



Alternative methods for water
Analytical performances certified

VALIDATION CERTIFICATE FOR ALTERNATIVE ANALYTICAL METHOD

According to protocol for the validation of a commercial method versus a reference method.

(AFNOR Certification – Rev. 0)

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The company **BIO-RAD**
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Is hereby authorized to refer to this **NF VALIDATION** certificate for the following quantitative analysis method:

RAPID'E.coli 2 + Supplement (Water Testing)

**Enumeration of β D-galactosidase positive coliform
and positive β D-glucuronidase *E coli***

PROTOCOL REFERENCE :

- RAPID'E.coli 2 Supplement (for water control) : 355-5298 V5
- RAPID'E.coli 2 Agar (for water control) : 356-3982 / 355-5296 V3

SCOPE : Water for human consumption, with low suspended matters, treated or non-treated

RESTRICTIONS FOR USE : None

REFERENCE METHOD :

Norme NF EN ISO 9308-1 : Water Quality – Detection and enumeration of *Escherichia coli* and coliform bacteria. Part 1 : Membrane filtration method

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PRINCIPLE OF THE METHOD

RAPID'E.coli 2 + Supplement (Water Testing) method is a chromogenic method which allows the direct and simultaneous detection and enumeration of *Escherichia coli*, and total coliforms, by membrane filtration method. The principle of the complete medium (supplemented RAPID'E coli 2) relies on the simultaneous detection of 2 enzymatic activities : β -DGalactosidase (GAL) and β -D-Glucuronidase (GLUC), by two chromogenic substrates :

- cleavage of the GAL specific substrate leads to form a precipitate giving a green coloration of the positive colonies for this enzyme (Coliforms),
- cleavage of the GLUC specific substrate leads to form a precipitate giving a pink coloration of the positive colonies for this enzyme (*E. coli*).

Coliforms (GAL+/GLUC-) form green colonies, *E. coli* (GAL+/GLUC+) form blue to violet colonies due to the superposition of both colorations.

LINEARITY AND RELATIVE ACCURACY

Comparison of performances of the alternative method and the reference method

Accuracy study :

Tests were performed in 2010. The statistical interpretation was conducted on 50 results for « coliforms bacteria » parameter and 42 results for « *E coli* » parameter, coming from 66 samples of following kinds of waters :

- treated waters with low suspended matters : network tap water
- non-treated waters with low suspended matters : mineral bottle water, waters from wells, spring waters, rain/underground waters

Artificially contaminated samples were carried out by using either contaminated suspensions with *E coli* or coliforms, or waters naturally contaminated (contamination made by mixing).

Among exploited results, 48 results « coliforms » and 42 results « *E coli* » are from artificially contaminated samples, that is to say an artificial contaminated percentage, respectively, of 96% and 100%.

The samples (matrix / strains pairs) were analyzed **in duplicate** with each of the two methods (reference method and alternative method)

The equation of the regression line between alternative method and the reference method was determined for each matrix / strain pair, as per the modal $y = bx + a$, where « y » representing alternative method (**log(Alt)**) and x reference method (**log(Ref)**).

The bias (D) between the two methods (alternative- reference) was calculated by taking the median of difference values per level. The following results were obtained :

matrix/ strain pairs	Median bia in log	Regression line $\log(\text{Alt}) = b.\log(\text{Ref}) + a$
Treated water/ <i>E. coli</i>	-0,218	$\log(\text{Alt}) = 1,163.\log(\text{Ref}) - 0,471$
Non treated / <i>E. coli</i>	-0,074	$\log(\text{Alt}) = 0,977.\log(\text{Ref}) - 0,078$
Treated/coliformes	-0,045	$\log(\text{Alt}) = 0,990.\log(\text{Ref}) + 0,022$
Non treated/coliformes	-0,060	$\log(\text{Alt}) = 0,948.\log(\text{Ref}) - 0,020$

Conclusion for relative accuracy

Accuracy study shows that results obtained with alternative method are comparable to the ones obtained with reference method. For « treated water / *E coli* » pair, a slight but systematic bias is noticed between reference and alternative method.

Linearity study :

Tests were performed in 2010 for below matrix/strain pairs. For each matrix/strain pair, samples were analyzed in duplicate with each of the two methods (reference and alternative), with artificial contaminations ranges as below :

Contamination ranges	Tap water	Mineral bottle water
Range 1	5 to 10 cfu/100 mL	5 to 10 cfu/100 mL
Range 2	20 cfu/100 mL	20 cfu/100 mL
Range 3	200 cfu/100 mL	200 cfu/100 mL

The following results were obtained :

Couple matrix/strain	Regression line	R ²
Tap water / <i>E. coli</i>	$\text{Log(Alt)} = 0,9276 \cdot \text{log(Ref)} + 0,1695$	0,9756
Mineral bottle water / <i>E. coli</i>	$\text{Log(Alt)} = 1,1218 \cdot \text{log(Ref)} - 0,2128$	0,9797

Conclusion for linearity

Linearity study shows that results obtained with alternative method are comparable to the ones obtained with reference method, for each couple matrix/strain

LIMIT OF DETECTION (LOD) AND OF QUANTIFICATION (LOQ)

Implementation of the alternative method alone

A pure *E. coli* strain culture was analyzed with alternative method, at 5 contamination ranges, and repeated 6 times at each range in two kinds of waters : tap water and mineral bottle water.

Contamination ranges were as follows:

Tap water, UFC/100mL	0	0,180	0,224	0,500	1,440
Mineral bottle water UFC/250mL	0	0,050	0,520	0,705	1,030

The limit detection study is done according NF EN ISO 16140 standard protocol.

The following results were obtained:

	Formule	Tap water	Mineral bottle water	
		UFC/100 mL	UFC/250 mL	UFC/100 mL
Critical limit(CL)	$1,65s_0 + x_0$ for $\alpha = 5\%$ (and $1 - \beta = 50\%$)	3,77	2,24	0,90
Limit of detection (LOD)	$3,3s_0 + x_0$ for $\alpha = 5\%$ (and $1 - \beta = 95\%$)	6,05	3,48	1,40
Limit of quantification (LOQ)	$10s_0 + x_0$	15,30	8,52	3,40

- s_0 is the standard deviation and x_0 is the bias.
- α is the probability to detect differences which are not true (false positive).

- β is the probability to not detect a true difference (false negative).
- $1-\beta$ is the probability to detect a value $>$ to CL.

Conclusion

Alternative method LOD and LOQ are adapted to the scope.

SELECTIVITY (INCLUSIVITY / EXCLUSIVITY)

Implementation of the alternative method alone

Inclusivity test

Tests were performed on 43 target strains, including **20 *E. coli* strains** and **23 coliforms strains** (non *E. coli*).

- The 20 *E. coli* strains tested were detected by the alternative method.
- Regarding the 23 coliforms strains different from *E. coli*, one strain was not detected by the alternative method. The strain was *Hafnia alvei*, from foodstuff origin. This strain forms not typical white colonies (GAL-/GLUC-) on the RAPID'E Coli + supplement (water testing) medium. With TTC-tergitol agar-agar, colonies were typical (lactose +)

Exclusivity tests

- The study on 32 non coliform strains gave 3 positives results with the alternative method. Some positives results were noticed with a *Salmonella* strain and a *Shigella sonnei* which both have β -D-Galactosidase and β -D-Glucuronidase (GAL+/GLUC+) activities, and also with an *Erwinia* strain having a β -D-Galactosidase (GAL+/GLUC-) activity.

PRACTICABILITY

Implementation of the alternative method alone

- **Time to results :**
 - **Positive** results were obtained within **1 day with the alternative method** compared to 2 days with the reference method, or even 3 days if some colonies are doubtful.
 - **Negative** results were obtained within **1 day with the alternative method** compared to day with reference method

INTER-LABORATORY STUDY

An inter-laboratory study was carried out in 2011 with 16 collaborating laboratories. The used matrix was spring water, artificially contaminated with a *E. coli* strain (coming from not bottled spring water), at the 4 following contamination levels : 0, 5-10, 30 et 100 UFC/100 mL.

The laboratories analyzed 2 samples per contamination level using each of the two methods (alternative and reference).

The data of 3 laboratories were excluded from the final interpretation because the samples were not received on time.

Calculation of standards deviations of precision per concentration level

For each level of concentration, the standard deviations of repeatability, between series and of reproducibility were calculated from repetitions of the alternative method according to the ISO 5725-2 standard

Precision and accuracy criteria per level are found in the following table, results are in (UFC/100 mL) :

Concentration level	Low	Medium	High
Average theoretical target concentration (in Log N)	0,85	1,30	1,84
Average concentration found (in Log N)	0,68	1,05	1,63
Standard deviation of repeatability	0,23	0,13	0,10
Standard deviation between series	0,30	0,15	0,13
Standard deviation of accuracy	0,38	0,20	0,17
Absolute mean bias	0,17	0,25	0,20
Relative bias	-20%	-19%	-11%

Calculation of tolerance interval

The **tolerance interval** is the interval in which one is expected to find on average a β proportion of future results obtained by using the method in routine, i.e. in conditions of reproducibility.

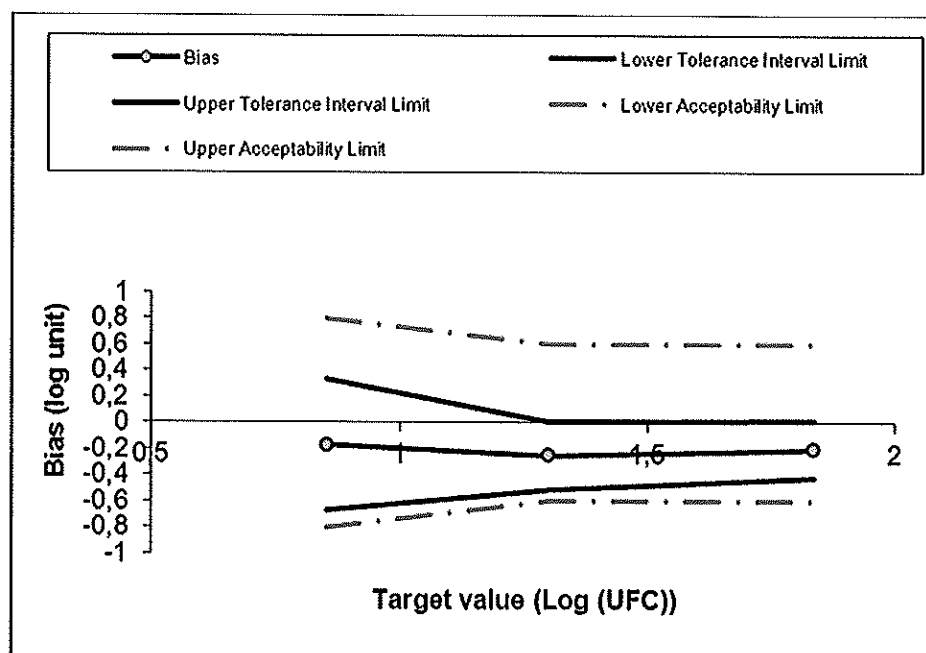
The method proposed by Mee [Mee, 1984] was selected for this protocol. The calculation is based on the data obtained with the alternative method for each level of concentration. The value selected for β must be at least 80%.

The following table unites the calculations of the limits of the tolerance intervals per level, for a margin probability of $\beta=80\%$:

Levels	Low	Medium	High
Average theoretical concentration (log N)	0,85	1,30	1,84
Lower tolerance limit	0,18	0,79	1,41
Upper tolerance limit	1,18	1,31	1,85
Differential lower tolerance limit	-0,67	-0,51	-0,42
Differential upper tolerance limit	0,33	0,01	0,01

Construction of accuracy profile

The data selected in the previous tables were plotted on a graph to build the following accuracy profile



Results interpretation

The horizontal axis of the graph represents the theoretical concentration of the levels and the vertical axis the difference between the theoretical concentration and the found concentration expressed in Log, as the absolute bias. The limits of the tolerance intervals define a domain in which a proportion $\beta=80\%$ of future results is situated.

Then the accuracy profile can be compared to the **acceptability interval** defined according to the objective of the method. The limits of the acceptability intervals are noted λ . The limit of acceptability λ depends on the context of use of the method and β proportion chosen.

The limits of the acceptability intervals, noted $\pm\lambda$ are here $\pm 0,8 \log (\text{UFC}/100\text{mL})$ for level 1 and $\pm 0,6 \log (\text{UFC}/100\text{mL})$ for level 2 and 3

The limits of the acceptability vary with the contamination level, in order to take into account the widespread of results, some enumeration being very few, for the highest contamination level (level 1)

In the scope delimited by the discontinuous vertical lines, the method is capable of producing a β proportion of results falling within the acceptability limits. The alternative method is deemed valid in the entire scope where the tolerance interval falls within the acceptability limits.

The scope of application represents the domain initially chosen to conduct the validation.

The **quantification limit** is defined as the point where the tolerance interval bisects one of the two acceptability limits. This is the limit beyond which the microbiologist can no longer guarantee a β percentage of results obtained using the alternative method that are acceptable. No quantification limit was underline for the contamination scope studied.

Conclusion

The accuracy profile with a margin probability $\beta=80\%$ leads to conclusion that the alternative method is valid for the whole scope of drinking waters.

- For $\lambda = \pm 0,8 \log$ (UFC/100mL), validity is 0,85 to 1,84 log (UFC/100mL)
- For $\lambda = \pm 0,6 \log$ (UFC/100mL), validity is 1,30 to 1,84 log (UFC/100mL).

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