

CONFIDENTIAL

**AFNOR CERTIFICATION
VALIDATION STUDY
HQS PCR CRONOBACTER TEST**

Preliminary study report
Collaborative study report

HQS PCR CRONOBACTER METHOD - S.R.(V0) - DECEMBER 2010

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For the AFNOR Certification validation according to the standard
ISO 16140 about the HQS PCR *Cronobacter* test with
confirmation according to the NF EN ISO 16140 standard

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1. Introduction

1.1. Validation referential

The aim of this validation study is to evaluate the performance of the alternative method against the reference method ISO/TS 22964 (fév. 2006). It consists in a preliminary study and a collaborative study.

1.2. Alternative method

HQS PCR *Cronobacter* test is based on a real-time Polymerase Chain Reaction technology. The test permits the presumptive detection of *Cronobacter* by identification of DNA sequences after enrichment in buffered peptone water. The test uses a SYBER[®] Green technology and the amplified sequence is located between 16S RNA gene and the 23S RNA gene (16S-23S rDNA ITS). The protocol of the method is showed in figure 1.

A Realplex thermocycler from Eppendorf was used during the preliminary study.

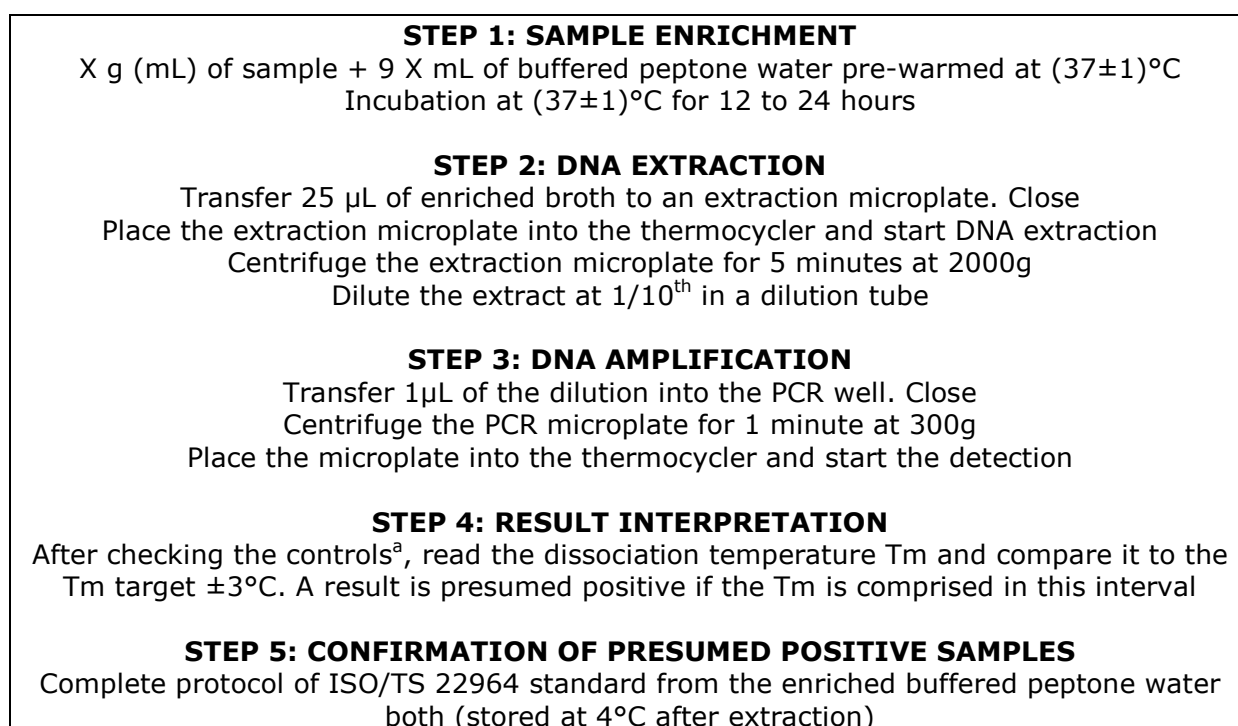


Figure 1: alternative method protocol (a=inhibition control, contamination control and external positive control)

1.3. Scope of application

The alternative method has been tested on one category of products: dairy products and milk powder especially.

1.4. Reference method (*)

The standard ISO/TS 22964 (fév. 2006): milk and dairy products – Detection of *Cronobacter* was applied. The protocol of this method is shown in figure 2.

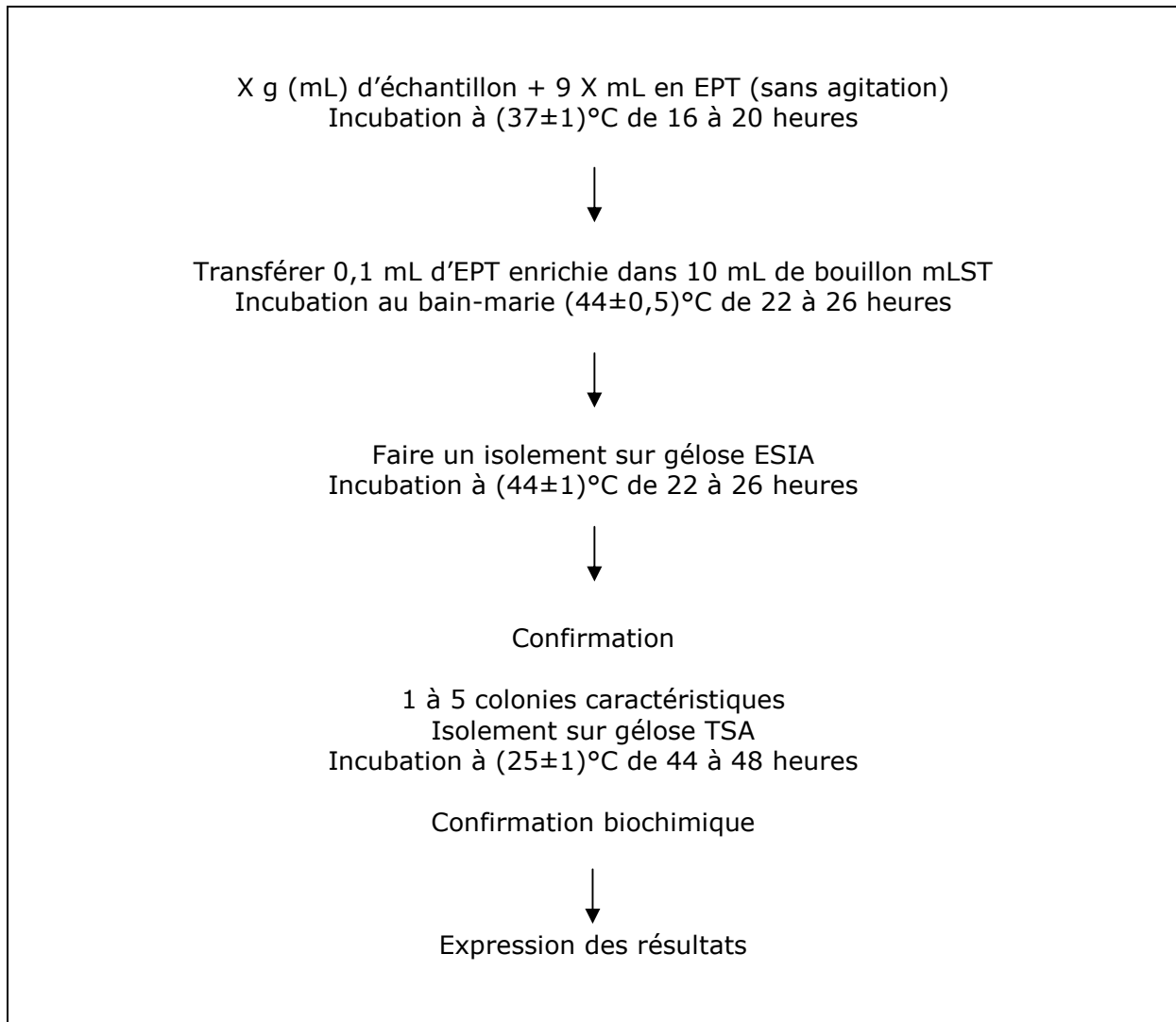


Figure 2: reference method protocol

2. Comparative study

The following characteristics are studied during the preliminary study:

- Relative accuracy (AC), relative specificity (SP) and relative sensitivity (SE)
- Relative detection level of the alternative method and the reference method
- Selectivity of the alternative method
- Practicability of the alternative method

2.1. Relative accuracy, relative specificity, relative sensitivity

The relative accuracy is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples.

The relative specificity is the ability of the alternative method to not detect the target microorganism when it is not detected by the reference method.

The relative sensitivity is the ability of the alternative method to detect the analyte when it is detected by the reference method.

The objective of this study is to evaluate the performance of both methods on contaminated and non-contaminated samples.

2.1.1. Number and nature of samples

Only one category was studied; dairy products with focus on milk powder for baby. A number of 68 samples was analysed. Types of products are indicated in table 1.

Category	Type	Number of positive*	Number of negative	Total
Dairy products	Milk powder	18	18	36
	Milk	5	8	13
	Condensed milk	1	1	2
	Yoghurt	3	4	7
	Cheese	4	6	10
	Total		31	37

Table 1: nature and number of analysed samples (*=positive results by either method)

2.1.2. Artificial contamination of samples

Naturally contaminated samples are seldom available. Therefore, artificial contaminations of food samples were almost performed. For spiking, several strains were stressed using different treatments and the stress intensity was evaluated (logarithmic difference between enumeration on non selective agar –TSA- and selective agar –ESIA).

One naturally contaminated sample was analysed, so 96,8% of positive samples are the results of artificial spiking.

2.1.3. Confirmation protocol

The confirmation of presumed positive results obtained by the alternative method was realized from the enriched buffered water according to the reference method.

2.1.4. Results

Each sample was analysed once by the alternative method and once by the reference method. Table 2 presents paired results of both methods for incubation of 12 hours of AM and table 3 presents paired results of both methods for incubation of 24 hours of AM.

Alternative method with 12 hours of incubation		
Response	Reference method ^(*) positive (R+)	Reference method ^(*) négative (R-)
Alternative method positive (A+)	PA=30	PD=0
Alternative method négative (A-)	ND=1 including PPND=0	NA=37 including PPNA=0

Table 2: results of relative accuracy for both methods (PA: positive agreement, NA: negative agreement, ND: negative deviation, PD: positive deviation, PP: presumed positive before confirmation, A+: confirmed positive, A-: negative immediately and negative after confirmation when presumed positive)

Alternative method with 24 hours of incubation		
Response	Reference method ^(*) positive (R+)	Reference method ^(*) négative (R-)
Alternative method positive (A+)	PA=31	PD=0
Alternative method négative (A-)	ND=0 including PPND=0	NA=37 including PPNA=0

Table 3: results of relative accuracy for both methods (PA: positive agreement, NA: negative agreement, ND: negative deviation, PD: positive deviation, PP: presumed positive before confirmation, A+: confirmed positive, A-: negative immediately and negative after confirmation when presumed positive)

2.1.5. Calculation of relative accuracy (AC), relative specificity (SP) and relative sensitivity (SE)

These results permit to calculate the relative accuracy, relative specificity and relative sensitivity according to NF EN ISO standard. Results are indicated in table 4 and table 5.

Incubation AM: 12 hours									
PA	NA	ND	PD	N	Relative accuracy AC [(PA+NA)/N]	N+ PA+ND	Relative sensitivity SE [PA/N+]	N- NA+PD	Relative specificity SP [NA/N-]
30	37	1	0	68	98,5%	31	96,8%	37	100%

Table 4: relative accuracy, relative specificity and relative sensitivity of alternative method (PA: positive agreement, NA: negative agreement, ND: negative deviation, PD: positive deviation, AC = (PA+NA)/N x 100%, SE = PA/N+ x 100%, SP = NA/N- x 100%, N+ = PA+ND and N- = NA+PD)

Incubation AM: 24 hours									
PA	NA	ND	PD	N	Relative accuracy AC [(PA+NA)/N]	N+ PA+ND	Relative sensitivity SE [PA/N+]	N- NA+PD	Relative specificity SP [NA/N-]
31	37	0	0	68	100%	31	100%	37	100%

Table 5: relative accuracy, relative specificity and relative sensitivity of alternative method (PA: positive agreement, NA: negative agreement, ND: negative deviation, PD: positive deviation, AC = (PA+NA)/N x 100%, SE = PA/N+ x 100%, SP = NA/N- x 100%, N+ = PA+ND and N- = NA+PD)

Criteria values in percent are shown in table 6.

	Alternative method 12 hours	Alternative method 24 hours
Relative accuracy	98,5%	100%
Relative sensitivity	96,8%	100%
Relative specificity	100%	100%

Table 6: AC, SE and SP in percent for alternative method

Sensitivity of both methods was recalculated considering all confirmed positive (including alternative method positive deviations). Results are shown in table 7.

	Alternative method (PA+PD)/(PA+PD+ND) 12 hours	Alternative method (PA+PD)/(PA+PD+ND) 24 hours	Reference method (PA+ND)/(PA+PD+ND)
Sensitivity	96,8%	100%	100%

Table 7: sensitivity of both methods including all confirmed positive

2.1.6. Analysis of discordant results

Discordant results are examined according to annex F of NF EN ISO 16140 standard, with Y as the number of discordant results and m as the smallest of the two values of PD and ND.

For 12 hours of incubation, Y = 1 and for 24 hours incubation, Y = 0. In the both cases Y < 6, so the two methods are equivalent.

The only discordant result observed concerns a sample (RD 2157) who gives a negative result with the alternative method after 12 hours of incubation and a positive result with reference method. After 24 hours of incubation the same sample gives a positive result with alternative method.

2.2. Relative detection level

The objective of this study is to determine the level of contamination for which less than 50% of the responses obtained are positive and that for which more than 50% of the responses obtained are positive.

2.2.1. Matrices

A couple "matrix-strain" was studied in parallel with the reference method and the alternative method for each category. The total viable count of each matrix was enumerated. Characteristics of the strain and the matrix are shown in table 8.

Matrix	Strain	ISHA code	Origin
Baby powder milk	<i>Cronobacter spp</i>	ENTB.3.1	Powder milk

Table 8: "matrix-strain" couples of the relative detection level

2.2.2. Spiking protocol

Six levels of contamination were tested including the negative control.

Six replicates for each level of contamination were inoculated and analysed by the reference method and the alternative method.

As the two methods have no common step, 12 test portions of 25 g were prepared for each level of contamination and individually inoculated with a calibrated bacterial suspension. Bacterial suspension of about 10 cells per mL was prepared. From this initial suspension, volumes of 0.9 mL, 0.3 mL and 0.1 mL were used to spike 30 g of sample respectively for the 3 first levels. In parallel, the initial suspension was diluted ratio 1/2 and 1/4 in order to inoculate the lower levels of contamination with 0.1 mL. For all the levels of contamination, homogeneity of the inoculums was checked by enumeration on

30 TSA Petri dishes. Then, the confidence interval was determined according to Poisson law.

2.2.3. Results

Tables 9 and 10 present the relative detection level for each method.

		Relative detection level according to the Spearman-Kärber model (cells in 30 g)		
Souche	Matrice	Reference method^(*)	Alternative method 12 h	Alternative method 24 h
ENTB.3.	Baby powder milk	0,335 [0,228 ; 0,491]	0,335 [0,228 ; 0,491]	0,335 [0,228 ; 0,491]

Table 9: relative detection level (3 significant numbers)

		Relative detection level according to the Spearman-Kärber model (cells in 30 g)		
Souche	Matrice	Reference method^(*)	Alternative method 12 h	Alternative method 24 h
ENTB.3.	Baby powder milk	0,3 [0,2 ; 0,5]	0,3 [0,2 ; 0,5]	0,3 [0,2 ; 0,5]

Table 10: relative detection level (1 significant number)

The alternative and the reference method show similar detection levels. The detection limit obtained with both methods is comprised between 0.2 and 0.5 CFU in 30 g.

2.3. Inclusivity / exclusivity (selectivity)

The objective of this study is to test:

- the inclusivity: the detection of the target microorganism from a wide range of strains,
- the exclusivity: the lack of interference from a relevant range of non-target microorganisms.

According to the requirements of NF EN ISO 16140, 50 strains of *Cronobacter* and 32 non-target strains were tested. A list of the strains figures in annex 1.

2.3.1. Test protocols

- **Inclusivity**

Each *Cronobacter* strain was cultivated twice before inoculation in BPW (about 1 to 100 CFU/225 mL). The complete protocol of alternative method was applied with the minimum time of incubation.

- **Exclusivity**

Each non-target strain was cultivated twice before inoculation in growth medium (Trypticase Soy Broth) with a level of contamination expected to occur in the food matrices (about 10⁵ CFU/mL). After 24 hours of incubation, the HQS test was performed.

2.3.2. Results

The 50 target strains tested were detected by the alternative method. No non target strain was detected by the alternative method.

2.3.3. Conclusion

The selectivity of the method is satisfactory.

3. Collaborative study

The main object of the collaborative study is to determine the variability of the results obtained by different laboratories analysing identical samples and to compare these results within the framework of the comparative study of the methods.

3.1. Collaborative study implementation

3.1.1. Participating laboratories

The collaborative study was realized by the expert laboratory and twelve participating laboratories.

3.1.2. *Cronobacter* absence in the matrix

Before spiking, the absence of *Cronobacter* was verified in the batch of baby milk used according to the reference method (ISO/TS 22964).

3.1.3. Strain stability in the matrix

The strain stability in baby milk matrix was evaluated for 4 days at $(4\pm 2)^{\circ}\text{C}$. The strain used was *Cronobacter sakazakii* (ISHA code: ENTB.3.47) isolated from dairy product industry.

Inoculation of 10 cells in 30 mL of baby milk. The samples were analysed at D0, D+1, D+2 and D+3 by the reference method and by the alternative method. The results are summarized in table 11.

Day	Alternative method	Reference method
D0	Presence in 30 mL	Presence in 30 mL
D+1	Presence in 30 mL	Presence in 30 mL
D+2	Presence in 30 mL	Presence in 30 mL
D+3	Presence in 30 mL	Presence in 30 mL

Table 11: results of the stability study of the strain ESC.1.93 in minced meat

The results show that the *Cronobacter sakazakii* strain used is stable for 3 days at $(4\pm 2)^{\circ}\text{C}$ in baby milk matrix.

3.1.4. Samples preparation and spiking

The matrix was inoculated with the target strain suspension to obtain 3 contamination levels:

- L0: 0 cell in 30 mL
- L1: 3 cells in 30 mL
- L2: 30 cells in 30 mL

The matrix was distributed at 30 mL in sterile vials. Every vial was individually spiked and homogenized. Eight samples per level, per laboratory and per method were prepared. Each laboratory received 24 samples to analyse, 1 sample to quantify the endogenous microflora and 1 water sample containing a temperature probe.

The results of the enumerations of the TVC, the target levels and the real levels of contamination are presented in table 12.

Matrix	Total viable count (CFU/mL)	Target level (cells / 30 mL)	Real level (cells / 30 mL)	Confidence interval
Baby milk	$2,0 \cdot 10^5$	<10	0	0
			3	5
			30	32

Table 12: target level, real level and TVC of the matrix

3.1.5. Samples labeling

The labelling of the bags was realized as follows: a code to identify the laboratory: from A to L (cf. table 13) and a code to identify each sample, only known by the expert laboratory. The samples and the temperature control vials (water sample with a temperature probe) were stored at 4°C before shipping.

Contamination level	Sample code
L0	2/3/5/7/8/14/19/24
L1	4/6/9/12/15/16/20/22
L2	1/10/11/13/17/18/21/23

Table 13: sample code by contamination level

3.1.6. Samples shipping

The samples were shipped in a coolbox the 16th of November 2009.

3.1.7. Samples reception and analysis

The coolboxes were received the 17th of November 2009 by all the participating laboratories. The control temperature was recorded upon receipt of the package and the temperature probe sent to the expert laboratory. The samples were analysed the same day. The expert laboratory concurrently analysed a set of samples under the same conditions with both methods.

3.2. Results

3.2.1. Temperature and state of the samples

The temperature readings upon reception and the state of the samples are shown in table 14.

Laboratory	Temperature (°C)	State of the samples
A	5,5	Correct
B	4,3	Correct
C	8,2	Correct
D	3,2	Correct
E	5,0	Correct
F	3,6	Correct
G	4,2	Correct
H	8,1	Correct
I	5,6	Correct
J	0,0	Correct
K	4,2	Correct
L	3,7	Correct

Table 14: temperature and state of the samples upon reception

The temperature measurements are inferior to 8.4°C for all the laboratories. The analysis of thermal profiles is shown in table 15.

Laboratory	A	B	C	D	E	F	G	H	I	J	K	L	
Temperature (°C)	Mean	3,7	-0,2	-1,0	2,6	2,4	2,0	2,3	1,4	2,0	3,0	2,4	2,5
	SD	0,6	0,3	1,0	0,4	0,5	0,2	0,5	0,6	0,2	0,6	0,4	0,4

Table 15: data of the temperature probes for the transportation time of samples

3.2.2. Total viable counts

For the whole laboratories, the total viable counts at 30°C vary between < 1 and 50 CFU/mL.

3.2.3. Expert laboratory results

The results obtained by the expert laboratory are summarized in table 16.

Contamination level	Alternative method	Reference method (*)
L0	0/8	0/8
L1	8/8	8/8
L2	8/8	8/8

Table 16: positive results obtained by expert laboratory by both methods

The results are consistent with those expected.

3.2.4. Participating laboratories results

The results are summarized in tables 17 and 18

- Alternative method results

Laboratory	Contamination level		
	L0	L1	L2
A	2/8	8/8	8/8
B	0/8	8/8	8/8
C	0/8	8/8	8/8
D	0/8	7/8	8/8
E	0/8	8/8	8/8
F	0/8	8/8	8/8
G	0/8	8/8	8/8
H	0/8	8/8	8/8
I	0/8	8/8	8/8
J	0/8	8/8	8/8
K	0/8	8/8	8/8
L	0/8	8/8	8/8

Table 17: alternative method positive results for all laboratories

Only the laboratory I reported inhibitions of the PCR reaction for 4 samples (I2, I6, I10 and I16). The protocol which consists in diluting the extract was applied and permitted to "lift" this inhibition.

- Reference method results

Laboratory	Contamination level		
	L0	L1	L2
A	2/8	8/8	8/8
B	0/8	8/8	8/8
C	0/8	8/8	8/8
D	0/8	7/8	8/8
E	0/8	8/8	8/8
F	0/8	8/8	8/8
G	0/8	8/8	8/8
H	0/8	8/8	8/8
I	0/8	8/8	8/8
J	0/8	8/8	8/8
K	0/8	8/8	8/8
L	0/8	8/8	8/8

Table 18: reference method positive results for all laboratories

- Results analysis

Laboratory A gives two positive results at the negative level. A second PCR was performed and the results were identical. The collaborative laboratory confirms a cross contamination of these two samples and the expert laboratory decides to exclude the results of the laboratory A.

3.2.5. Specificity (SP) and sensitivity (SE) calculations

The specificity and sensitivity calculations of both methods are presented in table 19, with the low critical value (LCL). Formulas used are:

For level L0, $SP = [1 - (FP/N_-)] \times 100\%$, N_- : total number of L0 tests
 FP: number of false positive

For levels L1 and L2, $SE = (TP/N_+) \times 100\%$, N_+ : total numbers of L1 or L2 tests
 TP: number of true positive

Specificity / sensitivity	Alternative method	LCL	Reference method	LCL
SP (level L0)	100%	98%	100%	98%
SE (level L1)	99%	98%	99%	98%
SE (level L2)	100%	98%	100%	98%
SE (level L1+L2)	99%	98%	99%	98%

Table 19: specificity (SP), sensitivity (SE) and LCL of alternative and reference method

3.2.6. Relative accuracy calculations

Pairs of results of the different levels of contamination are presented in table 20.

Level	Alternative method	Reference method		
		RM+	RM-	Total
L0	AM+	PA=0	PD=0	0
	AM-	ND=0	NA=88	88
	Total	0	88	88
L1	AM+	PA=87	PD=0	87
	AM-	ND=0	NA=1	1
	Total	87	1	88
L2	AM+	PA=88	PD=0	88
	AM-	ND=0	NA=0	0
	Total	88	0	88
L0+L1+L2	AM+	PA=175	PD=0	175
	AM-	ND=0	NA=89	89
	Total	175	89	264

Table 20: tests results for both methods (PA: positive agreement, NA: negative agreement, ND: negative deviation, PD: positive deviation)

Relative accuracy values of the different contamination levels are presented in table 21 with their LCL. Formula used is the following:

$AC = (PA+NA)/N \times 100\%$, PA: number of positive agreements
 NA: number of negative agreements

Level	Relative accuracy (AC)	LCL (Low Critical Value)
L0	100%	98%
L1	100%	98%
L2	100%	98%
L1+L2	100%	98%
Total	100%	98%

Table 21: relative accuracy values (AC) and LCL of alternative method

3.2.7. Discordant results analysis

No discordant results are observed according to the annex F of ISO 16140 standard. The HQS *Cronobacter* test and the reference method can be considered as equivalent.

3.3. Interpretation

3.3.1. Accordance

The accordance is the percentage chance of finding the same result (i.e. both negative or both positive) from two identical test portions analysed in the same laboratory, under repeatability conditions (i.e. one operator using the same apparatus and same reagents within the shortest feasible time interval).

To derive the accordance from the results of an interlaboratory study, the probability that two samples give the same result is calculated for each participating laboratory in turn, and this probability is then averaged over all laboratories. Values of accordance are shown in table 22.

Level	Alternative method	Reference method
L0	100%	100%
L1	98%	98%
L2	100%	100%

Table 22: accordance by level and method

3.3.2. Concordance

The concordance is the percentage chance of finding the same result for two identical samples analysed in two different laboratories.

To calculate the concordance from the results of an interlaboratory study, take in turn each replicate in each participating laboratory, pair it with identical results of all the other laboratories. The concordance is the percentage of all pairings giving the same results on all the possible pairings of data. Values of concordance are shown in table 23.

Level	Alternative method	Reference method
L0	100%	100%
L1	98%	98%
L2	100%	100%

Table 23: concordance by level and method

3.3.3. Concordance odds ratio

If the concordance is smaller than the accordance, it indicates that two identical samples are more likely to give the same result if they are analysed by the same laboratory than if they are analysed by different ones, suggesting that there can be variability in performance between laboratories. Unfortunately, the magnitude of the concordance and accordance is strongly dependent on the level of accuracy, making it difficult to assess easily the degree of between-laboratory variation.

It is therefore helpful to calculate the concordance odds ratio (COR) defined as follows:

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

Values of COR for both methods are shown in table 24.

A value for the odds ratio of 1.00 would be expected if accordance and concordance were equal, and the larger the odds ratio is, the more inter-laboratory variation is predominant. Nevertheless, values above 1.00 can occur by chance variation, and so a statistical significance test should be used to confirm whether the evidence for extra variation between laboratories is convincing. The "exact test" is the best recommended test for this). The philosophy behind such tests is that the probabilities of occurrence are calculated for all sets of replicate results that could have produced the overall numbers of positives and negatives.

Level	Alternative method			Reference method		
	Accordance	Concordance		Accordance	Concordance	
L0	100	100	1,0	100	100	1,0
L1	98	98	1,0	98	98	1,0
L2	100	100	1,0	100	100	1,0

Table 24: COR values for each method by contamination level

3.3.4. AC, SP, SE comparison

Table 23 summarizes the values obtained for AC, SP and SE parameters for the preliminary study and the interlaboratory study.

Parameter	Preliminary study - 24 hours of incubation	Interlaboratory study
AC	100%	100%
SP	100%	100%
SE	100%	99%

Table 25: AC, SP and SE comparison between preliminary and interlaboratory study

The values obtained during the collaborative study are better than those obtained during the preliminary study, probably because of the greater variety of samples and strains tested during the preliminary study.

4. Practicability

The practicability was evaluated according to the 13 criteria defined by AFNOR Technical Committee.

1- Mode of packaging of test components

Barrettes of 8 micro tubes with extraction solution
Barrettes of 8 micro tubes with PCR solution
Barrettes of 8 micro tubes with control solutions

2- Volume of reagents

Not specified.

3- Storage conditions of components and shelf-life of unopened products (expiration of not opened products)

The HQS detection kits must be stored at – 20 °C.

4- Modalities after first use

Not specified.

5- Equipment and specific local requirements

Equipment

- Real-time PCR thermocycler
- Centrifuge
- Barrettes supports
- Two multi-channel pipettes
- Two single channel pipettes
- PCR encloser
- *Stomacher* (homogenizer)
- Incubators
- Dilutor
- Bunsen burner
- Serological pipette pump
- *Stomacher* bag holder
- Refrigerator 4°C (2 to 8°C)
- Colour printer
- Pipettes supports
- Tubes racks

6- Reagents ready to use or for reconstitution

Ready to use solutions.

7- Training period for operator with no experience with the method

4 day is required for technicians with microbiology knowledge.

8- Handling time and flexibility of the method in relation to the number of samples

Steps- Manipulation time	Time (minutes)			
	Alternative method		Reference method	
	1 analysis	20 analyses	1 analysis	20 analyses
Suspension	3	23	3	23
Sampling	1	15	/	/
Extraction	6	26	/	/
Amplification	14	38	/	/
PCR reading	2	5,5	/	/
Broth inoculation and streaking	3	36	6,5	36
Reading	0.7	2	0.2	2
Confirmation test	8	62	4	62
Total	38,7	207,5	13,7	121

9- Time required for results

Steps -Time for negative results	Alternative method	Reference method
Suspension	D0	D0
Sampling	D1	D0
Extraction	D1	/
Amplification	D1	/
PCR reading	D1	/
Broth inoculation and streaking	/	D2
Reading	/	D3

Steps -Time for positive results	Alternative method	Reference method
Suspension	D0	D0
Sampling	D1	D0
Extraction	D1	/
Amplification	D1	/
PCR reading	D1	/
Broth inoculation and streaking	D1	D1
Reading	D2	D2
Confirmation	D5	D5

10- Operator qualification

Identical as necessary for the reference method

11- Steps common with the reference method

None.

12- Traceability of analysis results

Traceability realized by paper sheet

13- Maintenance by laboratory

None.

5. Conclusion

Concerning the preliminary study, the performances of the HQS *Cronobacter* test for the detection of *Cronobacter* are comparable to those of the ISO/TS 22964. This study concerned 68 samples of five categories of products (dairy products).

Values obtained for the 3 criteria are the following:

	Alternative method 12 hours	Alternative method 24 hours
Relative accuracy	98,5 %	100,0 %
Relative sensitivity	96,8 %	100,0 %
Relative specificity	100,0 %	100,0 %

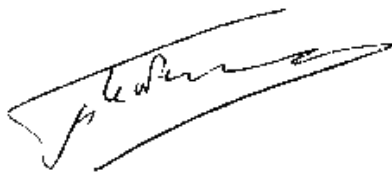
One discordant result was observed after 12 hours of incubation. After 24 hours of incubation no discordant was observed.

The relative level of detection of the alternative method and the reference method was evaluated. The detection limit obtained with the both methods is comprised between 0.2 and 0.5 CFU in 30 g of product.

The specificity of the method is satisfactory.

Concerning the interlaboratory study, the results obtained for the 12 selected laboratories showed that the values of relative accuracy, relative sensitivity and relative specificity are comparable to those obtained during the preliminary study. The variability of the alternative method, demonstrated by the calculations of accordance, concordance and concordance odds ratio, is similar to that of the reference method.

The study of the practicability of the alternative method shows a time-savings method compared to the reference method.



Massy, the 22th of December 2010
François Le Nestour
Research engineer

ANNEX 1: SELECTIVITY

Inclusivity

	Code	Microorganism	Origin
1	ENTB.3.1	<i>Cronobacter sakazakii subsp sakazakii</i>	Poudre de lait
2	ENTB.3.2	<i>Cronobacter sakazakii</i>	CIP 57.33
3	ENTB.3.3	<i>Cronobacter sakazakii</i>	Poudre de lait
4	ENTB.3.4	<i>Cronobacter sakazakii subsp sakazakii</i>	Institut Pasteur de Lille
5	ENTB.3.5	<i>Cronobacter sakazakii subsp sakazakii</i>	Institut Pasteur de Lille
6	ENTB.3.6	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement
7	ENTB.3.7	<i>Cronobacter sakazakii subsp sakazakii</i>	Eau de rivière
8	ENTB.3.8	<i>Cronobacter sakazakii</i>	Lait infantile en poudre
9	ENTB.3.10	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
10	ENTB.3.11	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
11	ENTB.3.12	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
12	ENTB.3.13	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
13	ENTB.3.14	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
14	ENTB.3.15	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
15	ENTB.3.16	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
16	ENTB.3.17	<i>Cronobacter sakazakii subsp malonaticus</i>	Environnement industriel
17	ENTB.3.19	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
18	ENTB.3.20	<i>Cronobacter sakazakii subsp malonaticus</i>	Environnement industriel
19	ENTB.3.21	<i>Cronobacter muytjensii</i>	CIP 103581
20	ENTB.3.22	<i>Cronobacter sakazakii</i>	CIP 103183
21	ENTB.3.23	<i>Cronobacter sakazakii</i>	CIP 104951
22	ENTB.3.24	<i>Cronobacter sakazakii</i>	CIP 104952
23	ENTB.3.25	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
24	ENTB.3.26	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
25	ENTB.3.27	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
26	ENTB.3.28	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
27	ENTB.3.29	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
28	ENTB.3.30	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
29	ENTB.3.31	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
30	ENTB.3.32	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
31	ENTB.3.33	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
32	ENTB.3.34	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
33	ENTB.3.35	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
34	ENTB.3.36	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
35	ENTB.3.37	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
36	ENTB.3.38	<i>Cronobacter sakazakii subsp malonaticus</i>	Environnement industriel
37	ENTB.3.39	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
38	ENTB.3.40	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
39	ENTB.3.41	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
40	ENTB.3.42	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
41	ENTB.3.43	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
42	ENTB.3.44	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
43	ENTB.3.45	<i>Cronobacter sakazakii subsp malonaticus</i>	Environnement industriel
44	ENTB.3.46	<i>Cronobacter sakazakii subsp malonaticus</i>	Environnement industriel
45	ENTB.3.47	<i>Cronobacter sakazakii subsp malonaticus</i>	Environnement industriel
46	ENTB.3.48	<i>Cronobacter sakazakii subsp sakazakii</i>	Environnement industriel
47	ENTB.3.49	<i>Cronobacter sakazakii</i>	Industrie laitière
48	ENTB.3.50	<i>Cronobacter sakazakii</i>	Industrie laitière
49	ENTB.3.51	<i>Cronobacter sakazakii</i>	Industrie laitière
50	ENTB.3.52	<i>Cronobacter sakazakii</i>	Industrie laitière

Exclusivity

	Code	Microorganism	Origin
1	ENTB.1.1	<i>Enterobacter aerogenes</i>	industrie laitière
2	ENTB.1.2	<i>Enterobacter aerogenes</i>	CIP 60.86 T
3	ENTB.2.1	<i>Enterobacter cloacae</i>	Eaux usagées
4	ENTB.2.2	<i>Enterobacter cloacae</i>	Eau de puits
5	ENTC.2.1	<i>Enterococcus faecium</i>	industrie laitière
6	ESC.1.3	<i>Escherichia coli</i>	industrie laitière
7	ESC.1.5	<i>Escherichia coli</i>	Camembert (isol. TBX)
8	HAF.1.1	<i>Hafnia alvei</i>	taboulé
9	HAN.1.1	<i>Hansenula anomala</i>	industrie laitière
10	LACB.1.1	<i>Lactobacillus casei</i>	produit laitier
11	SAL.1.121	<i>Salmonella enterica Salamae</i>	Lait cru
12	STA.2.1	<i>Staphylococcus epidermidis</i>	produit laitier
13	PSE.1.2	<i>Pseudomonas aeruginosa</i>	omelette gruyère
14	PSE.2.2	<i>Pseudomonas fluorescens</i>	CIP102127
15	SAL.1.98	<i>Salmonella enterica Newport</i>	Fromage au lait cru
16	SAL.1.163	<i>Salmonella enterica Infantis</i>	Lait (alim. humaine)
17	SER.1.1	<i>Serratia ficaria</i>	CIP 79.23
18	SER.2.1	<i>Serratia fonticola</i>	CIP 103580
19	SHI.1.1	<i>Shigella flexneri</i>	CIP 82.48T
20	SHI.2.1	<i>Shigella sonnei</i>	ATCC 9290
21	ESC.2.1	<i>Escherichia hermanii</i>	CIP 103176
22	CIT.1.1	<i>Citrobacter freundii</i>	CIP 53.62
23	KLE.2.1	<i>Klebsiella pneumoniae</i>	pâtisserie
24	KLE.1.1	<i>Klebsiella oxytoca</i>	salade soja
25	SAL.1.6	<i>Salmonella enterica Arizonae</i>	Saucisson sec
26	CIT.2.1	<i>Citrobacter koseri</i>	CIP 72.11
27	ENTB.4.1	<i>Enterobacter hormaechei</i>	CIP 104956
28	ENTB.5.1	<i>Enterobacter amnigenus</i>	CIP 104982
29	ENTB.6.1	<i>Enterobacter gergoviae</i>	CIP 76.1
30	ENTB.7.1	<i>Enterobacter nimipressuralis</i>	CIP 105047
31	ENTB.8.1	<i>Enterobacter pyrinus</i>	CIP 104019
32	ENTB.2.3	<i>Enterobacter cloacae</i>	CIP 106422