



**Alternative methods for agribusiness
Analytical performances certified**

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

Certificate N°: 3M 01/5 – 03/97 C

Validation date: 18.03.1997
1st Renewal date*: 13.12.2001
2nd Renewal date*: 04.05.2006
3rd Renewal date : 28.11.2008
End of validity : 18.03.2013

** The EN ISO 16140 protocol has been implemented for the 2nd renewal in 2006*

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

3M™ Petrifilm™ Rapid Coliform Count Plate
 Enumeration of gas producing coliforms after 24 h

Protocol reference : 34-8702-8710-8

SCOPE

All human food products (processed pork meat excepted).

RESTRICTIONS OF USE

Insufficient dilution.

REFERENCE METHOD

- **NF EN ISO 4831** (2006) – Horizontal method for the detection and enumeration of coliforms – Most probable number technique
- **NF EN ISO 4832** (2006) – Horizontal method for the enumeration of coliforms – Colony-count technique (VRBL)

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PRINCIPLE OF THE METHOD

3M™ Petrifilm™ Rapid Coliform Count plate consists of a medium for coliforms containing a pH indicator which allows one to detect acid production, and a tetrazolium red indicator which facilitates the count.

During metabolism, coliforms produce acid and / or gas from lactose. While colonies grow and produce acid, pH indicator turns from orange-red to yellow. Acidification zone may appear before the colonies. Gas is trapped around coliform colonies thus allows detecting gas producing coliforms. For the enumeration after 24 h, gas producing coliforms appear as red colonies surrounded with gas bubbles.

HISTORY OF THE VALIDATION

NOTE 1 : The protocol described in EN ISO 16140 standard has been conducted during 2006 renewal. Some results obtained at the previous studies have been maintained for a further use. Here they are :

- Relative accuracy : results obtained in 1997 from the expert laboratory and results obtained in 1996 from an external laboratory (taken into account within the AFNOR validation)
- Practicability : 1997 data
- Selectivity : 1997 data
- Inter-laboratory study : re-use of results obtained in 1997 on milk food type.

The validation concerned the 2 following protocols :

- Sea-foods products (incubation at 30°C)
- Other food products (incubation at 35°C)

NOTE 2 : In November 2008, The validation of 3M™ Petrifilm™ Rapid Coliform Count Plate was renewed, as the method has not been modified since previous validation, no additional test was performed.

LINEARITY and RELATIVE ACCURACY

Comparison of performances of the alternative method and the reference method

Note : EN ISO 4832 standard was used as a reference for the **linearity** study.

Only one standard is available for gas producing coliform enumeration : **EN ISO 4831**. It is based on Most Probable Number calculation, principle giving specific confidence intervals around the result, whereas the Petrifilm Rapid Coliform Count plate is based on colonies enumeration.

Though, during reference methods and Petrifilm RCC comparative study, linearity study was run comparing Petrifilm RCC results with EN ISO 4832 ones, as this method is also based on colonies enumeration. The linearity study being run only with **artificially contaminated** products with known inoculation levels, it is preferable to run linearity tests with method based on the **same principles**. The **limit of repeatability** for **EN ISO 4831** was obtained during **accuracy study** for two food categories, and during **inter-laboratory study**, this allows to draw conclusions for the accuracy study run with naturally contaminated samples.

Linearity study (1996/97 and 2005 studies) :

Tests were performed in **1996** on 3 food categories (dairy products, vegetables, ready meals) artificially contaminated with 3 strains (*Escherichia coli*, *Enterobacter cloacae*, *Klebsiella oxytoca*) ; 4 contamination levels were tested : 0, 100 to 1 000, 1 000 to 10 000, 10 000 to 100 000 CFU/g.

The samples were analysed **in double** with Petrifilm plates and **in single** with EN ISO 4831.

The statistical interpretation of 1996 results is the following :

$$\log(N \text{ reference method}) = 1.00 \log(N \text{ alternative method}) + 0.16$$

A complementary study was conducted in **2006** by comparison with **EN ISO 4832**. Samples were contaminated with 2 strains (*Escherichia coli*, *Citrobacter freundii*) at the five following artificial levels : 10, 500, 1000, 5000, 10000 CFU/g.

The samples were analysed **in duplicate with both methods**.

1996 data were analysed following ISO 16140 standard , for the three first food categories in the following table. For the 2 categories tested later on, statistical interpretation was also done following EN ISO 16140 standard.

The following regression lines were obtained by food category (indication on inoculated strain is given for the 2 categories tested in 2006). The following results were obtained :

Food category	Couple food/ strain	Regression line
Dairy products	Pasteurized milks	Y = 0.971+ 0.145X
Miscellaneous	Ready meals	Y = 1.001+ 0.050X
Vegetables	potatoes	Y = 0.966 + 0.098X
Sea-food	Fish / <i>Escherichia coli</i>	Y = 0.967+ 0.151X
Meat products	Minced meat / <i>Citrobacter freundii</i>	Y= 1.053 - 0.185X

Y = log(N alternative method)

X = log(N reference method)

Accuracy study (1996/97 and 2006 studies) :

Tests were performed in 1996 and 2005. The statistical interpretation was conducted on 65 countable results from samples which were all naturally contaminated, belonging to the major food categories described in the table below.

The samples were analysed **in duplicate** with Petrifilm RCC method and **in single** with **EN ISO 4831 method**.

For information, the levels of contamination (concentration) ranged as follows :

Food categories	Contamination range (log)
Meat products	1.36 to 5.04
Dairy products	0.97 to 4.41
Seafood products	1.63 to 4.63
Vegetables	1.38 to 6.32
Egg products	1.63 to 5.04

Note : An aberrant value was obtained in the egg products category for reference method. Interpretation was done by eliminating the concerned sample.

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

$$Y = 0.811 X + 0.542$$

Y = log(N alternative method)

X = log(N reference method)

The repeatability for both methods and the bias between the two methods were determined according to the method of calculation used for the inter-laboratory study (see sections 6.3.5 and 6.3.6 of EN ISO 16140 standard). These results provide additional information for the accuracy criterion.

Regarding 1996 datas, as the reference method was done in single, its repeatability is not available for all categories. Repeatability limits were calculated in 2006 for seafood and meat products :

	Alternative method	Reference method
Meat products	$r = 0.293$	$r = 0.795$
Seafoods	$r = 0.323$	$r = 0.998$

The bias (in log) between both methods (alternative – reference) is included **between -0.183 and 0.1 log CFU/g**.

Conclusion for linearity and relative accuracy :

Statistical tests confirm the correlation between the 2 methods for the following food categories : dairy products, egg products, vegetable and miscellaneous food

Some discordants results observed for sea-foods products and meats products categories were due to repeatability limits of the reference method.

SELECTIVITY (INCLUSIVITY / EXCLUSIVITY) – 1997 study

Use of alternative method only

- 20 strains of coliforms from 20 tested were detected, But 8 strains did not produce gas after 24 hours (they belonged to the following species : *Citrobacter freundii*, *Citrobacter diversus*, *Enterobacter cloacae*, *Hafnia alvei*, *Serratia lignefactors*). However, those 8 strains do not produce either gas on BGLB broth and would not be either detected with the reference methods.
- Study on 17 non coliform strains showed crossed reaction with 2 *Aeromonas hydrophila* strains.

Conclusion

3M™ Petrifilm™ Rapid Coliform Count Plate specificity with gas forming colonies reading after 24 hours is equivalent to reference method specificity.

PRACTICABILITY (1997 study)

Use of alternative method only

- **Response time :**
 - **Positive** and **negative** results are obtained within 24 hours with the alternative method instead of 48-96 hours with the reference method for a positive test.
- **Other criteria :** space saving, time saving for preparation of media, easier waste management.

INTER-LABORATORY STUDY

The inter-laboratory study was conducted in 1997 with 12 participating laboratories. Analyses were done with half-skimmed pasteurized milk samples, artificially contaminated with a strain of *Enterobacter cloacae*, at the 4 following levels :

- Level 0
- Level 10 to 100 log CFU/g
- Level 100 to 1 000 log CFU/g
- Level 1 000 to 10 000 log CFU/g

The laboratories tested, using each of the **two methods, two replicates per contamination level**.

The following results, interpreted following EN ISO 16140 were obtained :

Contamination level log CFU/g	Number of laboratories with countable results*	Reference method		Alternative method		
		Repeatability r	Reproducibility R	Repeatability r	Reproducibility R	Bias
10 to 100	10	1.507	1.266	0.221	0.249	-0.16
100 to 1000	10	0.983	0.830	0.264	0.262	-0.12
1000 to 10 000	10	1.111	1.09	0.235	0.235	-0.05

** 1 laboratory has received samples at a temperature higher than 8°C and 2 laboratories have not done enough dilutions to obtain accurate results. Their results have not been used.*

Conclusion

The inter-laboratory study shows comparable results between 3M™ Petrifilm™ Rapid Coliform Count Plate and the reference method.

Please send any queries concerning the performance of the validated method to
AFNOR Certification.

You may download a summary document on the preliminary and inter-laboratory
studies on www.afnor-validation.com