



Alternative methods for agribusiness
Analytical performances certified

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

Certificate No.: BIO 12/30 – 05/10

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The company **BIOMERIEUX**
Chemin de l'Orme
69280 MARCY L'ETOILE
FRANCE

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

CampyFood Agar (CFA) – Ref. 43 471

Protocol reference: 13250 version F

SCOPE

Meat products and production environmental samples

RESTRICTIONS FOR USE

None

REFERENCE METHOD

EN ISO 10272-1 (2006) - Microbiology of food and animal feeding stuffs - Horizontal method for detection and enumeration of *Campylobacter* spp. - Part 1: detection method

A handwritten signature in black ink, appearing to read "JBESLIN", written over a horizontal line.

Deputy General Manager
Jacques BESLIN

PRINCIPLE OF THE METHOD

CampyFood Agar (CFA) is a selective medium for the isolation of most *Campylobacter* species. CFA contains a mixture of activators which favors the growth of *Campylobacter* strains, a selective mixture which inhibits most contaminants and a color indicator giving red typical colonies.

Food samples, 1/10 diluted into the CampyFood broth, are enriched for 48 hours at 41.5°C prior to plating on CFA. Microaerobic atmosphere is generated directly into a specific stomacher type bag, by addition of a gas generator in a small pocket in the top of the bag

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

- By identification of 1 to 5 typical colonies according to classical tests described in methods standardized by CEN or ISO (including purification step),
- If typical colonies are present after 40-48 hours, collect 1 to 5 of them and isolate each one as follows :
 - One half of the colony on Columbia blood agar. Incubate the plate in an aerobic atmosphere
 - The other half of the colony on Columbia blood agar. Incubate the plate microaerobic atmosphere. A microscopic examination and an oxidase test should be performed on colonies which have grown exclusively in a microaerobic atmosphere.
- From colonies by implementing VIDAS CAM test (respecting the conditions described in the user guide of CFA method)

In the event of discordant results (positive with alternative method, by unconfirmed by one of the confirmation methods 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

Assays were carried out in 2010 on 208 product samples, of which 44 were naturally contaminated, 55 artificially contaminated, and 109 uncontaminated, belonging to the principal following food categories : "Meat products (raw meats, frozen meats and food preparations with meat) and environmental samples".

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

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

Answers	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	Positive agreement A+ / R+ PA = 83 ⁽¹⁾	Positive deviation A+ / R- PD = 14 ⁽¹⁾
Alternative method negative (A-)	Negative deviation A- / R+ ND = 2 ⁽²⁾	Negative agreement A- / R- NA = 109 ⁽³⁾

(1) Confirmed positives

(2) Of which none of the alternative method presumptive positive samples were negative after confirmation

Percentages obtained compared to the reference method are as follows:

Relative accuracy (AC)	92.3%
Relative specificity (SP)	88.6%
Relative sensitivity (SE)	97.6%

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

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

$$\text{Alternative method:} \\ (PA + PD) / (PA + PD + ND) = 98.0\%$$

$$\text{Reference method:} \\ (PA + ND) / (PA + PD + ND) = 85.9\%$$

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

$$PD = 14, ND = 2, Y = PD + ND = 16 ; 6 \leq Y \leq 22 ; m = 2, M = 3 ; m < M$$

Conclusion

The alternative method is statistically different from the reference method. This Result is explained by a significant number of additional confirmed positive results by the alternative method (PD = 14).

Relative DETECTION LEVEL

Comparison of performances of the alternative method and the reference method

Tests were carried out in 2010, on the 3 combinations of food products/strains described in the table below.

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	Reference method
Poultry meat	<i>Campylobacter jejuni</i>	0.7 [0.3 – 1.8]	0.9 [0.3 – 2.4]
Pork meat	<i>Campylobacter jejuni</i>	0.9 [0.4 – 1.8]	0.9 [0.4 – 1.8]
Water process	<i>Campylobacter coli</i>	0.3 [0.2 – 0.4]	0.3 [0.2 – 0.4]

(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 alternative method is between 0.2 and 1.8 CFU/25 g.
The relative detection level of the reference method is between 0.2 and 2.4 CFU/25 g.

INCLUSIVITY / EXCLUSIVITY

Implementation of alternative method only

- Fifty strains of *Campylobacter* (*C.coli*, *C.jejuni*, *C.upsaliensis*, *C.lari*) were detected out of the 50 strains tested.
- The study of 30 strains not belonging to the genus *Campylobacter* did not detect the presence of any cross-reaction.

Remark 1: Certain strains others than *Campylobacter* developed on CFA Agar but were not characteristic: a strain of *Enterobacter cloacae*, one of *E.coli* and one of *Acinetobacter baumannii* gave wine-red colonies with irregular edges. A strain of *Aeromonas hydrophila* gave purplish red colonies without metallic appearance.

Remark 2: non thermotolerant *C. fetus* strains did not grow on the CFA Agar incubated at 41.5°C.

PRACTICABILITY

Implementation of alternative method only

- **Response time:**
 - **Positive** results are obtained in 4 to 7 days using the alternative method against 6 to 8 days using the reference method.
 - **Negative** results are obtained in 4 days using the alternative method as for the reference method.
 - For presumptive positive results using the alternative method, that are negative following confirmation, these negative results are obtained in 4 to 7 days.
- **Other characteristics:** The CFA method, allowing a better identification of characteristic colonies reduces the need of confirmation..

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2010 with 17 participating laboratories. The analyses were carried out on minced poultry meat samples, artificially contaminated with a *Campylobacter jejuni* strain at the 3 following levels of contamination:

- 0 CFU/25g
- 3 CFU/25g
- 30 CFU/25g

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	136	128	88	73	81	15	7
1	136	128	88	65	2	23	86
2	136	128	88	47	0	41	88

* A laboratory did not run assays because the samples were received out of time and temperature of samples at delivery was not in accordance with the one expected.

** Results of 5 laboratories were not taken into account (they received samples too late and/or the temperature at delivery was not correct)

Calculations

- Relative accuracy = **52.3%**

NB: This percentage is explained by a better detection of positive samples with the alternative method compared to the reference method, as shown by the significant number (114) of additional positive results obtained by the alternative method. The high bacterial background flora, consisted of multi-resistant *Escherichia coli* and lactic flora, normally inhibited in the Bolton broth and on selective agars of *Campylobacter* spp. was able to grow and inhibit and/or mask the presence of *Campylobacter* spp.

- % specificity = **92.1%**

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

- % sensitivity = **98.8%**

Interpretation

Inter-laboratory study shows non equivalent results between the reference method and the alternative method, because of higher performances obtained with the alternative method.

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

Alternative method :	Reference method :
$(PA + PD) / (PA + PD + ND) = 93.8\%$	$(PA + ND) / (PA + PD + ND) = 40.9\%$

Taking into account all the confirmed positives results, the sensitivity of the alternative method obtained during inter-laboratory study is similar to that obtained during preliminary study. For the reference method, the sensitivity was reduced by at least a factor 2.

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:

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

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

Contamination level	Accordance	Concordance	COR
L0	87%	85.2%	1.02
L1	96%	95.5%	1.13
L2	100%	100.0%	1.00

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

Contamination level	Accordance	Concordance	COR
L0	84%	70.5%	1.20
L1	76%	59.9%	2.17
L2	75%	47.7%	3.33

Conclusion

Results of accordance, concordance and odds ratio of the alternative method are not equivalent to those of the reference method. Variability of the alternative method is lower than that of the reference method.

In this unfavourable condition of "target strain-competitor flora" ratio (background microflora was about 10^9 CFU/g), the CampyFood agar method showed better performance than 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