM et al., *Appl. Environ. Microbiol.* October 2011 vol. 77 no. 19 6954-6963)

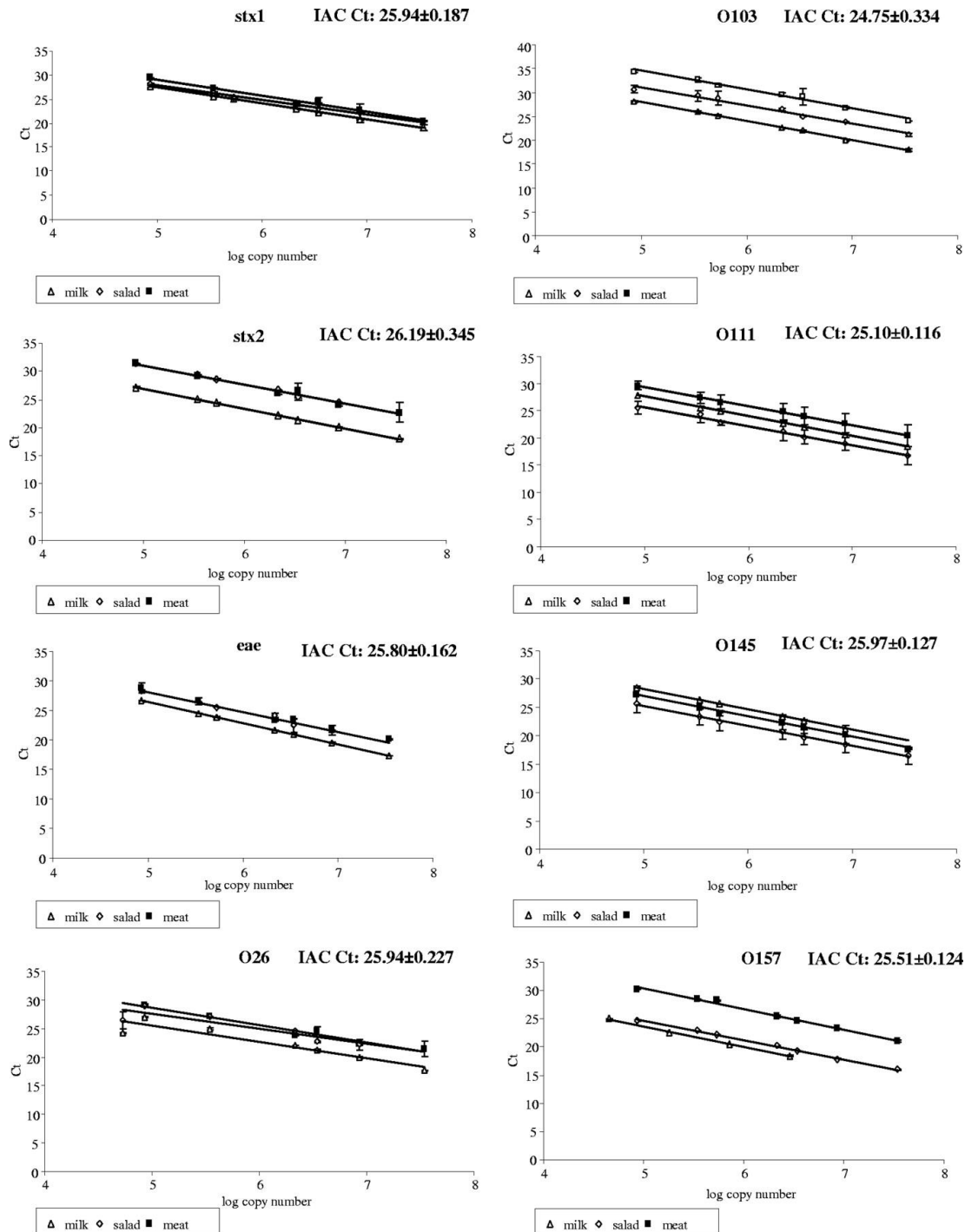


Table 1. LOD_{100%} of CEN ISO/TS 13136:2012 PCR (from: Kagkli DM *et al.*, *Appl. Environ. Microbiol.* October 2011 vol. 77 no. 19 6954-6963)

TABLE 3.								
LOD determinations for all VTEC PCR methods ^a								
Copy no./method	Mean <i>C_T</i> value ± SD (% positive)							
	<i>stx1</i>	<i>stx2</i>	<i>eae</i>	O103	O111	O145	O157	O26
20	NA ^b	NA	NA	38.64 ± 0.70 (100)	35.11 ± 0.61 (100)	35.7 ± 0.49 (100)	36.5 ± 1.14 (95)	36.2 ± 0.61 (100)
15	34 ± 0.90 (100)	34.1 ± 0.78 (100)	34.8 ± 1.49 (100)	36.8 ± 0.53 (97)	38.4 ± 0.98 (100)	37.5 ± 1.10 (100)	38.6 ± 1.01 (95)	38.7 ± 0.57 (98)
10	36.5 ± 1.22 (95)	36.4 ± 1.34 (98)	35.9 ± 1.04 (100)	39.86 ± 1.34 (98)	37.9 ± 0.97 (93)	36.1 ± 0.95 (98)	36.4 ± 1.09 (98)	37.7 ± 1.22 (97)
5	37.5 ± 1.29 (87)	38.3 ± 1.61 (93)	38.0 ± 0.85 (98)	38.9 ± 0.81 (98)	39.7 ± 1.07 (97)	39.0 ± 1.01 (95)	40.1 ± 1.58 (82)	40.2 ± 1.28 (97)
0.1	42.3 ± 1.47 (5)	41.4 ± 1.07 (12)	41.1 ± 1.94 (8)	Undetermined	41.6 ± 1.7	39.9 ± 0.56 (6.7)	Undetermined	41.5 ± 0.08 (5)

↙^a Based on 60 repetitions with genomic target DNA as the template. The copy number per reaction and the maximum likelihood estimates of the mean *C_T* value ± SD are shown. The percentages of positives are presented in parentheses.

↙^b NA, not applicable.

2. Performance of the CEN ISO/TS 13136:2012 in the proficiency testing program organized by the EU-RL VTEC in the period 2009-2020

The performance of the method has been evaluated by analysing the results obtained in 15 proficiency testing schemes involving the NRLs for *E. coli* of the European Union Member States and other European countries. The participating laboratories were required to use the method as in CEN ISO/TS 19036:2012 or its drafts whose principle was the same than the published version. In particular, the studies' design was as follows:

- i. Swab study (2009) **(PT3)**: 16 laboratories participating, 4 samples per laboratory.
 - **Simulated carcass swabs. Sample 1:** 2 CFU STEC O157; **Sample 2:** 2×10^3 CFU STEC O157 + 40 CFU STEC O26; **Sample 3:** 4×10^3 CFU STEC O26 + 20 CFU STEC O157; **Sample 4:** 40 CFU STEC O26.
- ii. Milk study (2010) **(PT4)**: 29 laboratories participating, 2 samples per laboratory.
 - **UHT treated milk. Sample 1:** STEC O103 *stx2*, *eae* 40 CFU/ml; **Sample 2:** negative. **Background flora added:** *E. coli* ATCC® 35218™ 10^2 CFU/ml and *E. faecium* ATCC® L565™ 10^2 CFU/ml. The uncertainty of measurement (UM) was calculated for each of the bacterial suspensions used to spike the samples according to CEN ISO TS 19036:2006. The UM values were the following: STEC O103: 0.24 log CFU/ml *E. faecium* ATCC L565: 0.38 log CFU/ml *E. coli* ATCC35218: 0.22 log CFU/ml.
- iii. Vegetable study (2011) **(PT7)**: 27 laboratories participating, 3 samples per laboratory.
 - **Spinach packaged at retail. Sample 1:** 40 CFU/ml STEC O157, *stx1*, *stx2*, *eae*; **Sample 2:** 40 CFU/ml STEC O145, *stx1*, *eae*; **Sample 3:** negative. **Background flora naturally present in the samples.** The uncertainty of measurement (UM) was calculated for each of the bacterial suspensions used to spike the samples according to CEN ISO TS 19036:2006. The UM values were the following: STEC O157: 0.27 log CFU/ml; VTEC O145: 0.37 log CFU/ml.

iv. Water study (2012) **(PT8)**: **32** laboratories participating, 2 samples per laboratory.

- **Tap water (not drinkable). Sample 1:** 1.2×10^3 CFU/ml STEC O157, *stx1*, *stx2*, *eae*; **Sample 2:** negative. **Background flora naturally present in the samples.** The uncertainty of measurement (UM) was calculated for each of the bacterial suspensions used to spike the samples according to CEN ISO TS 19036:2006. The UM value was the following: STEC O157: 0.27 log CFU/ml.

v. Seed study (2012) **(PT9)**: **35** laboratories participating, 2 samples per laboratory.

- **Beet seeds. Sample 1:** 8×10^4 CFU/g* STEC O157, *stx1*, *eae*; **Sample 2:** negative. **Background flora naturally present in the samples.** The uncertainty of measurement (UM) was calculated for each of the bacterial suspensions used to spike the samples according to CEN ISO TS 19036:2006. The UM value was the following: STEC O157: 0.27 log CFU/ml.

*= one single contaminated seed was added to each 50 g sample. The seeds were shown to contain an average of 4×10^6 CFU of contaminating STEC O157.

vi. Sprout study (2013) **(PT12)**: **37** laboratories participating, 3 samples per laboratory.

- **Sample 1:** 1.5×10^3 CFU/g STEC O157, *stx1*, *stx2*, *eae*; **Sample 2:** 1.8×10^2 CFU/g of an EIEC strain; **Sample 3:** negative. **Background flora naturally present in the samples.** The UM value was the following: 0.270 log CFU/ml.

vii. Sprout study (2014) **(PT14)**: **40** laboratories participating, 3 samples per laboratory.

- **Sample A:** 10^3 CFU/g STEC O104, *stx1*; **Sample B:** 10^2 CFU/g STEC O104, *stx1*; **Sample C:** negative. **Background flora naturally present in the samples.** The UM value was the following: 0.125 log CFU/ml.

viii. An inter-laboratory study on sprouts run in 2014 in the framework of the CEN Validation Study of ISO 16654 evaluating the performance parameters of the ISO/TS 13136: **13** laboratories participating, 3 samples per laboratory (eight replicates each).

- **Sample 1:** negative, **Sample 2:** 10 CFU/g STEC O157, *stx1*, *stx2*, *eae*; **Sample 3:** 100 CFU/g STEC O157, *stx1*, *stx2*, *eae*. **Background flora naturally present in the samples.** The UM value was the following: 0.270 log CFU/ml.
- ix. Sprout study (2015) **(PT15): 36** laboratories participating, 3 samples per laboratory.
- **Sample A:** 10³ CFU/g STEC O111, *stx1*, *stx2*, *eae*; **Sample B:** 10² CFU/g STEC O111, *stx1*, *stx2*, *eae*; **Sample C:** negative. **Background flora naturally present in the samples.** The UM value was the following: 0.138 log CFU/ml.
- x. Spent irrigation water study (2015) **(PT16): 51** laboratories participating, 3 samples per laboratory.
- **Sample A:** 500 CFU/ml STEC O157, *stx1*, *stx2*, *eae*; **Sample B:** 200 CFU/ml STEC O157, *stx1*, *stx2*, *eae*; **Sample C:** negative. **Background flora naturally present in the samples.** The UM value was the following: 0.270 log CFU/ml.
- xi. Ground beef study (2016) **(PT17): 37** laboratories, 3 samples per laboratory.
- **Sample A:** negative; **Sample B:** 5 CFU/g STEC O91, *stx1*, *stx2*; **Sample C:** 50 CFU/g STEC O91, *stx1*, *stx2*. **Background flora naturally present in the samples.** The UM value was the following: 0.121 log CFU/ml.
- xii. Rocket salad (2017) **(PT20): 37** laboratories, 3 samples per laboratory.
- **Sample 1:** negative, **Sample 2:** 4 CFU/g STEC O111, *stx1*, *stx2*, *eae*; **Sample 3:** 40 CFU/g STEC O111, *stx1*, *stx2*, *eae*. **Background flora naturally present in the samples.** The UM value was the following: 0.138 log CFU/ml.
- xiii. Sprout study (2018) **(PT21): 39** laboratories participating, 3 samples per laboratory.
- **Sample 1:** negative; **Sample 2:** 2 CFU/g STEC O26, *stx1*, *stx2*, *eae*; **Sample 3:** 20 CFU/g STEC O26, *stx1*, *stx2*, *eae*. **Background flora naturally present in the samples.** The UM value was the following: 0.209 log CFU/ml.

xiv. Spent irrigation water study (2018) (**PT22**): **56** laboratories participating, 2 samples per laboratory.

- **Sample 1:** 50 CFU/ml STEC O103, *stx2*, *eae*; **Sample 2:** negative. **Background flora naturally present in the samples.** The UM value was the following: 0.24 log CFU/ml.

xv. Flour study (2019) (**PT25**): **25** laboratories participating, 3 samples per laboratory.

- **Sample 1:** negative; **Sample 2:** 1 CFU/25 g STEC O121, *stx2*, *eae*; **Sample 3:** 5 CFU/25 g STEC O121, *stx2*, *eae*. The UM value was the following: 0.4 log CFU/ml.

xvi. Herbs study (run on basil in 2020) (**PT27**): **27** laboratories participating, 3 samples per laboratory.

- **Sample 1:** negative; **Sample 2:** 5 CFU/g STEC O88, *stx1*, *stx2*; **Sample 3:** 50 CFU/g STEC O88, *stx1*, *stx2*. **Background flora naturally present in the samples.** The UM value was the following: 0,22 log CFU/ml.

xvii. Cheese study (**PT33**): 26 laboratories participated, 3 samples per laboratory.

- **Sample 1:** negative; **Sample 2:** negative for STEC (2 CFU/g EAEC O104 *aggR*, *aaiC*) ; **Sample 3:** 2 CFU/g STEC O78 *stx1*. The UM value of the STEC strain was 0.4 log CFU/ml.

xviii. Sprout spent irrigation water study (**PT34**): **27** laboratories participated, 2 samples per laboratory.

- **Sample 1:** negative; **Sample 2:** 100 CFU/ml STEC O157, *stx1 stx2*, *eae*. **Background flora naturally present in the samples.** The UM value of the STEC strain was 0.24 log CFU/ml.

The performance of the method as derived from the analysis of the results obtained in the above-indicated studies is summarized per matrix in the tables below.

2.1. Sensitivity: $SE = [\text{true positives} / (\text{true positives} + \text{false negatives})] \times 100$

Evaluation of the performance of the ISO/TS 13136 during the inter-laboratory study run in 2014 in the framework of the validation of ISO 16654 on sprout samples – Sensitivity: Sample 2 (**Low - 10 CFU/g STEC O157**): **96 % -12 labs (1 outlier)**, Sample 3 (**High – 100 CFU/g STEC O157**): **99.04 % - 13 labs**

Evaluation of the performance of the ISO/TS 13136 during the inter-laboratory studies run in 2018 on spent irrigation water (PT22), in 2019 on flour (PT25, only for the high level of contamination, since the low level was lower than LOD₅₀) and in 2020 (PT27) , in 2022 on cheese (PT33) and sprout spent irrigation water (PT34) – Sensitivity (%):

Target/Matrix	Spent water PT 22 Sample 1	Flour PT 25 Sample 3 High level	Herbs PT 27 Sample 2 Low level	Herbs PT 27 Sample 3 High level	Cheese PT 33 Sample 3	Spent water PT 34 Sample 2
<i>vtx1/stx1</i>	NA	NA	100	96.3	100	96.4
<i>vtx2/stx2</i>	84.7	96.2	100	96.3	NA	96.4
<i>eae</i>	100	100	NA	NA	NA	96.3
<i>wzxO103</i>	93.3	NA	NA	NA	NA	NA
<i>rfbEO157</i>	NA	NA	NA	NA	NA	83.3
Isolation O103	74.1	NA	NA	NA	NA	NA
Isolation O121	NA	88.8	NA	NA	NA	NA
Isolation O88	NA	NA	96.1	96.3	NA	NA
Isolation O78	NA	NA	NA	NA	100	NA
Isolation O157	NA	NA	NA	NA	NA	NA
	50 CFU/ml O103 <i>stx2, eae</i>	5 CFU/25 g O121 <i>stx2, eae</i>	5 CFU/g O88 <i>stx1, stx2</i>	50 CFU/g O88 <i>stx1, stx2</i>	2 CFU/g O78 <i>stx1</i>	100 CFU/ml O157 <i>stx1 stx2, eae</i>

Evaluation of the performance of the ISO/TS 13136 during the inter-laboratory studies run in 2015 on spent irrigation water (PT16), in 2016 on ground beef (PT17), in 2017 on rocket salad (PT20) and in 2018 on sprouts (PT21) – Sensitivity (%):

Target/Matrix	Spent water PT 16 Sample 1 High level	Spent water PT 16 Sample 2 Low level	Ground beef PT 17 Sample 2 Low level	Ground beef PT 17 Sample 3 High level	Rocket salad PT20 Sample 2 Low level	Rocket salad PT20 Sample 3 High level	Sprouts PT21 Sample 2 Low level	Sprouts PT21 Sample 3 High level
<i>vtx1/stx1</i>	97.9	97.9	100	100	97.2	100	88.1	97.4
<i>vtx2/stx2</i>	100	97.9	100	100	100	100	95.0	95.0
<i>eae</i>	97.9	97.9	NA	NA	94.6	100	100	100
<i>rfBE_{O157}</i>	97.9	97.9	NA	NA	NA	NA	NA	NA
<i>wbdl_{O111}</i>	NA	NA	NA	NA	97.3	100	NA	NA
<i>wzx_{O26}</i>	NA	NA	NA	NA	NA	NA	94.6	97.3
Isolation O157	89.8	85.7	NA	NA	NA	NA	NA	NA
Isolation O91	NA	NA	78.3	78.3	NA	NA	NA	NA
Isolation O111	NA	NA	NA	NA	94.4	97.2	NA	NA
Isolation O26	NA	NA	NA	NA	NA	NA	76.1	88.1
	500 CFU/ml O157 <i>stx1, stx2, eae</i>	200 CFU/ml O157 <i>stx1, stx2, eae</i>	5 CFU/g O91 <i>stx1, stx2</i>	50 CFU/g O91 <i>stx1, stx2</i>	4 CFU/g O111 <i>stx1, stx2, eae</i>	40 CFU/g O111 <i>stx1, stx2, eae</i>	2 CFU/g O26 <i>stx1, stx2, eae</i>	20 CFU/g O26 <i>stx1, stx2, eae</i>

Evaluation of the performance of the ISO/TS 13136 during the inter-laboratory studies run in 2013, 2014 and 2015 (PT12, PT14 and PT15) on sprouts– Sensitivity (%):

Target/Matrix	Sprouts PT12 Sample A	Sprouts PT14 Sample 1 High level	Sprouts PT14 Sample 2 Low level	Sprouts PT15 Sample 1 High level	Sprouts PT15 Sample 2 Low level
<i>vtx1/stx1</i>	97.3	97.4	94.7	94.3	88.9
<i>vtx2/stx2</i>	100	NA	NA	94.3	88.9
<i>eae</i>	97.3	NA	NA	100	100
<i>rfBE_{O157}</i>	100	NA	NA	NA	NA
<i>wzx_{O111}</i>	NA	NA	NA	94.1	94.1
<i>wzx_{O104}</i>	NA	100	94.7	NA	NA
Isolation O157	89.2	NA	NA	NA	NA
Isolation O111	NA	NA	NA	63.9	44.4
Isolation O104	NA	94.6	77.1	NA	NA
	1,5x10 ³ CFU/g O157 <i>stx1, stx2, eae</i>	10 ³ CFU/g O104 <i>stx1</i>	10 ² CFU/g O104 <i>stx1</i>	10 ³ CFU/g O111 <i>stx1, stx2, eae</i>	10 ² CFU/g O111 <i>stx1, stx2, eae</i>

Evaluation of the performance of the ISO/TS 13136 during the inter-laboratory studies run in 2010 on milk (PT4), 2011 on vegetables (PT7) and 2012 (PT8 and PT9) on water and seeds– Sensitivity (%):

Target/Matrix	Milk PT 4	Spinach PT7	Water PT8	Seeds PT9
<i>vtx1/stx1</i>	NA	94.4	100	97.1
<i>vtx2/stx2</i>	100	96.2	100	100
<i>eae</i>	100	96.2	100	97.1
<i>rfBEO157</i>	NA	100	100	100
<i>wzxO26</i>	NA	NA	NA	NA
<i>wbdO111</i>	NA	NA	NA	NA
<i>wzxO103</i>	93.1	NA	NA	NA
<i>ihp1O145</i>	NA	100	NA	NA
Isolation O103	89.6	NA	NA	NA
Isolation O157	NA	91.7	100	100
Isolation O145	NA	100	NA	NA
	40 CFU/g O103 <i>stx2, eae</i>	40 CFU/g O157 <i>stx1, stx2, eae</i> and 40 CFU/g O145 <i>stx1, eae</i>	1,2x10 ³ CFU/g O157 <i>stx1, stx2, eae</i>	8x10 ⁴ CFU/g O157 <i>stx1, eae</i>

Evaluation of the performance of the ISO/TS 13136 during the inter-laboratory study run in 2009 (PT3), on the detection of STEC in carcass swabs– Sensitivity (%):

Target/Matrix	Swab PT 3 Sample A	Swab PT 3 Sample B	Swab PT3 Sample C	Swab PT3 Sample D
<i>vtx1/stx1</i>	93.7	93.7	100	100
<i>vtx2/stx2</i>	100	100	31.3*	100
<i>eae</i>	100	100	100	100
<i>rfBEO157</i>	100	100	100	NA
<i>wzxO26</i>	NA	100	100	100
<i>wbdO111</i>	NA	NA	NA	NA
<i>wzxO103</i>	NA	NA	NA	NA
<i>ihp1O145</i>	NA	NA	NA	NA
Isolation O26	NA	92.8	100	100
	2 CFU O157 <i>stx1, stx2, eae</i>	2x10 ³ O157 <i>stx1, stx2, eae</i> 40 CFU O26 <i>stx1, eae</i>	20 CFU O157 <i>stx1, stx2, eae</i> 4x10 ³ CFU O26 <i>stx1, eae</i>	40 CFU O26 <i>stx1, eae</i>

*A large excess of *stx1* was present in the sample. The system uses the same primers' set, differentiating the two *stx* gene types by means of different probes.

2.2. Specificity: $SP = [\text{True negatives} / (\text{true negatives} + \text{false positives})] \times 100$

Evaluation of the performance of the ISO/TS 13136 during the inter-laboratory study run in 2014 in the framework of the validation of ISO 16654 on sprout samples – **Specificity: Sample 1 100%**

Evaluation of the performance of the ISO/TS 13136 during the inter-laboratory studies run in the period 2018 - 2019 (PT22, PT25) – Specificity (%):

Target/Matrix	Spent irrigation water PT22	Flour PT 25	Herbs PT 27	Cheese PT33	Spent irrigation water PT34
<i>vtx1/stx1</i>	99	93.7	96.3	100	100
<i>vtx2/stx2</i>	100	89	96.3	100	100
<i>eae</i>	NA	NA	98.1	NA	NA

Evaluation of the performance of the ISO/TS 13136 during the inter-laboratory studies run in in the years 2013 2015 on sprouts (PT12, PT14 and PT15) and on spent irrigation water (PT16), in 2016 on ground beef (PT17), in 2017 on rocket salad (PT20) and in 2018 on sprouts (PT21) – Specificity (%):

Target/Matrix	Sprouts PT12	Sprouts PT14	Sprouts PT15	Spent irrigation water PT16	Ground beef PT17	Rocket salad PT20	Sprouts PT21
<i>vtx1/stx1</i>	98.7	100	97.2	100	97.2	97.3	100
<i>vtx2/stx2</i>	97.3	94.8	97.2	97.3	94.7	97.3	100
<i>eae</i>	97.3	100	NA	NA	NA	NA	NA

Evaluation of the performance of the ISO/TS 13136 during the inter-laboratory studies run in the period 2009 - 2015 (PT4, PT7, PT8, PT9)– Specificity (%):

Target/Matrix	Milk PT 4	Spinach PT7	Water PT8	Seeds PT9
<i>vtx1/stx1</i>	98.2	96.2	100	97.1
<i>vtx2/stx2</i>	93.1	98.1	100	100
<i>eae</i>	93.1	96.2	100	97.1
<i>rfBEO157</i>	NA	100	100	100
<i>wzxO26</i>	100	100	100	100
<i>wbdO111</i>	100	100	100	100
<i>wzxO103</i>	93.1	100	100	100
<i>ihp1O145</i>	100	100	100	100
Isolation	93.1	100	NA	NA

Evaluation of the performance of the ISO/TS 13136 during the inter-laboratory study run in 2009 (PT3), on the detection of STEC in carcass swabs– Specificity (%):

Target/Matrix	Swab PT 3 Sample A	Swab PT 3 Sample B	Swab PT3 Sample C	Swab PT3 Sample D
<i>vtx1/stx1</i>	100	100	100	100
<i>vtx2/stx2</i>	100	100	100	100
<i>eae</i>	93.7	93.7	93.7	93.7
<i>rfBEO157</i>	100	100	100	100
<i>wzxO26</i>	100	100	100	100
<i>wbdO111</i>	100	100	100	100
<i>wzxO103</i>	100	100	100	100
<i>ihp1O145</i>	100	100	100	100
Isolation O26	NA	100	100	100
	2 CFU O157 <i>stx1, stx2, eae</i>	2x10 ³ CFU O157 <i>stx1, stx2, eae</i> 40 CFU O26 <i>stx1, eae</i>	20 CFU O157 <i>stx1, stx2, eae</i> 4x10 ³ CFU O26 <i>stx1, eae</i>	40 CFU O26 <i>stx1, eae</i>

2.3. Accuracy: $AC = [(true\ positives + true\ negatives) / No.\ of\ samples\ tested] \times 100$

Target/Matrix	Milk	Spinach	water	seeds
vtx1	98,2	95	100	97,1
vtx2	96,5	96,2	100	100
eae	96,5	96,2	100	97,1
O157	NA	98,1	100	100
O26	100	100	100	100
O111	100	100	100	100
O103	93,1	100	100	100
O145	100	100	100	100
Isolation O157	NA	96	100	100
Isolation O145	NA	100	NA	NA
Isolation O103	91,3	NA	NA	NA

Target/Matrix	Swab 2 CFU O157	Swab 2*10 ³ O157 + 40 O26	Swab 4*10 ³ O26 + 20 O157	Swab 40 CFU O26
vtx1	96,8	96,8	100	100
vtx2	100	100	65,6*	100
eae	96,8	96,8	96,8	96,8
O157	100	100	100	100
O26	100	100	100	100
O111	100	100	100	100
O103	100	100	100	100
O145	100	100	100	100
Isolation O26	NA	100	100	100

*A large excess of *stx1* was present in the sample. The system uses the same primers' set, differentiating the two *stx* gene types by means of different probes.

2.4. Number of samples per each parameter (No. of correct results/number of tests)

The following tables detail the number of samples analyzed to determine the sensitivity and specificity parameters described in the previous paragraph.

2.4.1. Sensitivity

Target/Matrix	Milk	Spinach	water	seeds
vtx1	NA	51/54	32/32	34/35
vtx2	29/29	26/27	32/32	70/70
eae	29/29	52/54	32/32	34/35
O157	NA	25/27	32/32	33/33
O26	NA	NA	NA	NA
O111	NA	NA	NA	NA
O103	27/29	NA	NA	NA
O145	NA	26/26	NA	NA
Isolation O103	26/29	NA	NA	NA
Isolation O157	NA	23/25	32/32	34/34
Isolation O145	NA	24/24	NA	NA

Target/Matrix	Swab 2 CFU O157	Swab 2*10 ³ O157 + 40 O26	Swab 4*10 ³ O26 + 20 O157	Swab 40 CFU O26
vtx1	15/16	15/16	16/16	16/16
vtx2	16/16	16/16	5/16*	16/16
eae	16/16	16/16	16/16	16/16
O157	15/15	15/15	15/15	NA
O26	NA	16/16	16/16	16/16
O111	NA	NA	NA	NA
O103	NA	NA	NA	NA
O145	NA	NA	NA	NA
Isolation O26	NA	13/14	14/14	14/14

*A large excess of *stx1* was present in the sample. The system uses same primers but different probes.

2.4.2. Specificity

Target/Matrix	Milk	Spinach	water	seeds
vtx1	57/58	26/27	32/32	34/35
vtx2	27/29	53/54	32/32	70/70
eae	27/29	26/27	32/32	34/35
O157	NA	26/26	32/32	NA
O26	58/58	52/52	32/32	33/33
O111	58/58	52/52	32/32	33/33
O103	27/29	52/52	32/32	33/33
O145	58/58	26/26	32/32	33/33
Isolation	27/29	25/25	NA	NA

Target/Matrix	Swab 2 CFU O157	Swab 2*10 ³ O157 + 40 O26	Swab 4*10 ³ O26 + 20 O157	Swab 40 CFU O26
vtx1	16/16	16/16	16/16	16/16
vtx2	16/16	16/16	16/16	16/16
eae	15/16	15/16	15/16	15/16
O157	15/15	15/15	15/15	15/15
O26	16/16	16/16	16/16	16/16
O111	16/16	16/16	16/16	16/16
O103	16/16	16/16	16/16	16/16
O145	16/16	16/16	16/16	16/16
Isolation O26	NA	5/5	14/14	14/14

2.5. Calculation of the confidence intervals.

When SE, SP or AC is comprised between 10 % and 90 %, the two-tailed confidence interval (CI) is obtained by applying the following formulae:

$$CI_{95\%} = p \pm 2\sqrt{p(1-p)/n}$$

Where

- p corresponds to SE, SP or AC, in %
- n corresponds to:
 - o true positives + false negatives when CI for SE is calculated;
 - o true negatives + false positives when CI for SP is calculated;
 - o number of samples tested when CI for AC is calculated;

When SE, SP or AC is $(p) \geq 90 \%$, the one-tailed confidence interval (CI) is obtained by using the following binomial table:

CI_{95% inf} p ≥ 90 % and n = 10, 20, 30, etc

	n =	10	20	30	40	50	60
p = 0,90		0,75	0,83	0,82	0,84	0,83	0,84
0,92		0,85	0,83	0,85	0,86	0,87	0,88
0,94		0,85	0,88	0,88	0,89	0,89	0,89
0,96		0,85	0,93	0,92	0,91	0,93	0,93
0,98		0,95	0,93	0,95	0,96	0,95	0,96
0,99		0,95	0,98	0,98	0,96	0,97	0,98