



Alternative methods for agribusiness
Analytical performances certified

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

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

BAX[®] Real-Time PCR Assay *E.coli* O157:H7

Protocol reference: Part D14203648 – Rev 23QC-017.3-1110

SCOPE

Raw beef and vegetables

RESTRICTIONS FOR USE

None

REFERENCE METHOD

EN ISO 16654 (2001) – Microbiology of food and animal feeding stuffs: Horizontal method for the detection of *Escherichia coli* O157

A handwritten signature in black ink, appearing to read "Jacques Beslin".

Deputy General Manager
Jacques BESLIN

PRINCIPLE OF THE METHOD

The BAX[®] Real-Time PCR Assay *E.coli* O157:H7 method for the detection of *E.coli* O157:H7 uses the Polymerase Chain Reaction (PCR) to amplify specific DNA fragments. The method consists in a 3 step protocol: Preparation of DNA, Amplification, and Detection. After a lysis step, the BAX[®] cyclor/detector performs both amplification and automated detection.

In the context of AFNOR VALIDATION, all samples identified as positive by the BAX[®] Real-Time PCR Assay *E.coli* O157:H7 method must be confirmed from the MP enrichment broth by one of the following means:

- According to classical tests described in methods standardized by GEN or ISO from colonies (including a purification step).
- Streak 50 µL of enrichment onto CT-SMAC plates and incubate for 18-24 hours at 35-37°C. Check plates for typical *E. coli* O157:H7 colonies and confirm 1-5 characteristic colonies with the Wellcolex[™] latex test for *E. coli* O157:H7 (Oxoid reference R30959601).

If no typical colonies appear on CT-SMAC plates, follow the confirmation protocol for *E. coli* O157:H7 described in DuPont Qualicon technical bulletin 23C-013-0804 to recover *E. coli* O157:H7 with an IMS step. In some cases, a second IMS step may be necessary.

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.

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

In 2010 tests were carried out on 148 product samples, of which 4 were naturally contaminated, 81 artificially contaminated and 63 non-contaminated, belonging to the following principal food product categories:

- Raw meat (fresh, frozen, seasoned)
- Vegetables (raw, frozen, ready-to-eat, ready-to-cook)

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

Two incubation times were tested (7/8 hours – 7 hours for raw beef and 8 hours for vegetables – and 24 hours).

Incubation in 7/8 hours

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 = 39 ⁽¹⁾ | Positive deviation A+ / R- PD = 26 ⁽¹⁾ |
| Alternative method negative (A-) | Negative deviation A- / R+ ND = 12 ⁽²⁾ | Negative agreement A- / R- NA = 71 ⁽³⁾ |

(1) Confirmed positives

(2) (3) Of which none sample presumed positive by the alternative method was negative after confirmation

Incubation in 24 hours

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 = 46 ⁽¹⁾ | Positive deviation A+ / R- PD = 34 ⁽¹⁾ |
| Alternative method negative (A-) | Negative deviation A- / R+ ND = 5 ⁽²⁾ | Negative agreement A- / R- NA = 63 ⁽³⁾ |

(1) Confirmed positives

(2) (3) Of which none sample presumed positive by the alternative method was negative after confirmation

Percentages obtained compared to the reference method are as follows:

| | <u>Incubation in 7h/8h</u> | <u>Incubation in 24h</u> |
|---------------------------|----------------------------|--------------------------|
| Relative accuracy (AC) | 74.3% | 73.6% |
| Relative specificity (SP) | 73.2% | 64.9% |
| Relative sensitivity (SE) | 76.5% | 90.2% |

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 supplementary positives by alternative method):

| | Alternative method: (PA + PD) / (PA + PD + ND) | Reference method: (PA + ND) / (PA + PD + ND) |
|----------------------------|---|---|
| <u>Incubation in 7h/8h</u> | 84.4% | 66.2% |
| <u>Incubation in 24h</u> | 94.1% | 60.0% |

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

| | |
|----------------------------|--|
| <u>Incubation in 7h/8h</u> | Y = PD + ND = 38 : Y > 22 McNemar Test: $D = PD - ND = 14$; $x^2 = d^2/y = 5,15$; $x^2 > 3,841$ |
| <u>Incubation in 24h</u> | Y = PD + ND = 39 : Y > 22 McNemar Test: $D = PD - ND = 29$; $x^2 = d^2/y = 21,6$; $x^2 > 3,841$ |

Conclusion

Results show that alternative method performances are higher than the reference method ones.

Relative DETECTION LEVEL

Comparison of performances of the alternative method and the reference method

Tests were carried out in 2010, on 2 combinations of food products/strains described in the table below. Products samples were belonging to the following food categories:

- Raw meat (fresh, frozen, seasoned)
- Vegetables (raw, frozen, ready-to-eat, ready-to-cook)

Products were analysed **6 times** by the **2 methods** at **4 levels** of contamination.

Results obtained are as follows:

| Matrix | Strain | Relative detection level (CFU/25g or 25 ml) With confidence interval (3) LOD ₅₀ | |
|----------------|------------------------|--|------------------|
| | | Alternative method (Results for both incubation times) | Reference method |
| Ground beef | <i>E.coli</i> O157 :H7 | 0.6 [0.4 – 1.0] | 0.2 [0.1 – 0.7] |
| Frozen spinach | <i>E.coli</i> O157 :H7 | 0.3 [0.1 – 0.8] | 0.4 [0.2 – 0.9] |

(3) LOD₅₀: estimation of level of contamination enabling positive detection by alternative method in 50% of cases. FDA. 2006. *Final Report and Executive Summaries from the AOAC International Presidential Task Force on Best Practices in Microbiological Methodology. Appendix K. Statistics Working Group Tholen, D. W., D. S. Paulson, B. Jarvis, D. M. Mettler, B. Lombard, K. Newton, M. A. Mozola, and A. D. Hitchins.) Report Part 4a - LOD50.*

Conclusion

The relative detection level of the reference method is between 0.1 and 0.9 CFU/25g.

The relative detection level of the alternative method is between 0.1 and 1.0 CFU/25g (whatever the incubation time protocol used).

INCLUSIVITY / EXCLUSIVITY

Implementation of alternative method only

- 50 strains of *E.coli* O157:H7 were detected out of 50 tested.
- The study of 50 strains not belonging to the genus *E.coli* O157:H7 did not detect the presence of any cross-reaction.

PRACTICABILITY

Implementation of alternative method only

- **Time of results:**
 - **Positive** results are obtained in 2 to 3 days using the alternative method against 3 to 4 days using the reference method.
 - **Negative** results are obtained in the day or in 1 day 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 3 to 4 days.
- **Personnel training:** A training of at least 2 days on PCR analysis and on manipulation with the automated system is recommended.

INTERLABORATORY STUDY

The interlaboratory study was conducted in 2010 with 14 participating laboratories. The analyses was carried out on samples of ground beef, artificially contaminated with an *E.coli* O157:H7 strain at the following levels of contamination:

- 0 CFU/25 ml
- 5 CFU/25ml
- 25 CFU/25ml

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

The following results were obtained:

| 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 | 96 | 80 | 78 | 80 | 2 | 0 |
| 1 | 112 | 96 | 80 | 7 | 0 | 73 | 80 |
| 2 | 112 | 96 | 80 | 2 | 0 | 78 | 80 |

* Two laboratories did not realize the assays (one never received the samples and the other can not run the test)

** Results of two laboratories were excluded (alternative method protocol not correctly followed).

Calculations

- Relative accuracy = 95.4%
- % specificity = 100%
- % sensitivity = 100%

Interpretation

Results of the interlaboratory study are comparable to those obtained during the preliminary study.

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 | 100% | 100% | 1.00 |
| L1 | 100% | 100% | 1.00 |
| L2 | 100% | 100% | 1.00 |

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

| Contamination level | Accordance | Concordance | COR |
|---------------------|------------|-------------|------|
| L0 | 96.3% | 95.0% | 1.01 |
| L1 | 87.2% | 83.7% | 1.04 |
| L2 | 95.6% | 95.1% | 1.01 |

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.com