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AFNOR Validation of Alternative Methods
FOOD MICROBIOLOGY

REPORT

*(Preliminary and Collaborative studies according to the
NF EN ISO 16140 standard)*

**ISO 16140 validation of
3M™ Petrifilm™ Coliforms count plates (CC)
for thermotolerant coliforms enumeration**

Quantitative method

This report includes 30 pages, with 3 annexes.
Only copies including the totality of this report are authorised.

**ER Petrifilm CC plate thermotolerant coliforms Synthesis
(Version 2) - September 3, 2008**

*Cancel and replace the previous version which must be returned
to ADRIA Développement or destroyed internally*

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Summary

| | | |
|----------|---|-----------|
| 1 | INTRODUCTION _____ | 4 |
| | 1.1 Validation standard _____ | 4 |
| | 1.2 Protocol and principle of the alternative method _____ | 4 |
| | 1.3 Application area _____ | 4 |
| | 1.4 Routine method _____ | 5 |
| 2 | COMPARATIVE STUDY _____ | 5 |
| | 2.1 Bibliographical study _____ | 5 |
| | 2.2 Linearity _____ | 5 |
| | 2.3 Relative accuracy _____ | 9 |
| | 2.4 Detection limit (LOD) and quantification limit (LOQ) _____ | 14 |
| | 2.5 Relative sensitivity _____ | 15 |
| | 2.6 Specificity _____ | 16 |
| | 2.7 Practicability _____ | 16 |
| 3 | COLLABORATIVE STUDY _____ | 17 |
| | 3.1 Organisation study _____ | 17 |
| | 3.2 Results _____ | 18 |
| 4 | CONCLUSION _____ | 24 |
| | <i>Annex 1 - Alternative method use instruction</i> _____ | <i>25</i> |
| | <i>Annex 2 - Linearity: regression straight lines</i> _____ | <i>29</i> |
| | <i>Annex 3 - Relative accuracy: regression straight lines</i> _____ | <i>30</i> |

The modifications brought to the report are indicated by a double line in the margin on the left.

Foreword

Competences of the laboratory are certified by COFRAC accreditation for the analysis marked with symbol♦.

Any element which allows analysis quality certification can be consulted by 3M SANTE.

The results are presented according to the NF EN ISO 16140 standard.

- ✓ **Firm:** 3M SANTE
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- ✓ **Expert laboratory:** ADRIA Développement
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- ✓ **Studied method:** ISO 16140 validation of 3M™ Petrifilm™ Coliforms count plates (CC) for thermotolerant coliforms enumeration

- ✓ **Validation standard:** NF EN ISO 16140 (October 2003) : Food microbiology – Protocol for the validation of alternative methods

- ✓ **Standard method♦ :** NF V08-060 (March 1996): Microbiology of food and animal feedings stuffs. Enumeration of thermotolerant coliforms by colony-count technique at 44°C. Routine method

- ✓ **Validation area:** All food products

♦ NF V08-060 : analysis performed according to the COFRAC accreditation
ADRIA Développement 3/30
**Petrifilm CC Plate -
Thermotolerant coliforms Synthesis (Version 2)**

1 INTRODUCTION

1.1 Validation standard

The validation study has been performed according to the NF EN ISO 16140 (October 2003) : Food microbiology – Protocol for the validation of alternative methods.

1.2 Protocol and principle of the alternative method

General use instructions are shown in Annex 1.

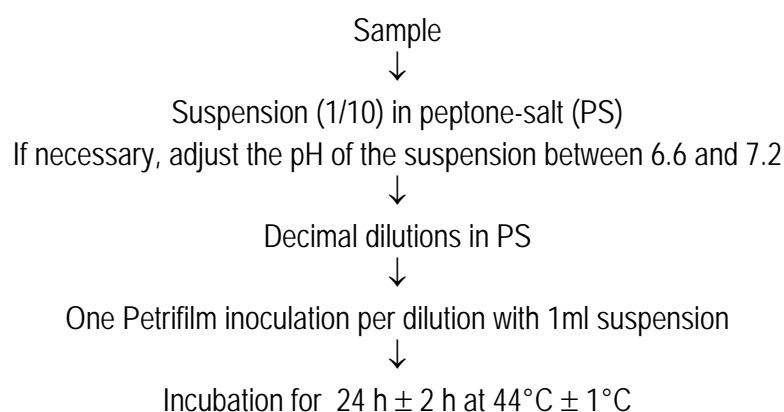
1.2.1 Principle of the alternative method

The Petrifilm CC plate is a ready-to-use system for the enumeration of coliform colonies. It is constituted by a cold water soluble dehydrated gel fixed between a support of polyethylene and a sheet leaf in polypropylene. The Petrifilm CC plate is based on the VRBL medium formulation.

After an incubation for 24 h \pm 2 h at 44°C \pm 1°C, thermotolerant coliforms appear as red colonies associated with gas

1.2.2 Protocol

The general protocol is presented below :



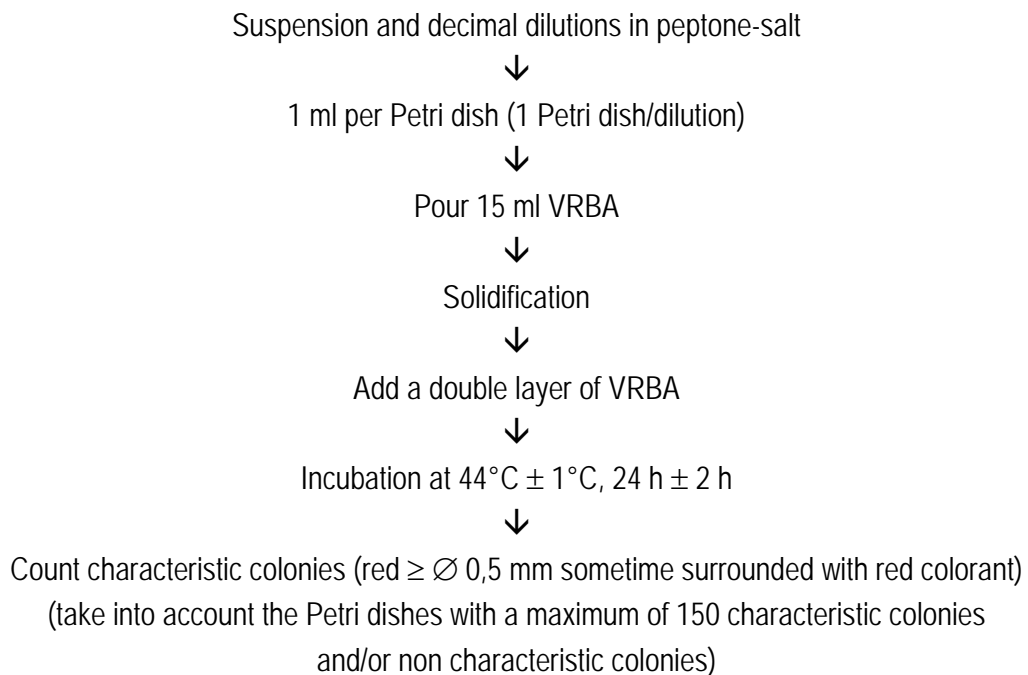
1.3 Application area

All food products

1.4 Routine method

The routine method is the NF V08-060 routine method: Microbiology of food and animal feedings stuffs. Enumeration of thermotolerant coliforms by colony-count technique at 44°C. The protocol is presented below:

Figure 1 - NF V08-060 method



2 COMPARATIVE STUDY

2.1 Bibliographical study

No papers related to the alternative method have been published since the last study which has been done in May 2002.

2.2 Linearity

Linearity is the ability of the method when used with a given matrix to give results that are in proportion to the amount of analyte present in the sample, that is an increase in analyte corresponds to a linear or proportional increase in results.

2.2.1 Food matrices and protocols

The linearity was investigated with 5 couples (category / strain and matrix / category).

Five contamination levels were analysed in duplicate.

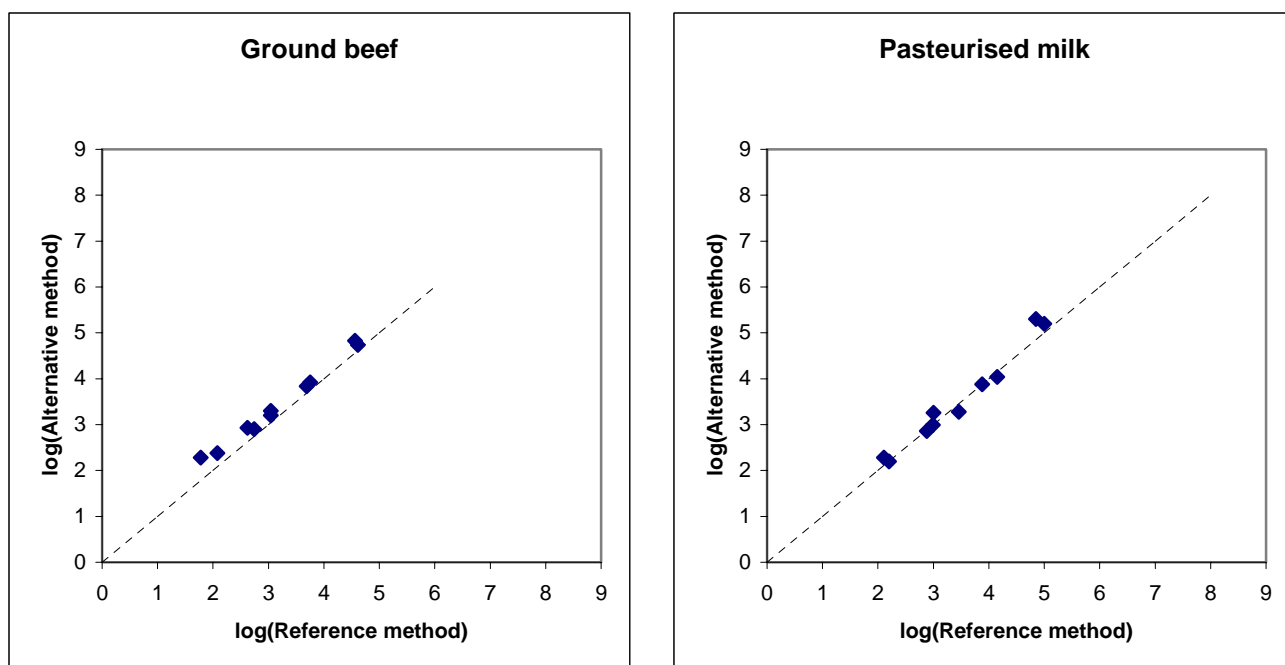
The contamination levels, the tested samples and the inoculated strains are presented in the table below :

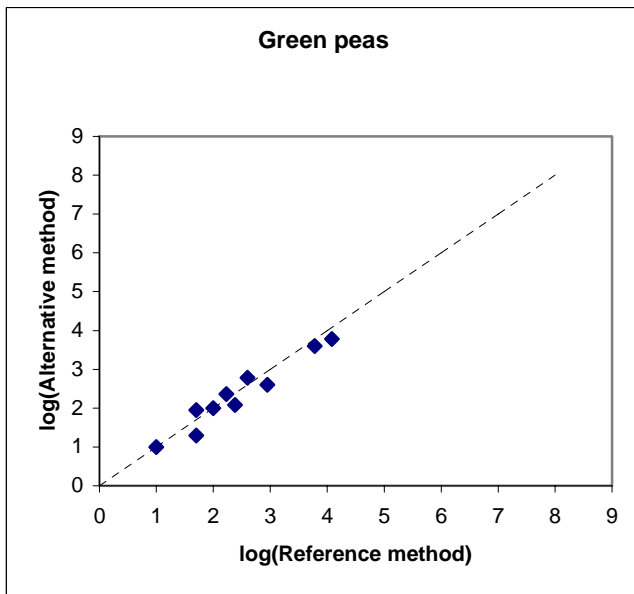
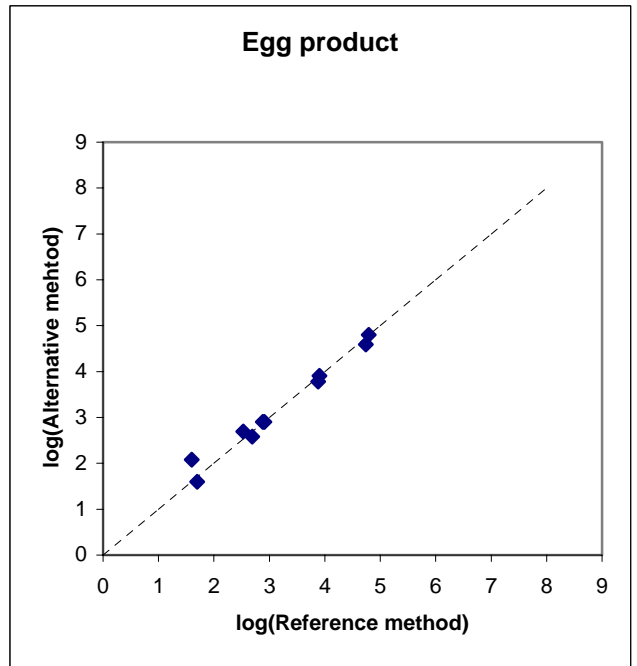
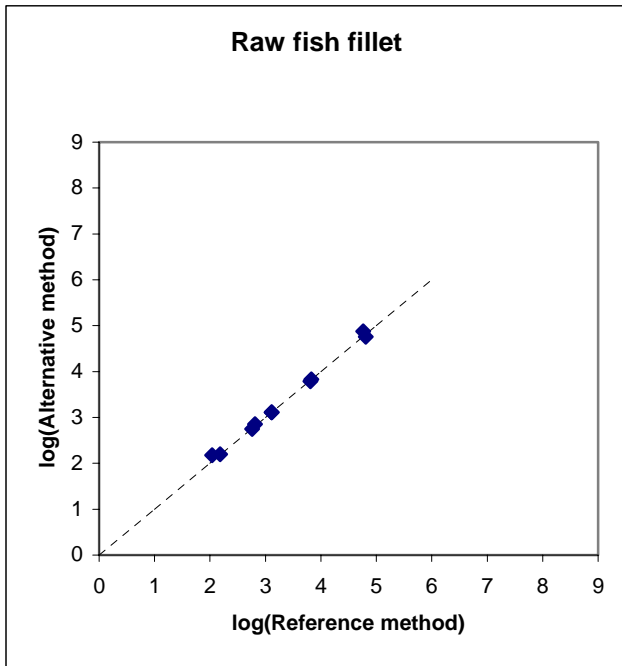
| Category | Strain |
|------------------|----------------------------------|
| Ground beef | <i>Enterobacter cloacae</i> 58 |
| Pasteurised milk | <i>Enterobacter sakazakii</i> 95 |
| Egg product | <i>Klebsiella pneumoniae</i> 89 |
| Raw fish fillet | <i>Escherichia coli</i> Ad 228 |
| Green peas | <i>Escherichia coli</i> 19 |

2.2.2 Results

The bi-dimensional graphs are shown figure 2.

Figure 2 - Linearity: bi-dimensional graphs





2.2.3 Statistical interpretations

Table 1 - Comparison Petrifilm CC plate (44°C) / V08-060

| Matrix | R | Selected Regression | Rob.F | Critical value | P% | Correlation coefficient | Regression equation* |
|-------------|------|---------------------|-------|----------------|----|-------------------------|----------------------------------|
| Ground beef | 1,50 | GMFR | 1,05 | 5,41 | 45 | 0,998 | log Alt = 0,930 log Ref. + 0,463 |
| Milk | 0,67 | GMFR | 8,57 | 5,41 | 2 | 0,994 | log Alt = 1,081 log Ref. - 0,202 |
| Raw fish | 0,80 | GMFR | 2,63 | 5,41 | 16 | 0,999 | log Alt = 0,987 log Ref. + 0,069 |
| Egg product | 2,60 | OLS1 | 4,26 | 5,41 | 8 | 0,992 | log Alt = 0,922 log Ref. + 0,267 |
| Green peas | 0,60 | GMFR | 1,70 | 5,41 | 28 | 0,990 | log Alt = 0,950 log Ref. + 0,025 |

* x-axis and y-axis choice depends on the selected regression.

Statistical Interpretation :

P > 5 % : not significant

1 % < P < 5 % : significant

P < 1 % : highly significant

The regression lines are shown in Annex 2.

2.2.4 Conclusion

The determined correlation coefficients are all upper or equal to 0,99 whatever the tested matrix.

The linearity test is not significant for the following matrices : ground beef, raw fish fillet, egg product and green peas. The correlation coefficient observed for pasteurised milk, associated to a P value under 5%, shows a high value which decreases the non linearity test robustness.

The Petrifilm CC plate shows satisfying linearity.

2.3 Relative accuracy

The accuracy is the closeness of agreement between a test result and the accepted reference value.

The bias is the difference between the expectation of the test results and an accepted reference value.

2.3.1 Number and nature of the samples

Categories and types of analysed food are presented in table 1.

Table 2 – Number and nature of the samples

| Categories | Types | Number of analysed samples | Number of results used in statistical tests |
|---------------------------|---|----------------------------|---|
| Meat products | Raw meats, raw and cooked delicatessen, ready-to-eat food | 22 | 16 |
| Milk products | Raw milk, cheeses, creams, milk powder | 15 | 12 |
| Egg products and pastries | Egg product, pastries, cooked eggs | 12 | 10 |
| Vegetables | Frozen vegetables, salads, cooked vegetables, ready-to-eat food | 26 | 11 |
| Fish products | Raw fish and shellfish, ready-to-eat food | 15 | 11 |
| All products | | 90 | 60 |

Cross contaminations with egg products have been done for three samples.

2.3.2 Results

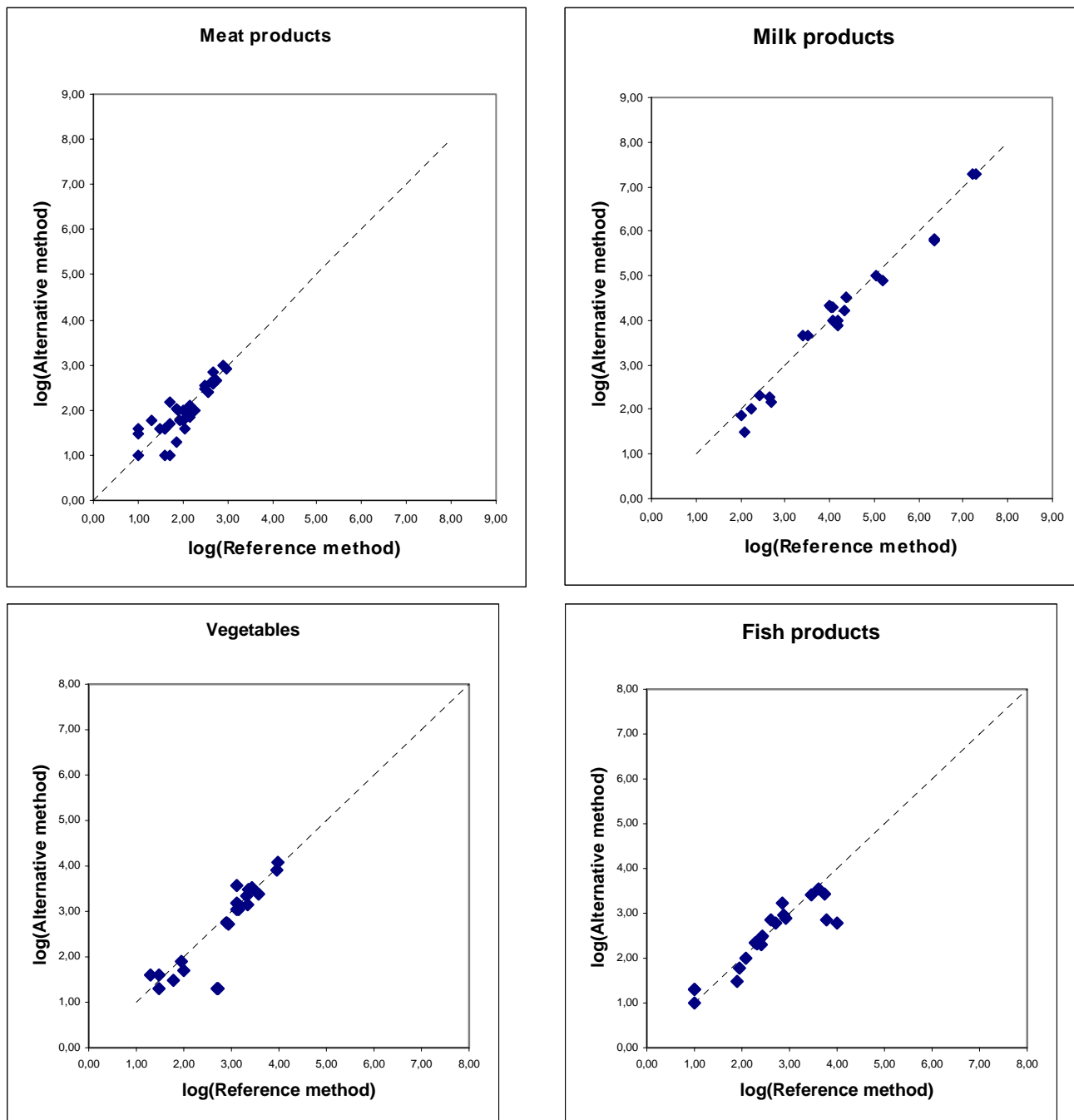
Samples have been analysed in duplicate for both methods.

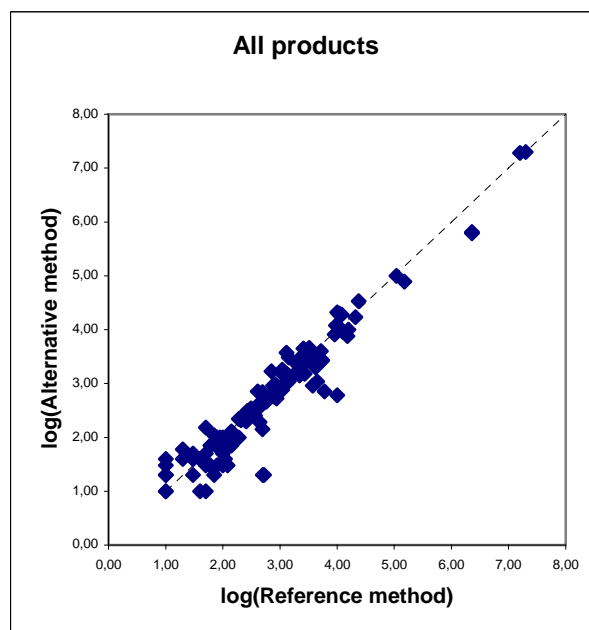
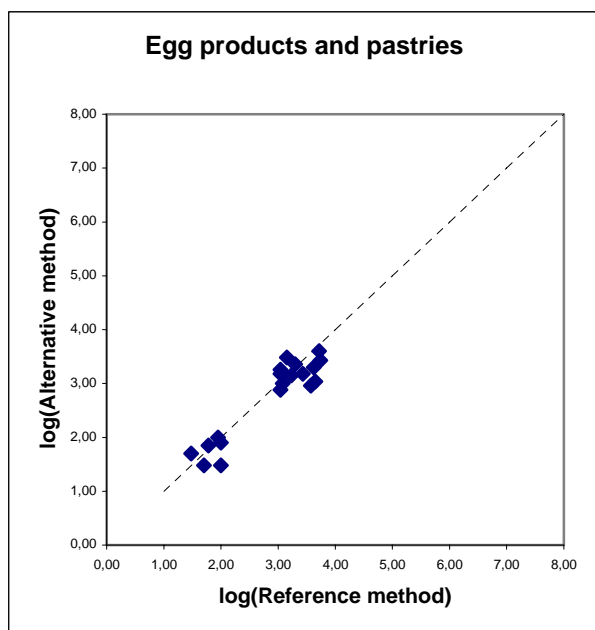
Table 3 - Petrifilm CC plate / NF V08-060

| Food category | Contamination scale (log CFU/g) |
|---------------------------|---------------------------------|
| Meat products | 1,00 to 2,99 |
| Milk products | 1,48 to 7,30 |
| Egg products and pastries | 1,48 to 3,74 |
| Vegetables | 1,30 to 4,08 |
| Fish products | 1,00 to 3,74 |

Bi-dimensional graphs for each category and for all samples are presented figure 3.

Figure 3 - Relative accuracy : bi-dimensional graphs





Comments on samples showing enumeration differences between the two methods

✓ **Vegetables category: sample n° 242**

The results obtained by both methods are the following:

| | NF V08-60 (log CFU/g) | Petrifim CC plate (log CFU/g) |
|-------------|-----------------------|-------------------------------|
| Duplicate 1 | 2,70 | 1,30 |
| Duplicate 2 | 2,72 | 1,30 |

API 20E profiles from 4 colonies isolated from VRBA and Petrifilm CC plate, have been performed.

| Colonies | NF V08-060 | Petrifim CC plate |
|----------|------------------------------|-------------------------------|
| 1 | <i>Serratia liquefaciens</i> | <i>Enterobacter sakazakii</i> |
| 2 | Oxydase + | <i>Enterobacter sakazakii</i> |
| 3 | <i>Klebsiella pneumoniae</i> | <i>Enterobacter sakazakii</i> |
| 4 | <i>Klebsiella pneumoniae</i> | <i>Enterobacter sakazakii</i> |

✓ **Fish products category: sample n° 243**

The results obtained with both methods are presented in the table below. The results obtained with the routine method (V08-060) using VRBA from an other manufacturer are also given:

| | NF V08-60 (VRBA A) log CFU/g | Petrifim CC plate log CFU/g | NF V08-60 (VRBA B) log CFU/g |
|-------------|------------------------------------|--------------------------------|------------------------------------|
| Replicate 1 | 3,78 | 2,85 | 2,00 |
| Replicate 2 | 4,00 | 2,78 | 3,92 |

The API 20E profiles obtained for 4 colonies, isolated from VRBA and Petrifim CC plate, have been performed: all the colonies were identified to *Klebsiella pneumoniae* species.

The minimal, optimal and maximal growth temperatures for two *Klebsiella pneumoniae* strains and for two *Serratia liquefaciens* strains are presented in the table forward¹. These parameters are presented for three fecal coliform group species: *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter kobei*.

| | T _{min} | T _{opt} | T _{max} |
|---|--------------------------|--------------------------|--------------------------|
| <i>Klebsiella pneumoniae</i> (2 strains) | 2,77 [- 2,44 ; 7,96] | 36,28 [32,41 ; 40,16] | 44,00 [37,41 ; 52,58] |
| | 1,07 [- 0,89 ; 3,04] | 37,20 [35,76 ; 38,64] | 45,00 [41,64 ; 48,36] |
| <i>Serratia liquefaciens</i> (2 strains) | -2,16 [-3,61 ; -0,76] | 32,91 [29,51 ; 34,30] | 37,30 [37,15 ; 37,44] |
| | -1,22 [-3,37 ; 0,92] | 31,91 [29,51 ; 34,30] | 38,08 [36,73 ; 39,44] |
| <i>Enterobacter cloacae</i> | 2,05 [0,69 ; 3,40] | 38,17 [37,28 ; 39,06] | 43,07 [42,40 ; 43,74] |
| <i>Enterobacter kobei</i> | 2,16 [- 2,92 ; 7,24] | 36,58 [33,99 ; 39,16] | 46,44 [38,84 ; 54,04] |
| <i>Escherichia coli</i> (2 strains) | 5,52 [3,24 ; 7,80] | 38,89 [37,83 ; 41,95] | 47,00 [44,69 ; 49,31] |
| | 5,34 [3,63 ; 7,06] | 40,85 [39,09 ; 42,61] | 45,87 [44,95 ; 46,80] |

T_{min}: minimal growth temperature

T_{opt}: optimal t growth emperature

T_{max}: maximal growth temperature

Klebsiella pneumoniae and *Serratia liquefaciens* cardinal temperatures are clearly different from fecal coliforms ones. Depending on VRBA media formulation, these strains may have some difficulties to grow.

¹ (ACTIA 01.5 « Coliformes : diversité des souches, diversité des comportements en fonction de la température et signification hygiénique » - Report n°3)

One colony on four tested, isolated from sample n° 242 VRBA analyse, is oxidase positive. Being oxidase negative is an *Enterobacteria* or coliforms group phenotype characteristic. Therefore, the colony isolated from the n° 242 sample which shows an oxidase positive phenotype can not belong to *Enterobacteriaceae* or coliforms group.

2.3.3 Statistical interpretations

Regression straight lines (graph and equation representations) for each food category and for all matrices are presented in Annex 3

Table 4 - Petrifilm CC plate / NF V08-060

| Categories | N | R | Selected regression | A | t(a) | b | t(b) | Critical T | P% | |
|----------------------------|-----------|-------------|---------------------|---------------|--------------|--------------|--------------|--------------|---------------|------------|
| | | | | | | | | | Ordinate at 0 | Slope at 1 |
| Meat products | 16 | 0,73 | GMFR | - 0,096 | 0,382 | 1,028 | 0,278 | 2,144 | 71 | 82 |
| Milk products | 12 | 1,00 | GMFR | - 0,230 | 0,951 | 1,029 | 0,528 | 2,228 | 36 | 61 |
| Egg products and pastries | 10 | 0,71 | GMFR | - 0,023 | 0,088 | 0,970 | 0,336 | 2,306 | 94 | 77 |
| Vegetables (all results) | 11 | 3,50 | OLS1 | -0,272 | 0,531 | 1,043 | 0,243 | 2,262 | 61 | 81 |
| Fish products (all points) | 11 | 0,70 | GMFR | -0,313 | 1,010 | 0,844 | 1,341 | 2,262 | 34 | 21 |
| All products | 60 | 0,80 | GMFR | -0,061 | 1,048 | 0,994 | 0,332 | 2,001 | 30 | 74 |

Statistical Interpretation :

P > 5 % : not significant

1 % < P < 5 % : significant

P < 1 % : highly significant

| Category | Bias D | Alternative method repeatability limit | Reference method repeatability limit |
|----------------------------|----------------|--|--------------------------------------|
| Meat products | - 0,020 | 0,405 | 0,543 |
| Milk products | - 0,168 | 0,300 | 0,294 |
| Egg products and pastries | - 0,168 | 0,352 | 0,499 |
| Vegetables (all results) | -0,035 | 0,411 | 0,117 |
| Fish products (all points) | 0,055 | 0,206 | 0,294 |
| All products | - 0,040 | 0,360 | 0,440 |

2.3.4 Conclusion

Bias between both methods show low values whatever the categories and vary -0,168 to 0,055 Log CFU/g.

Alternative method and reference method repeatability limits are similar. The intercept close to 0 and the slope close to 1 are validated for all categories.

For all products, the regression straight line is the following:

$$\text{Log (reference method)} = 0,994 \text{ Log (alternative method)} - 0,061.$$

The Petrifilm CC plate shows satisfying relative accuracy.

2.4 Detection limit (LOD) and quantification limit (LOQ)

The critical level is defined as the smallest amount which can be detected (not null), but not quantified as an exact value. Below this value, it cannot be sure that the true value is not null.

The detection limit is defined as being higher than the critical level because it involves a power, the probability $1-\beta$, which has to be well over 50 %, for example 95 %.

The quantification limit is defined as the smallest amount of analyte (that is the lowest actual number of organisms) which can be measured and quantified with defined precision and accuracy under the experimental conditions by the method under validation.

2.4.1 Protocol

The detection limit of the alternative method has been done with an *Escherichia coli* pure cultures. Three different levels of inoculation have been tested, with six replicates per level, i.e. a total of 18 analyses by the alternative method.

Quantification limit has been calculated for six independent blank sample determinations.

2.4.2 Results

Data are intrinsic to the method used and are presented in the following tables:

Table 5 - Petrifilm CC plate

| Level | Positive samples nb | Standard deviation | Bias |
|-------|---------------------|--------------------|------|
| 0,5 | 4/6 | 0,753 | 1 |
| 1 | 3/6 | 0,816 | 0,5 |
| 5 | 6/6 | 1,033 | 3 |

| | Formulas | Obtained values |
|-----|-------------|-----------------|
| LC | $S_0 + X_0$ | 2,2 |
| LOD | $S_0 + X_0$ | 3,5 |
| LOQ | $S_0 + X_0$ | 8,5 |

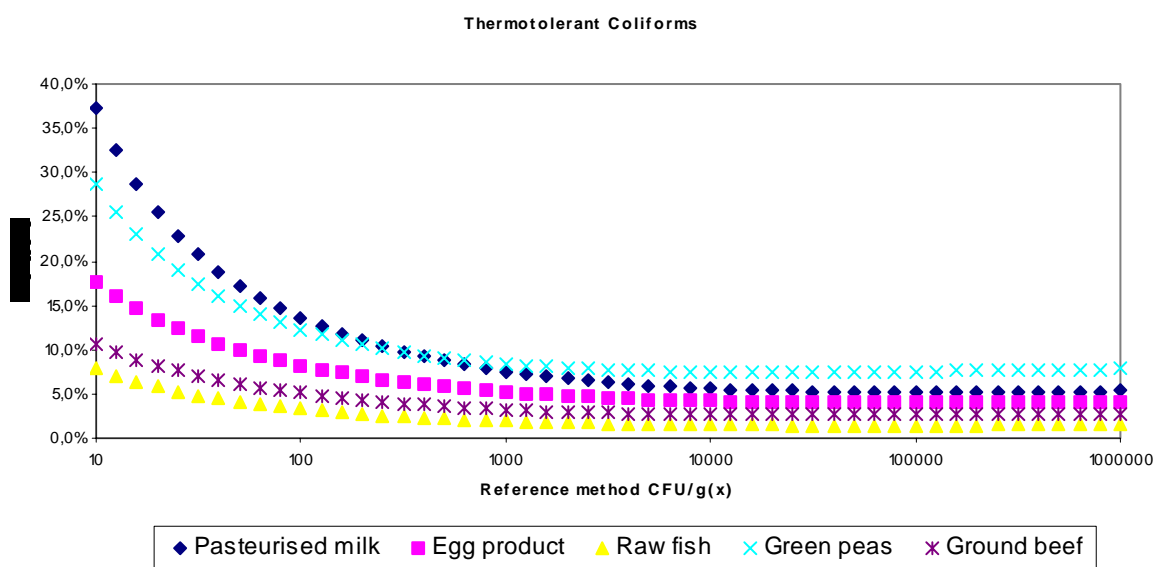
2.5 Relative sensitivity

The relative sensitivity is defined as the ability of the alternative method to detect two different amounts of analyte measured by the reference method within a given matrix, at a specified average value, or over the whole measurement range ; that is, it is the minimal quantity variation (increase of the analyte concentration x) which gives a significant variation of the measured signal (response y).

Data are intrinsic to the method used and are obtained from the results of the linearity study.

Accuracy patterns obtained for different matrices are presented in figure 4.

Figure 4 - Accuracy patterns for the different matrices used



2.6 Specificity

The specificity is defined as the degree to which a method is affected (or not) by the other components present in a multi-component sample. That is the ability of a method to measure exactly a given analyte, or its amount, within the sample without interference from non-target components such as a matrix effect, or background noise.

The results obtained during the previous validation have been completed in order to fit with the ISO 16140 standard requirements.

2.6.1 Exclusivity

All the 20 tested strains show a negative result by both method.

2.6.2 Inclusivity

30 targeted strains have been tested. The results are similar between both method, except for *Enterobacter sakazakii* 90 strain which shows non growth on VRBA, but which was enumerated by the Petrifilm CC plate.

The Petrifilm CC plate shows a satisfying specificity and selectivity.

2.7 Praticability

The saving of time is mainly obtained by the fact that:

- the Petrifilm CC plate is ready-to-use and allows to avoid the medium preparation.
- it is not necessary to pour a double layer of medium.

The time delays to obtain results are equivalent between both method, i.e. 24 h \pm 2 h. The space needed for incubation is much smaller for the Petrifilm CC plate than for NF V08-060 method. The Petrifilm CC plate minimizes the volume of wastes and the manipulation between two different analysis steps.

3 COLLABORATIVE STUDY

3.1 Organisation study

✓ *Collaborator laboratories*

14 laboratories have participated to this study.

✓ *Instructions for collaborator laboratories*

Detailed instructions have been transmitted to the collaborators by the expert laboratory.

✓ *Samples*

Pasteurised semi-skimmed milk has been inoculated by *Escherichia coli* 94, isolated from dairy product.

✓ *Inoculation*

Inoculation levels targeted are :

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

Each laboratory received eight flasks of 25 ml sample, i.e. two flasks per inoculation level. Furthermore, one non-inoculated sample have been added to the package for total viable count microflora (NF ISO 4833 method).

✓ *Labelling and shipping*

Coded samples (code is only known by the expert laboratory) have been placed in isothermal boxes which contained cooling blocks, and express-shipped to the different laboratories.

A temperature control flask containing temperature register has been added to the package in order to register temperature profile during transport and at reception.

Samples have been shipped in 24 h to laboratories of the collaborative study. Sample temperature should be lower or equal to 8°C during transport, and between 0°C - 8°C at arrival.

✓ **Analyses**

Collaborative study laboratories and the expert laboratory have carried out the analyses with the alternative and reference methods.

A stability study of the strain inoculated has been realised in order to verify there is no evolution during the transport.

3.2 Results

3.2.1 Results obtained by the expert laboratory

✓ **Strain stability during transport**

In order to evaluate the *Escherichia coli* 94 strain variability during transport, bacterial count of all inoculated flasks have been checked at different time, i.e. inoculation time, after 24 h and 48 h of conservation at 2°C. Results are reported in table 6.

Table 6 - *Escherichia coli* 94 count with the alternative method and reference method (in log CFU/ml)

| | Level 1 | | Level 2 | | Level 3 | |
|-------|------------------|--------------------|------------------|--------------------|------------------|--------------------|
| | Reference method | Alternative method | Reference method | Alternative method | Reference method | Alternative method |
| Day 0 | 71 / 76 | 75 / 78 | 580 / 670 | 540 / 450 | 5 900 / 6 700 | 3 700 / 4 000 |
| Day 1 | 65 / 45 | 64 / 60 | 560 / 620 | 360 / 390 | 3 900 / 5 400 | 4 000 / 5 500 |
| Day 2 | 68 / 96 | 58 / 56 | 830 / 910 | 570 / 470 | 6 500 / 1 900 | 4 600 / 4 200 |

No evolution of the strain has been observed after 48 h of storage at 4°C in the isothermal box.

✓ **Results obtained for both methods**

The mesophilic aerobic microflora has been done on the matrix with ISO 4833 method. The results varied from 70 to 3 600 CFU/ml.

The results obtained by the expert laboratory for *Escherichia coli* count for the both method are given in table 7.

Table 7 - Expert laboratory results (in log CFU/g)

| Targeted rate (log CFU/g) | Reference method | | Alternative method | |
|------------------------------|------------------|-------------|--------------------|-------------|
| | Duplicate 1 | Duplicate 2 | Duplicate 1 | Duplicate 2 |
| 0 | 0 | 0 | 0 | 0 |
| 1 to 2 | 1,81 | 1,65 | 1,81 | 1,78 |
| 2 to 3 | 2,75 | 2,79 | 2,56 | 2,59 |
| 3 to 4 | 3,59 | 3,73 | 3,60 | 3,74 |

Contamination levels targeted have been reached.

3.2.2 Results obtained by the collaborative laboratories

3.2.2.1 Sample temperature on receipt

Measured temperatures on receipt are listed in table 8.

The packages have been chipped in 24 h for the 14 laboratories. Any temperature upper than 8°C has been noted at reception.

Table 8 – Sample temperature on receipt

| Laboratories | Temperature measured by the thermobutton (°C) |
|--------------|---|
| A | 2,2 (J2) |
| B | 2,0 |
| C | 5,3 |
| D | 1,7 |
| E | 2,7 |
| F | 3,3 |
| G | 2,8 |
| H | 4,9 |
| I | 3,5 |
| J | 1,5 |
| K | 1,8 |
| L | 3,9 |
| N | 2,5 |
| O | 5,0 |

3.2.2.2 *Sample temperature during transport*

Any temperature upper than 8°C has been noted during transport.

3.2.2.3 *Results*

The laboratory A has received the package at J1, but has realised the analyses only at J2 and the laboratory I has not realise two successive dilutions. These results were not taken into account for statistical interpretation.

The results from 12 laboratories have been retained for statistical interpretation.

A results synthesis is presented in table 9.

Table 9 - Results synthesis obtained by NF V08-060 method and Petrifilm CC plate

| Laboratories | Level 0 | | | | Level 1 | | | | Level 2 | | | | Level 3 | | | |
|--------------|------------------|---|---------------------|---|------------------|----|---------------------|----|------------------|------|---------------------|-----|------------------|-------|---------------------|------|
| | Reference method | | Alternative methode | | Reference method | | Alternative methode | | Reference Method | | Alternative methode | | Reference method | | Alternative methode | |
| A | 0 | 0 | 0 | 0 | 57 | 73 | 36 | 45 | 680 | 660 | 360 | 450 | 3100 | 8500 | 3600 | 3300 |
| B | 0 | 0 | 0 | 0 | 67 | 68 | 74 | 84 | 800 | 670 | 550 | 760 | 6300 | 6100 | 5000 | 4500 |
| C | 0 | 0 | 0 | 0 | 35 | 39 | 57 | 57 | 470 | 490 | 480 | 380 | 5200 | 3900 | 3900 | 2900 |
| D | 0 | 0 | 0 | 0 | 70 | 73 | 57 | 77 | 490 | 630 | 560 | 500 | 6300 | 7800 | 4800 | 4000 |
| E | 0 | 0 | 0 | 0 | 51 | 88 | 85 | 68 | 960 | 680 | 580 | 680 | 7900 | 5900 | 7000 | 6000 |
| F | 0 | 0 | 0 | 0 | 55 | 45 | 64 | 66 | 530 | 470 | 540 | 510 | 6700 | 6500 | 5500 | 4500 |
| G | 0 | 0 | 0 | 0 | 67 | 69 | 78 | 70 | 660 | 780 | 620 | 490 | 6800 | 9600 | 5800 | 5600 |
| H | 0 | 0 | 0 | 0 | 59 | 49 | 86 | 78 | 780 | 870 | 560 | 600 | 8200 | 8700 | 5000 | 5400 |
| J | 0 | 0 | 0 | 0 | 80 | 72 | 63 | 78 | 650 | 780 | 560 | 570 | 5800 | 10000 | 6100 | 3600 |
| K | 0 | 0 | 0 | 0 | 62 | 63 | 58 | 61 | 670 | 750 | 580 | 660 | 4500 | 5800 | 4500 | 4900 |
| L | 0 | 0 | 0 | 0 | 25 | 31 | 66 | 72 | 390 | 510 | 540 | 500 | 4700 | 5400 | 3300 | 5400 |
| N | 0 | 0 | 0 | 0 | 65 | 35 | 84 | 95 | 760 | 1000 | 600 | 650 | 10000 | 8000 | 7700 | 7000 |
| O | 0 | 0 | 0 | 0 | 94 | 93 | 73 | 92 | 680 | 990 | 590 | 730 | 9500 | 6500 | 4800 | 6600 |

3.2.2.4 Statistical interpretation

↳ Bias calculation

For each level, difference between duplicate means (d_i) obtained by the alternative method and reference method has been calculated as : $d_i = (M_{i,alt} - M_{i,ref})$.

Median (MED $\{d_i\}$) of d_i allows the determination of bias D (MED $\{d_i\}$ = biais D).

Bias D and robust standard deviation $S \{d_i\} = K1Sn$ give t statistic ($t(d) = \text{MED}\{d_i\} \sqrt{n}/S\{d_i\}$). This t value obtained is compared to a critical value found in Student table (for $n = 12$, critical $t = 2,201$).

$t(d)$ values obtained by level are reported in table 10.

Table 10 - t(d) values obtained by level

| Level | Bias D | t(d) | Critical t ddl (n-1) | Conclusion |
|------------|--------|---------|-------------------------|----------------------|
| 1 (n = 12) | + 0,06 | 1,569 | 2,201 | non significant bias |
| 2 (n = 12) | - 0,08 | + 3,968 | 2,201 | significant bias |
| 3 (n = 12) | - 0,12 | + 6,116 | 2,201 | significant bias |

Critical level : t(d) < critical t

↪ **Repeatability calculation**

Values obtained for the repeatability limit, as well as values obtained for F test are reported in table 11.

Table 11 – Obtained values for repeatability limit and values for F test

| Level | Repeatability limit | | Calculated F (or 1/F*) | Critical F (0,05 ; n ; n) | P % |
|------------|---------------------|--------------------|---------------------------|------------------------------|-----|
| | Reference method | Alternative method | | | |
| 1 (n = 12) | 0,136 | 0,147 | 1,173 | 2,69 | 39 |
| 2 (n = 12) | 0,229 | 0,155 | 2,199 | 2,69 | 9 |
| 3 (n = 12) | 0,304 | 0,214 | 2,009 | 2,69 | 12 |

Statistical Interpretation

P > 5% : non significant

0,1 % < P < 1 % : highly significant

1 % < P < 5 % : significant

P < 0,1 % : very highly significant

↪ **Reproducibility**

Values obtained for reproducibility limit, as well as values obtained for F test are given in table 12.

Table 12 - Obtained values for reproducibility limit and F Test values

| Level | Reproducibility limit | | Calculated F (or 1/F*) | Critical F (0,05 ; n - 1 ; n - 1) | P % |
|------------|-----------------------|--------------------|---------------------------|--------------------------------------|-----|
| | Reference method | Alternative method | | | |
| 1 (n = 12) | 0,354 | 0,223 | 2,512 | 2,82 | 7 |
| 2 (n = 12) | 0,269 | 0,212 | 1,610 | 2,82 | 22 |
| 3 (n = 12) | 0,367 | 0,297 | 1,529 | 2,82 | 25 |

Statistical Interpretation

P > 5% : non significant

1 % < P < 5 % : significant

0,1 % < P < 1 % : highly significant

P < 0,1 % : very highly significant

↪ Ratio reproducibility limit / repeatability limit

The ratio reproducibility limit / repeatability limit are given in table 13.

Table 13 - Ratio of reproducibility limit / repeatability limit

| Level | Reference method | Alternative method |
|------------|------------------|--------------------|
| 1 (n = 12) | 2,599 | 1,513 |
| 2 (n = 12) | 1,173 | 1,371 |
| 3 (n = 12) | 1,206 | 1,383 |

Critical level : reproducibility / repeatability < 2

↪ Dispersion between laboratories

| Level | Reference method F | Alternative method F ou 1/F | Critical F (0,05 ; n-1 ; n) |
|------------|-----------------------|--------------------------------|--------------------------------|
| 1 (n = 12) | 12,504 | 3,578 | 2,79 |
| 2 (n = 12) | 1,753 | 2,759 | 2,79 |
| 3 (n = 12) | 1,911 | 2,827 | 2,79 |

Critical level : F or 1/F < F Critical

Discussion

- ✓ The bias between both methods were are very low, comprised between - 0,12 and + 0,06 log CFU/g.
- ✓ The repeatability and the reproducibility limits are similar between both methods.
- ✓ The ratio repeatability / reproducibility are below 2 as expected with the ISO 16140 method for the two methods, excepted for level 1 for the reference method.
- ✓ The labs results dispersion for level 1 is much more important by the reference method than the Petrifilm CC plate.

4 CONCLUSION

Conclusions of comparative study are the following:

The Petrifilm CC plate shows satisfying linearity.

The Petrifilm CC plate shows satisfying relative accuracy for all products.

The Petrifilm CC plate and NF V08-060 standard show equivalent specificity and selectivity.

The time delays to obtain results are equivalent between both method, i.e. 24 h \pm 2 h. The Petrifilm CC plate minimizes the incubation space, the volume of wastes and the manipulation between two different analysis steps.

Conclusions of the interlaboratory study are the following:

The bias between the two methods are small for the three level tested : + 0,6, - 0,08 and - 0,12 log CFU/g).

The repeatability and the reproducibility limits are similar between both methods.

Annex 1 - Alternative method use instruction

CC ins 38-9018-1244-6 8/20/04 4:43 PM Page 1



3M™ Petrifilm™

(English)

Coliform Count Plate

DESCRIPTION

The 3M™ Petrifilm™ Coliform Count (CC) Plate is a sample-ready-culture-medium system which contains Violet Red Bile (VRB) nutrients, a cold-water-soluble gelling agent, and a tetrazolium indicator that facilitates colony enumeration. Petrifilm CC plates are used for the enumeration of coliforms in the food and beverage industries. Petrifilm CC plate components are decontaminated though not sterilized. 3M Microbiology is certified to ISO (International Standards Organization) 9001.

AOAC INTERNATIONAL and the U.S. FDA Bacteriological Analytical Manual (BAM) define coliforms as gram-negative rods, which produce acid and gas from lactose fermentation. Coliform colonies growing on the Petrifilm CC plate produce acid, which causes the pH indicator to deepen the gel color; gas trapped around red colonies indicates coliforms.

ISO defines coliforms by their ability to grow in method-specific, selective media. ISO method 4832, enumerating coliforms by colony-count technique, defines coliforms as acid producers on VRB with lactose (VRBL) agar. On Petrifilm CC plates these acid-producing coliforms are indicated by red colonies with or without gas production. ISO method 4831, enumerating coliforms by the most probable number (MPN) method, defines coliforms by their ability to grow and produce gas from lactose in a selective broth. On Petrifilm CC plates these coliforms are indicated by red colonies associated with gas. AFNOR has validated Petrifilm CC plate in comparison to ISO method 4831 and ISO method 4832 for enumeration of total coliforms. AFNOR has also validated Petrifilm CC plate in comparison to NF V08-017, for enumeration of thermotolerant coliforms.

CAUTIONS

3M has not documented Petrifilm CC plates for use in industries other than food and beverage. For example, 3M has not documented Petrifilm CC plates for testing water, pharmaceuticals or cosmetics.

Petrifilm CC plates have not been tested with all possible food products, food processes, testing protocols or with all possible strains of coliform or other bacteria.

The Petrifilm CC plates have been evaluated following AOAC / AFNOR / NordVal guidelines and met validation criteria in representative samples of the following food categories: vegetables, meat, poultry, seafood, dairy, and processed foods.

1

Do not use Petrifilm CC plates in the diagnosis of conditions in humans or animals.

For information on documentation of product performance contact your official 3M Microbiology representative.

USER RESPONSIBILITY

No one culture medium will always recover the exact same strains or enumerate a particular strain exactly as does another medium. In addition, external factors such as sampling methods, testing protocols, preparation time and handling may influence recovery and enumeration. The food sample itself may influence results. For example, foods with high sugar content may increase the potential for gas production from non-coliform Enterobacteriaceae.

It is the user's responsibility in selecting any test method to evaluate a sufficient number of samples with particular foods and microbial challenges to satisfy the user that the chosen test method meets the user's criteria.

It is also the user's responsibility to determine that any test methods and results meet its customers' or suppliers' requirements.

As with any culture medium, Petrifilm CC plate results do not constitute a guarantee of quality of food or beverage products or processes that are tested with the plates.

The user must train its personnel in proper testing techniques: for example, Good Laboratory Practices (U.S. Food and Drug Administration, Title 21, Part 58 of the Code of Federal Regulations) or ISO 17025.

DISCLAIMER OF WARRANTIES / LIMITED REMEDY

UNLESS OTHERWISE PROHIBITED BY LAW, 3M DISCLAIMS ALL EXPRESS AND IMPLIED WARRANTIES, INCLUDING, BUT NOT LIMITED TO, ANY WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR USE. If any 3M Petrifilm plate is proven to be defective, 3M or its authorized distributor will replace or, at its option, refund the purchase price of any plate. These are your exclusive remedies. You must promptly notify 3M within sixty days of discovery of any suspected defect in a product and return the product to 3M. Please call Customer Service (1-800-328-1671 in the U.S.) or your official 3M Microbiology representative for a Returned Goods Authorization.

LIMITATION OF 3M LIABILITY

UNLESS OTHERWISE PROHIBITED BY LAW, 3M WILL NOT BE LIABLE TO USER OR OTHERS FOR ANY LOSS OR DAMAGE, WHETHER DIRECT, INDIRECT, SPECIAL,

2



INCIDENTAL OR CONSEQUENTIAL DAMAGES, INCLUDING, BUT NOT LIMITED TO, LOST PROFITS. Except where prohibited by law, in no event shall 3M's liability under any legal theory exceed the purchase price of the plates alleged to be defective. Customer may have additional rights and should seek advice in country of purchase.

STORAGE AND DISPOSAL

Store unopened Petrifilm plate pouches refrigerated or frozen at temperatures $\leq 8^{\circ}\text{C}$ (46°F). Just prior to use, allow unopened pouches to come to room temperature before opening. Return unused plates to pouch. Seal by folding the end of the pouch over and taping shut. To prevent exposure to moisture, do not refrigerate opened pouches. Store resealed pouches in a cool dry place for no longer than one month. It is recommended that resealed pouches of Petrifilm plates be stored in a freezer (see below) if the laboratory temperature exceeds 25°C (77°F) and/or the laboratory is located in a region where the relative humidity exceeds 50% (with the exception of air-conditioned premises).

To store opened pouches in a freezer, place Petrifilm plates in a sealable container. To remove frozen Petrifilm plates for use, open the container, remove the plates that are needed and immediately return remaining plates to the freezer in the sealed container. Plates should not be used past their expiration date. The freezer that is used for open pouch storage must not have an automatic defrost cycle as this would repeatedly expose the plates to moisture which can damage the plates.

Do not use plates that show discoloration. Expiration date and lot number are noted on each package of Petrifilm plates. The lot number is also noted on individual plates.

After use, Petrifilm CC plates may contain microorganisms that may be a potential biohazard. Follow current industry standards for disposal.

INSTRUCTIONS FOR USE

Sample Preparation

1. Use appropriate sterile diluents:

Butterfield's phosphate buffer^{1,2} 2 0.1% peptone water, peptone salt diluent,³ buffered peptone water,⁴ saline solution (0.85-0.90%), bisulfite-free letheen broth or distilled water.

Do not use diluents containing citrate, bisulfite or thiosulfate with Petrifilm plates; they can inhibit growth. If citrate buffer is indicated in the standard procedure, substitute with one of the buffers listed above, warmed to $40\text{--}45^{\circ}\text{C}$.

AFNOR validated methods:

3

Only ISO diluents listed above and referenced are in the scope of the validation.

2. Blend or homogenize sample.
3. For optimal growth and recovery of microorganisms, adjust the pH of the sample suspension to 6.6 - 7.2. For acidic products, adjust the pH with 1N NaOH. For alkaline products, adjust the pH with 1N HCl.

Plating

1. Place the Petrifilm CC plate on a flat, level surface (see figure a).
2. Lift the top film and with the pipette perpendicular dispense 1 mL of sample suspension onto the center of bottom film (see figure b).
3. Roll the top film down onto the sample to prevent trapping air bubbles (see figure c).
4. Place the plastic spreader with the flat side down on the center of the plate (see figure d). Press gently on the center of the spreader to distribute the sample evenly. Spread the inoculum over the entire Petrifilm plate growth area before the gel is formed. Do not slide the spreader across the film.
5. Remove the spreader and leave the plate undisturbed for at least one minute to permit the gel to form.

Incubation

Incubate plates in a horizontal position with the clear side up in stacks of no more than 20 plates. Several incubation times and temperatures can be used depending on current local reference methods.

For example:

AOAC® Official Methods™ (986.33 Bacteria and Coliform Counts in Milk, Dry Rehydratable Film Methods and 989.10 Bacterial and Coliforms Counts in Dairy Products, Dry Rehydratable Film Methods)

Incubate Petrifilm CC plates 24 h \pm 2 h at $32^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

AOAC® Official Methods™ (991.14 Coliform and Escherichia coli Counts in Foods, Dry Rehydratable Film Methods)

Incubate Petrifilm CC plates 24 h \pm 2 h at $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

AFNOR validated methods

Total coliforms: in comparison to ISO 4832 (3M-01/2-09/89 A) and in comparison to ISO 4831 (3M-01/2-09/89 B)

All food except raw shellfish.

- Incubate Petrifilm CC plates 24 h \pm 2 h at $30^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$ or $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

4



- As recommended in ISO 4831 and ISO 4832, these temperatures should be agreed upon between concerned parties and mentioned in the analysis certificate.

Thermotolerant coliforms: in comparison to NF V08-060, VRBL incubated at 44.5°C ± 1°C (3M-01/2-09/89 C).
All food.

- Incubate Petrifilm CC plates 24 h ± 2 h at 44°C ± 1°C.

Nordic System for validation of alternative microbiological methods, NordVal Validation (Ref. No.: 2003-20-5408-00011)

Refer to NordVal validation for Petrifilm CC plate method details.

Interpretation

1. Petrifilm CC plates can be counted using a standard colony counter or other illuminated magnifier. Gas produced by coliform may disrupt the colony so that the colony "outlines" the bubble. This should be counted as a single coliform. Do not count colonies on the foam dam since they are removed from the selective influence of the medium. Do not count artifact bubbles that may be present.

The interpretation of coliform colonies on the Petrifilm CC plate varies by method. For example:

AOAC Official Methods

Coliform colonies are red and closely associated (within one colony diameter) with entrapped gas. Colonies not associated with gas (a distance greater than one colony diameter between colony and gas bubble) are not counted as coliforms.

OR AFNOR validated methods

- As compared to ISO method 4831 (MPN method), coliform colonies are red and closely associated (within one colony diameter) with entrapped gas (see figure e).

Note: a high ratio of non gas-producing gram-negative rods may require a higher dilution in order to enumerate gas producing coliforms within the recommended counting range.

- As compared to ISO method 4832 and NF V08-060 (VRBL methods), count all red colonies with or without gas.

2. The circular growth area is approximately 20 cm². Estimates can be made on plates containing greater than 150 colonies by counting the number of colonies in one or more representative squares and determining the average number per square. Multiply the average number by 20 to determine the estimated count per plate (see figure f); estimations are outside of the AFNOR validation scope.

5

3. When present in large numbers, Petrifilm CC plates may have a deepening of the gel color and either or both of the following characteristics: many small, indistinct colonies and/or many gas bubbles. High concentrations of coliforms will cause the growth area to turn dark red (see figure g). When this occurs, record results as too numerous to count (TNTC). When an actual count is required, plate at a higher dilution.
4. Where necessary, colonies may be isolated for further identification. Lift the top film and pick the colony from the gel (see figure h). Test using standard procedures.
5. If the plates cannot be counted within 1 hour of removal from the incubator, they may be stored for later enumeration by freezing in a sealable container at temperatures ≤ minus 15°C for no longer than one week.

For further information refer to the appropriate Petrifilm plate "Interpretation Guide." If you have questions about specific applications or procedures, please contact your official 3M Microbiology representative nearest you.

References

1. FDA. 1998. Bacteriological Analytical Manual, 8th ed., Revision A, Appendix 3.64.
2. International Standards Organization, ISO 6887-1:1999. Microbiology of food and animal feeding stuffs – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination
3. International Standards Organization, ISO 8261:2001. Milk and milk products – General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination

6

Explanation of symbols



- Attention, see instructions for use



- The lot in a box and the hourglass symbols are symbols that represent lot number and expiration date. The hourglass is followed by a year and month which represents the expiration date (year and month: 2010-10). The entire line after the hourglass represents the lot number. (2010 - 10 AZ).

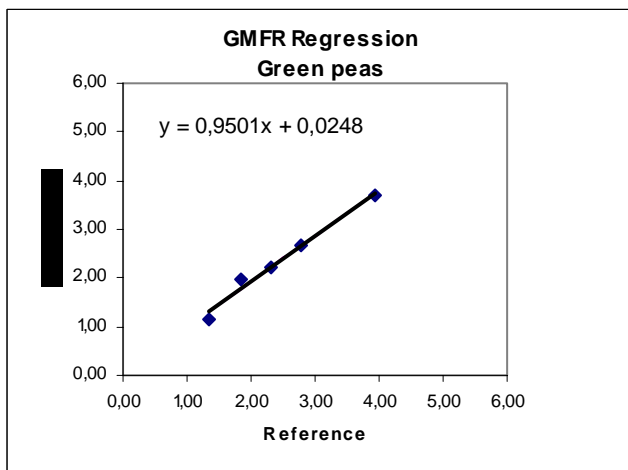
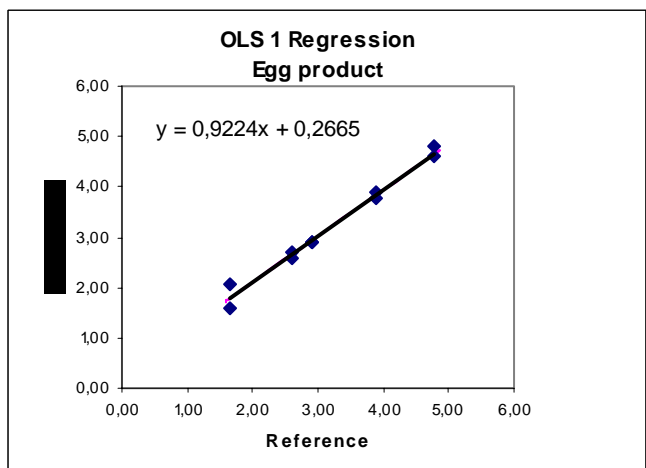
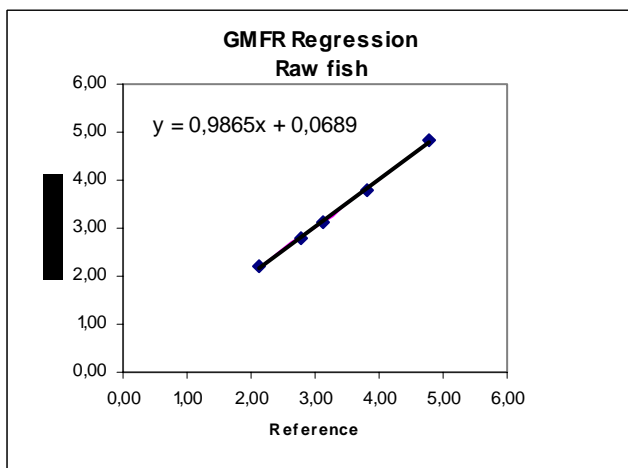
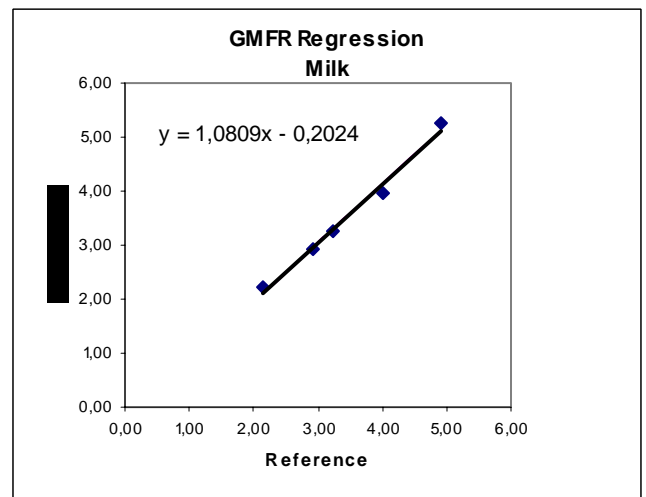
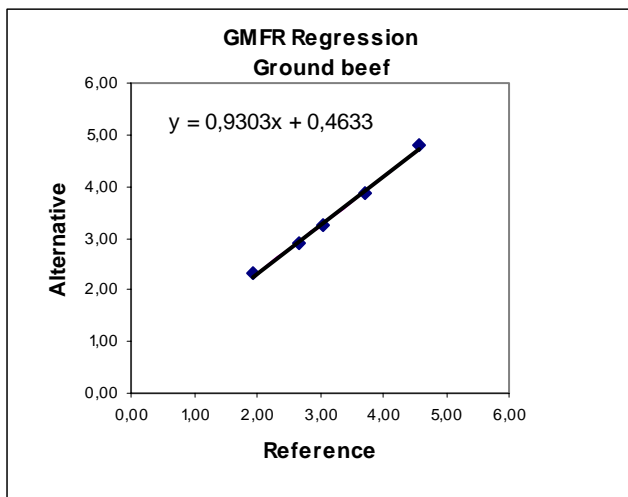


- Store below given temperature.

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Annex 2 - Linearity: regression straight lines



Annex 3 - Relative accuracy: regression straight lines

