



Alternative methods for agribusiness
Analytical performances certified

**VALIDATION CERTIFICATE FOR ALTERNATIVE ANALYTICAL METHOD
ACCORDING TO STANDARD EN ISO 16140: 2003**

Certificate No.: BRD 07/15-06/08

Validation date :	30.06.2008
Extension dates:	25.09.2008 26.01.2009 05.02.2010
End of validity :	30.06.2012

The company
(head office, distribution
and production site)

BIO-RAD
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FRANCE

is hereby authorized to refer to this **AFNOR Validation certificate** for the following alternative **qualitative** analysis method:

iQ-Check™ *E.coli* O157:H7 (Cat. # : 357-8114)

Protocol reference: 808466 – Rev. E

SCOPE

Raw beef.

RESTRICTIONS OF USE

None.

REFERENCE METHOD

EN ISO 16654 (2001) – Food Microbiology: Horizontal method for the detection of *Escherichia coli* O157.

A handwritten signature in black ink, appearing to read "Jacques BESLIN". The signature is stylized and fluid, with a long horizontal stroke at the end.

Deputy General Manager
Jacques BESLIN

AFNOR Certification

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PRINCIPLE OF THE METHOD

The iQ-Check *E. coli* O157:H7 test is based on real-time PCR detection. Detection, amplification and analyses of results are performed in a thermal cycler (Chromo4™, iQ™ 5, iCycler iQ™, MiniOpticon™ and CFX96™).

In the context of AFNOR Validation, all samples identified as positive by the alternative method must be confirmed by one of the following means:

- According to classical tests described in methods standardized by CEN or ISO (including a purification step), starting from the buffered peptone water enrichment broth.
- By isolation on CT-SMAC agar (with or without an immunoseparation step first), followed by O157 and H7 latex tests.
- By isolation on the chromogenic medium RAPID'E.coli O157:H7, with an immunoseparation (IMS) step first, followed by O157 and H7 latex tests.

In the event of discordant results (positive with alternative method, non-confirmed by means of options described above) the laboratory must follow the necessary steps to ensure validity of the result obtained.

NOTE (History of validation)

1/ In September of 2008 the validation was extended to a new alternative enrichment protocol:

- 20 hours enrichment in buffered peptone water, at 37°C

The initial validation of June 2008 was supplemented with data for the relative accuracy, relative sensitivity, and relative specificity for 31 positive samples and 31 negative samples in the raw beef category, as well as the relative detection level. The EN ISO 16140 protocol was used for the analysis of these samples.

Warning: In this study, 16.6 % of samples tested could not be confirmed using a direct isolation on CT-SMAC agar, but were confirmed using a confirmation protocol which include an initial IMS step.

2/ In January 2009, a new study was conducted extending the validation to include the use of a new version of the Opticon Monitor™ software, which offers in addition to a manual analysis, the option of automated data analysis.

Tests were conducted internally and by a third party, and followed the Easy II extraction protocol after enrichment in buffered peptone water, for 8h and 24h. All iQ-Check tests were done in the Chromo4, and data analysed both manually and with the automated option of the Opticon Monitor™ software.

These assays demonstrated that manual and automated data analysis of samples gave equivalent results. For clarity, results of this study are not detailed in this certificate.

3/ In February of 2010, the following extensions were validated by the AFNOR VALIDATION technical committee:

- Modification of the extraction step, using a new "Deepwell plate" format (in addition to the "tube" format validated before). Internal assays showed that these modifications did not have any impact on rendered results.
- The CFX Manager™ software can be used for a complete automated analysis for the CFX96™ and the Mini Opticon™ real-time PCR instruments. Internal assays showed that results obtained with these new combinations of automated systems were equivalent to those obtained with instruments and software validated before.

Relative ACCURACY, relative SPECIFICITY and relative SENSITIVITY Comparison of performances of the alternative method and the reference method

In March 2008 tests were carried out on 67 product samples, of which 1 was naturally contaminated, 35 artificially contaminated, and 31 non-contaminated, belonging to the following principal food product category: raw beef.

The enrichment in buffered peptone water at 8 hours and at 24 hours, at 41.5°C, and the following three confirmation protocols were tested:

- Direct isolation on CT-SMAC agar, followed by O157 and H7 latex tests on colonies.
- IMS followed by isolation on RAPID'E.coli O157:H7, followed by O157 and H7 latex tests on colonies.
- IMS followed by isolation on CT-SMAC agar, followed by O157 and H7 latex tests on colonies.

All samples were analysed **in single** by the **two methods**.

8 hours protocol, 41.5°C

Table of results (Cf. Table 1 of the EN ISO 16140 standard):

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 28 ⁽¹⁾	Positive deviation A+ / R- PD = 4 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 4 ⁽²⁾	Negative agreement A- / R- NA = 31 ⁽³⁾

(1) Confirmed positives

(2)(3) Of which no sample presumed positive by the alternative method was negative after confirmation

24 hours protocol, 41.5°C

Table of results (Cf. Table 1 of the EN ISO 16140 standard):

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 31 ⁽¹⁾	Positive deviation A+ / R- PD = 4 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 1 ⁽²⁾	Negative agreement A- / R- NA = 31 ⁽³⁾

(1) Confirmed positives

(2)(3) Of which no sample presumed positive by the alternative method was negative after confirmation

Additional tests were performed in September 2008 on 62 product samples, of which 1 was naturally contaminated, 30 artificially contaminated, and 31 non-contaminated, belonging to the following principal food product category: raw beef.

The enrichment in buffered peptone water at 20 hours, at 37°C, and the following three confirmation protocols were tested:

- Direct isolation on CT-SMAC agar, followed by a latex test on colonies, with a TCS isolation step first ;
- IMS followed by isolation on RAPID'E.coli O157:H7, followed by a latex test on colonies, with a TCS isolation step first ;
- IMS followed by isolation on CT-SMAC agar, followed by a latex test on colonies, with a TCS isolation step first.

All samples were analysed **in single** by the **two methods**.

20 hours protocol, 37°C

Table of results (Cf. Table 1 of the EN ISO 16140 standard):

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 28 ⁽¹⁾	Positive deviation A+ / R- PD = 1 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 2 ⁽²⁾	Negative agreement A- / R- NA = 31 ⁽³⁾

(1) Confirmed positives

(2) Of which one sample presumed positive by the alternative method was negative after confirmation

(3) Of which no sample presumed positive by the alternative method was negative after confirmation

Percentages obtained compared to the reference method are as follows:

	Relative accuracy AC (%)	Relative specificity SP (%)	Relative sensitivity SE (%)
8 hours Protocol (41.5°C)	88.1	88.6	87.5
24 hours Protocol (41.5°C)	92.5	88.6	96.9
20 hours Protocol (37°C)	95.2	96.9	93.3

NB: **relative specificity** below 100% results from a number of confirmed supplementary positives and not from false positives

Sensitivity was also recalculated taking into account all confirmed positives (including additional positives with the alternative method):

	Alternative method (PA + PD) / (PA+ PD + ND) =	Reference method (PA + ND) / (PA+ PD + ND) =
8 hours Protocol (41.5°C)	88.9	88.9
24 hours Protocol (41.5°C)	97.2	88.9
20 hours Protocol (37°C)	93.5	96.8

Analysis of discrepant results (according to appendix F of the EN ISO 16140 standard):

Protocol	Results	Conclusion
8 hours Protocol (41.5°C)	PD = 4 ; ND = 4 ; Y = PD + ND = 8 ; 6 ≤ Y ≤ 22; M = 0 ; M = 4 ; so m > M	Statistically, the two methods are not different.
24 hours Protocol (41.5°C)	PD = 4 ; ND = 1 ; Y = PD + ND = 5 ; Y < 6	No statistical test is available.
20 hours Protocol (37°C)	PD = 1 ; ND = 2 ; Y = PD + ND = 3 ; Y < 6	No statistical test is available.

Conclusion

The two methods are not different in statistical terms, regardless of the protocol used.

Relative DETECTION LEVEL

Comparison of performances of the alternative method and the reference method

Tests were carried out in **March and September 2008**, on the combination raw beef / *E.coli* O157:H7. Products were analysed **6 times** by the **2 methods** at **4 levels** of contamination.

Results obtained are as follows:

	Matrix	Strain	Relative detection level LOD ₅₀ ⁽³⁾ with confidence interval (CFU/25g ou 25 ml)	
			Alternative method	Reference method
8 hours Protocol (41.5°C)	Ground beef	<i>E.coli</i> O157:H7	0.7 [0.2 – 2.2]	0.5 [0.2 – 1.9]
24 hours Protocol (41.5°C)	Ground beef	<i>E.coli</i> O157:H7	0.4 [0.1 – 1.7]	0.5 [0.2 – 1.9]
20 hours Protocol (37°C)	Ground beef	<i>E.coli</i> O157:H7	0.2 [0.1 – 0.7]	0.3 [0.1 – 1.0]

(3) **LOD₅₀**: estimation of level of contamination enabling positive detection by alternative method in 50% of cases.

"Hitchins A. Proposed Use of a 50% Limit of detection Value in Defining Uncertainty Limits in the Validation of Presence-Absence Microbial detection Methods, Draft 10th December, 2003"

Conclusion

The detection level of the alternative method is between 0.1 and 2.2 CFU/25 g.

The detection level of the reference method is between 0.1 and 1.9 CFU/25 g.

INCLUSIVITY / EXCLUSIVITY

Implementation of alternative method only

- 50 strains of *E.coli* O157:H7 were detected out of 50 tested.
- The study of 36 *E.coli* non-O157:H7 or non-*E.coli* strains resulted in no cross-reactions.

PRACTICABILITY

Implementation of alternative method only

- **Response time:**
 - **Positive** results are obtained in 1 day (8 hours protocol) or 2 days (24 hours protocol) using the alternative method (*including confirmation according to classical tests of the reference method, with purification step included*) against 3 to 4 days using the reference method.
 - **Negative** results are obtained the same day (8 hours protocol) or in 1 day (24 hours protocol) using the alternative method against 1 day using the reference method.
 - In the case of results presumed positive using the alternative method, but rendered negative following confirmation, these negative results are obtained in 1 day (8 hours protocol) or 2 days (24 hours protocol).

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in **June 2008** with 14 participating laboratories. The analyses were carried out on samples of raw ground beef, artificially contaminated with an *E.coli* O157:H7 strain at the following three levels of contamination:

- 0 CFU/25g
- 1 - 10 CFU/25g
- 5 - 50 CFU/25g

The laboratories tested, **8 replicate samples** for **each level** of contamination, giving a total of 24 analyses for each method, for each participating laboratory.

Results:

Contamin- ation level	Total number of samples	Number of samples analysed*	Number of results processed **	Number of negative results		Number of positive results	
				REF	ALT	REF	ALT
0	112	104	104	96**	101**	8**	3**
1	112	104	104	3	0	101	104
2	112	104	104	0	0	104	104

* One laboratory could not perform the tests.

** For three laboratories, there were most likely inter-contaminations as the positive results obtained on un-contaminated samples were confirmed.

Calculations

- Relative accuracy = 97.4 %
- Specificity = 97.1 %
- Sensitivity = 100 %

NB: **relative specificity** below 100% results from a number of confirmed supplementary positives and not from false positives

Interpretation

Results of the inter-laboratory study are comparable to those obtained during the preliminary study.

Sensitivity was also recalculated taking into account all confirmed positive results (this includes supplementary positives with alternative method):

$$\text{Alternative method :} \\ (PA + PD) / (PA + PD + ND) = 97.7\%$$

$$\text{Reference method :} \\ (PA + ND) / (PA + PD + ND) = 98.6\%$$

Accordance, concordance and concordance odds ratio:

Accordance : percentage chance of finding the same result (i.e. both negative or both positive) from two identical test portions analysed in the same laboratory, under repeatability conditions (i.e. one operator using the same apparatus and same reagents within the shortest feasible time interval). The accordance is the average (mean) of the probabilities that two replicates give the same result for each laboratory.

Concordance : percentage chance of finding the same result for two identical samples analysed in two different laboratories. The concordance is the percentage of all pairings of duplicates giving the same result.

Concordance odds ratio (COR) : defined by the following formula:

$$\text{COR} = \frac{\text{accordance} \times (100 - \text{concordance})}{\text{concordance} \times (100 - \text{accordance})}$$

The following table indicates values for the **alternative method**:

Contamination level	Accordance	Concordance	COR
L0	95%	89%	1.1
L1	100%	100%	1.0
L2	100%	100%	1.0

The following table indicates values for the **reference method**:

Contamination level	Accordance	Concordance	COR
L0	91%	85%	1.1
L1	95%	94%	1.0
L2	100%	100%	1.0

Conclusion

Variability of the alternative method (accordance, concordance, concordance odds ratio) is equivalent to that of the reference method.

Please send any queries concerning the performance of the validated method to AFNOR Certification.

You may download a summary document on the preliminary and inter-laboratory studies on www.afnor-validation.org