



**Alternative methods for agribusiness
Analytical performances certified**

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

Certificate No.: BRD 07/17 – 01/09

**Validation date: 26.01.2009
End of validity: 26.01.2013**

The company
(head office, distributor,
and production site)

Bio-Rad
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FRANCE

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

AL – enumeration method

Protocol reference: AL short protocol / Agar – V2
356-3695 / 356-4041
356-4042 / 356-4043

SCOPE

All human food products and environmental samples.

RESTRICTIONS OF USE

None.

REFERENCE METHOD

EN ISO 11290-1 (1997) including **amendment A1** (2004): Microbiology – Horizontal method for the detection and enumeration of *Listeria monocytogenes* – Part 2: Enumeration method.

**Deputy General Manager
Jacques BESLIN**

A handwritten signature in black ink, appearing to be "Jacques Beslin", written over a horizontal line.

AFNOR Certification

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PRINCIPLE OF THE METHOD

The enumeration A.L. method is a chromogenic agar medium, that permits the simultaneous detection of *Listeria* genus by formation of a blue to blue-green colour (β -D-glucosidase activity) and to differentiate the *Listeria monocytogenes* by production of an opaque halo around the colony (phospholipase C activity). The final result count is reached after 48 hours \pm 3hours (with formation of *Listeria monocytogenes* characteristic colonies from 24 hours).

In the context of AFNOR Validation, all samples identified as positive by the alternative method must be confirmed by one of the following means (only one colony confirmation is required):

- According to classical tests described in methods standardized by CEN or ISO (including a purification step).
- Using nucleic probes as described in EN ISO 7218 standard (including or not the purification step).
- A colony isolated on A.L. agar may be confirmed by means of spot sub-culture on RAPID'L. *Mono* agar, without previous purification.
- Using any other AFNOR VALIDATION certified method, the principle of which is different from the AL enumeration method. The protocol of the second validated method shall be followed entirely. All steps that are before the step from which the confirmation is done shall be common to both methods.

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.

LINEARITY AND relative ACCURACY

Comparison of performances of the alternative method and the reference method

Linearity study:

Tests were performed in 2008 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 à 100 CFU/g
- 100 à 500 CFU /g
- 500 à 1 000 CFU /g
- 1 000 à 5 000 CFU /g
- 5 000 à 10 000 CFU /g

The following results were obtained:

- AL enumeration - Buffered peptone water (BWP) with revivification - Inoculation on the surface:

Food category	Food product/strain pair	Regression line
Meat products	Potted meat / <i>Listeria monocytogenes</i> 1/2b	Y = X
Dairy products	Raw milk / <i>Listeria monocytogenes</i> 4b	
Seafood products	Smoked salmon / <i>Listeria monocytogenes</i> 1/2a	
Vegetables	Raw vegetables / <i>Listeria monocytogenes</i> 4b	
Environmental samples	Water process / <i>Listeria monocytogenes</i> 1/2c	

[y = log(N alternative method) and x = log(N reference method)]

- AL enumeration - BPW with revivification - in-depth inoculation:

Food category	Food product/strain pair	Regression line
Meat products	Potted meat / <i>Listeria monocytogenes</i> 1/2b	$X = 1,031Y - 0,229$
Dairy products	Raw milk / <i>Listeria monocytogenes</i> 4b	$Y = 0,967X + 0,046$
Seafood products	Smoked salmon / <i>Listeria monocytogenes</i> 1/2a	$Y = 1,009X - 0,068$
Vegetables	Raw vegetables / <i>Listeria monocytogenes</i> 4b	$Y = 0,958X + 0,092$
Environmental samples	Water process / <i>Listeria monocytogenes</i> 1/2c	$Y = 0,978X - 0,087$

[Y = log(N alternative method) and X = log(N reference method)]

- AL enumeration - BPW without revivification step - Inoculation on the surface :

Food category	Food product/strain pair	Regression line
Meat products	Potted meat / <i>Listeria monocytogenes</i> 1/2b	$Y = 1,032X - 0,322$
Dairy products	Raw milk / <i>Listeria monocytogenes</i> 4b	$Y = 1,042X - 0,295$
Seafood products	Smoked salmon / <i>Listeria monocytogenes</i> 1/2a	$Y = 1,013X - 0,153$
Vegetables	Raw vegetables / <i>Listeria monocytogenes</i> 4b	$Y = 0,952X + 0,054$
Environmental samples	Water process / <i>Listeria monocytogenes</i> 1/2c	$Y = 1,044X - 0,426$

[Y = log(N alternative method) and X = log(N reference method)]

- AL enumeration - BPW without revivification step - in-depth inoculation:

Food category	Food product/strain pair	Regression line
Meat products	Potted meat / <i>Listeria monocytogenes</i> 1/2b	$Y = 1,016X - 0,309$
Dairy products	Raw milk / <i>Listeria monocytogenes</i> 4b	$Y = 0,988X - 0,140$
Seafood products	Smoked salmon / <i>Listeria monocytogenes</i> 1/2a	$Y = 1,084X - 0,443$
Vegetables	Raw vegetables / <i>Listeria monocytogenes</i> 4b	$Y = 0,952X - 0,013$
Environmental samples	Water process / <i>Listeria monocytogenes</i> 1/2c	$Y = 1,008X - 0,327$

[Y = log(N alternative method) and X = log(N reference method)]

- AL enumeration - Fraser ½ - Inoculation on surface:

Food category	Food product/strain pair	Regression line
Meat products	Potted meat / <i>Listeria monocytogenes</i> 1/2b	$Y = 1,106X - 0,645$
Dairy products	Raw milk / <i>Listeria monocytogenes</i> 4b	$Y = 0,995X + 0,012$
Seafood products	Smoked salmon / <i>Listeria monocytogenes</i> 1/2a	$Y = 1,064X - 0,286$
Vegetables	Raw vegetables / <i>Listeria monocytogenes</i> 4b	$Y = 0,993X - 0,100$
Environmental samples	Water process / <i>Listeria monocytogenes</i> 1/2c	$Y = 1,013X - 0,163$

[Y = log(N alternative method) and X = log(N reference method)]

- AL enumeration - Fraser ½ - in-depth inoculation:

Food category	Food product/strain pair	Regression line
Meat products	Potted meat / <i>Listeria monocytogenes</i> 1/2b	$Y = 0,974X - 0,083$
Dairy products	Raw milk / <i>Listeria monocytogenes</i> 4b	$Y = 0,991X - 0,150$
Seafood products	Smoked salmon / <i>Listeria monocytogenes</i> 1/2a	$Y = 1,024X - 0,227$
Vegetables	Raw vegetables / <i>Listeria monocytogenes</i> 4b	$Y = 0,956X - 0,002$
Environmental samples	Water process / <i>Listeria monocytogenes</i> 1/2c	$Y = 0,979X - 0,134$

[Y = log(N alternative method) and X = log(N reference method)]

Accuracy study:

Tests were performed in 2008. The statistical interpretation was conducted on 52 results, including 37 naturally contaminated samples and 15 artificially contaminated samples, belonging to the following major food categories:

Meat products, dairy products, seafood products, vegetables and environmental samples.

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 products	1.78 – 6.90
Dairy products	1.48 – 5.59
Seafood products	1.70 – 5.73
Vegetables	1.30 – 6.22
Environmental samples	1.96 – 5.57

* write values obtained for both methods, keeping max and min values expressed in log

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

	Regression line : Y = a X+ b
BPW with revivification step - inoculation on surface	Y = X
BPW with revivification step - in-depth inoculation	Y = 0,954 X + 0,165
BPW without revivification step - inoculation on surface	Y = 0,965 X + 0,142
BPW without revivification step - in-depth inoculation	Y = 0,945 X + 0,184
Fraser ½ - Inoculation on surface	Y = 0,970 X + 0,039
Fraser ½ - in-depth inoculation	Y = 0,920 X + 0,202

Y = log(N alternative method)

X = log(N 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 are as follows:

	Limit of repeatability (log)	
	Alternative method	Reference method
BPW with revivification step - inoculation on surface	0.17 UFC/g	
BPW with revivification step - in-depth inoculation	0.14 UFC/g	0.17 UFC/g
BPW without revivification step - inoculation on surface	0.23 UFC/g	0.17 UFC/g
BPW without revivification step - in-depth inoculation	0.16 UFC/g	0.17 UFC/g
Fraser ½ - Inoculation on surface	0.28 UFC/g	0.17 UFC/g
Fraser ½ - in-depth inoculation	0.23 UFC/g	0.17 UFC/g

The bias (in log) between the two methods (alternative method - reference method) is as follows:

	p	D
BPW <u>with</u> revivification step - inoculation on surface	0	0
BPW with revivification step - in-depth inoculation	- 0.026	- 0.010
BPW <u>without</u> revivification step - inoculation on surface	+ 0.010	+ 0.012
BPW without revivification step - in-depth inoculation	- 0.012	- 0.019
Fraser ½ - Inoculation on surface	- 0.057	- 0.073
Fraser ½ - in-depth inoculation	- 0.087	- 0.096

Conclusion for linearity and relative accuracy:

The linearity study and the relative accuracy study showed comparable results for the alternative method and the reference method.

The correlations between the reference method and the alternative method, using buffered peptone as diluent, with or without revivification, are satisfactory, about of the mode of inoculation. The bias between the two methods is about ± 0.01 log CFU/g.

For the alternative method, using the Fraser ½ broth as diluent, the statistical hypothesis are accepted. The limits of repeatability are highest for the alternative method than for the reference method. The bias are about $- 0.1$ log CFU/g. Nevertheless, the sample sizes used for the reference method and the alternative method were not the same.

SELECTIVITY (INCLUSIVITY/EXCLUSIVITY)

Use of alternative method only

- 60 strains of *Listeria monocytogenes* were detected out of 60 tested.
- The study of 18 strains not belonging to the *Listeria* genus did not detect the presence of cross-reactions.

The study of 19 strains of *Listeria* not *monocytogenes* showed that *Listeria ivanovii* had characteristic aspect before 24 hours of incubation, with halos littlest.

PRACTICABILITY

Use of alternative method only

- **Time of response:**
 - **Positive** results are obtained with the alternative method in 2 days (if PCR confirmation) to 9 days (confirmation by classical tests) as opposed to 4 to 9 days with the reference method.
 - **Negative** results are obtained in 2 days with the alternative method as opposed to (number) days with the reference method.

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 2008 with 16 participating laboratories. The analyses were carried out on samples of pasteurized milk artificially contaminated with a *Listeria monocytogenes* strain at the 4 following levels:

- 0 UFC/ml
- 100 UFC/ml
- 1 000 UFC/ml
- 10 000 UFC/ml

The laboratories tested, using **both methods, two replicates per contamination level**.

The following results were obtained:

Contamination level	Number of samples taken into account*	Reference method		Alternative method		
		Repeatability r	Reproducibility R	Repeatability r	Reproducibility R	Bias
Level 1	11	0.513	0.727	0.320	0.489	0.064
Level 2	11	0.231	0.427	0.079	0.265	0.028
Level 3	11	0.232	0.302	0.122	0.235	0.026

* Three laboratories did not realize the analysis because of delivery problems. Another laboratory did not send the results obtained to the expert laboratory. A last laboratory obtained incoherent results.

Conclusion

The results obtained with the alternative method during the interlaboratory study 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