



**Validation of the  
iQ-Check™ *Listeria monocytogenes* method**

**Summary report**

*Comparative and interlaboratory studies according to the EN ISO  
16140 Standard*

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## 1 Introduction

### 1.1 Validation references

The iQ-Check™ *Listeria monocytogenes* method was fully validated (comparative study of the methods and interlaboratory study) according to the EN ISO 16140: 2003 standard.

### 1.2 Protocol and principle of the alternative method

#### 1.2.1 Principle

The iQ-Check *Listeria monocytogenes* test is based on gene amplification and detection using the real-time PCR technique of a sequence specific to *Listeria monocytogenes*. Detection is possible due to a specific oligonucleotide probe, called molecular Beacon, labeled with a fluorophore (FAM) which emits fluorescence only when hybridized to the amplicons. This fluorescence is measured by the optical module of the thermal cycler iCycler iQ™ or Chromo4 system™.

A synthetic DNA called "internal control" is included in each reaction and is amplified at the same time as the target sequence of *Listeria monocytogenes*, but detected by a probe labeled with a different fluorophore than that of *Listeria monocytogenes*. It enables to verify the absence of inhibition in the PCR reaction and to validate a negative result.

The software program associated with the iCycler iQ or Chromo4 system automatically calculates the optical relationship between the intensity of the fluorescence and the amplification cycle. Results are viewed as curves, which are then used to determine whether the result is positive or negative.

#### 1.2.2 Protocols

Several protocols have been validated:

##### Tests performed in 2005

- a protocol using half Fraser broth and standard lysis

##### Tests performed in 2006

- a protocol using a specific broth (LSB) and standard lysis
- a protocol using a specific broth (LSB) and easy lysis
- a protocol using half Fraser broth and easy lysis, specific for environmental samples

These different protocols are described in detail below and flowcharts can be found in Appendix A.

### 1.2.2.1 Protocol validated in 2005 (using half Fraser broth and standard lysis)

The different **analytical steps** are the following:

- Enrichment  
**25 hours +/- 1 hour at 30 °C** in half Fraser broth (1/10)
- Bacterial lysis of to release bacterial DNA
- Amplification - detection  
with the iCycler iQ™ thermal cycler or the Chromo4™ system
- Confirmations  
Positive samples with the iQ-Check™ *Listeria monocytogenes* test are confirmed:
  - 1) According to the tests of standardized methods, after isolation from the enrichment broth on selective agars such as *Listeria* agar according to Ottaviani and Agosti or PALCAM or Oxford agars;
  - 2) By spreading-isolation of 0.1 ml of half Fraser broth on RAPID'L.mono agar.  
If typical colonies are present on RAPID'L.mono, the iQ-Check™ result is considered to be confirmed.

### 1.2.2.2 Protocols tested in the 2006 extension study (use of specific LSB broth)

A specific broth, *Listeria* Special Broth (LSB), has been developed by Bio-Rad to provide better revivification of *Listeria* and the use of a simplified lysis protocol (easy lysis) eliminating the first centrifugation step.

- Enrichment  
**24 hours +/- 2 hours at 30 °C** in LSB broth for the standard lysis protocol  
**25 hours +/- 1 hour at 30 °C** in LSB broth for the easy lysis protocol
- Bacterial lysis of to release bacterial DNA  
Two bacterial lysis protocols are possible:
  - 1) *Standard lysis protocol (protocol identical to the one tested during the initial validation study using half Fraser broth)*
  - 2) *Easy lysis protocol*
- Amplification – detection  
with the iCycler iQ™ thermal cycler or the Chromo4™ system
- Confirmations  
Identical to the initial validation

### 1.2.2.3 Protocol specific to the environmental samples, tested during the 2006 extension study

Inhibitions related to environmental samples can be observed in LSB broth, it is thus interesting to have an alternative protocol for this type of sample.

The initially validated protocol using a half Fraser broth as the enrichment broth is still valid, but it uses the standard lysis protocol.

It was therefore worthwhile to test the easy lysis protocol with these samples.

Thus, along with enrichment in specific LSB broth, environmental samples were also tested with enrichment in half Fraser broth and an easy lysis protocol, with the following enrichment protocol:

- Enrichment  
**24 hours +/- 2 hours at 30°C** in half Fraser broth,  
then subculturing 1 ml in TSB broth, incubate **3 hours to 5 hours at 30°C**, in order to eliminate the risk of inhibition of the PCR reaction due to the half Fraser broth and the samples.

then,

- Easy bacterial lysis protocol to release bacterial DNA
- Amplification - detection
- Confirmations

## 1.3 Requested field of application

The protocols described above (paragraphs 1.2.2.1 and 1.2.2.2) can be applied to all human food products as well as to environmental samples.

In parallel, a specific protocol for environmental samples was also developed.

## 1.4 Reference method

The validation study was conducted in relation to the reference method ISO 11290-1/A1: 2005: "Horizontal method for the detection of *Listeria monocytogenes*".

## 2 Comparative study

The following criteria were determined for the different existing protocols:

- relative accuracy, specificity and sensitivity;
- relative detection level;
- specificity: inclusivity and exclusivity.

In addition, practicability criteria were established according to AFNOR requirements.

### 2.1 Relative accuracy, relative specificity and relative sensitivity

The aim of this study is to compare the two methods:

- the reference method, ISO 11290-1: 2004,
  - the iQ-Check™ *Listeria monocytogenes* method (Bio-Rad),
- on uncontaminated samples and samples contaminated with *Listeria monocytogenes*.

## 2.1.1 Number and nature of samples

A minimum of 60 products per category were analyzed, with approximately 50% of positive products and 50% negative products per category, and a minimum of 30 positive samples per category.

### 2.1.1.1 Tests performed in 2005 (half Fraser + standard lysis protocol)

Categories	Types	Positive*	Negative	Total
Meat products	Raw meats	11	22	33
	Prepared & seasoned (raw)meats	6	13	19
	Sausage meats	14	10	24
	<b>Total</b>	<b>31</b>	<b>45</b>	<b>76</b>
Dairy products	Raw cow's milk cheeses	11	16	27
	Raw goat's/sheep's milk cheeses	10	11	21
	Miscellaneous: raw milk, pastries	9	9	18
	<b>Total</b>	<b>30</b>	<b>36</b>	<b>66</b>
Fish products	Smoked fish	14	9	23
	Raw fish	9	14	23
	Prepared fish and shellfish	9	8	17
	<b>Total</b>	<b>32</b>	<b>31</b>	<b>63</b>
Vegetable products	Raw vegetables	8	10	18
	Frozen vegetables	8	9	17
	Cooked & seasoned vegetables	15	11	26
	<b>Total</b>	<b>31</b>	<b>30</b>	<b>61</b>
Environmental samples	Miscellaneous waters	16	8	24
	Surface samples	15	17	32
	Residues	6	15	21
	<b>Total</b>	<b>37</b>	<b>40</b>	<b>77</b>
<b>TOTAL</b>		<b>161</b>	<b>182</b>	<b>343</b>

\* these are positive results for either one method or the other

### 2.1.1.2 Tests performed in 2006 (extension)

Categories	Types	Positive*	Negative	Total
Meat products	raw	14	10	24
	raw and prepared, ready to cook	17	5	22
	Sausage meats, prepared dishes, etc.	7	17	24
	<b>Total</b>	<b>38</b>	<b>32</b>	<b>70</b>
Dairy products	cow's milk cheeses	14	14	28
	goat's or sheep's milk cheeses	8	10	18
	desserts, powdered milk, raw milk	14	7	21
	<b>Total</b>	<b>36</b>	<b>31</b>	<b>67</b>
Fish products	fresh fish filets and shellfish	16	11	27
	smoked fish	11	10	21
	fish-based prepared dishes	9	11	20
	<b>Total</b>	<b>36</b>	<b>32</b>	<b>68</b>
Vegetable products	frozen	10	5	15
	fresh or ready prepared	10	10	20
	prepared cooked vegetables	11	16	27
	<b>Total</b>	<b>31</b>	<b>31</b>	<b>62</b>
Environmental samples	miscellaneous waters	5	6	11
	surface samples	16	18	34
	residues	9	7	16
	<b>Total</b>	<b>30</b>	<b>31</b>	<b>61</b>
<b>TOTAL</b>		<b>171</b>	<b>157</b>	<b>328</b>

\* these are positive results for either one method or the other

## 2.1.2 Artificial contamination of samples and percentage

The contamination method was as follows: contamination with a strain of *Listeria monocytogenes* having undergone a combination of heating and cooling or freezing cycles.

Stress was evaluated by calculating the log difference between enumeration on TSAYE agar and enumeration obtained on PALCAM agar. This difference needed to be at least 0.5 log.

### Study performed in 2005

Products were contaminated by these stressed strains at levels varying from 1 to 12 cells per 25 grams, depending on the strains used. Eight *Listeria monocytogenes* strains of different origins and serotypes were used for these artificial contaminations.

In all, 38 positive results out of 161 were obtained following the artificial contaminations, i.e. 24%.

### Study performed in 2006

Nine *Listeria monocytogenes* strains of different origins and serotypes were used for these artificial contaminations.

In all, 30 positive results out of 171 results were obtained following the artificial contaminations, i.e. 18%.

## 2.1.3 Test results

### 2.1.3.1 Tests performed in 2005 (half Fraser + standard lysis protocol)

Results obtained for the 343 samples analyzed are summarized as follows.

It is important to note that no inhibitions were observed for all of the results obtained.

	Positive reference method (R+)	Negative reference method (R-)	Total
Positive alternative method (A+)	Positive agreement (A+/R+) <b>PA = 155</b>	Positive deviation (R-/A+) <b>PD = 2</b>	157
Negative alternative method (A-)	Negative deviation (A-/R+) <b>ND = 4<sup>(1)</sup></b>	Negative agreement (A-/R-) <b>NA = 182<sup>(2)</sup></b>	186
Total	159	184	<b>343</b>

Legend:

A+ = confirmed positives

A- = immediate negatives **and** negatives after confirmation of presumptive positives

(1) : including no samples presumed positive with the alternative method, negative after confirmation

(2) : including 9 presumed positive with the alternative method, negative after confirmation

The same tables of results but for each sample category are included in Appendix B.

### 2.1.3.2 Tests performed in 2006 (extension)

Results obtained for the 328 samples analyzed are summarized as follows.

- 1) Results obtained with LSB broth incubated 22 hours and standard lysis protocol (all categories)

	Positive reference method (R+)	Negative reference method (R-)	Total
Positive alternative method (A+)	Positive agreement (A+/R+) <b>PA = 138</b>	Positive deviation (R-/A+) <b>PD = 22</b>	160
Negative alternative method (A-)	Negative deviation (A-/R+) <b>ND = 11<sup>(3)</sup></b>	Negative agreement (A-/R-) <b>NA = 157<sup>(4)</sup></b>	168
Total	149	179	<b>328</b>

Legend:

A+ = confirmed positives

A- = immediate negatives **and** negatives after confirmation of presumptive positives

(3) : including 1 sample presumed positive with the alternative method, negative after confirmation

(4) : including 7 samples presumed positive with the alternative method, negative after confirmation

The same tables of results but for each sample category are included in Appendix C.

2) Results obtained with LSB broth incubated 24 hours and easy lysis protocol (all categories)

	<b>Positive reference method (R+)</b>	<b>Negative reference method (R-)</b>	<b>Total</b>
<b>Positive alternative method (A+)</b>	Positive agreement (A+/R+) <b>PA = 135</b>	Positive deviation (R-/A+) <b>PD = 21</b>	156
<b>Negative alternative method (A-)</b>	Negative deviation (A-/R+) <b>ND = 14</b> <sup>(5)</sup>	Negative agreement (A-/R-) <b>NA = 158</b> <sup>(6)</sup>	172
<b>Total</b>	149	179	<b>328</b>

Legend:

A+ = confirmed positives

A- = immediate negatives **and** negatives after confirmation of presumptive positives

(5) : including no samples presumed positive with the alternative method, negative after confirmation

(6) : including 4 samples presumed positive with the alternative method, negative after confirmation

The same tables of results but for each sample category are included in Appendix C.

3) Results obtained for the specific “environmental samples” protocol (half Fraser broth + TSB broth and easy lysis protocol)

	<b>Positive reference method (R+)</b>	<b>Negative reference method (R-)</b>	<b>Total</b>
<b>Positive alternative method (A+)</b>	Positive agreement (A+/R+) <b>PA = 30</b>	Positive deviation (R-/A+) <b>PD = 0</b>	30
<b>Negative alternative method (A-)</b>	Negative deviation (A-/R+) <b>ND = 0</b>	Negative agreement (A-/R-) <b>NA = 31</b> <sup>(7)</sup>	31
<b>Total</b>	30	31	<b>61</b>

Legend:

A+ = confirmed positives

A- = immediate negatives **and** negatives after confirmation of presumptive positives

(7) : including 2 samples presumed positive with the alternative method, negative after confirmation

## 2.1.4 Calculation of relative accuracy (AC), relative specificity (SP) and relative sensitivity (SE)

All of these results are used to calculate relative accuracy, relative sensitivity and relative specificity for each category and for all of the categories, according to the equations in the EN ISO 16140 standard.

1) Results obtained with half Fraser broth and standard lysis protocol (all categories)

Category	PA	NA	ND	PD	Sum N	Relative accuracy AC (%) [100x(PA+NA)]/N	N+ PA + ND	Relative sensitivity SE (%) [100xPA]/N+	N- NA + PD	Relative specificity SP (%) [100xNA]/N-
Meat products	29	45	1	1	76	97.4	30	96.7	46	97.8
Dairy products	30	36	0	0	66	100	30	100	36	100
Fish products	29	31	3	0	63	95.2	32	90.6	31	100
Vegetables	30	30	0	1	61	98.4	30	100	31	96.8
Environmentals	37	40	0	0	77	100	37	100	40	100
<b>TOTAL</b>	<b>155</b>	<b>182</b>	<b>4</b>	<b>2</b>	<b>343</b>	<b>98.3</b>	<b>159</b>	<b>97.5</b>	<b>184</b>	<b>98.9</b>

2) Results obtained with LSB broth incubated 22 hours and standard lysis protocol (all categories)

Category	PA	NA	ND	PD	Sum N	Relative accuracy AC (%) [100x(PA+NA)]/N	N+ PA + ND	Relative sensitivity SE (%) [100xPA]/N+	N- NA + PD	Relative specificity SP (%) [100xNA]/N-
Meat products	23	32	4	11	70	78.6	27	85.2	43	74.4
Dairy products	31	31	2	3	67	92.5	33	93.9	34	91.2
Fish	30	32	1	5	68	91.2	31	96.8	37	86.5
Vegetables	26	31	2	3	62	91.9	28	92.9	34	91.2
Environmentals	28	31	2	0	61	96.7	30	93.3	31	100
<b>TOTAL</b>	<b>138</b>	<b>157</b>	<b>11</b>	<b>22</b>	<b>328</b>	<b>89.9</b>	<b>149</b>	<b>92.6</b>	<b>179</b>	<b>87.7</b>

3) Results obtained with LSB broth incubated 24 hours and easy lysis protocol (all categories)

Category	PA	NA	ND	PD	Sum N	Relative accuracy AC (%) [100x(PA+NA)]/N	N+ PA + ND	Relative sensitivity SE (%) [100xPA]/N+	N- NA + PD	Relative specificity SP (%) [100xNA]/N-
Meat products	23	33	4	10	70	80.0	27	85.2	43	76.7
Dairy products	29	31	4	3	67	89.6	33	87.9	34	91.2
Fish	29	32	2	5	68	89.7	31	93.5	37	86.5
Vegetables	26	31	2	3	62	91.9	28	92.9	34	91.2
Environmentals	28	31	2	0	61	96.7	30	93.3	31	100
<b>TOTAL</b>	<b>135</b>	<b>158</b>	<b>14</b>	<b>21</b>	<b>328</b>	<b>89.3</b>	<b>149</b>	<b>90.6</b>	<b>179</b>	<b>88.3</b>

4) Results obtained for the environmental samples with half Fraser -TSB broths and easy lysis protocol

Category	PA	NA	ND	PD	Sum N	Relative accuracy AC (%) [100x(PA+NA)]/N	N+ PA + ND	Relative sensitivity SE (%) [100xPA]/N+	N- NA + PD	Relative specificity SP (%) [100xNA]/N-
Environmentals (2006 results)	30	31	0	0	61	100	30	100	31	100

All of these results are used to calculate the following three criteria for the alternative method:

	Half Fraser broth (2005)	LSB broth	
	Standard lysis protocol	Standard lysis protocol	Easy lysis protocol
relative accuracy: <b>AC</b>	<b>98.3 %</b>	<b>89.9 %</b>	<b>89.3 %</b>
relative specificity: <b>SP</b>	<b>98.9 %</b>	<b>87.7 %</b>	<b>88.3 %</b>
relative sensitivity: <b>SE</b>	<b>97.5 %</b>	<b>92.6 %</b>	<b>90.6 %</b>

According to the following calculations required by the Technical Committee, sensitivity was recalculated taking into account all confirmed positives (these include the additional positives with the alternative method):

	Alternative method	Reference method
Half Fraser broth (2005): Standard lysis protocol	$(PA + PD) / (PA + PD + ND) = 97.5 \%$	$(PA + ND) / (PA + PD + ND) = 98.6 \%$
LSB broth: Standard lysis protocol	$(PA + PD) / (PA + PD + ND) = 93.6 \%$	$(PA + ND) / (PA + PD + ND) = 87.1 \%$
Easy lysis protocol	$(PA + PD) / (PA + PD + ND) = 91.8 \%$	$(PA + ND) / (PA + PD + ND) = 87.6 \%$

## 2.1.5 Analysis of discordant results

According to Appendix F of the NF EN ISO 16140 standard, the number of discordant results above which a statistical test must be conducted in order to compare both methods is 6.

This statistical test was carried out for the iQ Check™ *Listeria monocytogenes* method, since the number of discordant results was:

- 6 with half Fraser broth and the standard lysis protocol,
- 33 with LSB broth and the standard lysis protocol,
- 35 with LSB broth and the easy lysis protocol.

*Reminder: in the case of the LSB broth, the alternative method and the reference method use different enrichment broths and the percentage of naturally contaminated samples is high (82%), which is a factor that strongly influences the number of discordant results.*

### 2.1.5.1 Tests performed in 2005 (half Fraser + standard lysis protocol)

It is a question of determining M as a function of the total number of discordants, according to the ISO 16140 standard (Appendix F), and comparing M with a value, m, the smaller of the two values of PD and ND.

The two methods will be considered to be equivalent if  $m > M$ .

Number of discordant results	M	m	Conclusion
6	0	2	Equivalence

### 2.1.5.2 Tests performed in 2006 (extension)

*NB: for the "Environmental Samples" category tested with the half Fraser - TSB protocol, no discordance was observed. The two methods were considered to be equivalent for this specific protocol.*

When the number of discordant results is greater than 22, the McNemar test with  $\chi^2$  distribution is used for one degree of freedom.

It is a question of determining  $d = |PD - ND|$  and comparing d with a minimum d value defined for each number of discordant results.

The two methods will be considered to be different if  $d \geq$  minimum d.

	Number of discordant results	minimum d	d	Conclusion
<i>Standard lysis protocol</i>	33	12	$22 - 11 = 11$	Equivalence
<i>Easy lysis protocol</i>	35	12	$21 - 14 = 7$	Equivalence

The alternative method using LSB broth is equivalent to the reference method.

During the tests performed in the context of this study, however, the alternative method resulted in a large number of confirmed additional positives. Given these results, we can conclude that it provides performances superior to those of the reference method.

## 2.1.6 Comments on inhibitions

It should be noted that no cases of inhibition were observed for any of the results obtained with the half Fraser and standard lysis protocol.

Inhibited results were only obtained with the LSB broth protocol.

- 11 results inhibited after the iQ Check™ *Listeria monocytogenes* test using pure DNA were observed with the standard lysis protocol:
  - one meat product;
  - four dairy products;
  - two fish products;
  - one sample of vegetable products;
  - three environmental samples.
- 3 results inhibited after the iQ Check™ *Listeria monocytogenes* test using pure DNA were observed with the easy lysis protocol:
  - a sample of chocolate ice cream;
  - two surface samples.

All of these inhibitions were removed by diluting the DNA to 1/10<sup>th</sup> before performing the test and the results all complied with those expected (notably, no result became false negative).

## 2.1.7 Comments on the confirmation protocol

### 2.1.7.1 Tests performed in 2005 (half Fraser broth protocol)

Positive samples from the iQ-Check™ *Listeria monocytogenes* test are confirmed:

- 1) according to the reference method protocol (half Fraser broth isolation on selective agars, 'Agar *Listeria* according to Ottaviani and Agosti' and PALCAM and inoculation of a whole Fraser broth followed, where necessary, by isolations on selective agars, then identification of the typical colonies);
- 2) by spreading-isolation on RAPID'L. *mono* agar: if typical colonies are present on RAPID'L. *mono*, the iQ-Check result is considered to be confirmed.

#### 1) With the reference method

Confirmations carried out from half Fraser broth on agars used for the reference method (PALCAM and *Listeria* Agar inoculated with a 10µl calibrated loop) for the most part enabled the detection of *Listeria monocytogenes*. For six samples, however, *Listeria monocytogenes* was not detected on any of the three agars after half Fraser, but was detected after whole Fraser.

For other samples, *Listeria monocytogenes* was only detected on some media inoculated from half Fraser broth, but it was detected on the three media after whole Fraser.

Thus, *Listeria monocytogenes* was not detected after half Fraser broth incubation, on:

- PALCAM agar for 8 samples;
- Agar *Listeria* for 6 samples;
- ALOA® agar for 8 samples.

#### 2) With RAPID'L. *mono*

The confirmation of an iQ-Check™ *Listeria monocytogenes* test was also carried out by spreading-isolation of 100 µl of half Fraser broth on RAPID'L. *mono* chromogenic agar.

Of the 157 positive results confirmed obtained with the alternative method, only one result was not confirmed on RAPID'L. *mono* agar. This consisted of a "diced smoked bacon" sample which was only found to be positive on PALCAM agar isolations, and not on Agar *Listeria* according to Ottaviani and Agosti.

It is important to note that the two additional positive samples are due to the presence of characteristic *Listeria monocytogenes* colonies, identified as such, on RAPID'L. *mono* agar only.

### 2.1.7.2 Tests performed in 2006 (LSB broth protocol)

Positive samples with iQ-Check™ *Listeria monocytogenes* are confirmed by isolation of LSB broth on RAPID'L.mono agar: if characteristic colonies are present on RAPID'L. mono, the iQ-Check result is considered to be confirmed.

LSB broth isolation on RAPID'L.mono agar always enabled detection of *Listeria monocytogenes* (except for specific cases of false positive results which did not contain *Listeria monocytogenes*).

For a diced smoked salmon sample, 48 hours of incubation of the RAPID'L.mono agar was necessary to detect characteristic blue colonies of *Listeria monocytogenes*.

In a single case, subculturing of the LSB broth in Fraser broth before isolation on RAPID'L.mono agar was necessary to detect *Listeria monocytogenes*: this was a smoked haddock sample, also contaminated with *Listeria innocua* in which only *Listeria innocua* was detected with the first isolation (no blue colonies present).

In another case (fresh salmon brochettes sample), *Listeria monocytogenes* was not detected on RAPID'L.mono agar, even after subculturing the LSB broth in Fraser broth. On the other hand, *Listeria monocytogenes* was detected on RAPID'Listeria spp. agar.

### 2.1.8 Comments on storing LSB broths at 2-8°C for 72 hours

LSB broths were stored for 72 hours at 2-8°C and a second iQ Check™ *Listeria monocytogenes* test was performed. Some results were different:

#### False positive results:

- one meat product and an environmental sample, negative, became false positives;  
and
- one fish product, a false positive with the easy lysis, became a concordant negative;
- one environmental sample, a false positive with the standard lysis, became a concordant negative.

#### False negative results:

- one dairy product, a false negative with the easy lysis and concordant positive in standard lysis, became concordant positive;
- one positive dairy product became a false negative.

#### Inhibitions:

- two iQ Check™ *Listeria monocytogenes* tests had inhibitions, whereas the results were positive with pure DNA when the test was performed directly after LSB broth incubation.
- the chocolate ice cream sample was no longer inhibited with the easy lysis protocol.

## 2.2 Relative detection level of the iQ-Check™ *Listeria monocytogenes* method and the reference method

The aim of these tests is to determine the level of contamination for which less than 50% of the results obtained are positive and the level for which more than 50% of the results obtained are positive.

Different 'food matrix-strain' pairs were analyzed in parallel using the reference method and the iQ-Check™ *Listeria monocytogenes* method, for five product categories.

Artificial contaminations were performed in compliance with the requirements of the EN ISO 16140 standard and the microbiology technical committee.

The detection levels, calculated according to the Spearman-Kärber method\* (LOD<sub>50</sub>), for each "matrix-strain" combination, were the following:

### 1) Test performed in 2005 (half Fraser broth protocol)

Matrix	Strain	Relative detection level of reference method (CFU / 25 g or 25 ml)	Relative detection level of alternative method (CFU / 25 g or 25 ml)
Home-made rilette	<i>L. monocytogenes</i> 1/2 c	0.7 [0.4 – 1.2]	0.7 [0.4 – 1.2]
Raw milk	<i>L. monocytogenes</i> 1/2 b	0.5 [0.3 – 0.8]	0.5 [0.3 – 0.8]
Smoked salmon	<i>L. monocytogenes</i> 1/2 a	0.3 [0.2 – 0.5]	0.3 [0.2 – 0.5]
Cabbage, corn and lettuce mix	<i>L. monocytogenes</i> 4b	0.9 [0.5 – 1.6]	0.9 [0.5 – 1.6]
Process water	<i>L. monocytogenes</i> 1/2 c	0.6 [0.3 – 0.8]	0.6 [0.3 – 0.8]

### Conclusion

The detection level obtained for the alternative method is between 0.2 and 1.6 cells per 25 grams and is identical to that of the reference method.

### 2) Test performed in 2006 (LSB broth protocol)

Matrix	Strain	Relative detection level of reference method (CFU / 25 g or 25 ml)	Relative detection level of alternative method (CFU / 25 g or 25 ml)
Rilette	<i>L. monocytogenes</i> 1/2b	0.5 [0.3 – 0.9]	0.5 [0.3 – 0.9]
Raw milk	<i>L. monocytogenes</i> 1/2b	0.5 [0.3 – 0.8]	0.6 [0.4 – 0.9]
Smoked salmon	<i>L. monocytogenes</i> 4b	0.5 [0.3 – 0.8]	0.4 [0.2 – 0.8]
Vegetable mix	<i>L. monocytogenes</i> 1/2a	0.6 [0.3 – 1.1]	0.6 [0.3 – 1.2]
Process water	<i>L. monocytogenes</i> 1/2c	0.6 [0.3 – 1.1]	0.6 [0.3 – 1.2]

### Conclusion

The detection level obtained for the alternative method with the LSB broth is between 0.2 and 1.2 cells per 25 grams, whatever the lysis protocol, vs. 0.3 and 1.1 cells per 25 grams for the reference method.

### 3) Test performed in 2006 for the environmental samples with the half Fraser - TSB broths and the easy lysis protocol

Matrix	Strain	Relative detection level of reference method (CFU / 25 g or 25 ml)	Relative detection level of alternative method (CFU / 25 g or 25 ml)
Process water	<i>L. monocytogenes</i> 1/2c	0.6 [0.3 – 1.1]	0.6 [0.3 – 1.1]

### Conclusion

The relative detection level for the environmental samples with the new half Fraser protocol, followed by TSB and the easy lysis protocol is identical to that of the reference method: it is between 0.3 and 1.1 cells per 25 ml. It is also very close to that obtained with the tests performed in 2005 on this category, with the standard lysis protocol: it was between 0.3 and 0.8 cells per 25 ml.

\* "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 10th December, 2003".

## 2.3 Inclusivity/exclusivity

The inclusivity and exclusivity of the method are defined by the analysis of 50 positive strains and 30 negative strains, respectively.

### 2.3.1 Assay protocols

#### 2.3.1.1 Protocol for inclusivity

For each strain of *Listeria monocytogenes*, a culture in nutrient broth was prepared.

Three protocols were then carried out:

- inoculation of a half Fraser broth with approximately 10 *Listeria monocytogenes* per 225 ml incubated at 30 °C for 24 hours, followed by the standard lysis protocol and the iQ-Check™ *Listeria monocytogenes* test (2005 tests);
- inoculation of a LSB broth with approximately 10 *Listeria monocytogenes* per 225 ml and incubated at 30 °C for 24 hours, followed by the easy lysis protocol and the iQ-Check™ *Listeria monocytogenes* (2006 tests);
- inoculation of a half Fraser broth with approximately 10 *Listeria monocytogenes* per 225 ml and incubated at 30 °C for 22 hours, followed by secondary enrichment in TSB broth, then the easy lysis protocol and iQ-Check™ *Listeria monocytogenes* test (2006 tests).

#### 2.3.1.2 Protocol for exclusivity

During the tests performed in 2005, the different negative strains were cultured in nutrient broths, then:

- inoculated in half Fraser broth for the non-*monocytogenes* *Listeria* strains,
  - inoculated in non-selective nutrient broth for the non-*Listeria* strains,
- to obtain levels of approximately 10<sup>5</sup> cells per ml.

The standard lysis protocol and the test were then carried out using the broths previously incubated for 24 hours at 30 °C.

During the tests performed in 2006, all of the negative strains were cultured in nutrient broth for 24 hours at 30 °C, inoculated in 10 ml of nutrient broth to obtain levels of approximately 10<sup>5</sup> cells per ml, then incubated for 24 hours at 30 °C before carrying out the easy lysis protocol and the iQ-Check™ *Listeria monocytogenes* test.

### 2.3.2 Results

The results can be found in Appendix D.

All 50 strains of *Listeria monocytogenes* tested during the two studies gave a positive with the iQ-Check™ *Listeria monocytogenes* test.

Sixteen strains of non-*monocytogenes* *Listeria* in 2005 and 22 strains of non-*monocytogenes* *Listeria* in 2006 all gave negative results.

With the standard lysis protocol carried out after culture in nutrient broth, among the 17 strains other than *Listeria* tested in 2005, one strain of *Enterococcus faecium* gave a positive result after culture in nutrient broth. A second test with this same strain gave the same result.

This strain was therefore tested using the complete protocol of the alternative method, i.e. substituting the non-selective nutrient broth with a half Fraser broth. The test came out negative.

In parallel, the 'Agar *Listeria* according to Ottaviani and Agosti' and PALCAM media were inoculated (reference method) and no culture was observed on the selective agars. On the other hand, colonies were present on the non-selective TSAYE agar.

Furthermore, a second strain of *Enterococcus faecium* was tested and gave a negative result, as did two strains of *Enterococcus faecalis*.

Fourteen strains other than *Listeria* were tested in 2006, following culture in nutrient broth and the easy lysis protocol. They all gave a negative result, including the strains of *Enterococcus faecium* tested.

### 2.3.3 Conclusion

The iQ-Check™ *Listeria monocytogenes* test is indeed specific for *Listeria monocytogenes* and did not detect the other *Listeria* tested.

A non-repeatable cross-reaction was observed with *Enterococcus faecium* cultivated in nutrient broth, but the use of a selective broth did not confirm this result.

## 3 Interlaboratory study

The aim of the interlaboratory study is to determine the variability of the results obtained in different laboratories analyzing identical samples.

Participating laboratories carried out the analysis according to the iQ-Check™ *Listeria monocytogenes* method (half Fraser broth and standard lysis protocol) and the reference method, in 2005.

### 3.1 Implementation

- Number participating laboratories

Fifteen laboratories received samples.

- Matrix used

A “pasteurized milk” matrix was used to carry out the interlaboratory study.

Natural flora present in this matrix was approximately  $4.1 \times 10^1$  cells per ml.

- Strain used

The strain used for the contaminations was a strain of *Listeria monocytogenes* from “raw milk cheese”.

- Number of samples per laboratory

Twenty-four samples per laboratory were prepared, divided into 3 levels of contamination, with 8 samples per level.

### 3.2 Control of the experimental parameters

#### 3.2.1 Levels of contamination obtained after artificial contamination

The contamination levels obtained in the matrix and the precision estimates are given in the table below:

Level	Samples	Targeted theoretical level (b/25ml)	Real level (b/25ml)	Estimation of lower limit of contamination per 25 ml of sample	Estimation of the upper limit of contamination per 25 ml of sample
Level 0	1-4-7-10- 13-16-19-22	0	0		
Low level	2-5-8-11 14-17-20-23	3	4.4	3	5
High level	3-6-9-12 15-18-21-24	30	42.4	36	49

#### 3.2.2 Temperature problems detected during transport, reception temperature and reception times

Recorded temperature upon reception obtained by the laboratories varied from 0.1 °C to 4.9 °C.

These met the requirements (between 0 °C and 8 °C) for the 15 laboratories.

Temperature graphs obtained using data collected from the temperature indicators showed that the temperatures were stable during transport, and remained below or equal to the temperature at reception of samples.

### 3.2.3 Conclusion: description of possible problems encountered and reason for excluding laboratories

All of the laboratories received their samples the day after they were shipped.

Temperatures upon reception reported by the laboratories complied with the requirements (between 0°C and 8°C) for 13 out of the 15 laboratories.

One laboratory reported a temperature at reception of 9.0°C. After analysis of the temperature collected by the temperature indicator, the temperature upon reception was most likely approximately 4°C.

The conditions at reception for the samples for this laboratory are therefore valid and the results from this laboratory were included.

One laboratory was unable to carry out the analyses due to an organizational problem that occurred the day samples were received.

## 3.3 Test results

### 3.3.1 Results obtained by the collaborating laboratories

The positive results after confirmation obtained by the collaborating laboratories are presented in the following tables:

#### Positive results with the reference method

Laboratories	Contamination Levels					
	L0		L1		L2	
	Obtained	Nbr. of samples	Obtained	Nbr. of samples	Obtained	Nbr. of samples
Laboratory A	0	8	8	8	8	8
Laboratory B	0	8	8	8	8	8
Laboratory C	0	8	8	8	8	8
Laboratory D	0	8	8	8	8	8
Laboratory E	0	8	8	8	8	8
Laboratory F	0	8	8	8	8	8
Laboratory G	0	8	8	8	8	8
Laboratory H	0	8	8	8	8	8
Laboratory I	0	8	8	8	8	8
Laboratory J	0	8	8	8	8	8
Laboratory K	0	8	8	8	8	8
Laboratory L	0	8	8	8	8	8
Laboratory M	1	8	7	8	8	8
Laboratory N	0	8	8	8	8	8
Total	1	112	111	112	112	112

#### Positive results with the alternative method

Laboratories	Contamination Levels					
	L0		L1		L2	
	Obtained	Nbr. of samples	Obtained	Nbr. of samples	Obtained	Nbr. of samples
Laboratory A	0	8	8	8	8	8
Laboratory B	0	8	8	8	8	8
Laboratory C	0	8	8	8	8	8
Laboratory D	0	8	8	8	8	8
Laboratory E	0	8	7	8	8	8
Laboratory F	0	8	8	8	8	8
Laboratory G	0	8	8	8	8	8
Laboratory H	0	8	8	8	8	8
Laboratory I	0	8	8	8	8	8
Laboratory J	0	8	8	8	8	8
Laboratory K	0	8	8	8	8	8
Laboratory L	0	8	8	8	8	8
Laboratory M	1	8	7	8	8	8
Laboratory N	0	8	8	8	8	8
Total	1	112	110	112	112	112

### 3.3.2 Comments and conclusion (discordance in relation to expected results, exclusions, etc.)

Fourteen laboratories performed the analyses.

In general, the results obtained with the alternative method after confirmation, for the 14 laboratories included, were **concordant** with those obtained with the reference method.

One laboratory found one low-level contaminated sample, positive with the reference method but negative with the alternative method. This laboratory retested the extracted DNA and the result was definitely positive.

One laboratory found one of the low-level contaminated samples, negative with both methods and an uncontaminated sample positive with both methods (only on AL with the reference method and after confirmation on RAPID'L. *mono* agar with the alternative method).

Levels	Total number of samples	Number of samples analyzed*	Number of results used	Number of negative results		Number of positive results	
				Reference	Alternative	Reference	Alternative
0	120	112	112	111	111	1	1
1	120	112	112	1	2	111	110
2	120	112	112	0	0	112	112

\* one laboratory did not perform the analyses.

## 3.4 Calculations

### 3.4.1 Calculation of the specificity (SP) and sensitivity (SE) for the two methods, expressed as percentages

**For level L0**, it is required that the percentage of specificity be calculated for each method:

$$SP = \{1 - (FP/N_-)\} \times 100$$

where FP is the number of false positives  
N<sub>-</sub> is the total number of all L0 tests

**For levels L1 and L2**, it is required that the percentage of sensitivity be calculated for each method:

$$SE = (TP/N_+) \times 100$$

where TP is the number of true positives  
N<sub>+</sub> is the total number of L1 or L2 tests

The results are presented in the table below:

	Reference method		Alternative method	
		LCL*		LCL*
Level L0	<b>SP = 99.1%</b>	98%	<b>SP = 99.1%</b>	98%
Level L1	<b>SE = 99.1%</b>	98%	<b>SE = 98.2%</b>	96%
Level L2	<b>SE = 100%</b>	98%	<b>SE = 100%</b>	98%
Levels L1 and L2	<b>SE = 99.6%</b>	98%	<b>SE = 99.1%</b>	98%

\* LCL: low critical limit

### 3.4.2 Calculation of relative accuracy (AC), expressed as a percentage

Relative accuracy is calculated using the following equation:

$$AC = \{(PA + NA) / N\} \times 100$$

where PA is the number of positive agreements  
NA is the number of negative agreements

	Positive reference method (R+)	Negative reference method (R-)	Total
Positive alternative method (A+)	Positive agreement (A+/R+) PA = 223	Positive deviation (R-/A+) PD = 0	<b>(N+) = 223</b>
Negative alternative method (A-)	Negative deviation (A-/R+) ND = 1	Negative agreement (A-/R-) NA = 112 <sup>(1)</sup>	<b>(N-) = 113</b>
Total	<b>(N+) = 224</b>	<b>(N-) = 112</b>	<b>N = 336</b>

(1) including 5 samples found positive but negative after confirmation

In this study, relative accuracy is 99.7%.

### 3.4.3 Study of discordant results

- There being only one discordant result between the two methods, no statistical test exists to verify the equivalence of the methods in relation to sensitivity/specificity.
- it must be noted that two results were discordant with respect to expected results, but were in agreement between the two methods.

## 3.5 Interpretation

### 3.5.1 Comparison of the relative accuracy (AC), specificity (SP) and sensitivity (SE) values

Values obtained for both parts of the validation study are given in the table below:

	Interlaboratory study	Preliminary study (protocol used in the interlaboratory study)
Relative accuracy (AC)	<b>99.7%</b>	<b>98.3%</b>
Sensitivity (SE)	<b>99.1%</b>	<b>97.5%</b>
Specificity (SP)	<b>99.1%</b>	<b>98.9%</b>

Values obtained in the interlaboratory study are comparable to those obtained in the preliminary study.

The AFNOR Technical Committee requires that the sensitivity of the two methods be recalculated taking into account all of the confirmed positives (samples truly positive):

	Alternative method	Reference method
sensitivity	$(PA + PD) / (PA + PD + ND) = \mathbf{99.6\%}$	$(PA + ND) / (PA + PD + ND) = \mathbf{100\%}$

### 3.5.2 Accordance (DA)

Accordance is the percentage of chance of finding the same result for two identical test samples analyzed in the same laboratory under conditions of repeatability, i.e. a single operator using the same equipment and the same reagents within the shortest feasible time interval.

To calculate the accordance, it is necessary to calculate the probability that two identical samples give the same result. This is calculated for each participating laboratory; the average probability for all of the laboratories is then determined.

The different tables used to deduce the accordance are given in Appendix E and the accordance for each of the methods at each level are presented in the table below:

	Reference method	Alternative method
Level L0	DA = 98%	DA = 98%
Level L1	DA = 98%	DA = 97%
Level L2	DA = 100%	DA = 100%

### 3.5.3 Concordance

Concordance is the percentage of chance of finding the same result for two identical samples analyzed in two different laboratories.

It is therefore necessary to calculate the percentage of all pairs giving the same results out of all of the possible pairs of results.

The tables of results used for the calculations can be found in Appendix F and the percentage of concordance for each method, at each level are presented in the table below:

	Reference method	Alternative method
Level L0	Concordance = 98.2%	Concordance = 98.2%
Level L1	Concordance = 98.2%	Concordance = 96.5%
Level L2	Concordance = 100%	Concordance = 100%

### 3.5.4 Odds ratio (COR)

This is calculated using the following equation:

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

The Odds ratio for each of the methods and at each level is shown in the table below:

	Reference method	Alternative method
Level L0	COR = 1.00	COR = 1.00
Level L1	COR = 1.00	COR = 1.00
Level L2	COR = 1.00	COR = 1.00

An Odds ratio value of 1.00 means that accordance and concordance are equal.

The higher the Odds ratio, the more interlaboratory variation is predominant.

## 4 Practicability

Practicability is studied according to the 13 criteria defined by the technical committee, by comparing the reference method and the iQ-Check™ *Listeria monocytogenes* method (Bio-Rad).

The criteria defined by AFNOR are listed below:

<p>1. <i>Packaging of test components</i> (cf. instructions)  2. <i>Volume of reagents</i> (cf. instructions and packaging of bottles)</p>	<p>The kit contains the quantity of reagents necessary for 96 analyses:</p> <ul style="list-style-type: none"> <li>- one bottle of lysis reagent (20 ml)</li> <li>- one bottle of lysis beads</li> <li>- one tube of fluorescent probes (0.55 ml)</li> <li>- two tubes of amplification solution (2 x 2.2 ml)</li> <li>- one tube of negative PCR control (0.5 ml)</li> <li>- one tube of positive PCR control (0.25 ml)</li> </ul>
<p>3. <i>Storage conditions of the components</i> (cf. instructions) –  <i>Shelf-life of unopened products</i> (cf. instructions)</p>	<p>The kit should be stored between +2°C and +8°C.  Each reagent stored between +2°C and +8°C can be used until the expiry date indicated on the tube.</p> <p>The kit remains valid for approximately 1 year.</p>
<p>4. <i>Directions for use after initial use</i> (cf. instructions)</p>	<p>Each reagent should be stored between +2°C and +8°C.</p> <p>The reaction mix obtained by mixing the amplification solution and the fluorescent probes can be stored for 1h at 2 – 8°C after preparation.  The reconstituted lysis reagent (lysis buffer + lysis beads) can be stored for 6 months at 2 – 8°C.</p>
<p>5. <i>Specific equipment or premises required</i> (cf. instructions)</p>	<p>Equipment required:</p> <ul style="list-style-type: none"> <li>- 30 °C incubator</li> <li>- Cell disruptor</li> <li>- Heating block (95-100°C) for conical tubes</li> <li>- Laboratory centrifuge (10,000 to 12,000 g max.)</li> <li>- iCycler thermocycler with an optical module and iCycler iQ® or Chromo™ 4 system</li> </ul> <p>Premises:  4 distinct work <b>areas</b> are recommended: one for the extraction of nucleic acids, one reserved for the preparation of the reaction mix, one for the distribution of the extracted DNA in the PCR plate and a fourth one dedicated to amplification and detection.</p>
<p>6. <i>Ready to use reagents or to be reconstituted</i> (cf. instructions)</p>	<p>All of the reagents are ready to use, except for:</p> <ul style="list-style-type: none"> <li>- the reaction mix, obtained by mixing the amplification solution and the fluorescent probes;</li> <li>- the lysis reagent, to which lysis beads are added.</li> </ul>
<p>7. <i>Training period for an operator unfamiliar with the method</i></p>	<p>Two days are necessary for a technician trained in traditional microbiology and PCR techniques.  For technicians with no training in the PCR technique, 4 to 5 days of initial training would be necessary.</p> <p>Occasional contact with Bio-Rad is recommended for interpreting the results.</p>

## 8. Actual handling time – Flexibility of the method with respect to the number of samples to be analyzed

Steps	Average time for a sample (min)		Average time for 48 samples (min)			
	Standard	iQ-Check™	Standard	iQ-Check™		
Preparation, weighing, dilution in the enrichment broth and grinding	7	7	120	120		
Transfer from half Fraser to Fraser	1		30			
Performing the iQ-Check™ test: - centrifugations - lysis - transfer to the PCR tube - software programming		Standard lysis 10 Easy lysis 7		Standard lysis 120 Easy lysis 80		
Isolation on selective agars from half Fraser and Fraser	2		40			
Reading the agar plates and selection of colonies for identification	2		20			
Interpretation of results		1		10		
<b>Total</b>	12 (0h12)	18 (0h18)	15 (0h15)	210 (3h30)	260 (4h10)	210 (3h30)

The advantage of the alternative method particularly lies in the possibility of sorting negative samples from suspicious samples, thus reducing the number of confirmations

For positive samples, the time required for confirmations must be added.

For example, the confirmation of 5 colonies with the reference method tests can be evaluated in approximately 21 minutes, without preparation of the media.

For the alternative method using confirmation option 2, confirmations take less time: RAPID'L. *mono* agar isolation usually is sufficient to confirm the results of the iQ-Check™ *Listeria monocytogenes* test.

## 9. Time required to obtain results

negative samples:

Steps	Time required NF EN ISO 11290-1/A1 reference method	Time required iQ-Check™ <i>L. monocytogenes</i> method
Preparation of the enrichment broth	D0	D0
Inoculation of Fraser broth	D1	/
Performing the iQ-Check™ test Isolation of 10 µl on selective agars	D1 and D3	D1
<b>Obtaining negative results</b> - if there are no typical colonies - if there is confirmation of non- <i>monocytogenes Listeria</i> - if the test is positive and the confirmation negative	<b>D5</b> D9 to D11	<b>D1</b> D2* to D11**

\* if isolation on RAPID'L. *mono*

\*\* if confirmation by conventional tests described in NF EN ISO 11290-1/A1

positive samples:

<b>Steps</b>	<b>Time required NF EN ISO 11290-1/A1 reference method</b>	<b>Time required iQ-Check™ <i>L. monocytogenes</i> method</b>
Preparation of the enrichment broth	D0	D0
Inoculation of Fraser broth	D1	/
Performing the iQ-Check™ test Isolation on selective agars	D1 and D3	D1 D1
Confirmation tests:		
- Isolation on TSAYE	D2 to D5	D2
- CAMP test, hemolysis, TSBYE broth	D3 to D6	D3
- Use of carbohydrates	D3 to D7	D4
- Reading RAPID' <i>L. mono</i>		D2
<b>Obtaining positive results:</b>		
- after confirmation with the reference method tests	<b>D9 to D11</b>	<b>D9</b>
- after RAPID' <i>L. mono</i>		<b>D2</b>

<i>10. Operator qualifications</i>	The user should be trained in good laboratory practices for food microbiology and molecular biology.
<i>11. Steps in common with the reference method</i>	Where applicable, the half Fraser broth enrichment step.
<i>12. Traceability of analysis results</i>	All of the results are saved in electronic file format. Results can be transferred to spreadsheets or LIMS.
<i>13. Maintenance by the laboratory</i>	It is recommended that the alignment of the software mask be verified at regular 6-month intervals, and that it be realigned when the iCycler iQ thermocycler is moved.  A maintenance contract and a customer telephone hotline are available to iCycler iQ or Chromo4 system users.

## 5 Conclusion

The validation study was carried in accordance with the EN ISO 16140: 2003 reference.

**The comparative study** of the methods provided results for:

- relative accuracy, relative specificity and relative sensitivity;
- relative detection level;
- inclusivity and exclusivity.

The initial validation study was used to study a half Fraser broth enrichment protocol followed by a standard lysis protocol.

The performances of the iQ-Check™ *Listeria monocytogenes* method are equivalent to those of the reference method EN ISO 11290-1/A1. They were determined by analyzing 343 samples distributed in five product categories.

The relative accuracy obtained was 98.3%, relative sensitivity was 97.5% and relative specificity was 98.9%.

Six samples gave discordant results in relation to the reference method: four false negative samples and two confirmed additional positive samples.

The relative detection level of the iQ-Check™ *Listeria monocytogenes* method and the reference method was evaluated by artificial contaminations of five different products representative of the five categories tested. It was identical to that of the reference method.

The specificity of the iQ-Check™ *Listeria monocytogenes* method is good since all of the strains of *Listeria monocytogenes* tested were detected (inclusivity) and no cross-reactions were observed among the non-*Listeria monocytogenes* strains tested (exclusivity) when the method protocol is applied as a whole.

During the validation extension study, two lysis protocols were tested after enrichment in LSB broth on all of the food categories and environmental samples: a standard lysis protocol and an easy lysis protocol.

The performances of the iQ-Check™ *Listeria monocytogenes* method are equivalent to those of the reference method, NF EN ISO 11290-1/A1: 2005. They were determined by the analysis of 328 samples distributed in five product categories.

The results for relative accuracy, relative sensitivity and relative specificity are the following:

### 1) Standard lysis protocol

The relative accuracy obtained was 89.9%, relative sensitivity was 92.6% and relative specificity was 87.7%, according to the calculations required by the EN ISO 16140 standard.

Thirty-three discordant results were obtained: 22 additional positive results and 11 false negative results.

The positive samples with the alternative method having been confirmed positive samples, the sensitivities were recalculated in relation to all of the positive results and gave:

- 93.6% sensitivity for the alternative method,
- 87.1% sensitivity for the reference method.

### 2) Easy lysis protocol

The relative accuracy obtained was 89.3%, relative sensitivity was 90.6% and relative specificity was 88.3%, according to the calculations required by the EN ISO 16140 standard.

Thirty-five discordant results were obtained: 21 additional positive results and 14 false negative results.

Likewise, the positive samples with the alternative method having been confirmed positive samples, the sensitivities were recalculated in relation to all of the positive results and gave:

- 91.8% sensitivity for the alternative method,
- 87.6% sensitivity for the reference method.

The relative detection level for the iQ-Check™ *Listeria monocytogenes* method and the reference method was evaluated by artificial contaminations of five different products representative of the five categories tested. It was between 0.2 and 1.2 cells per 25 grams or 25 ml vs. 0.3 and 1.1 cells per 25 grams or 25 ml for the reference method.

The specificity of the method is good, since all of the strains of *Listeria monocytogenes* tested were detected (inclusivity).

In the exclusivity study, no cross-reactions were observed, neither with *Listeria* other than *monocytogenes*, nor with the strains of other genera tested.

Furthermore, the easy lysis protocol was also studied following half Fraser broth enrichment in particular for environmental samples.

The percentages of relative accuracy, relative sensitivity and relative specificity were 100%, since no discordance with the reference method was observed.

The relative detection level for the environmental samples with the new half Fraser protocol followed by TSB and the easy lysis protocol was identical to that of the reference method: it was between 0.3 and 1.1 cells per 25 ml.

The specificity of this protocol was also satisfactory, since all of the strains of *Listeria monocytogenes* tested were detected (inclusivity).

The results of the **interlaboratory study** obtained for all 14 laboratories accepted showed that the alternative method and the reference method had equivalent relative accuracy, specificity and sensitivity values, similar to those obtained during the preliminary study.

The variability of the alternative method (accordance, concordance, odds ratio) was comparable to that of the reference method.

The advantage of this method particularly lies in the possibility of sorting negative samples from suspicious samples, thus reducing the number of confirmations. Furthermore, handling time is reduced compared with the reference method if series of sample are used.

The time required for obtaining results is also of interest: 24 hours for a negative result and starting at 48 hours for a positive result if it is confirmed on RAPID'L. *mono* agar vs. 5 to 12 days for the reference method.

Lastly, the extension protocols developed provided an improvement to the currently validated protocol: reduced incubation times and simplified extraction protocol, where applicable.

Given these results, the iQ-Check™ *Listeria monocytogenes* method (Bio-Rad) was validated in April 2005, under number BRD 07/10-04/05.

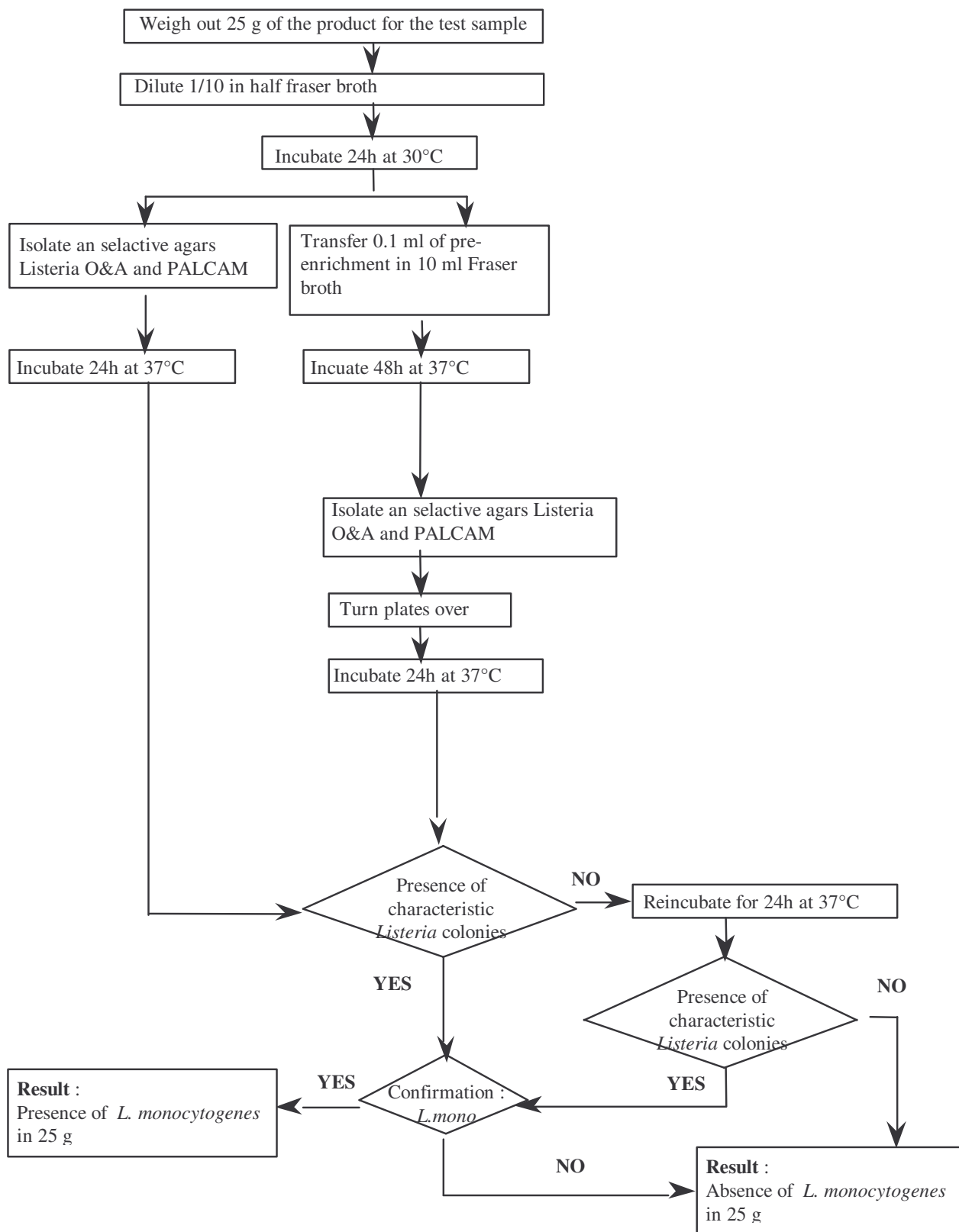
An extension of the validation for different protocols was accepted in December 2006.

# APPENDICES

## APPENDIX A

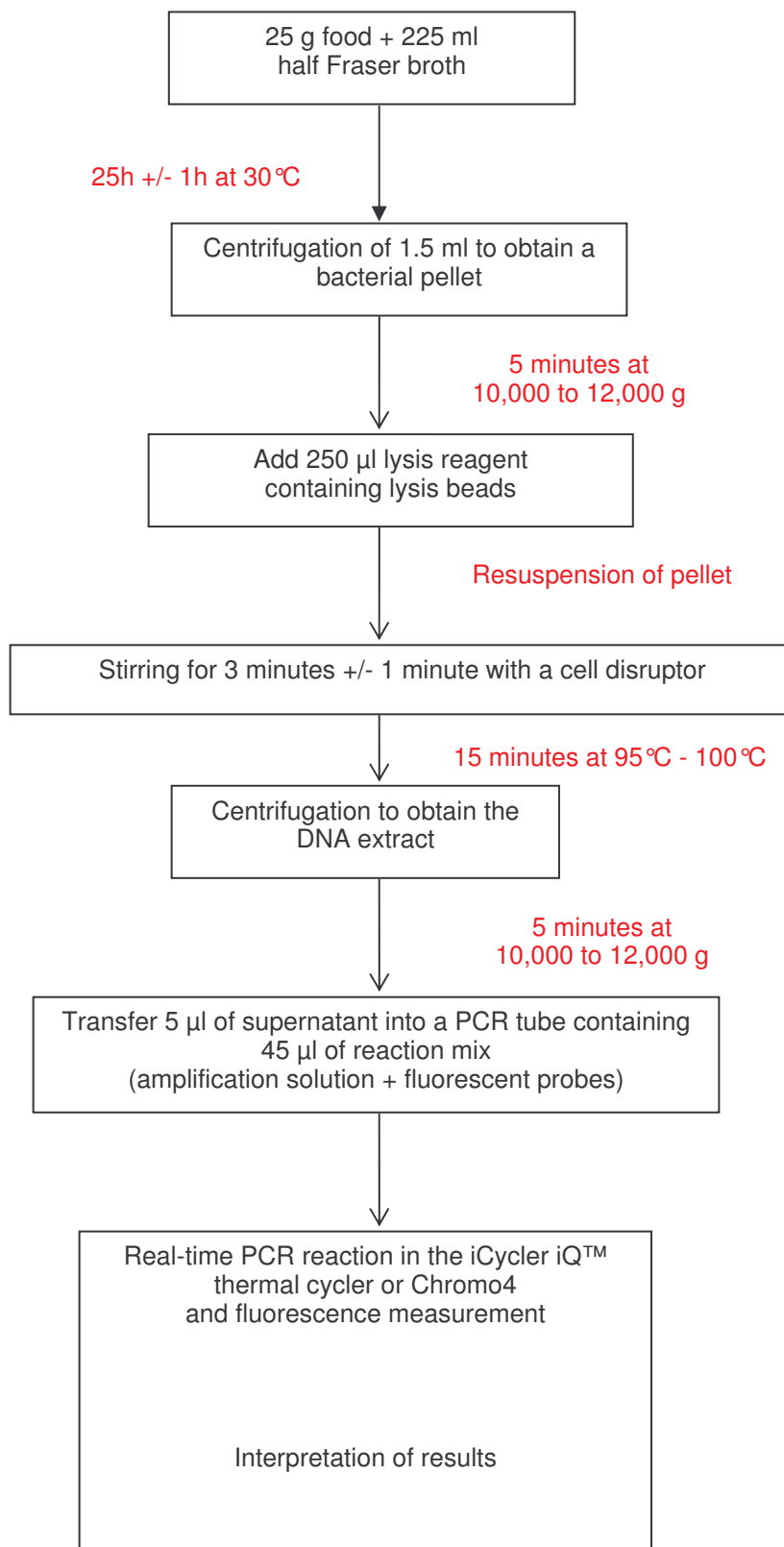
### ANALYTICAL PROTOCOLS

# ISO 11290-1/A1: 2005 STANDARD



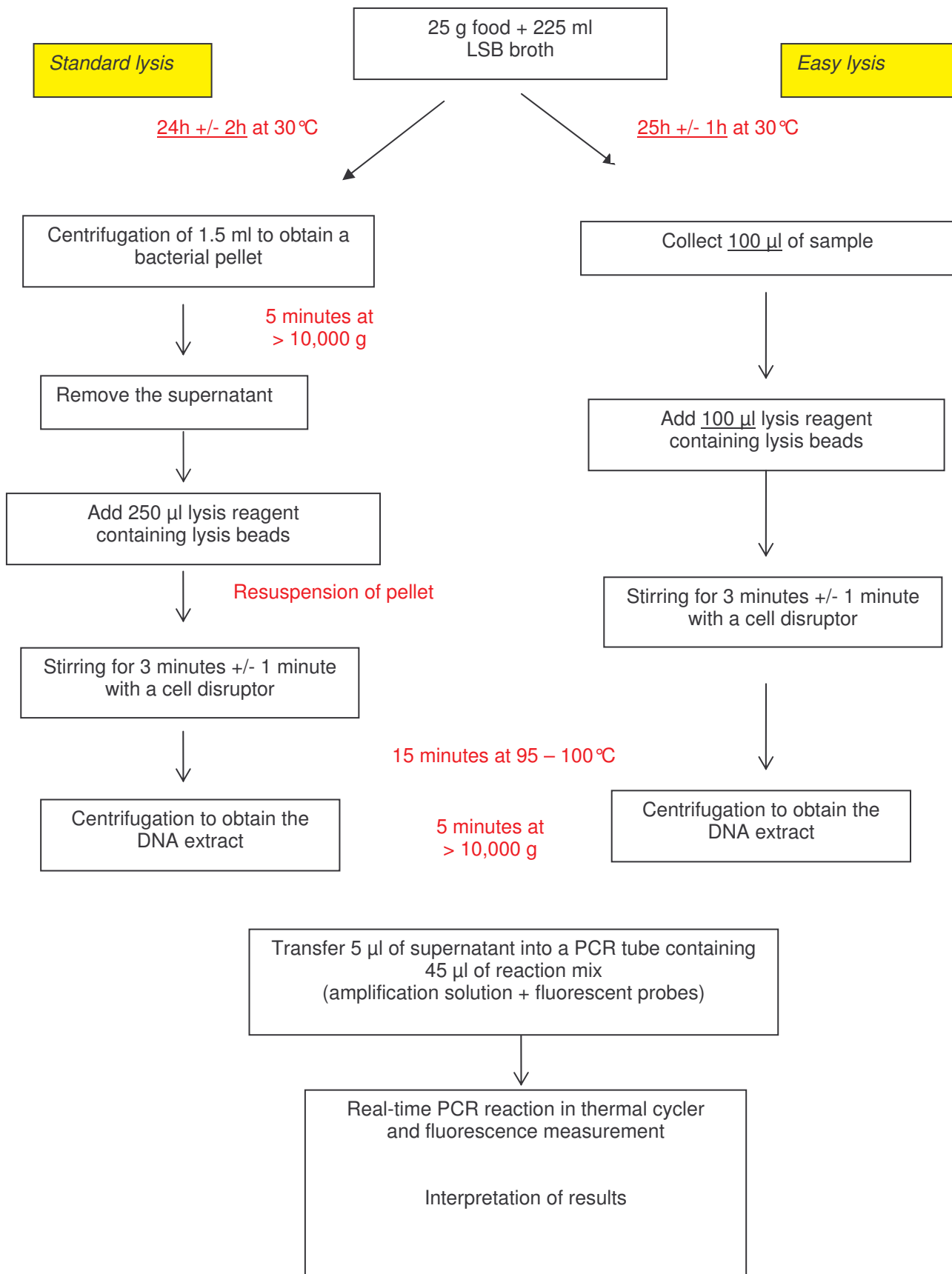
# iQ-Check™ *Listeria monocytogenes* (Bio-Rad) METHOD

## Protocol initially validated



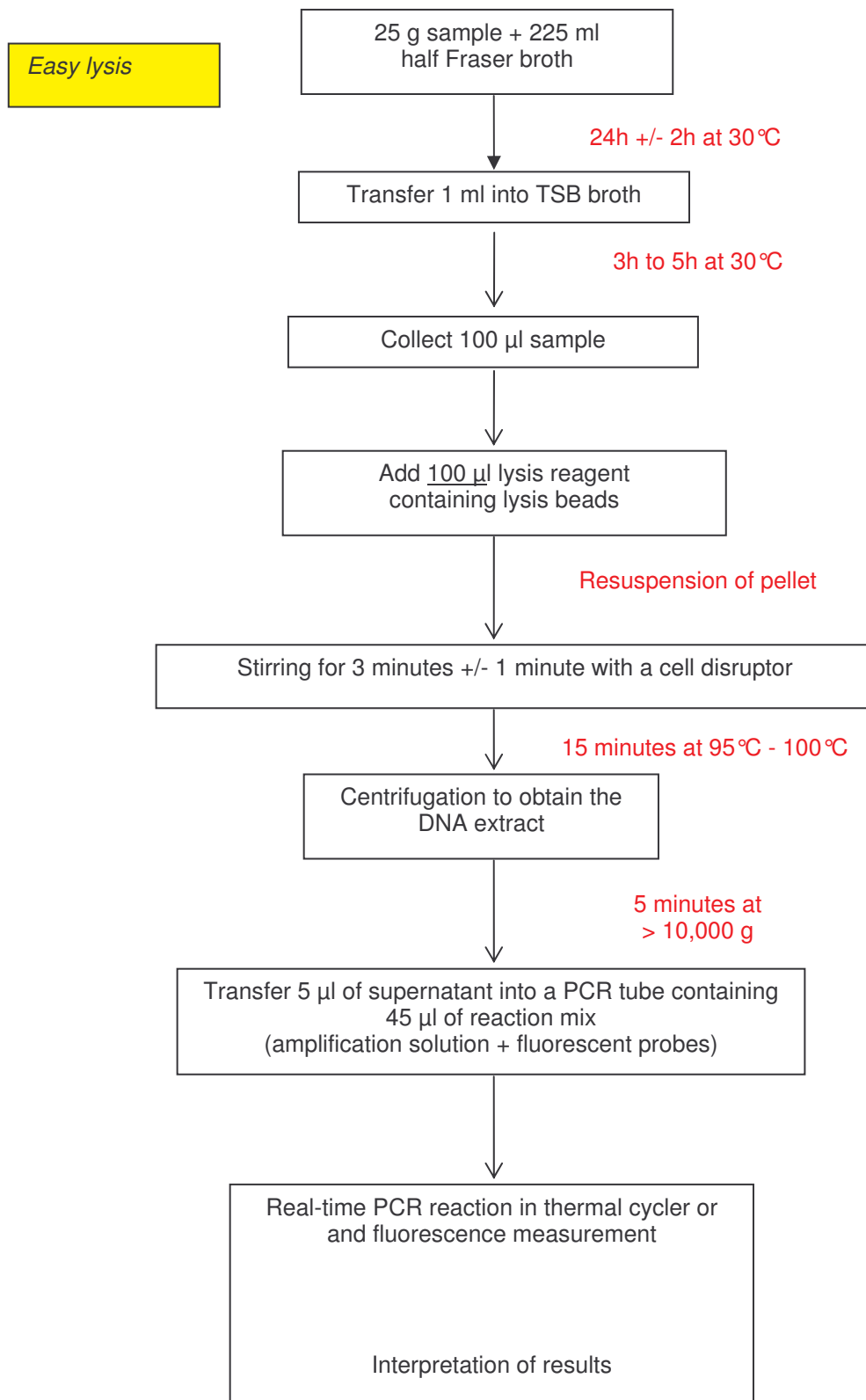
# iQ-Check™ *Listeria monocytogenes* (Bio-Rad) METHOD

## Complementary protocol, part of extension study



# iQ-Check™ *Listeria monocytogenes* (Bio-Rad) METHOD

## Complementary protocol for environmental samples



## APPENDIX B

### COMPARATIVE STUDY OF THE METHODS - SUMMARY OF RESULTS BY CATEGORY 2005 STUDY: half Fraser protocol

# TABLE OF RESULTS BY SAMPLE CATEGORY

## Half Fraser Protocol

<b>Meat products results (76)</b>	<b>Reference method Positive (R+)</b>	<b>Reference method Negative (R-)</b>
<b>Alternative method Positive (A+)</b>	Positive agreement (A+/R+) <b>PA = 29</b>	Positive deviation (R-/A+) <b>PD = 1</b>
<b>Alternative method Negative (A-)</b>	Negative deviation (A-/R+) <b>ND = 1</b>	Negative agreement (A-/R-) <b>NA = 45</b>

<b>Dairy products results (66)</b>	<b>Reference method Positive (R+)</b>	<b>Reference method Negative (R-)</b>
<b>Alternative method Positive (A+)</b>	Positive agreement (A+/R+) <b>PA = 30</b>	Positive deviation (R-/A+) <b>PD = 0</b>
<b>Alternative method Negative (A-)</b>	Negative deviation (A-/R+) <b>ND = 0</b>	Negative agreement (A-/R-) <b>NA = 36</b>

<b>Fish products results (63)</b>	<b>Reference method Positive (R+)</b>	<b>Reference method Negative (R-)</b>
<b>Alternative method Positive (A+)</b>	Positive agreement (A+/R+) <b>PA = 29</b>	Positive deviation (R-/A+) <b>PD = 0</b>
<b>Alternative method Negative (A-)</b>	Negative deviation (A-/R+) <b>ND = 3</b>	Negative agreement (A-/R-) <b>NA = 31</b>

<b>Vegetable products results (61)</b>	<b>Reference method Positive (R+)</b>	<b>Reference method Negative (R-)</b>
<b>Alternative method Positive (A+)</b>	Positive agreement (A+/R+) <b>PA = 30</b>	Positive deviation (R-/A+) <b>PD = 1</b>
<b>Alternative method Negative (A-)</b>	Negative deviation (A-/R+) <b>ND = 0</b>	Negative agreement (A-/R-) <b>NA = 30</b>

<b>Environmentals results (77)</b>	<b>Reference method Positive (R+)</b>	<b>Reference method Negative (R-)</b>
<b>Alternative method Positive (A+)</b>	Positive agreement (A+/R+) <b>PA = 37</b>	Positive deviation (R-/A+) <b>PD = 0</b>
<b>Alternative method Negative (A-)</b>	Negative deviation (A-/R+) <b>ND = 0</b>	Negative agreement (A-/R-) <b>NA = 40</b>

## APPENDIX C

COMPARATIVE STUDY OF THE METHODS

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SUMMARY OF RESULTS BY CATEGORY  
2006 STUDY: LSB protocol

## TABLE OF RESULTS BY SAMPLE CATEGORY

<u>Meat products</u>	Standard lysis protocol (LSB 22h)		Easy lysis protocol (LSB 24h)	
	Reference method Positive (R+)	Reference method Negative (R-)	Reference method Positive (R+)	Reference method Negative (R-)
<b>Alternative method Positive (A+)</b>	Positive agreement (A+/R+) <b>PA = 23</b>	Positive deviation (R-/A+) <b>PD = 11</b>	Positive agreement (A+/R+) <b>PA = 23</b>	Positive deviation (R-/A+) <b>PD = 10</b>
<b>Alternative method Negative (A-)</b>	Negative deviation (A-/R+) <b>ND = 4</b>	Negative agreement (A-/R-) <b>NA = 32 *</b>	Negative deviation (A-/R+) <b>ND = 4</b>	Negative agreement (A-/R-) <b>NA = 33 **</b>

\*: including 2 samples presumed positive with the alternative method, negative after confirmation

\*\* including 1 sample presumed positive with the alternative method, negative after confirmation

<u>Dairy products</u>	Standard lysis protocol (LSB 22h)		Easy lysis protocol (LSB 24h)	
	Reference method Positive (R+)	Reference method Negative (R-)	Reference method Positive (R+)	Reference method Negative (R-)
<b>Alternative method Positive (A+)</b>	Positive agreement (A+/R+) <b>PA = 31</b>	Positive deviation (R-/A+) <b>PD = 3</b>	Positive agreement (A+/R+) <b>PA = 29</b>	Positive deviation (R-/A+) <b>PD = 3</b>
<b>Alternative method Negative (A-)</b>	Negative deviation (A-/R+) <b>ND = 2</b>	Negative agreement (A-/R-) <b>NA = 31</b>	Negative deviation (A-/R+) <b>ND = 4</b>	Negative agreement (A-/R-) <b>NA = 31</b>

<u>Fish products</u>	Standard lysis protocol (LSB 22h)		Easy lysis protocol (LSB 24h)	
	Reference method Positive (R+)	Reference method Negative (R-)	Reference method Positive (R+)	Reference method Negative (R-)
<b>Alternative method Positive (A+)</b>	Positive agreement (A+/R+) <b>PA = 30</b>	Positive deviation (R-/A+) <b>PD = 5</b>	Positive agreement (A+/R+) <b>PA = 29</b>	Positive deviation (R-/A+) <b>PD = 5</b>
<b>Alternative method Negative (A-)</b>	Negative deviation (A-/R+) <b>ND = 1</b>	Negative agreement (A-/R-) <b>NA = 32 *</b>	Negative deviation (A-/R+) <b>ND = 2</b>	Negative agreement (A-/R-) <b>NA = 32 *</b>

\* including 1 sample presumed positive with the alternative method, negative after confirmation

<u>Vegetable products</u>	Standard lysis protocol (LSB 22h)		Easy lysis protocol (LSB 24h)	
	Reference method Positive (R+)	Reference method Negative (R-)	Reference method Positive (R+)	Reference method Negative (R-)
<b>Alternative method Positive (A+)</b>	Positive agreement (A+/R+) <b>PA = 26</b>	Positive deviation (R-/A+) <b>PD = 3</b>	Positive agreement (A+/R+) <b>PA = 26</b>	Positive deviation (R-/A+) <b>PD = 3</b>
<b>Alternative method Negative (A-)</b>	Negative deviation (A-/R+) <b>ND = 2</b>	Negative agreement (A-/R-) <b>NA = 31</b>	Negative deviation (A-/R+) <b>ND = 2</b>	Negative agreement (A-/R-) <b>NA = 31</b>

<u>Environmental samples</u>	Standard lysis protocol (LSB 22h)		Easy lysis protocol (LSB 24h)	
	Reference method Positive (R+)	Reference method Negative (R-)	Reference method Positive (R+)	Reference method Negative (R-)
<b>Alternative method Positive (A+)</b>	Positive agreement (A+/R+) <b>PA = 28</b>	Positive deviation (R-/A+) <b>PD = 0</b>	Positive agreement (A+/R+) <b>PA = 28</b>	Positive deviation (R-/A+) <b>PD = 0</b>
<b>Alternative method Negative (A-)</b>	Negative deviation (A-/R+) <b>ND = 2 *</b>	Negative agreement (A-/R-) <b>NA = 31 **</b>	Negative deviation (A-/R+) <b>ND = 2</b>	Negative agreement (A-/R-) <b>NA = 31 ***</b>

\* including 1 sample presumed positive with the alternative method, negative after confirmation

\*\* including 4 samples presumed positive with the alternative method, negative after confirmation

\*\*\* including 2 sample presumed positive with the alternative method, negative after confirmation

## APPENDIX D

### INCLUSIVITY / EXCLUSIVITY STUDY - TABLE OF RESULTS

## Half Fraser Inclusivity – 2005

Reference	Strain	Origin	Inoculation level in 225 ml half Fraser	undiluted DNA		Result
				Ct TEX	Ct Cint	
A20	<i>Listeria monocytogenes</i>	Raw milk cheese	6.9	21.4	35.4	Pos
L11	<i>Listeria monocytogenes</i>	Spinach	6.1	25.0	34.8	Pos
L121	<i>Listeria monocytogenes</i>	Cheese	6.5	26.7	35.5	Pos
L123	<i>Listeria monocytogenes</i>	Mozzarella cheese	7.4	27.1	34.6	Pos
L124	<i>Listeria monocytogenes</i>	Perch filet	7.4	27.8	34.0	Pos
L125	<i>Listeria monocytogenes</i>	Sauteed vegetables	10.9	28.6	33.9	Pos
L130	<i>Listeria monocytogenes</i>	Ground meat	6.9	25.6	34.3	Pos
L20	<i>Listeria monocytogenes</i>	Smoked salmon shavings	7.1	27.5	35.5	Pos
L25	<i>Listeria monocytogenes</i>	Hen	8.8	29.0	34.6	Pos
L69	<i>Listeria monocytogenes</i>	Salami	8.8	26.7	33.8	Pos
L70	<i>Listeria monocytogenes</i>	Smoked salmon	5.9	25.7	34.5	Pos
L7	<i>Listeria monocytogenes</i> 1/2a	Munster cheese	10.8	28.1	34.8	Pos
L10	<i>Listeria monocytogenes</i> 1/2a	Rillettes	7.6	28.1	35.0	Pos
L116	<i>Listeria monocytogenes</i> 1/2a	Scallop of fish	5.4	22.1	34.6	Pos
L119	<i>Listeria monocytogenes</i> 1/2a	Munster cheese	13.3	28.4	33.6	Pos
L12	<i>Listeria monocytogenes</i> 1/2a	Smoked salmon	7.1	30.6	34.5	Pos
L129	<i>Listeria monocytogenes</i> 1/2a	Sauteed apples	5.1	29.3	34.5	Pos
L4	<i>Listeria monocytogenes</i> 1/2a	ATCC 35152	8.3	27.0	33.9	Pos
L40	<i>Listeria monocytogenes</i> 1/2a	Munster cheese	6.9	27.0	35.9	Pos
L42	<i>Listeria monocytogenes</i> 1/2a	Chicken breast	9.5	29.0	34.4	Pos
L43	<i>Listeria monocytogenes</i> 1/2a	Ground meat	6.3	27.7	33.8	Pos
L44	<i>Listeria monocytogenes</i> 1/2a	Salami	5.7	27.9	33.6	Pos
L45	<i>Listeria monocytogenes</i> 1/2a	Rabbit terrine	6.1	25.9	34.1	Pos
L47	<i>Listeria monocytogenes</i> 1/2a	Sauteed apples	5.2	30.4	34.6	Pos
L5	<i>Listeria monocytogenes</i> 1/2a	Diced smoked salmon	5.5	25.9	35.6	Pos
L6	<i>Listeria monocytogenes</i> 1/2a	Pizza	5.4	30.7	34.5	Pos
L13	<i>Listeria monocytogenes</i> 1/2b	Pig ears	4.9	24.8	34.4	Pos
L37	<i>Listeria monocytogenes</i> 1/2b	Raw milk Maroille cheese	6.0	22.9	35.4	Pos
L48	<i>Listeria monocytogenes</i> 1/2b	Pig tongue	7.6	32.1	34.3	Pos
L49	<i>Listeria monocytogenes</i> 1/2b	Chicken liver cream	4.3	32.5	34.6	Pos
L51	<i>Listeria monocytogenes</i> 1/2b	Cured cheese	3.9	33.5	34.6	Pos
L52	<i>Listeria monocytogenes</i> 1/2b	SLCC 2755	2.7	33.4	33.7	Pos
L117	<i>Listeria monocytogenes</i> 1/2c	Montbéliard sausage	5.0	21.9	34.0	Pos
L14	<i>Listeria monocytogenes</i> 1/2c	Ground meat	4.9	33.2	33.8	Pos
L15	<i>Listeria monocytogenes</i> 1/2c	Beef	6.8	25.5	34.6	Pos
L17	<i>Listeria monocytogenes</i> 1/2c	Pork breast	9.2	29.5	34.5	Pos
L18	<i>Listeria monocytogenes</i> 1/2c	Munster cheese	2.7	30.8	34.1	Pos
L53	<i>Listeria monocytogenes</i> 1/2c	Ground meat	4.4	30.7	33.9	Pos
L54	<i>Listeria monocytogenes</i> 1/2c	Beef bourguignon	8.4	29.4	34.4	Pos
L55	<i>Listeria monocytogenes</i> 3b	SLCC 2540	8.7	30.7	33.0	Pos
L56	<i>Listeria monocytogenes</i> 3c	SLCC 2479	9.1	26.4	33.9	Pos
L57	<i>Listeria monocytogenes</i> 4a	ATCC 19114	5.4	42.3	33.5	Pos
L32	<i>Listeria monocytogenes</i> 4b	Munster cheese	7.1	25.6	34.3	Pos
L33	<i>Listeria monocytogenes</i> 4b	ATCC 19115	4.3	29.3	34.4	Pos
L58	<i>Listeria monocytogenes</i> 4b	Salad	6.1	27.1	34.6	Pos
L60	<i>Listeria monocytogenes</i> 4d	ATCC	7.8	30.7	33.6	Pos
L61	<i>Listeria monocytogenes</i> 4e	ATCC 19118	7.3	31.8	34.1	Pos
L62	<i>Listeria monocytogenes</i> 4e	Reblochon cheese	7.4	28.2	34.3	Pos
L63	<i>Listeria monocytogenes</i> 4e	Munster cheese	7.0	27.3	34.9	Pos
L67	<i>Listeria monocytogenes</i> 7	SLCC 2482	8.3	30.4	33.5	Pos

## Inclusivity – 2006

Reference	Strain	Origin	Inoculation level in 225 ml half Fraser or LSB broth	Easy lysis following LSB broth			Easy lysis following half Fraser broth then TSB		
				Ct C.int	Ct FAM	Result	Ct C.int	Ct FAM	Result
L5	<i>Listeria monocytogenes</i> 1/2a	Diced smoked salmon	13.0	24.73	23.75	+	25.46	29.75	+
L6	<i>Listeria monocytogenes</i> 1/2a	Pizza	12.0	24.24	22.64	+	25.95	29.52	+
L7	<i>Listeria monocytogenes</i> 1/2a	Munster cheese crust	13.0	25.10	26.62	+	25.84	30.07	+
L9	<i>Listeria monocytogenes</i> 1/2a	Munster cheese crust	7.0	25.05	26.18	+	25.50	33.07	+
L10	<i>Listeria monocytogenes</i> 1/2a	Rillettes	9.0	24.56	24.24	+	25.48	30.59	+
L11	<i>Listeria monocytogenes</i> 1/2a	Munster cheese crust	12.0	24.38	22.97	+	25.25	27.88	+
L12	<i>Listeria monocytogenes</i> 1/2a	Smoked salmon	11.0	25.09	26.23	+	26.22	31.69	+
L13	<i>Listeria monocytogenes</i> 1/2b	Pig's ear	7.0	25.90	34.33	+	25.81	29.15	+
L14	<i>Listeria monocytogenes</i> 1/2c	Raw ground beef	12.0	24.03	20.86	+	26.45	29.74	+
L15	<i>Listeria monocytogenes</i> 1/2c	Beef (raw material)	9.0	24.26	22.69	+	25.21	29.34	+
L16	<i>Listeria monocytogenes</i> 1/2c	Ground meat	7.0	23.13	17.00	+	25.57	23.57	+
L17	<i>Listeria monocytogenes</i> 1/2c	Breast	6.0	24.05	19.17	+	25.40	25.20	+
L18	<i>Listeria monocytogenes</i> 1/2c	Munster cheese crust	6.0	23.94	19.79	+	25.44	24.49	+
L20	<i>Listeria monocytogenes</i> 1/2	Smoked salmon shavings	5.0	24.70	22.72	+	25.97	25.69	+
L25	<i>Listeria monocytogenes</i> 1/2	Hen	10.0	24.68	21.80	+	26.00	28.22	+
L28	<i>Listeria monocytogenes</i> 1/2c	Surface sponge	6.0	24.82	20.86	+	26.25	26.17	+
L32	<i>Listeria monocytogenes</i> 4b	Munster cheese	10.0	23.53	17.14	+	26.10	25.23	+
L37	<i>Listeria monocytogenes</i> 1/2b	Raw milk Maroille cheese	8.0	23.10	17.38	+	26.00	24.48	+
L39	<i>Listeria monocytogenes</i>	Ham salami	6.0	23.91	17.99	+	26.10	27.66	+
L40	<i>Listeria monocytogenes</i> 1/2a	Farm Munster cheese	9.0	24.06	19.18	+	26.07	26.82	+
L42	<i>Listeria monocytogenes</i> 1/2a	Chicken breast	6.0	24.32	22.49	+	25.60	25.97	+
L43	<i>Listeria monocytogenes</i> 1/2a	Raw ground beef	6.6	24.68	23.47	+	26.21	27.04	+
L44	<i>Listeria monocytogenes</i> 1/2a	Salami	5.0	24.83	24.02	+	26.63	25.67	+
L45	<i>Listeria monocytogenes</i> 1/2a	Rabbit terrine	6.0	25.35	24.70	+	26.74	27.29	+
L47	<i>Listeria monocytogenes</i> 1/2a	Sauteed apples	6.0	25.22	24.59	+	26.53	28.91	+
L48	<i>Listeria monocytogenes</i> 1/2b	Pig's tongue	5.4	24.26	23.19	+	26.15	28.35	+
L49	<i>Listeria monocytogenes</i> 1/2b	Chicken liver cream	8.0	24.59	24.21	+	25.92	26.35	+
L51	<i>Listeria monocytogenes</i> 1/2b	Cured Germain cheese	5.5	24.75	24.40	+	25.99	27.70	+
L53	<i>Listeria monocytogenes</i> 1/2c	Raw ground beef	6.6	24.54	23.23	+	26.01	26.53	+
L54	<i>Listeria monocytogenes</i> 1/2c	Beef bourguignon	5.0	25.06	26.10	+	25.98	28.52	+
L55	<i>Listeria monocytogenes</i> 3b	SLCC 2540	7.0	25.17	29.90	+	26.54	32.29	+
L56	<i>Listeria monocytogenes</i> 3c	SLCC 2479	6.6	25.63	27.12	+	27.11	28.77	+
L58	<i>Listeria monocytogenes</i> 4b	Salad	10.0	24.98	23.83	+	25.98	26.85	+
L62	<i>Listeria monocytogenes</i> 4e	Reblochon cheese	7.2	24.33	22.90	+	26.37	26.66	+
L63	<i>Listeria monocytogenes</i> 4e	Munster cheese	6.5	23.67	20.15	+	24.53	25.40	+
L116	<i>Listeria monocytogenes</i> 1/2a	Scallop of fish	4.5	24.28	20.08	+	24.90	25.13	+
L117	<i>Listeria monocytogenes</i> 1/2c	Montbéliard sausage	5.5	24.74	20.76	+	25.38	26.01	+
L119	<i>Listeria monocytogenes</i>	Spinach	6.5	23.50	19.79	+	24.93	24.87	+
L121	<i>Listeria monocytogenes</i>	Neuchâtel cheese	6.0	23.98	20.07	+	26.05	25.30	+
L123	<i>Listeria monocytogenes</i>	Mozzarella cheese	5.5	24.31	20.64	+	25.13	25.35	+
L124	<i>Listeria monocytogenes</i>	Perch filet	5.5	24.05	20.25	+	25.89	25.52	+
L128	<i>Listeria monocytogenes</i> 1/2a	Soya cakes	4.5	23.99	20.01	+	26.14	25.38	+
L129	<i>Listeria monocytogenes</i> 1/2a	Sauteed apples	7.0	24.74	20.80	+	25.27	26.32	+
L130	<i>Listeria monocytogenes</i>	Raw ground beef	6.0	24.12	20.43	+	24.66	25.87	+
L137	<i>Listeria monocytogenes</i>	Raw milk Coulommier cheese	6.0	25.02	23.61	+	24.73	26.64	+
L141	<i>Listeria monocytogenes</i>	Environmental sample	6.0	24.43	22.62	+	24.19	27.55	+
L149	<i>Listeria monocytogenes</i>	Environmental sample	5.0	24.07	20.91	+	24.52	25.77	+
L152	<i>Listeria monocytogenes</i>	Environmental sample	6.5	24.19	20.75	+	23.78	26.29	+
L156	<i>Listeria monocytogenes</i>	Fries	6.0	24.03	20.05	+	23.25	23.57	+
L176	<i>Listeria monocytogenes</i>	Rib steak	4.5	24.40	21.22	+	26.39	25.75	+

## Exclusivity – 2005

Reference	Strain	Origin	Inoculation level in 225 ml half Fraser	undiluted DNA		Result
				Ct TEX	Ct Cint	
L64	<i>Listeria innocua</i>	Epoisses cheese	6.70E+04	N/A	33.5	Neg
L72	<i>Listeria innocua</i>	Boulette d'Avesnes cheese	6.70E+04	N/A	33.0	Neg
L78	<i>Listeria innocua</i>	Cockereel	3.40E+04	N/A	31.6	Neg
L77	<i>Listeria innocua 6a</i>	Toulouse sausage	6.80E+04	N/A	33.3	Neg
L76	<i>Listeria innocua 6b</i>	Hamburger patty	1.80E+04	N/A	33.4	Neg
L80	<i>Listeria ivanovii</i>	Collection	2.60E+04	N/A	33.2	Neg
L151	<i>Listeria ivanovii</i>	Raw ground beef	6.20E+04	N/A	33.2	Neg
L133	<i>Listeria ivanovii</i>	Roquefort	7.90E+04	N/A	33.2	Neg
L146	<i>Listeria grayi</i>	Collection	3.60E+04	N/A	33.7	Neg
L143	<i>Listeria grayi</i>	Frozen fries	2.20E+04	N/A	33.7	Neg
L142	<i>Listeria seeligeri</i>	Raw milk cheese	5.90E+04	N/A	34.3	Neg
L84	<i>Listeria seeligeri</i>	Raw ground beef	7.80E+04	N/A	34.4	Neg
L91	<i>Listeria welshimeri</i>	Salami	1.25E+05	N/A	34.9	Neg
L99	<i>Listeria welshimeri</i>	Sausages	1.02E+05	N/A	34.8	Neg
L87	<i>Listeria welshimeri</i>	Raw ground beef	7.70E+04	N/A	33.9	Neg
L83	<i>Listeria seeligeri</i>	Jellied pig's tongue	7.50E+05	N/A	33.7	Neg

Reference	Strain	Origin	Inoculation level in 225 mL non-selective nutrient broth (CFU)	undiluted DNA		Result	Isolation on TSAYE agar	Inoculation level in 225 ml <b>half Fraser</b> broth (CFU)	undiluted DNA		Result	Isolation on TSAYE agar
				Ct TEX	Ct Cint				Ct TEX	Ct Cint		
BA1	<i>Bacillus cereus</i>	Egg product	9.00E+04	N/A	31.2	Neg	+					
BA5	<i>Bacillus sphaericus</i>	Meat product	2.00E+02	N/A	33.8	Neg	+					
BA4	<i>Bacillus stearothermophilus</i>	Dairy product	1.10E+05	N/A	31.2	Neg	+					
15	<i>Brochotrix</i>	Ground meat	4.80E+04	N/A	33.8	Neg	+					
Le3	<i>Candida albicans</i>	Collection	7.30E+04	N/A	31.4	Neg	+					
26	<i>Corynebacterie aquaticum</i>	Raw milk cheese	6.70E+04	N/A	31.0	Neg	+					
3	<i>Corynebacterie spp.</i>	Collection	1.60E+05	N/A	31.9	Neg	+					
E1	<i>Enterococcus faecalis</i>	Egg product	7.30E+04	N/A	34.0	Neg	+					
E6	<i>Enterococcus faecalis</i>	Collection ATCC 19433	2.40E+05	N/A	31.2	Neg	+					
E2	<i>Enterococcus faecium</i>	Collection ATCC 3286	2.50E+05 1.40E+05	37.6 38.8	31.5 33.7	Pos Pos	+	4.60E+04	N/A	34.5	Neg	+
E7	<i>Enterococcus faecium</i>	Collection CIP 5433	2.60E+05	N/A	31.3	Neg	+					
L139	<i>Jonesia denitrificans</i>	Collection	3.20E+05	N/A	33.6	Neg	+					
22	<i>Lactobacillus plantarum</i>	Dairy product	2.00E+05	N/A	31.4	Neg	+					
M3	<i>Micrococcus spp.</i>	Environmental sample	6.80E+04	N/A	33.9	Neg	+					
32	<i>Rhodococcus equi</i>	Meat product	3.80E+04	N/A	33.7	Neg	+					
Le5	<i>Saccharomyces cerevisiae</i>	Coffee extract	1.10E+05	N/A	33.9	Neg	+					
ST17	<i>Staphylococcus aureus</i>	Yogurt	2.20E+04	N/A	33.8	Neg	+					

## Exclusivity – 2006

Reference	Strain	Origin	Level in 10mL of non-selective nutrient broth (CFU/mL)	Easy lysis protocol		
				Ct C.int	Ct FAM	Result
L1	<i>Listeria grayi</i>	Frozen fries	1.0E+05	25.75	N/A	-
L140	<i>Listeria grayi</i>	ATCC 19120	1.0E+05	25.71	N/A	-
L142	<i>Listeria innocua</i>	Gorgonzola cheese	1.0E+05	25.87	N/A	-
L146	<i>Listeria innocua</i>	Smoked halibut	1.0E+06	25.23	N/A	-
L147	<i>Listeria innocua</i>	Epoisses cheese	3.0E+05	24.92	N/A	-
L154	<i>Listeria innocua</i>	Epoisses cheese	4.5E+05	25.79	N/A	-
L155	<i>Listeria innocua</i>	Spinach	4.0E+05	25.88	N/A	-
L157	<i>Listeria innocua</i>	Boulettes d'Avesnes cheese	4.0E+05	25.85	N/A	-
L158	<i>Listeria innocua</i>	Cockerel	3.5E+05	25.96	N/A	-
L159	<i>Listeria innocua</i> 6a	Toulouse sausage	4.0E+05	25.44	N/A	-
L173	<i>Listeria innocua</i> 6b	Raw ground beef	4.5E+05	26.39	N/A	-
L175	<i>Listeria innocua</i> 6a	ATCC 33090	1.5E+05	26.26	N/A	-
L180	<i>Listeria ivanovii</i>	Raw ground beef	4.5E+05	25.98	N/A	-
L182	<i>Listeria ivanovii</i>	Environmental sample	1.2E+05	25.71	N/A	-
L3	<i>Listeria ivanovii</i>	Collection	3.7E+05	25.05	N/A	-
L82	<i>Listeria ivanovii</i>	Bird net	1.5E+05	26.55	N/A	-
(C11)	<i>Listeria ivanovii</i>	Environmental residue	1.0E+06	30.27	N/A	-
L85	<i>Listeria seeligeri</i>	Drain water	1.0E+05	24.98	N/A	-
L86	<i>Listeria seeligeri</i>	Raw ground beef	1.4E+05	25.51	N/A	-
L87	<i>Listeria seeligeri</i> 1/2b	Tongue	3.0E+05	25.20	N/A	-
L90	<i>Listeria welshimeri</i>	Spread	1.0E+05	25.52	N/A	-
L91	<i>Listeria welshimeri</i>	Ham	1.0E+05	25.40	N/A	-
BA2	<i>Bacillus cereus</i>	Beets	3.0E+05	25.70	N/A	-
BA4	<i>Bacillus stearothermophilus</i>	Dairy product	3.0E+05	24.72	N/A	-
BA5	<i>Bacillus sphaericus</i>	Meat product	2.0E+05	25.37	N/A	-
BA7	<i>Bacillus coagulans</i>	Collection	5.0E+05	25.49	N/A	-
E1	<i>Enterococcus faecalis</i>	Egg product	3.0E+05	25.97	N/A	-
E2	<i>Enterococcus faecium</i>	Collection ATCC 3286	3.0E+05	26.30	N/A	-
E3	<i>Streptococcus bovis</i>	Collection	2.3E+05	25.24	N/A	-
E6	<i>Enterococcus faecalis</i>	Collection ATCC 19433	4.0E+05	26.40	N/A	-
E7	<i>Enterococcus faecium</i>	Collection CIP 5433	6.7E+05	25.47	N/A	-
33	<i>Lactobacillus casei</i>	Dairy product	2.3E+05	25.63	N/A	-
L139	<i>Jonesia denitrificans</i>	Collection	1.6E+05	25.62	N/A	-
Le1	<i>Rhodotorula rubra</i>	Pastry	2.0E+05	25.66	N/A	-
Le3	<i>Candida albicans</i>	Collection	9.0E+05	25.22	N/A	-
ST3	<i>Staphylococcus epidermidis</i>	Yogurt	4.8E+05	25.35	N/A	-

## APPENDIX E

# INTERLABORATORY STUDY - ACCORDANCE

ALTERNATIVE METHOD

**Level L0**

Laboratory	Expected # of negatives	Actual # of negatives	Probability of negatives	Probability of pairs of negatives	Probability of positives	Probability of pairs of positives	Probability of identical result pairs
Laboratory A	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory B	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory C	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory D	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory E	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory F	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory G	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory H	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory I	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory J	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory K	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory L	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory M	8	7	0.88	0.77	0.13	0.02	0.78
Laboratory N	8	8	1.00	1.00	0.00	0.00	1.00
<b>Average:</b>							<b>0.98</b>
<b>Accordance:</b>							<b>98%</b>

**Level L1**

Laboratory	Expected # of positives	Actual # of positives	Probability of positives	Probability of pairs of positives	Probability of negatives	Probability of pairs of negatives	Probability of identical result pairs
Laboratory A	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory B	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory C	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory D	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory E	8	7	0.88	0.77	0.13	0.02	0.78
Laboratory F	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory G	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory H	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory I	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory J	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory K	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory L	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory M	8	7	0.88	0.77	0.13	0.02	0.78
Laboratory N	8	8	1.00	1.00	0.00	0.00	1.00
<b>Average:</b>							<b>0.97</b>
<b>Accordance:</b>							<b>97%</b>

**Level L2**

Laboratory	Expected # of positives	Actual # of positives	Probability of positives	Probability of pairs of positives	Probability of negatives	Probability of pairs of negatives	Probability of identical result pairs
Laboratory A	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory B	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory C	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory D	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory E	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory F	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory G	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory H	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory I	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory J	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory K	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory L	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory M	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory N	8	8	1.00	1.00	0.00	0.00	1.00
<b>Average:</b>							<b>1.00</b>
<b>Accordance:</b>							<b>100%</b>

REFERENCE METHOD

Level L0

Laboratory	Expected # of negatives	Actual # of negatives	Probability of negatives	Probability of pairs of negatives	Probability of positives	Probability of pairs of positives	Probability of identical result pairs
Laboratory A	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory B	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory C	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory D	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory E	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory F	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory G	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory H	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory I	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory J	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory K	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory L	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory M	8	7	0.88	0.77	0.13	0.02	0.78
Laboratory N	8	8	1.00	1.00	0.00	0.00	1.00
<b>Average:</b>							<b>0.98</b>
<b>Accordance:</b>							<b>98%</b>

Level L1

Laboratory	Expected # of positives	Actual # of positives	Probability of positives	Probability of pairs of positives	Probability of negatives	Probability of pairs of negatives	Probability of identical result pairs
Laboratory A	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory B	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory C	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory D	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory E	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory F	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory G	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory H	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory I	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory J	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory K	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory L	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory M	8	7	0.88	0.77	0.13	0.02	0.78
Laboratory N	8	8	1.00	1.00	0.00	0.00	1.00
<b>Average:</b>							<b>0.98</b>
<b>Accordance:</b>							<b>98%</b>

Level L2

Laboratory	Expected # of positives	Actual # of positives	Probability of positives	Probability of pairs of positives	Probability of negatives	Probability of pairs of negatives	Probability of identical result pairs
Laboratory A	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory B	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory C	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory D	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory E	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory F	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory G	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory H	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory I	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory J	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory K	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory L	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory M	8	8	1.00	1.00	0.00	0.00	1.00
Laboratory N	8	8	1.00	1.00	0.00	0.00	1.00
<b>Average:</b>							<b>1.00</b>
<b>Accordance:</b>							<b>100%</b>

## APPENDIX F

# INTERLABORATORY STUDY - CONCORDANCE

## ALTERNATIVE METHOD

Number of laboratories 14

Number of negatives per laboratory 8

### Level L0

Laboratory	# of expected negatives	# of actual negatives	Interlaboratory pairs with the same result	Total # of interlaboratory pairs
Laboratory A	8	8	824	832
Laboratory B	8	8	824	832
Laboratory C	8	8	824	832
Laboratory D	8	8	824	832
Laboratory E	8	8	824	832
Laboratory F	8	8	824	832
Laboratory G	8	8	824	832
Laboratory H	8	8	824	832
Laboratory I	8	8	824	832
Laboratory J	8	8	824	832
Laboratory K	8	8	824	832
Laboratory L	8	8	824	832
Laboratory M	8	7	728	832
Laboratory N	8	8	824	832
<b>Total</b>			<b>11440</b>	<b>11648</b>
<b>Concordance</b>	98.21%			

Number of laboratories 14

Number of positives per laboratory 8

### Level L1

Laboratory	# of expected positives	# of actual positives	Interlaboratory pairs with the same result	Total # of interlaboratory pairs
Laboratory A	8	8	816	832
Laboratory B	8	8	816	832
Laboratory C	8	8	816	832
Laboratory D	8	8	816	832
Laboratory E	8	7	722	832
Laboratory F	8	8	816	832
Laboratory G	8	8	816	832
Laboratory H	8	8	816	832
Laboratory I	8	8	816	832
Laboratory J	8	8	816	832
Laboratory K	8	8	816	832
Laboratory L	8	8	816	832
Laboratory M	8	7	722	832
Laboratory N	8	8	816	832
<b>Total</b>			<b>11236</b>	<b>11648</b>
<b>Concordance</b>	96.46%			

Number of laboratories 14

Number of positives per laboratory 8

### Level L2

Laboratory	# of expected positives	# of actual positives	Interlaboratory pairs with the same result	Total # of interlaboratory pairs
Laboratory A	8	8	832	832
Laboratory B	8	8	832	832
Laboratory C	8	8	832	832
Laboratory D	8	8	832	832
Laboratory E	8	8	832	832
Laboratory F	8	8	832	832
Laboratory G	8	8	832	832
Laboratory H	8	8	832	832
Laboratory I	8	8	832	832
Laboratory J	8	8	832	832
Laboratory K	8	8	832	832
Laboratory L	8	8	832	832
Laboratory M	8	8	832	832
Laboratory N	8	8	832	832
<b>Total</b>			<b>11648</b>	<b>11648</b>
<b>Concordance</b>	100.00%			

## REFERENCE METHOD

Number of laboratories 14  
 Number of negatives per laboratory 8

### Level L0

Laboratory	# of expected negatives	# of actual negatives	Interlaboratory pairs with the same result	Total # of interlaboratory pairs
Laboratory A	8	8	824	832
Laboratory B	8	8	824	832
Laboratory C	8	8	824	832
Laboratory D	8	8	824	832
Laboratory E	8	8	824	832
Laboratory F	8	8	824	832
Laboratory G	8	8	824	832
Laboratory H	8	8	824	832
Laboratory I	8	8	824	832
Laboratory J	8	8	824	832
Laboratory K	8	8	824	832
Laboratory L	8	8	824	832
Laboratory M	8	7	728	832
Laboratory N	8	8	824	832
<b>Total</b>			<b>11440</b>	<b>11648</b>
<b>Concordance</b>	98.21%			

Number of laboratories 14  
 Number of positives per laboratory 8

### Level L1

Laboratory	# of expected positives	# of actual positives	Interlaboratory pairs with the same result	Total # of interlaboratory pairs
Laboratory A	8	8	824	832
Laboratory B	8	8	824	832
Laboratory C	8	8	824	832
Laboratory D	8	8	824	832
Laboratory E	8	8	824	832
Laboratory F	8	8	824	832
Laboratory G	8	8	824	832
Laboratory H	8	8	824	832
Laboratory I	8	8	824	832
Laboratory J	8	8	824	832
Laboratory K	8	8	824	832
Laboratory L	8	8	824	832
Laboratory M	8	7	728	832
Laboratory N	8	8	824	832
<b>Total</b>			<b>11440</b>	<b>11648</b>
<b>Concordance</b>	98.21%			

Number of laboratories 14  
 Number of positives per laboratory 8

### Level L2

Laboratory	# of expected positives	# of actual positives	Interlaboratory pairs with the same result	Total # of interlaboratory pairs
Laboratory A	8	8	832	832
Laboratory B	8	8	832	832
Laboratory C	8	8	832	832
Laboratory D	8	8	832	832
Laboratory E	8	8	832	832
Laboratory F	8	8	832	832
Laboratory G	8	8	832	832
Laboratory H	8	8	832	832
Laboratory I	8	8	832	832
Laboratory J	8	8	832	832
Laboratory K	8	8	832	832
Laboratory L	8	8	832	832
Laboratory M	8	8	832	832
Laboratory N	8	8	832	832
<b>Total</b>			<b>11648</b>	<b>11648</b>
<b>Concordance</b>	100.00%			