# AFNOR CERTIFICATION VALIDATION STUDY
# HQS PCR ESCHERICHIA COLI O157:H7
# TEST SYSTEM

## SYNTHESIS REPORT

HQS PCR ESCHERICHIA COLI O157:H7 TEST SYSTEM - S.R.(V0)
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For the AFNOR Certification validation of the HQS PCR *Escherichia coli* O157:H7 test kit with confirmation according to the NF EN ISO 16140 standard

# SUMMARY

# 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 according to the ISO 16140 referiantial. It consists in a preliminary study and a collaborative study.

## 1.2. Alternative method

The principle of the HQS PCR *E. coli* O157:H7 test system lies on real-time Polymerase Chain Reaction (PCR) technology. The system provides rapid detection of *Escherichia coli* O157:H7 by specifically identifying the DNA sequence after an enrichment time in PCRone® broth. The SYBR® Green system is used with this method and the sequence amplified is located between the genes coding for the RNA 16S and RNA 23S (16S-23S rDNA ITS).

The protocol of the method is showed in figure 1.

**STEP 1: ENRICHMENT**
X g (mL) sample + 9X mL PCRone ® broth pre-warmed at 37°C
Incubate at (37±1)°C for 12 to 24 hours

**STEP 2 : DNA EXTRACTION**
Thaw the extarction barrettes and centrifuge them for 30 s at 300 g
Transfer 100 µL of enriched broth in an extraction tube of a microplate
Close the microplate, place it in a thermocycler and begin the extraction step

**STEP 3: DNA PURIFICATION**
Purify the DNA by transferring it in a purification column and by applying two washings

**STEP 4 : DNA AMPLIFICATION**
Transfer 3 µL of extract in the amplification pins
Close and spin-dry pins during 1 minute in 300 g
Deposit the extraction microplate in the thermocycleur and launch the detection.

**STEP 5 : READING AND INTERPRETATION OF THE RESULTS**
Check the different controls, read the dissociation temperature Tm and compare it at the Tm interval
If the temperature is in the Tm interval the result is presumed positive

**STEP 6 : CONFIRMATION**
The confirmation consists in an isolation of enriched PCRone® broth according to the reference method

**Figure 1**: alternative method protocol

## 1.3. Scope of application

The alternative method was tested for two food categories: meat products and dairy products.

## 1.4. Reference method(*)

The standard NF EN ISO 16654 (2001), horizontal method for the detection of *E. coli* O157:H7 was applied. The protocol of this method is shown in figure 2.

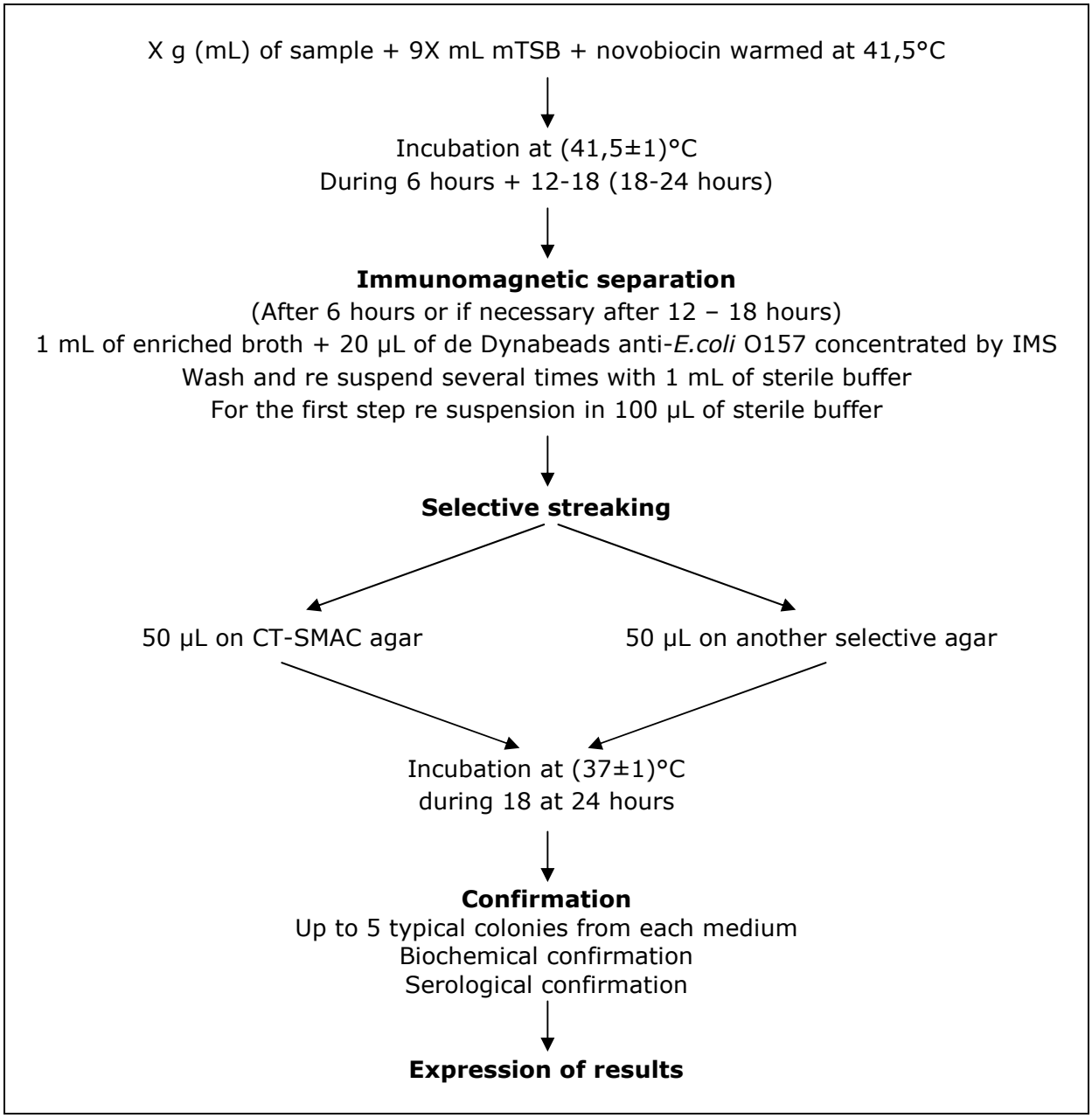



**Figure 2**: reference method protocol

# 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

The following categories are studied: meat products and dairy products.

A number of 121 samples was analysed. Types of products are indicated in table 1.

| Category | Type | Number of positive* | Number of negative | Total |
|---|---|---|---|---|
| **Meat products** | Raw meat | 15 | 15 | 30 |
| | Cooked meat | 4 | 0 | 4 |
| | Delicatessen | 4 | 9 | 13 |
| | Meat dishes | 8 | 6 | 14 |
| | **Total** | **31** | **30** | **61** |
| **Dairy products** | Raw milk cheese | 15 | 6 | 21 |
| | Pasteurized milk cheese | 7 | 10 | 17 |
| | Yoghurts and puddings | 2 | 9 | 11 |
| | Milks and creams | 6 | 5 | 11 |
| | **Total** | **30** | **30** | **60** |
| **Total** | | **61** | **60** | **121** |

**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 –CT-SMAC).

3 naturally contaminated samples were analysed, so 95,1% of positive samples are the results of artificial spikings.

### 2.1.3. Confirmation protocol

The confirmation of presumed positive results obtained by the alternative method was realized from the enriched PCRone® broth according to the reference method after 24 hours of incubation

### 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.

**Incubation AM broth: 12 hours**

| Category | Response | Reference method(*) positive (R+) | Reference method (*) négative (R-) |
|---|---|---|---|
| **Meat products** | **Alternative method positive (A+)** | PA=30 | PD=0 |
| | **Alternative method négative (A-)** | ND=1 including 0 PPND | NA=30 including 0 PPNA |
| **Dairy products** | **Alternative method positive (A+)** | PA=27 | PD=1 |
| | **Alternative method négative (A-)** | ND=2 including 0 PPND | NA=30 including 0 PPNA |
| **All products** | **Alternative method positive (A+)** | PA=57 | PD=1 |
| | **Alternative method négative (A-)** | ND=3 including 0 PPND | NA=60 Including 0 PPNA |

**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)

**Incubation AM broth: 24 hours**

| Category | Response | Reference method(*) positive (R+) | Reference method (*) négative (R-) |
|---|---|---|---|
| **Meat products** | **Alternative method positive (A+)** | PA=30 | PD=0 |
| | **Alternative method négative (A-)** | ND=1 including 0 PPND | NA=30 including 0 PPNA |
| **Dairy products** | **Alternative method positive (A+)** | PA=29 | PD=1 |
| | **Alternative method négative (A-)** | ND=0 including 0 PPND | NA=30 including 0 PPNA |
| **All products** | **Alternative method positive (A+)** | PA=59 | PD=1 |
| | **Alternative method négative (A-)** | ND=1 including 0 PPND | NA=60 Including 0 PPNA |

**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)

For all products categories, 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 broth: 12 hours**

| **Category** | **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-] |
|---|---|---|---|---|---|---|---|---|---|---|
| Meat products | 30 | 30 | 1 | 0 | 61 | 98,4 % | 31 | 96,8 % | 30 | 100,0 % |
| Dairy products | 27 | 30 | 2 | 1 | 60 | 95,0 % | 29 | 93,1 % | 31 | 96,8 % |
| All products | 57 | 60 | 3 | 1 | 121 | 96,7 % | 60 | 95,0 % | 61 | 98,4 % |

**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 broth: 24 hours**

| **Category** | **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-] |
|---|---|---|---|---|---|---|---|---|---|---|
| Meat products | 30 | 30 | 1 | 0 | 61 | 98,4 % | 31 | 96,8 % | 30 | 100,0 % |
| Dairy products | 29 | 30 | 0 | 1 | 60 | 98,3 % | 29 | 100,0 % | 31 | 96,8 % |
| All products | 59 | 60 | 1 | 1 | 121 | 98,3 % | 60 | 98,3 % | 61 | 98,4 % |

**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 4.

| | **Alternative method 12 hours** | **Alternative method 24 hours** |
|---|---|---|
| **Relative accuracy** | 96,7 % | 98,3 % |
| **Relative sensitivity** | 95,0 % | 98,3 % |
| **Relative specificity** | 98,4 % | 98,4 % |

**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 5.

| | **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** | 95,1 % | 98,4 % | 98,4 % |

**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 = 4 and for 24 hours incubation, Y = 2. In both cases Y< 6, so the two methods are equivalent.

- Negative deviations

-Sample numbers: RD 2673
A positive result is obtained by the reference method whereas a negative result is obtained by the alternative method from the 2 incubation times. However the isolation of the PCRone® broth on selective agar medium didn't allow finding typical colonies. This result is due to the difference of sampling between both methods, no cell of *E. coli* O157 may have been taken in the sampling for the reference method.

-Sample numbers RD 2717 / RD 2732
A positive result is obtained by the reference method whereas a negative result is obtained by the alternative method from 12 hours of incubation. However the 24 hours of incubation gives positive results.

Positive deviations
-Sample numbers: RD 2740
A positive result is obtained by the alternative medium whereas a negative result is obtained by the reference method. Due to the difference of sampling between both methods, no cell of *E. coli* O157 may have been taken in the sampling for the reference 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 |
|---|---|---|---|
| Minced meat | *E. coli* O157:H7 | ESC 1.109 | Ham |
| Raw milk | *E. coli* O157:H7 | ESC 1.93 | CIP 105917 |

**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 25 g of sample respectively for the 3 first levels. In parallel, the initial suspension was diluted ratio ½ and ¼ 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 25 g) | | |
|---|---|---|---|---|
| **Souche** | **Matrice** | **Reference method(*)** | **Alternative method 12 h** | **Alternative method 24 h** |
| ESC 1.109 | Minced meat | 0,899 [0,530 ; 1,525] | 1,153 [0,680 ; 1,956] | 1,011 [0,596 ; 1,715] |
| ESC 1.93 | Raw milk | 0,814 [0,554 ; 1,196] | 1,255 [0,776 ; 2,030] | 1,011 [0,625 ; 1,634] |

**Table 9**: relative detection level (3 significant numbers)

| | | Relative detection level according to the Spearman-Kärber model(cells in 25 g) | | |
|---|---|---|---|---|
| **Souche** | **Matrice** | **Reference method(*)** | **Alternative method 12 h** | **Alternative method 24 h** |
| ESC 1.109 | Minced meat | 0,9 [0,5 ; 1,5] | 1,2 [0,7 ; 2,0] | 1,0 [0,6 ; 1,7] |
| ESC 1.93 | Raw milk | 0,8 [0,6 ; 1,2] | 1,3 [0,8 ; 2,0] | 1,0 [0,6 ; 1,6] |

**Table 10**: relative detection level (1 significant number)

The alternative and the reference method show similar detection levels. The detection limit obtained with the alternative method is comprised between 0.7 and 2.0 CFU in 25 g.at 12 hours and is comprised between 0.6 and 1.7 CFU in 25 g. at 24 hours The detection limit obtained with the reference method is comprised between 0.5 and 1.5 CFU in 25 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 *E. coli* O 157:H7 and 33 non-target strains were tested. A list of the strains figures in annex 1.

### 2.3.1. Test protocols

- **Inclusivity**

Each *E. coli* O157:H7 strain was cultivated twice before inoculation in PCRone broth (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 (Tryticase Soy Broth) with a level of contamination expected to occur in the food matrices (about $10^5$ CFU/mL). After 24 hours of incubation, the HQS test was performed.
In cases where the target strains or non-target strains results were unexpected to interpret by the alternative method, the analysis was conducted once again in parallel with the alternative method and the reference method (complete protocol).

### 2.3.2. Results

The 50 *E. coli* O157:H7 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. *E. coli* O157 :H7 absence in the matrix

Before spiking, the absence of *E. coli* O157:H7 was verified in the batch of minced meat used according to the reference method.

### 3.1.3. Strain stability in the matrix

The strain stability in minced meat matrix was evaluated for 4 days at (4±2)°C. The strain used was *E. coli* O157:H7 (ISHA code: ESC.1.93) CIP strain number 105917.

Inoculation of 10 cells in 25 g minced meat. 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 25 g | Presence in 25 g |
| D+1 | Presence in 25 g | Presence in 25 g |
| D+2 | Presence in 25 g | Presence in 25 g |
| D+3 | Presence in 25 g | Presence in 25 g |

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

The results show that the *E. coli* O157: H7 strain used is stable for 3 days at (4±2)°C in the minced meat 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 25 g
-L1: 3 cells in 25 g
-L2: 30 cells in 25 g

The matrix was distributed at 25 g in sterile bags. Every bag was individually spiked and homogenized. Eight samples per level, per laboratory and per method were prepared. Each laboratory received 48 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 / 25 g) | Real level (cells / 25 g) | Confidence interval |
|---|---|---|---|---|
| Minced meat | $2{,}0.10^5$ | 0 | 0 | 0 |
| | | 3 | 4 | [ 0 ; 8 ] |
| | | 30 | 37 | [ 25 ; 49 ] |

**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 | 1/2/7/8/11/12/14/24 |
| L1 | 4/6/10/13/15/17/19/21 |
| L2 | 3/5/9/16/18/20/22/23 |

**Table 13**: sample code by contamination level

### 3.1.6. Samples shipping

The samples were shipped in a coolbox the 7$^{th}$ of December 2009.

### 3.1.7. Samples reception and analysis

The coolboxes were received the 8$^{th}$ of December 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 | 6,9 | Correct |
| B | 3,1 | Correct |
| C | 4,3 | Correct |
| D | 6,0 | Correct |
| E | 4,1 | Correct |
| F | 3,6 | Correct |
| G | 6,5 | Correct |
| H | 6,6 | Correct |
| I | 3,6 | Correct |
| J | 4,2 | Correct |
| K | 2,3 | Correct |
| L | 6,7 | 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 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tempera-ture (°C) | Mean | 1,8 | 2,4 | 2,1 | 2,5 | 1,7 | 2,6 | 1,9 | 2,0 | 1,9 | 3,0 | 1,7 | 2,1 |
| | SD | 0,2 | 0,9 | 2,1 | 0,7 | 0,3 | 0,3 | 0,4 | 0,4 | 1,6 | 0,4 | 0,3 | 0,8 |

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

The thermal profiles analysis indicates for all laboratories mean temperatures comprises between 1.7 and 3.0°C.

### 3.2.2. Total viable counts

For the whole laboratories, the total viable counts at 30°C vary between $2,5.10^7$ and $>10^5$ 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 17and 18

- Alternative method results

| | Contamination level | | |
|---|---|---|---|
| **Laboratory** | **L0** | **L1** | **L2** |
| **A** | 0/8 | 8/8 | 8/8 |
| **B** | 0/8 | 8/8 | 8/8 |
| **C** | 0/8 | 8/8 | 8/8 |
| **D** | 0/8 | 8/8 | 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 | 7/8 | 8/8 |
| **I** | 0/8 | 5/8 | 8/8 |
| **J** | 0/8 | 6/8 | 8/8 |
| **K** | 0/8 | 7/8 | 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

| | Contamination level | | |
|---|---|---|---|
| **Laboratory** | **L0** | **L1** | **L2** |
| **A** | 0/8 | 8/8 | 8/8 |
| **B** | 0/8 | 8/8 | 8/8 |
| **C** | 0/8 | 8/8 | 8/8 |
| **D** | 0/8 | 8/8 | 8/8 |
| **E** | 0/8 | 7/8 | 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 | 7/8 | 8/8 |

**Table 18**: reference method positive results for all laboratories

- Results analysis

The results are consistent with those expected for the level L0 and the level L2.
All discordant results are found at the L1 level, the contamination of this level is comprise between 0 and 8 CFU/g. No typical colonies are observed after streaking enriched PCRone broth on CT-SMAC agar. This result is due to the difference of sampling between both methods, no cell of *E. coli* O157 may have been taken in the sampling for the methods regarding the low level of contamination.

### 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-)] x 100%, N-: total number of L0 tests
FP: number of false positive

For levels L1 and L2, SE = (TP/N+) x 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)** | 93% | 88% | 98% | 96% |
| **SE (level L2)** | 100% | 98% | 100% | 98% |
| **SE (level L1+L2)** | 96% | 93% | 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=96 | 96 |
| | **Total** | 0 | 96 | 96 |
| **L1** | **AM+** | PA=87 | PD=2 | 89 |
| | **AM-** | ND=7 | NA=0 | 7 |
| | **Total** | 94 | 2 | 96 |
| **L2** | **AM+** | PA=96 | PD=0 | 96 |
| | **AM-** | ND=0 | NA=0 | 0 |
| | **Total** | 96 | 0 | 96 |
| **L0+L1+L2** | **AM+** | PA=183 | PD=2 | 185 |
| | **AM-** | ND=7 | NA=96 | 103 |
| | **Total** | 190 | 98 | 288 |

**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 19 with their LCL. Formula used is the following:

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

| Level | Relative accuracy (AC) | LCL (Low Critical Value) |
|---|---|---|
| **L0** | 100% | 98% |
| **L1** | 91% | 84% |
| **L2** | 100% | 98% |
| **L1+L2** | 95% | 93% |
| **Total** | 97% | 93% |

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

#### 3.2.7. Discordant results analysis

Discordant results are analysed according to the annex F of ISO 16140 standard. The total number of discordant results is given by the following formula: Y = PD + ND.
In the present case, Y = 7 + 2 = 9, the binomial law test was used to compare the two methods:

| | Alternative method |
|---|---|
| **Y = PD + ND** | Y = 7 + 2 = 9 |
| **m** | 2 |
| **M (for 9≤Y≤11)** | 1 |
| **Conclusion** | m > M the two methods are not different for α = 0,05 |

The HQS *E. coli* O157:H7 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** | 89% | 96% |
| **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** | 86% | 96% |
| **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:
COR = accordance x (100-concordance)/concordance x (100 – 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** | 89 | 86 | 1,3 | 96 | 96 | 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** | 98,3% | 97% |
| **SP** | 98,4% | 100% |
| **SE** | 98,3% | 96% |

**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.

The sensitivity of both methods is recalculated in table 26 by including all confirmed positive results.

| Alternative method (PA+PD)/(PA+PD+ND) | Reference method (PA+ND)/(PA+PD+ND) |
|---|---|
| 96% | 99% |

**Table 26**: sensitivity recalculated by both methods

# 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
None informed

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
None informed

5- Equipment and specific local requirements

Equipment

- Real-time PCR thermocycler
- Centrifuge
- Capping tool
- 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 | 0.5 | 15 | / | / |
| IMS | / | / | 2 | 120 |
| Extraction | 4.5 | 46 | / | / |
| Amplification | 22 | 105 | / | / |
| PCR reading | 2 | 5,5 | / | / |
| IMS | 20 | 120 | 20 | 120 |
| Reading | 0.2 | 2 | 0.2 | 2 |
| Confirmation test | 8 | 70 | 8 | 70 |
| **Total** | **59,7** | **371,5** | **51,2** | **335** |

9- Time required for results

| Steps –Time for negative results | Alternative method | Reference method |
|---|---|---|
| Suspension | D0 | D0 |
| Sampling | D1 | D0 |
| IMS | / | D0 |
| Extraction | D1 | / |
| Amplification | D1 | / |
| PCR reading | D1 | / |
| IMS | / | D1 |
| Reading | / | D2/D3 |

| Steps –Time for positive results | Alternative method | Reference method |
|---|---|---|
| Suspension | D0 | D0 |
| Sampling | D1 | D0 |
| IMS | / | D0 |
| Extraction | D1 | / |
| Amplification | D1 | / |
| PCR reading | D1 | / |
| IMS | D1 | D1 |
| Reading | D2 | D2/D3 |
| Confirmation | D4 | D4/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 *E. coli* O157: H7 test for the detection of *E. coli* O157: H7 are comparable to those of the standard NF EN ISO 16654 (2001).
This study concerned 121 samples of five categories of products (meat, dairy products).

Values obtained for the 3 criteria are the following:

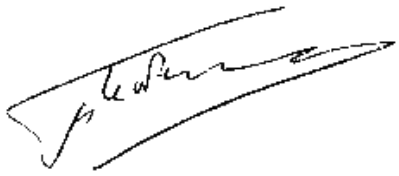
| | **Alternative method 12 hours** | **Alternative method 24 hours** |
|---|---|---|
| **Relative accuracy** | 96,7 % | 98,3 % |
| **Relative sensitivity** | 95,0 % | 98,3 % |
| **Relative specificity** | 98,4 % | 98,4 % |

Several discordant results were observed. Mostly of them may be explained because the first culture step of each method differs. Consequently, 2 replicates were prepared for each sample. Because of the low level of artificial contamination, is it possible that no cell of *E. coli* O157:H7 was present in the test portion replicate analysed with either method.

The relative level of detection of the alternative method and the reference method was evaluated for two product categories. The detection limit obtained with the alternative method is comprised between 0.7 and 2.0 CFU in 25 g. at 12 hours and is comprised between 0.6 and 1.7 CFU in 25 g. at 24 hours  The detection limit obtained with the reference method is comprised between 0.5 and 1.5 CFU in 25 g.
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 simple and easy-to-use method and a time savings compared to the reference method.



Massy, the 17th of December 2010
François Le Nestour
*Research engineer*

# Annex 1 : selectivity

## Inclusivity list

| | Code | Microorganism | Origin |
|---|---|---|---|
| 1 | ESC.1.70 | *Escherichia coli* O157:H7 | Fécès |
| 2 | ESC.1.71 | *Escherichia coli* O157:H7 | Clinique |
| 3 | ESC.1.72 | *Escherichia coli* O157:H7 | Clinique |
| 4 | ESC.1.73 | *Escherichia coli* O157:H7 | Clinique |
| 5 | ESC.1.74 | *Escherichia coli* O157:H7 | Environnement |
| 6 | ESC.1.75 | *Escherichia coli* O157:H7 | Clinique |
| 7 | ESC.1.76 | *Escherichia coli* O157:H7 | Clinique |
| 8 | ESC.1.77 | *Escherichia coli* O157:H7 | Clinique |
| 9 | ESC.1.78 | *Escherichia coli* O157:H7 | Clinique |
| 10 | ESC.1.81 | *Escherichia coli* O157:H7 | ATCC 700728 |
| 11 | ESC.1.82 | *Escherichia coli* O157:H7 | NCTC 12900 |
| 12 | ESC.1.89 | *Escherichia coli* O157:H7 | CIP 105214 |
| 13 | ESC.1.90 | *Escherichia coli* O157:H7 | CIP 105243 |
| 14 | ESC.1.91 | *Escherichia coli* O157:H7 | CIP 105190 |
| 15 | ESC.1.92 | *Escherichia coli* O157:H7 | CIP 106327 |
| 16 | ESC.1.93 | *Escherichia coli* O157:H7 | CIP 105917 |
| 17 | ESC.1.94 | *Escherichia coli* O157:H7 | USDA C7927 |
| 18 | ESC.1.95 | *Escherichia coli* O157:H7 | USDA 45753-32 |
| 19 | ESC.1.96 | *Escherichia coli* O157:H7 | R&F 224 (porc) |
| 20 | ESC.1.97 | *Escherichia coli* O157:H7 | USDA 505b (bœuf) |
| 21 | ESC.1.98 | *Escherichia coli* O157:H7 | R&F 219 (cidre) |
| 22 | ESC.1.99 | *Escherichia coli* O157:H7 | ATCC 51657 (fécès humains) |
| 23 | ESC.1.100 | *Escherichia coli* O157:H7 | ATCC 35150 (fécès humains) |
| 24 | ESC.1.101 | *Escherichia coli* O157:H7 | Abattoir bovin |
| 25 | ESC.1.102 | *Escherichia coli* O157:H7 | Fécès humains |
| 26 | ESC.1.103 | *Escherichia coli* O157:H7 | Fécès humains |
| 27 | ESC.1.104 | *Escherichia coli* O157:H7 | Steack |
| 28 | ESC.1.105 | *Escherichia coli* O157:H7 | Environnement |
| 29 | ESC.1.106 | *Escherichia coli* O157:H7 | Fécès bovins |
| 30 | ESC.1.107 | *Escherichia coli* O157:H7 | Environnement |
| 31 | ESC.1.108 | *Escherichia coli* O157:H7 | Viande de bœuf |
| 32 | ESC.1.109 | *Escherichia coli* O157:H7 | Viande de bœuf |
| 33 | ESC.1.110 | *Escherichia coli* O157:H7 | Viande de bœuf |
| 34 | ESC.1.51 | *Escherichia coli* O157:H7 | CIP 105917 |
| 35 | ESC.1.52 | *Escherichia coli* O157:H7 | Souche clinique |
| 36 | ESC.1.53 | *Escherichia coli* O157:H7 | Souche clinique |
| 37 | ESC.1.54 | *Escherichia coli* O157:H7 | Souche clinique |
| 38 | ESC.1.55 | *Escherichia coli* O157:H7 | Souche clinique |
| 39 | ESC.1.56 | *Escherichia coli* O157:H7 | Souche clinique |
| 40 | ESC.1.57 | *Escherichia coli* O157:H7 | Souche clinique |
| 41 | ESC.1.58 | *Escherichia coli* O157:H7 | Souche clinique |
| 42 | ESC.1.59 | *Escherichia coli* O157:H7 | Souche clinique |
| 43 | ESC.1.60 | *Escherichia coli* O157:H7 | Souche clinique |
| 44 | ESC.1.61 | *Escherichia coli* O157:H7 | Souche clinique |
| 45 | ESC.1.83 | *Escherichia coli* O157:H7 | Steack haché |
| 46 | ESC.1.88 | *Escherichia coli* O157:H7 | CIP 105181 |
| 47 | ESC.1.129 | *Escherichia coli* O157:H7 | Viande hachée |
| 48 | ESC.1.130 | *Escherichia coli* O157:H7 | Viande hachée |
| 49 | ESC.1.131 | *Escherichia coli* O157:H7 | Viande hachée |
| 50 | ESC.1.132 | *Escherichia coli* O157:H7 | Souche clinique |

## Exclusivity list

| | Strain code | Microorganism | Origin |
|---|---|---|---|
| 1 | ESC.1.62 | *Escherichia coli* O26:K60(B6) | CIP 52.171 |
| 2 | ESC.1.63 | *Escherichia coli* O26:K60(B6):H11 | CIP 52.172 |
| 3 | ESC.1.64 | *Escherichia coli* O55:H7 | CIP 105228 |
| 4 | ESC.1.65 | *Escherichia coli* O111:H21 | CIP 107192 |
| 5 | ESC.1.66 | *Escherichia coli* O121 | CIP 105992 |
| 6 | ESC.1.67 | *Escherichia coli* O128:H7 | CIP 107199 |
| 7 | ESC.1.68 | *Escherichia coli* O157:H43 | CIP 107193 |
| 8 | ESC.1.69 | *Escherichia coli* O157:K88a,c:H19 | CIP 105185 |
| 9 | ESC.1.84 | *Escherichia coli* O111 | CIP 105935 |
| 10 | ESC.1.3 | *Escherichia coli* | Industrie laitière |
| 11 | ESC.1.5 | *Escherichia coli* | Camembert |
| 12 | ESC.1.10 | *Escherichia coli* | Poulet mariné |
| 13 | ESC.1.11 | *Escherichia coli* | Blanc de poulet |
| 14 | ESC.1.12 | *Escherichia coli* | Collier d'agneau |
| 15 | ESC.1.14 | *Escherichia coli* | Escalope de dinde crue |
| 16 | ESC.1.15 | *Escherichia coli* | Bœuf muscle 80/20 |
| 17 | ESC.1.16 | *Escherichia coli* | Viande bœuf hachée |
| 18 | ESC.1.30 | *Escherichia coli* | Camembert |
| 19 | ESC.1.35 | *Escherichia coli* | Laguiole au lait cru |
| 20 | ESC.1.36 | *Escherichia coli* | Cantal jeune au lait cru |
| 21 | CIT.1.2 | *Citrobacter freundii* | ATCC 8090 |
| 22 | CIT.2.2 | *Citrobacter diversus* | CIP 82.87 T |
| 23 | HAF.1.1 | *Hafnia alvei* | Taboulé |
| 24 | KLE.1.1 | *Klebsiella oxytoca* | Salade soja |
| 25 | PAN.1.2 | *Pantoa agglomerans* | CIP 57.51 T |
| 26 | PRO.1.1 | *Proteus mirabilis* | CIP 103181 |
| 27 | SAL.1.133 | *Salmonella* Typhimurium | Ground beef |
| 28 | SER.1.1 | *Serratia ficaria* | CIP 79.23 |
| 29 | SHI.2.1 | *Shigella sonnei* | ATCC 9290 |
| 30 | PSE.2.1 | *Pseudomonas fluorescens* | CIP 69.13 T |
| 31 | SAL.1.174 | *Salmonella* Soerenga | Soja |
| 32 | SAL.1.175 | *Salmonella* Urbana | Bœuf |
| 33 | SAL.1.176 | *Salmonella* Hilversum | / |