



Alternative methods for agribusiness
Analytical performances certified

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

Certificate No.: AES 10/10 – 07/10

Validation date: 2010.07.02

End of validity: 2014.07.02

The company AES CHEMUNEX
rue Maryse Bastié
Ker Lann / CS 17219
35172 BRUZ CEDEX
FRANCE

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

Bacillus Cereus Rapid Agar (BACARA®)
Selective medium for the enumeration of presumptive *Bacillus cereus*

Protocol reference: £520100 : 02/07/10- B

SCOPE

Human food and animal feeding stuffs

RESTRICTIONS FOR USE

None

REFERENCE METHOD

EN ISO 7932 (2005) – Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of presumptive *Bacillus cereus* - Colony-count technique at 30 °C.

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

Deputy General Manager
Jacques BESLIN

PRINCIPLE OF THE METHOD

BACARA® agar is a selective chromogenic medium that allows the enumeration of presumptive strains of *Bacillus* of the *cereus* group without confirmation. Strains belonging to *B. cereus* group give characteristic colonies after 24 hrs ± 2 hrs of incubation. Typical colonies show a pink / orangey colour due to the metabolism of the chromogenic substrate and are surrounded with an opaque halo due to the phospholipase activity.

Surface and pour-plate procedures are both applicable in the context of AFNOR VALIDATION.

Note: Confirmation tests are not required on typical colonies obtained using BACARA® method. Nevertheless, confirmations by classical tests described in standardized methods by CEN or ISO (including a purification step, and at the rate of 5 colonies per plate) were tested during AFNOR VALIDATION study. All characteristic colonies gave positive results.

LINEARITY AND relative ACCURACY

Comparison of performances of the alternative method and the reference method

Linearity study:

Tests were performed in 2010 on the 5 food product/strain combinations and for the food categories given in the table below.

The samples were analyzed **in duplicate** with each of the **two methods**, at the five following artificial contamination levels:

- 50 to 100 CFU/g
- 100 to 500 CFU /g
- 500 to 1, 000 CFU /g
- 1, 000 to 5, 000 CFU /g
- 5, 000 to 10, 000 CFU /g

Both surface and pour-plate protocols of the alternative method were tested. The following results were obtained:

- Surface protocol -

Food category	Food product/strain pair	Regression line
Meat & seafood products	Potted meat / <i>Bacillus weihenstephanensis</i>	$y = 1.107 x - 0.310$
Dairy products	Raw milk / <i>Bacillus cereus</i>	$x = 1.006 y + 0.075$
	Dried milk / <i>Bacillus mycoïdes</i>	$x = 0.933 y + 0.141$
Vegetables	Instant mashed potato / <i>Bacillus mycoïdes</i>	$y = 1.068 x - 0.264$
Miscellaneous	Dehydrated confectioner's custard / <i>Bacillus cereus</i>	$y = 0.988 x + 0.110$
	Tabbouleh / <i>Bacillus cereus</i>	$y = 0.990 x + 0.102$
Animal feeding	Cattle cake / <i>Bacillus mycoïdes</i>	$x = 1.129 y - 0.515$

$y = \log(N \text{ alternative method})$

$x = \log(N \text{ reference method})$

- Pour-plate protocol -

Food category	Food product/strain pair	Regression line
Meat & seafood products	Potted meat / <i>Bacillus weihenstephanensis</i>	$y = 0.978 x - 0.080$
Dairy products	Raw milk / <i>Bacillus cereus</i>	$x = 1.000 y - 0.146$
	Dried milk / <i>Bacillus mycoïdes</i>	$y = 1.064 x - 0.306$
Vegetables	Instant mashed potato / <i>Bacillus mycoïdes</i>	$y = 0.987 x + 0.023$
Miscellaneous	Dehydrated confectioner's custard / <i>Bacillus cereus</i>	$y = 0.878 x + 0.454$
	Tabbouleh / <i>Bacillus cereus</i>	$y = 0.929 x + 0.352$
Animal feeding	Cattle cake / <i>Bacillus mycoïdes</i>	$y = 0.839 x + 0.557$

$y = \log(N \text{ alternative method})$

$x = \log(N \text{ reference method})$

Accuracy study:

Tests were performed in 2010. The statistical interpretation was conducted on 52 results, including 20 naturally contaminated samples and 32 artificially contaminated samples, belonging to the following major food categories: Meat and seafood products, dairy products, vegetables, miscellaneous, animal feeding.

Both surface and pour-plate protocols of the alternative method were tested. The samples were analyzed **in duplicate** with each of the **two methods**.

As an indication, the contamination (concentration) ranges were as follows:

Food category	Contamination range* (in log CFU/g)
Meat & seafood products	1.95 à 4.10
Dairy products	2.70 à 4.03
Vegetables	1.00 à 4.28
Miscellaneous	2.00 à 4.00
Animal feeding	1.00 à 4.07

The equations of the regression line between the alternative method and the reference method, for all categories combined, are as follows:

Surface protocol:

$$Y = 1.030 X - 0.146$$

Pour-plate protocol:

$$Y = 1.006 X - 0.041$$

$y = \log(N \text{ alternative method})$

$x = \log(N \text{ reference method})$

The repeatability for both methods and the bias between the two methods were determined according to the method of calculation used for the interlaboratory study (see sections 6.3.5 and 6.3.6 of the standard EN ISO 16140). These results provide additional information for the accuracy criterion.

The limits of repeatability (in log) obtained for the alternative method and the reference method, and the bias (in log) between the two methods (alternative method - reference method) are as follows:

	Limit of repeatability (in log)		<i>Bias D</i> (individual bias average)
	Alternative method	Reference method	
Surface protocol	0.248	0.209	- 0.071
Pour-plate protocol	0.171	0,209	- 0.025

Conclusion for linearity and relative accuracy:

Linearity and accuracy studies show that results obtained with alternative method are comparable to the ones obtained with reference method. Bias between both methods is low whatever the alternative method protocol used.

Storage of incubated agar plates during 48 hrs at 3°C ±2°C

Results obtained after 24 hrs ±2 hrs of incubation at 30°C were compared to those obtained on the same agar plates stored during 48 hrs at 3°C±2°C. The cold storage of agar plates does not modify the results observed just after incubation.

SELECTIVITY (INCLUSIVITY/EXCLUSIVITY)

Use of alternative method only

- 30 strains of *Bacillus cereus* were detected out of 30 tested.
- The study of 27 strains not belonging to the genus *Bacillus cereus* did not detect the presence of cross-reactions. Among the strains tested, two *Enterococcus faecalis* have grown on BACARA[®] Agar, without forming halo.

PRACTICABILITY

Use of alternative method only

- **Positive** and **negative** results are obtained in 1 day with the alternative method, against to 2 to 4 days with the reference method.
- BACARA[®] method which does not require a confirmation step allows an **important labour saving** compared to the reference method.

INTERLABORATORY STUDY

The inter-laboratory study was conducted in 2010 with 14 participating laboratories. The analyses were carried out on samples of pasteurized milk, artificially contaminated with a *Bacillus cereus* strain at the 4 following levels:

- 0 CFU/ml
- 100 CFU/ml
- 1, 000 CFU/ml
- 10, 000 CFU/ml

The laboratories tested, using each of the **two methods**, **two replicates per contamination level**.

The results calculated in accordance with the draft amendment 1 to EN ISO 16140 standard (version prA1: 2009) were the following:

Contamination level	Number of laboratories with results taken into account*	Reference method		Alternative method		Bias
		Repeatability standard deviation S_r	Reproducibility standard deviation S_R	Repeatability standard deviation S_r	Reproducibility standard deviation S_R	
Level 1	13	0.219	0.219	0.315	0.315	-0.24
Level 2	13	0.041	0.077	0.078	0.090	-0.07
Level 3	13	0.058	0.102	0.031	0.068	-0.04

*A laboratory did the assays out of time. Their results were not taken into account.

NB: Limit of repeatability $r = 2.8 S_r$, with S_r : repeatability standard deviation
Limit of reproducibility $R = 2.8 S_R$, with S_R : reproducibility standard deviation

Conclusion

The interlaboratory study shows that the results obtained with the alternative method are comparable to those obtained with 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