



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<sup>®</sup> System *E.coli* O157:H7 MP**

Protocol reference :

- User Guide : 2CQ-049.4-0307/ FR0908-2 (BAX Q7)
- Technical Notice: BAX QB0673-0908
- Protocol summary for *E. coli* O157:H7 : Technical Bulletin 23C-013-0804 FR0908
- Ready Reference : 2C-016.7-0306/FR1207
- Software version : 2.4 (BAX Q7)

**SCOPE**

Raw beef meat, raw milk, fruits, vegetables and Ready-to-Eat meals, raw pork meat, raw ovine meat and raw chicken meat.

**RESTRICTIONS OF USE**

It is remembered to the User that PCR method requires strict manipulations in order to prevent any contamination issue. It is recommended to follow carefully the User Guide.

**REFERENCE METHOD**

ISO 16654 – Food Microbiology - Horizontal method for the detection of *Escherichia coli* O157.

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## PRINCIPLE OF THE METHOD

The BAX® system for detection of *E.coli* O157:H7 is a detection kit using PCR (Polymerase Chain Reaction) technology. There is a 3 steps protocol: preparation of DNA, amplification and detection.

The PCR technology allows the BAX® system to perform a specific and rapid amplification of the DNA. After lysis step, the BAX® cycler/detector is doing both amplification and automated detection.

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

- From the enrichment broth, on selective agar CT SMAC, with confirmation of suspect colonies by the Latex Wellcolex *E.coli* O157:H7 test
- By the confirmation protocol of Qualicon (cf. reference on the first page) in the case of non-confirmation by the precedent protocol.

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

According to the products to be analyzed, 2 protocols are defined; both were tested in validation studies :

For raw beef meat, an enrichment broth Bax *E.coli* during 8h to 24h and transfer of 20 microliters for lysis.

For all other matrices, an enrichment broth mTSB + novobiocin during 18h to 24h and transfer of 5 microliters for lysis.

The protocol "raw beef meat" has been tested at 8h and at 24h for all samples. Equally, the two PCR cycles have been tested: *E. Coli* MP (3h30) and *E.Coli* MP Express (2h30)

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

In **2007** tests were carried out on 266 product samples, of which 3 were naturally contaminated, 150 artificially contaminated, and 123 non-contaminated, belonging to the following principal food product categories:

Raw beef meat, raw milk, fruits and vegetables, other (Ready-to-Eat meals, raw pork meat, raw ovine meat and raw chicken meat).

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

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

1/ with mTSB or Bax 8 hours, and MP protocol :

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = <b>110</b> <sup>(1)</sup>	Positive deviation A+ / R- PD = <b>22</b> <sup>(1)</sup>
Alternative method negative (A-)	Negative deviation A- / R+ ND = <b>7</b> <sup>(2)</sup>	Negative agreement A- / R- NA = <b>127</b> <sup>(3)</sup>

(1) Confirmed positives

(2) Of which 1 sample presumed positive by the alternative method was negative after confirmation

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

**2/ with mTSB or Bax 8 hours, and MPE protocol :**

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 103 <sup>(1)</sup>	Positive deviation A+ / R- PD = 14 <sup>(1)</sup>
Alternative method negative (A-)	Negative deviation A- / R+ ND = 13 <sup>(2)</sup>	Negative agreement A- / R- NA = 136 <sup>(3)</sup>

(1) Confirmed positives

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

**3/ with mTSB or Bax 24 hours, and MP protocol :**

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 113 <sup>(1)</sup>	Positive deviation A+ / R- PD = 27 <sup>(1)</sup>
Alternative method negative (A-)	Negative deviation A- / R+ ND = 3 <sup>(2)</sup>	Negative agreement A- / R- NA = 123 <sup>(3)</sup>

(1) Confirmed positives

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

**4/ with mTSB or Bax 24 hours, and MPE protocol :**

	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 110 <sup>(1)</sup>	Positive deviation A+ / R- PD = 19 <sup>(1)</sup>
Alternative method negative (A-)	Negative deviation A- / R+ ND = 6 <sup>(2)</sup>	Negative agreement A- / R- NA = 131 <sup>(3)</sup>

(1) Confirmed positives

(2) Of which 1 sample presumed positive by the alternative method was negative after confirmation

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

The percentages obtained, regarding the reference method, are :

Protocol	Relative accuracy : AC	Relative specificity : SP	Relative sensitivity : SE
mTSB / Bax 8 h / MP	89,1	85,2	94,0
mTSB / Bax 8 h / MPE	89,8	90,7	88,8
mTSB / Bax 24 h / MP	88,7	82,0	97,4
mTSB / Bax 24 h / MPE	90,6	87,3	94,8

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) :

Protocol	Relative sensitivity : SE	
	Alternative method : (PA + PD) / (PA + PD + ND) = %	Reference method : (PA + ND) / (PA + PD + ND) = %
mTSB / Bax 8 h / MP	95,0	84,2
mTSB / Bax 8 h / MPE	90,0	89,2
mTSB / Bax 24 h / MP	97,9	81,1
mTSB / Bax 24 h / MPE	95,6	85,9

**Analysis of discrepancies** (following annex F of standard EN ISO 16140) :

	Y=PD+ ND	D=  PD-ND	X <sup>2</sup> = d <sup>2</sup> /y	Conclusion ( Mac Nemar's test)
mTSB / Bax 8 h / MP	30	15	7,5	The 2 methods are different* at $\alpha < 0,05$
mTSB / Bax 8 h / MPE	27	1	0,04	The 2 methods are not different at $\alpha < 0,05$
mTSB / Bax 24 h / MP	30	24	19,2	The 2 methods are different* at $\alpha < 0,05$
mTSB / Bax 24 h / MPE	25	13	6,76	The 2 methods are different* at $\alpha < 0,05$

\* The differences are in favor of the alternative method.

## Relative DETECTION LEVEL

### Comparison of performances of the alternative method and the reference method

Tests were carried out in **2007**, on 4 combinations of food products/strains. Those products represent the following food categories: raw beef meat, raw milk, fruits and vegetables, others.

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 <sub>50</sub>		
		Alternative method (MP)	Alternative method (MPE)	Reference method
Minced beef / Bax 8h	<i>E.coli</i> O157 :H7	1.1 [0.7 – 1.5]	1.1 [0.7 -1.5]	0.3 [0.1 – 0.8]
Minced beef / Bax 24 h	<i>E.coli</i> O157 :H7	0.9 [0.7 – 1.3]	1.2 [0.9 – 1.7]	0.3 [0.1 – 0.8]
Raw Milk	<i>E.coli</i> O157 :H7	0.4 [0.1 – 1.3]	0.4 [0.1 – 1.3]	0.4 [0.1 – 1.3]
Piedmontese ham (baked potatoes, fresh tomatoes, white ham, pickles)	<i>E.coli</i> O157 :H7	0.4 [0.1 – 1.6]	0.4 [0.1 – 1.6]	0.4 [0.1 – 1.6]
Farm cider	<i>E.coli</i> O157 :H7	0.3 [0.1 – 1.1]	0.3 [0.1 –1.1]	0.3 [0.1 – 1.1]

(3) LOD<sub>50</sub> : 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 10<sup>th</sup> December, 2003"

## Conclusion

The detection limit of the alternative method is between 0,1 and 1,7 CFU/gram.  
The detection limit of the reference method is between 0,1 and 1,6 CFU/gram.

## INCLUSIVITY / EXCLUSIVITY

### Implementation of alternative method only

- 50 strains of *E.coli* O157 :H7 tested gave all positive results, with characteristic aspect on CT SMAC agar.
    - In the 35 strains non *E.coli* O157 :H7 tested, one strain of *E.coli* O55 :H7 has given a positive PCR test, but presents non characteristic colonies on confirmation agar.
- It must be noticed that 2 strains of *E.coli* O157 :H-, tested with the inclusivity protocol, show a negative result with Bax test and give characteristic results on CT SMAC agar.

## PRACTICABILITY

### Implementation of alternative method only

- **Response time :**
  - **Positive** results are obtained in 3 days (Bax 8h) to 4 days (Bax 24h and mTSB) using the alternative method (including the confirmation) against 3 to 4 days using the reference method.
  - **Negative** results are obtained in less than 1 day (Bax 8h) to 1 day (Bax 24h and mTSB) 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 days.
- **Personnel training :**  
A training of at less 2 days on PCR analysis and on manipulation with the automated system is recommended.

## INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2008 with 15 participating laboratories. The analyses were carried out on samples of minced raw frozen spinach, spiked with a non pathogenic strain of *E.Coli* O157: H7 ATCC 43888 at 3 levels of contamination :

- 0 UFC/25 ml
- 1 – 10 UFC/25 ml
- 5 – 50 UFC/25 ml

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

That is the protocol relative to the analyse of vegetables with an enrichment in mTSB at 41,5°C during 18 to 24 hours which has been performed in all laboratories.

The following results were obtained :

Contamination 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	120	104	96	94	95	2	1
1	120	104	96	4	4	92	92
2	120	104	96	0	0	96	96

\* 2 laboratories did not receive in time the samples and didn't do the analysis.

\*\* 1 laboratory has found all non inoculated samples, positive by the reference method, so their results has been excluded

### Calculations

- Relative accuracy = **99,7%**
- % specificity = **99,0%**

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

- % sensitivity = **97,9 %**

### Interpretation

Results of the collaborative 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	98,0	97,0	1,0
L1	94,0	92,0	1,0
L2	100,0	100,0	1,0

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

Contamination level	Accordance	Concordance	COR
L0	96,0	96,0	1,0
L1	94,0	92,0	1,0
L2	100,0	100,0	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.com](http://www.afnor-validation.com)