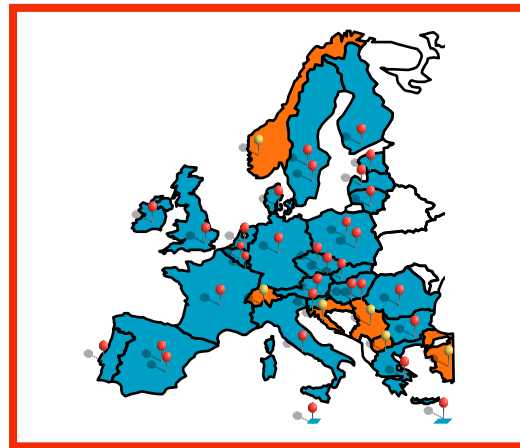


**The collaborative study for the validation of the
ISO 16654:2001
and the additional study for ISO/TS 13136:2012
validation in sprouts**

**Reported at the 21st meeting of CEN/TC 275/WG 6 25-27 June 2014
Washington – USA**



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History

- In 2006 WG6 assessed the equivalence between the ISO 16654 and the NMKL method No 164, 2. Ed. 2005
- NMKL method No 164 was validated in a collaborative study in 2002
- Project for validation in mandate M/381 included a reduced study as agreed by CEN in 2007 in Cairo (only one epidemiologically relevant matrix, milk)

Mandate M/381 Validation Study

Samples E.coli O157	
number of labs	15
stam	E.coli O157 NCCB 100282
method for homogeneity testing	ISO 16654
media	CT-SMAC
incubation	24h 37°C
matrix	milk
volume	10 ml
labelling	sample 1 to 24 coded ad random

blanco	120 sample code	3	4	5	8	12	13	14	24
low level (10-50 cfu/ml)	120 sample code	1	9	15	16	17	19	20	23
high level (100-500 cfu/ml)	120 sample code	2	6	7	10	11	18	21	22

Homogeneity testing	10x	low level (sample codes)	1	9	15	16	17	19	20	23	1	9
	10x	high level (sample codes)	2	6	7	10	11	18	21	22	2	6
blanco testing	10x	blanco (sample codes)	3	4	5	8	12	13	14	24	3	4

Samples stable for one year @-20°C!

Mandate M/381 Results

		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15
expected	Sample	Result	Result	Result	Result	Result	Result	Result	Result	Result	Result	Result	Result	Result	Result	Result
L	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O	3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
O	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
O	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
H	6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H	10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H	11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O	12	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
O	13	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
O	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
L	15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L	16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L	17	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H	18	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L	19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L	20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H	21	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
H	22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L	23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
O	24	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0

ISO 16654:2001 – Table of results (Milk)

	Milk (blank)	Milk (Low level)	Milk (High level)
No. Of laboratories having returned results	15	15	15
No. Of samples per laboratory	24	24	24
No. Of excluded laboratories	0	0	0
No. Of laboratories retained after the exclusion	15	15	15
NO. Of accepted samples	120	120	120
Accuracy (Sensitivity)	-	100%	100%
Accuracy (Specificity)	94,4%	-	-
LOD50		N.A	N.A.

ISO 16654:2001 – Performance on milk samples

- ✓ Se: 100%
- ✓ Sp: 94,4%

The method does not lack of Specificity in itself, but cross-contamination can occur if many samples are handled at the same time (e.g. collaborative study) - All the false positive samples actually contained the strain used to spike.

Mandate M/381: Further steps

- ✓ Meeting of participant Labs in Rome, March 2013:
 - ✓ Agreement on the possibility to run an additional study on sprouts
 - ✓ Provisional OK from 11 of the 15 labs to the new study on sprouts
 - ✓ March 28, 2013. Formal Invitation sent out (deadline May 30, 2013)

Mandate M/381: Further steps

- ✓ Acceptances collected from 11 out of the 15 labs (1 declined, 3 did not reply)
- ✓ Additional labs enrolled
- ✓ On July 10, 2013 Information that the validation project was *“properly registered to CEN”*
- ✓ The “green light” from EC/CEN arrived on 8 October 2013
- ✓ Agreement with the labs to run the tests within March 2014 (decision taken at the meeting. Confirmation by E-mail forum)

Additional study

One matrix epidemiologically relevant: Sprouts

Caused the German outbreak in 2011

Microcriteria released in 2013 (enforced on July)

Problematic matrix

Performance of the ISO TS 13136:2012 method on this matrix

ISO 16654 is basically a module of the ISO TS 13136:2012

Additional study: experimental design

14 Laboratories enrolled for the ISO 16654 additional study

15 Laboratories enrolled for the ISO TS 13136:2012

Study outline

Enrichment done by adding the medium to the stomacher bags containing the sprouts (25 gr)

Incubation

**1 ml taken for IMS
(ISO 16654:2001)**

**1 ml taken for template
preparation
(ISO TS 13136:2012)**

Isolation

**qPCR for all the
targets**

Results

Results

Additional study: experimental design

Mixture of alpha-alpha (90%) and watercress (10%) from the producer (10 Kg)

Eight blind replicates

Three levels of contamination
VTEC O157 *eae vtx1 vtx2*

Endogenous microflora: 10^5 - 10^7 CFU/gr

0 CFU/gr
10 CFU/gr (around the limit of detection)
100 CFU/gr (one log above LOD)

Uncertainty: 0.27 log CFU/ml

Additional study: experimental design

**Fractional recovery: 12 replicates tested (10 and 100 CFU/gr).
Six samples with 10 CFU/gr positive with the ISO 16654 method**

Stability

(assessed for the PT 12 of the EU RL on another batch of an identical mixture from the same producer):

Sample spiking: 31 October 2013

Tests: 31/10/13 - 05/11/13 - 07/11/13

**Samples stable for at least eight days when refrigerated
(ten days shelf life)**

Additional study: experimental design

Homogeneity:

Negative: 12 samples tested (with ISO/TS13136) on 20 March 2014 **Low:** 12 samples tested on 24-25 March 2014 (with ISO/TS13136 and ISO 16654)

High: 12 samples tested on 24-25 March 2014 (with ISO/TS13136 and ISO 16654)

Shipment done on March 24

Recommendation on the Tests to be initiated immediately upon arrival
(tests done on 26-27 March)

Results (ISO 16654)

Specificity: 99,1 %

Sample	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Results (ISO 16654) Sensitivity

High: 96,42%

Sample		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14
2	H	1	1	1	1	1	1	1	0	1	1	1	1	1	1
9	H	1	1	1	1	1	1	1	1	1	1	1	1	1	1
11	H	1	1	1	1	1	1	1	0	1	1	1	1	1	1
13	H	1	1	1	1	1	0	1	1	1	1	1	1	1	1
16	H	1	1	1	1	1	1	1	1	1	1	1	1	1	1
18	H	1	1	1	1	1	1	1	1	1	1	1	1	1	1
19	H	1	1	1	1	1	1	1	1	1	1	1	1	1	1
21	H	1	1	1	1	1	0	1	1	1	1	1	1	1	1

Low: 75,89%

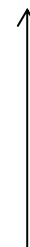
Sample		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14
3	L	1	1	1	1	1	0	1	1	1	1	1	1	1	1
5	L	1	1	1	1	1	1	0	1	0	1	1	1	0	0
10	L	1	1	1	1	1	1	0	0	0	1	1	1	1	0
14	L	1	1	1	1	1	0	0	1	1	1	1	1	1	0
17	L	1	1	1	1	1	0	0	1	1	1	1	1	1	0
22	L	1	1	1	1	1	0	0	1	0	1	1	1	0	1
23	L	1	1	1	1	1	0	0	1	0	1	1	1	1	0
24	L	1	1	1	1	1	0	0	1	0	1	1	1	0	1

ISO 16654 – Table of results (Sprouts)

	Sprouts (blank)	Sprouts (Low level)	Sprouts (High level)
No. Of laboratories having returned results	14	14	14
No. Of samples per laboratory	24	24	24
No. Of excluded laboratories	0	0	0
No. Of laboratories retained after the exclusion	14	14	14
NO. Of accepted samples	112	112	112
Accuracy (Sensitivity)	-	75,89%	96,42%
Accuracy (Specificity)	99,10%	-	-

ISO 16654 – Table of results (Sprouts)

No. of matrix i	Name of matrix matrix _i	Matrix effect F _i	SD of log Matrix effect S _{fi}	Detection limit d _{0.5,i}	LOD _{50%} ¹		Detection limit d _{0.95,i}	LOD _{95%} ²		Matrix effect ³ z _i
					lower conf. limit d _{0.5,i,L}	upper conf. limit d _{0.5,i,U}		lower conf. limit d _{0.95,i,L}	upper conf. limit d _{0.95,i,U}	
1	Sprouts	0,003	0,127	8,141	6,316	10,494	35,186	27,298	45,353	0,000



Results (ISO TS 13136)

Specificity: 100%

Sample	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15
1	0	0	0	0	0	0	0	not done	0	0	0	0	0	not done	0
4	0	0	0	0	0	0	0	not done	0	0	0	0	0	not done	0
6	0	0	0	0	0	0	0	not done	0	0	0	0	0	not done	0
7	0	0	0	0	0	0	0	not done	0	0	0	0	0	not done	0
8	0	0	0	0	0	0	0	not done	0	0	0	0	0	not done	0
12	0	0	0	0	0	0	0	not done	0	0	0	0	0	not done	0
15	0	0	0	0	0	0	0	not done	0	0	0	0	0	not done	0
20	0	0	0	0	0	0	0	not done	0	0	0	0	0	not done	0

Results (ISO TS 13136) Sensitivity

Low: 96% -12 labs (1 outlier)

Sample		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15
3	L	1	1	1	1	1	1	1	not done	1	1	1	1	1	not done	1
5	L	1	1	1	1	1	1	0	not done	0	1	1	1	1	not done	1
10	L	1	1	1	1	1	1	0	not done	1	1	1	1	1	not done	1
14	L	1	1	1	1	1	1	0	not done	1	1	1	1	1	not done	1
17	L	1	1	1	1	1	1	0	not done	1	1	1	1	1	not done	1
22	L	1	1	1	1	1	1	0	not done	0	1	1	1	0	not done	1
23	L	1	1	1	1	1	1	0	not done	1	1	1	1	1	not done	1
24	L	1	1	1	1	1	1	0	not done	0	1	1	1	1	not done	1

High: 99,04% - 13 labs

Sample		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15
2	H	1	1	1	1	1	1	1	not done	1	1	1	1	1	not done	1
9	H	1	1	1	1	1	1	1	not done	1	1	1	1	1	not done	1
11	H	1	1	1	1	1	1	0	not done	1	1	1	1	1	not done	1
13	H	1	1	1	1	1	1	1	not done	1	1	1	1	1	not done	1
16	H	1	1	1	1	1	1	1	not done	1	1	1	1	1	not done	1
18	H	1	1	1	1	1	1	1	not done	1	1	1	1	1	not done	1
19	H	1	1	1	1	1	1	1	not done	1	1	1	1	1	not done	1
21	H	1	1	1	1	1	1	1	not done	1	1	1	1	1	not done	1

Comparison between the two approaches

SENSITIVITY (paired study-same labs-same enrichment)

Culture (Low) 88,23%

PCR (Low) 100%

Culture (High) 96,84%

PCR (High) 100%



Vs

