

MICA Advance

Total Coliforms



**SCIENTIFIC
REPORT**

CONTENTS

I.	Introduction.....	3
II.	Principle and procedure	3
	a. Principle	3
	b. Procedure.....	3
	c. Comparison with standard method	3
III.	Performances	4
	1. Inclusivity/exclusivity	4
	a. Selected strains	4
	b. Inclusivity	5
	c. Exclusivity.....	6
	2. Limit of detection and quantification.....	6
	a. LOD and LOQ determination method	6
	b. MICA Advance Total Coliforms LOD and LOQ.....	7
	c. LOD and LOQ performance comparison with other methods.....	7
	3. Sensitivity and specificity of real matrices	8
	a. Listing of tested matrices.....	8
	b. Sensitivity/specificity	8
	4. Comparison of MICA enumeration compared with standard method	10
	5. Accuracy profile.....	11
	6. Repeatability of the method	12
	7. Robustness of the solution: impact of the incubation time.....	13
IV.	Conclusion	14
	Appendix I: Strains tested – comparison enumeration MICA Advance Total Coliforms vs ISO 9308-1	16
	Appendix II: Raw data – accuracy profile	17

I. Introduction

MICA Advance Total Coliforms is a simple and rapid solution that detects and enumerates Total Coliforms in bottled and tap water after 10 hours of incubation.

MICA Advance Total Coliforms gives an enumeration of Total Coliforms in the sample in colony-forming units.

II. Principle and procedure

a. Principle

The principle of MICA Advance Total Coliforms solution procedure is based on the standard method ISO 9308-1 and uses membrane filtration method. The advantages are that MICA Advance Total Coliforms has a shorter incubation time (10 hours) and the enumeration is done automatically.

b. Procedure

Protocol based on the ISO 9308-01 method

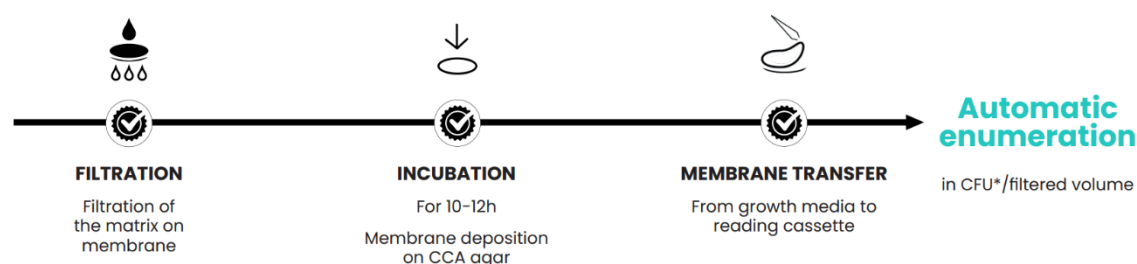


Figure 1: MICA Advance Total Coliforms drinking water process based on membrane filtration

c. Comparison with standard method

ISO 9308-1:2014 is a standard method used for enumeration of Total Coliforms for waters with low bacterial numbers like drinking water. This method is particularly accurate and suitable for microbial analyses in drinking water such as bottled and tap waters.

The culture media used in this standard method and addressed by MICA solution is chromogenic coliform agar (CCA). Incubation conditions are 37°C with both standard and MICA solution. The time to result is 48 hours (including culture incubation and confirmation tests) for standard method compared to 10 hours for MICA Advance method.

	ISO 9308-1:2014	MICA Advance Total Coliforms
Target samples	Drinking waters (bottled and tap waters)	
Target microorganisms	<i>E. coli</i> and other coliforms	Total Coliforms including <i>E. coli</i>
Temperature	37°C	37°C
Incubation	48h	From 10 to maximum 12h

Table 1: ISO 9308-1:2014 vs MICA Advance Total Coliforms

III. Performances

The results shown below were acquired using 0.45µm sterile MCE membranes (gridded Merck Millipore HAWG047S6 and EZHAWG474) and chromogenic coliform agar (Oxoid CM1205B).

1. Inclusivity/exclusivity

a. Selected strains

For inclusivity and exclusivity, we tested strains representative of those found in drinking water samples. The strains tested were collection strains or strains isolated from real matrices from customers or prospects around the world.

- For inclusivity, 20 strains of coliforms were tested: 5 strains of *E. coli* and 15 strains of other coliforms. The bacterial load was 30-50 bacteria/membrane.
- For exclusivity, 20 strains of contaminants were tested. The bacterial load was 10^3 bacteria/membrane.

The species selected in the data set are well documented and representative of the strains in drinking water samples¹⁻⁶.

Collection strains come from Leibniz Institute in Germany (DSMZ), the Biological Resources Center of the Pasteur Institute in France (CIP) and American Type Culture Collection (ATCC).

For inclusivity and exclusivity testing, all strains were diluted in 0.9% sterile NaCl, filtered and analyzed with MICA Advance Total Coliforms method developed by Diamidex and the results were compared to those obtained with the standards. The strains were stored in 20% glycerol stocks prepared from stationary phase cultures to imitate stressful environmental conditions.

b. Inclusivity

Number of strains tested	Origin	MICA Total Coliforms	ISO 9308-1 : 2014 21h
20 coliforms	<i>E. coli</i> ATCC 11775	+	+
	<i>E. coli</i> ATCC 8739	+	+
	<i>E. coli</i> ATCC 25922	+	+
	<i>E. coli</i> ATCC 13706	+	+
	<i>E. coli</i> ATCC 23631	+	+
	<i>Klebsiella variicola</i> ATCC 31488	+	+
	<i>Citrobacter freundii</i> ATCC 8090	+	+
	<i>Citrobacter koseri</i> ATCC 27028	+	+
	<i>Citrobacter sakazakii</i> CIP 103183T	+	+
	<i>Enterobacter aerogenes</i> ATCC 35028	+	+
	<i>Klebsiella oxytoca</i> ATCC 13182	+	+
	<i>Klebsiella pneumoniae</i> ATCC 13883	+	+
	<i>Raoultella planticola</i> CIP 100751T	+	+
	<i>Serratia marcescens</i> environmental	+	+
	<i>Enterobacter cloacae cplx</i> environmental	+	+
	<i>Raoultella (klebsiella) ornithinolytica</i> environmental	+	+
	<i>Citrobacter braakii</i> environmental	+	+
	<i>Enterobacter hormaeche</i> environmental	+	+
	<i>Kluyvera sp.</i> DSM 30112	+	+
	<i>Leclercia sp.</i> DSM 22903	+	+

Table 2: Inclusivity testing

Conclusion: MICA Advance Total Coliforms **detects 100% of the coliform** strains tested as the standard method.

c. Exclusivity

Number of strains tested	Origin	MICA Total Coliforms	ISO 9308-1 : 2014 21h
20 contaminants	<i>Acinetobacter baumannii</i> ATCC 19606	-	-
	<i>Acinetobacter calcoaceticus lwoffii</i> CIP 70.17	-	-
	<i>Acinetobacter haemolyticus</i> CIP 64.3T	-	-
	<i>Acinetobacter radioresistens</i> CIP 64.3T	-	-
	<i>Aeromonas allosaccharophila</i> DSM 11576	-	-
	<i>Alcaligenes faecalis</i> environmental	-	-
	<i>Buttiauxella sp.</i> DSM 105071	-	-
	<i>Bacillus cereus</i> environmental	-	-
	<i>Comamonas aquatica</i> environmental	-	-
	<i>Enterococcus faecalis</i> ATCC 19433	-	-
	<i>Proteus mirabilis</i> ATCC 29906	-	-
	<i>Providencia rettgeri</i> ATCC 29944	-	-
	<i>Providencia rettgeri</i> CIP 103182T	-	-
	<i>Providencia stuartii</i> ATCC 29914	-	-
	<i>Salmonella enteritidis</i> CIP 82.97	-	-
	<i>Salmonella typhimurium</i> CIP 58.58	-	-
	<i>Shigella flexneri</i> ATCC 12022	-	-
	<i>Staphylococcus aureus</i> ATCC 6538	-	-
	<i>Staphylococcus pasteurii</i> environmental	-	-
	<i>Vibrio parahaemolyticus</i> ATCC 17802	-	-

Table 3: Exclusivity testing

Conclusion: MICA Advance Total Coliforms have negative detection for 100% of non-coliforms strains*.

*Note: As the standard method, MICA Advance Total Coliforms gave false positive result for beta-glucuronidase positive microorganisms other than *E. coli* such as some *Shigella sonnei* strains.

2. Limit of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) have been determined according to ISO 16140-4.

a. LOD and LOQ determination method

The relative detection and quantification level using the 50% cut-off point known as LOD₅₀ and LOQ₅₀ were used to estimate the limit of detection and quantification of MICA Advance Total Coliforms. These calculations were performed using the Spearman-Kärber method (calculated using formula 3.3 $S_0 + X_0$ and $10 S_0 + X_0$ of the first level with 50% positive results for LOD and LOQ respectively). LOD₅₀ can also be interpreted as the level of contamination that would theoretically correspond to a recovery rate of 50%.

b. MICA Advance Total Coliforms LOD and LOQ

To estimate the LOD₅₀ and LOQ₅₀, *Klebsiella variicola* ATCC 31488 was spiked in tap water with 4 different contamination levels. For each level, 6 membranes were analyzed.

Spiking level	Analyzed samples	MICA Total Coliforms positive results	Standard deviation (S ₀)	Biais (X ₀)
0 CFU/ 100mL	6	0	0	0
0.5 CFU/ 100mL	6	3	0.548	0.5
0.75 CFU/ 100mL	6	4	0.516	1
2.2 CFU/ 100mL	6	6	1.52	2.5

Table 4: Results of MICA Advance Total Coliforms for different replicates and levels of contamination for *Klebsiella variicola* ATCC 31488

Using the Spearman-Kärber method, the relative level of detection LOD₅₀ of the MICA Advance Total Coliforms method is 3 CFU/100 mL and the relative level of quantification LOQ₅₀ is 7.4 CFU/mL.

c. LOD and LOQ performance comparison with other methods

The LOD₅₀ and LOQ₅₀ of the following solutions were calculated using the same method as for MICA Advance Total coliforms:

Solution	LOD ₅₀	LOQ ₅₀	Reference
Compass cc Agar	6 CFU/250 mL	16 CFU/100 mL	Afnor validation Certificate BKR 23/08-06-12 by microsept ⁷
Colilert-18	2.3 CFU/100 mL	6.3 CFU/100 mL	Afnor validation Certificate 33/01-11-09 by microsept ⁸

Table 5: Performance of LOD and LOQ of other solutions for Total Coliforms detection

MICA Advance Total Coliforms **LOD and LOQ performances are better or equivalent of other solutions** for Total coliforms detection in drinking waters.

3. Sensitivity and specificity of real matrices

We tested real matrices of bottled waters and mains waters.

As naturally contaminated real matrices are difficult to obtain, we artificially contaminated drinking water with surface water to obtain positive samples of real matrices.

250 mL and 100 mL of sample were analyzed for bottled waters and mains waters respectively.

a. Listing of tested matrices

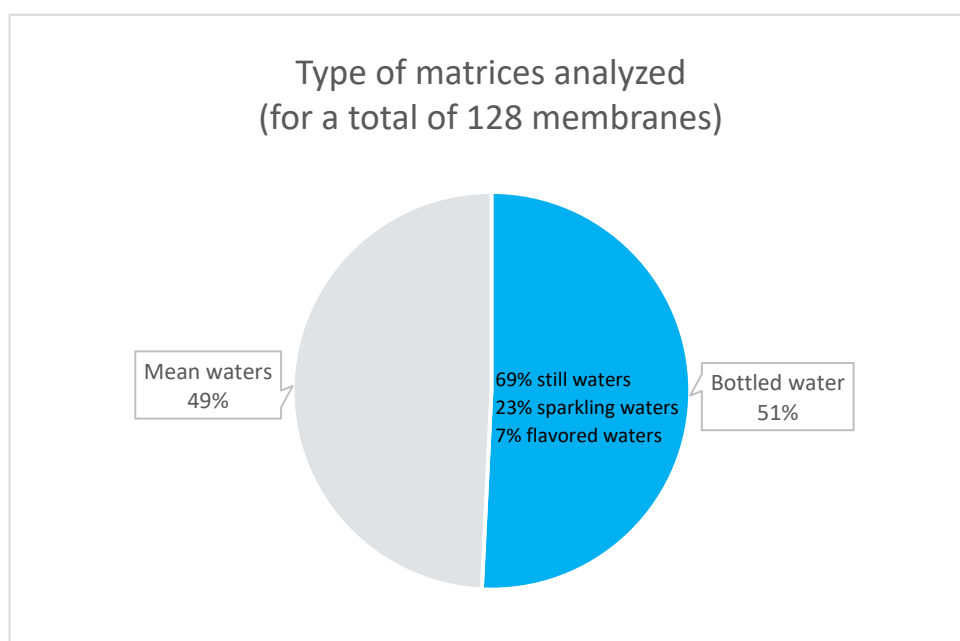


Figure 2: Type of matrices analyzed for MICA Advance Total Coliforms enumeration

For bottled waters, 24 different brands were tested (18 of still water, 6 of sparkling waters including one flavored sparkling water and one flavored still water). Waters came from Europe (France, Norway, Italy, Spain, Austria, Portugal, Germany) and around the world (Fiji Islands, Iceland, ...).

b. Sensitivity/specificity

For positive samples, between 100µL-10mL of surface waters (containing *E. coli* and other coliforms) were spiked in drinking water matrices (see 3.a) to obtain positive samples with a range concentration of total coliforms between 5-100 CFU/volume filtered.

Each sample was tested with both MICA and the standard method. Every analysis with a result >0 CFU/volume filtered was considered positive, otherwise it was considered negative.

		MICA	
		+	-
ISO	+	75	3
	-	0	50

Table 6: Contingency table of the MICA Advance Total Coliforms real samples analysis

Performances	
Sensitivity	0.96
Specificity	1
Positive predictive value	1
Negative predictive value	0.94

Table 7: Performances of the MICA Advance Total Coliforms

We calculated the Kappa Cohen coefficient K which measures the agreement between two measurements. The coefficient K is calculated as follow:

$$K = \frac{Po - Pe}{1 - Pe}$$

With:

		MICA		
		+	-	
ISO	+	a	b	n ₁
	-	c	d	n ₂
		t ₁	t ₂	N

$$Po = \frac{a + d}{N}$$

$$Pe = \frac{n_1}{N} \times \frac{t_1}{N} + \frac{n_2}{N} \times \frac{t_2}{N}$$

The calculated **Kappa coefficient K for MICA Advance Total coliforms** compared to ISO 9308-1 is:

$$K = 0.95$$

which corresponds to a **level of agreement almost perfect** according to the interpretation of the Cohen Kappa coefficient⁹:

Value of Kappa	Level of agreement	% of reliable data
0-0.2	None	0-4%
0.21-0.39	Minimal	4-15%
0.4-0.59	Weak	15-35%
0.60-0.79	Moderate	35-63%
0.80-0.90	Strong	64-81%
0.91-1	Almost perfect	82-10%

Table 8: Interpretation of Cohen's Kappa coefficient

4. Comparison of MICA enumeration compared with standard method

For the comparison of MICA enumeration with standard method, we tested strains representative of those found in drinking water samples. The strains tested were collection strains or strains isolated from real matrices from customers or prospects around the world (see Appendix I: Strains tested - comparison MICA Advance Total Coliforms – ISO 9308-1:2014).

All strains were diluted in drinking water or 0.9% sterile NaCl, filtered and analyzed with MICA Advance Total Coliforms method developed by Diamidex and the results were compared to those obtained with the standard method (ISO 9308-1:2014). The strains used were microorganisms stored in 20% glycerol stocks prepared from stationary phase cultures to imitate stressful environmental conditions or microorganisms from Bioréférence pellets (used for quality control in water testing).

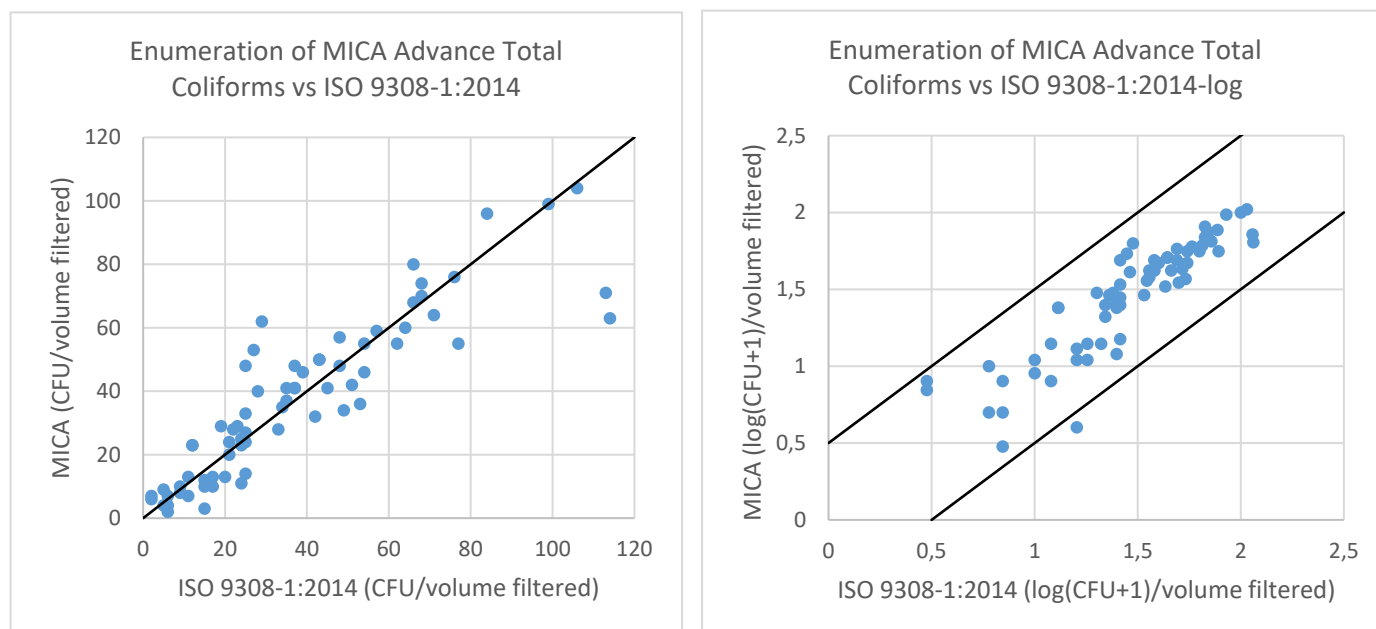


Figure 3: Comparison of the enumeration of MICA Advance Total Coliforms vs ISO 9308-1:2014

MICA Advance Total Coliforms shows **good correlation** for the enumeration compared to the standard method ISO 9308-1.

5. Accuracy profile

We determine the accuracy profile according to the ISO 16140-2: Protocol for validation of alternative methods against a reference method.

To determine the accuracy profile one strain of *Citrobacter freundii* (ATCC 8090) and one strain of *Klebsiella oxytoca* (ATCC 1382) were spiked in drinking water at 3 different levels of contamination (high, medium, low) as recommended in the ISO 16140-2. 5 replicates of each strain for each level of contamination were analyzed with both MICA and standard method. Raw data are shown in appendix II.

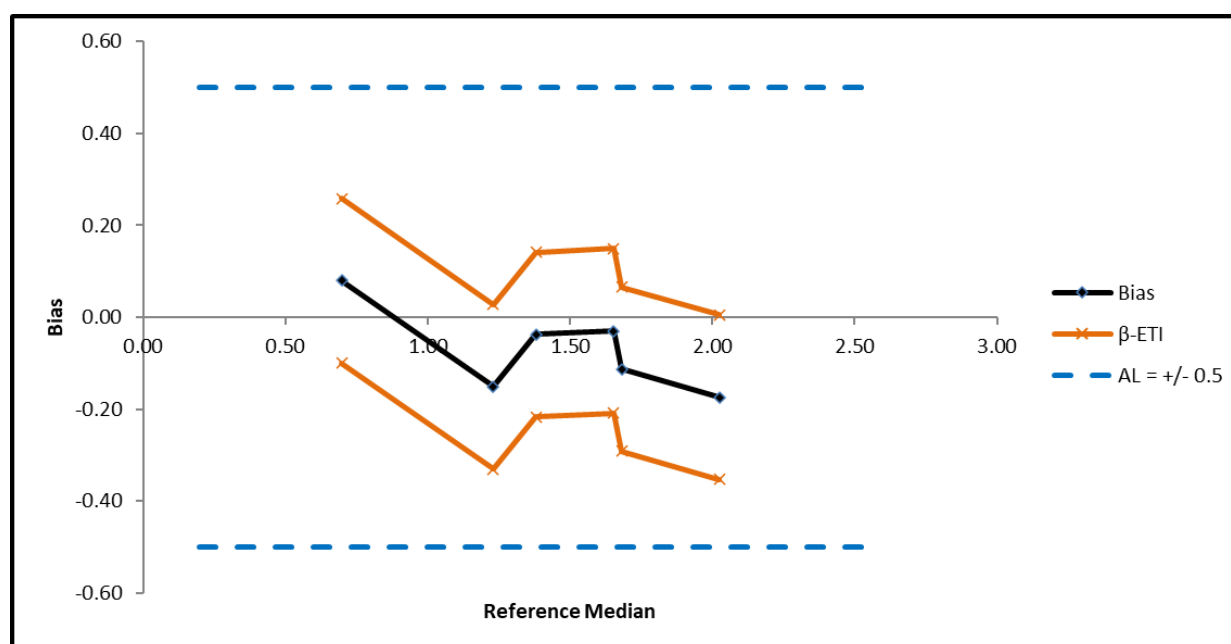


Figure 4: Accuracy profile of the MICA Advance Total compared to ISO 9308-1:2014

Sample Name	Reference Central value	Bias	Lower β-ETI*	Upper β-ETI	β-ETI compared to AL*=±0.5 Acceptable	β-ETI compared to final AL Acceptable
Low	0.70	0.079	-0.100	0.258	YES	YES
Low	1.23	-0.151	-0.330	0.028	YES	YES
Intermediate	1.38	-0.038	-0.217	0.141	YES	YES
Intermediate	1.65	-0.030	-0.209	0.149	YES	YES
High	1.68	-0.113	-0.292	0.066	YES	YES
High	2.03	-0.174	-0.353	0.005	YES	YES

Table 9: Accuracy profile results

*β-ETI: Esperance tolerance interval

*AL: Acceptable limits

The tolerance intervals β-ETI of MICA Advance Total coliforms are within acceptable limits (AL ± 0.5 log). **According to the accuracy profile, MICA Advance Total Coliforms is equivalent to ISO 9308-1 for drinking water.**

6. Repeatability of the method

The repeatability of our method has been determined following ISO 13843 procedure.

We assessed the repeatability of our method. It is expressed by the repeatability overdispersion factor u_r^2 , which represents the excess dispersion of the results compared with the dispersion predicted by Poisson's law.

To calculate the u_r^2 factor, we used the strains *Klebsiella aerogenes* ATCC 13048, *Citrobacter freundii* ATCC 43864, *E. coli* ATCC 8739 recovered from Bioréférence pellets, Eurofins (calibrated pellet of microorganisms). Under repeatable conditions, we made 10 replicates of each strain using the MICA Total Coliforms protocol.

Strain	Replicates (CFU/volume filtered)									
<i>E. coli</i> ATCC 8739	7	7	7	6	9	4	13	7	3	7
<i>Klebsiella aerogenes</i> ATCC 13048	8	10	2	7	6	12	7	11	9	11
<i>Citrobacter freundii</i> ATCC 43864	23	14	8	15	13	7	12	22	13	11

Table 10: Raw results for the repeatability uncertainty measurement

For each strain i , we calculate the mean $\bar{x}_i$, the standard deviation s_i , the coefficient of variation $CV_i = \frac{s_i}{\bar{x}_i}$ and the overdispersion factor $u_{r,i}^2 = CV_i^2 - \frac{1}{\bar{x}_i}$. Then, we calculated $A = \text{mean}(u_{r,i}^2)$. If $A < 0$, $u_r^2 = 0$, otherwise $u_r^2 = A$.

For the MICA Advance Total Coliforms solution:

$$u_r^2 = 0.029$$

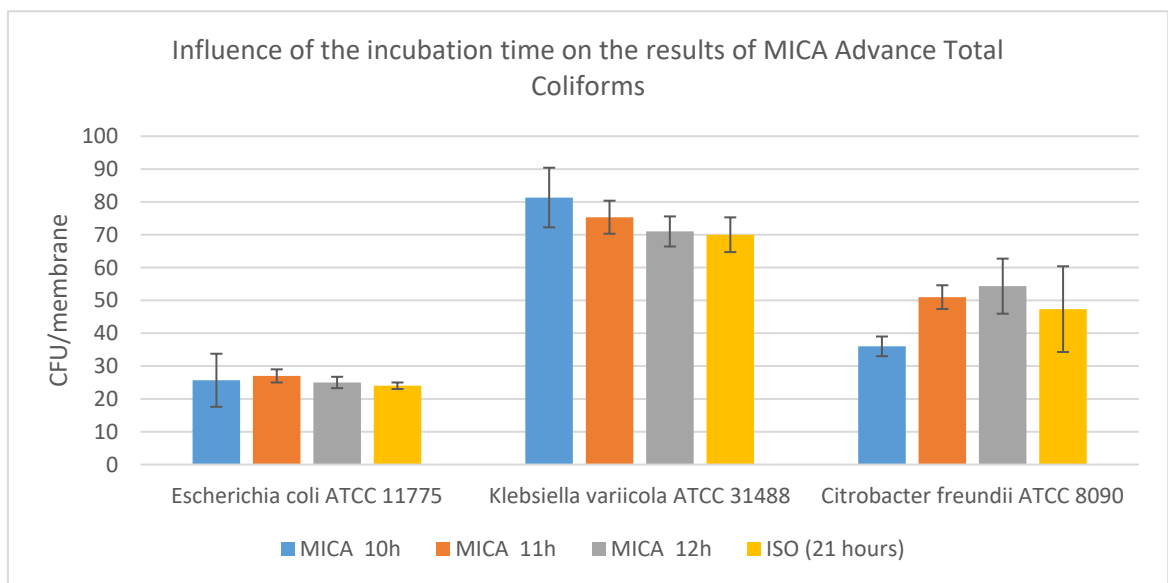
(<0.05) which corresponds to a **weak uncertainty for repeatability**¹⁰.

7. Robustness of the solution: impact of the incubation time

The robustness of our method has been determined following ISO 13843 procedure.

We evaluated the impact of the incubation time (between 10 to 12 hours) on the performance of the solution.

3 coliforms strains (*E. coli* ATCC 11775, *Klebsiella variicola* ATCC 31488 and *Citrobacter freundii* ATCC 8090) were spiked in sterile NaCl 0.9% and analyzed after 10, 11 and 12 hours of incubation (different membranes were used for each incubation time).



All the results are equivalent at 10, 11 and 12 hours of incubation and are also equivalent to the results of the standard method. **The MICA Advance solution demonstrates robust performance regarding incubation time variability.**

IV. Conclusion

MICA Advance Total Coliforms solution detects and enumerates total coliforms (including *E. coli*) in bottled and tap waters. The method was compared to the ISO 9308-1:2014.

The comparative study results show that:

- MICA Advance Total Coliforms **detects 100% of the coliform strains** of the inclusivity testing and **detects 0% of the non-coliforms strains** of the exclusivity testing compared to the reference method.
- The **LOD and LOQ** of our method are **3 CFU/100mL and 7.4 CFU/100mL** which are better or equivalent of the performances of other alternative methods (CC Compass Agar, Colilert-18).
- For real matrices, the **sensitivity and specificity of our method are respectively 0.96 and 1** which correspond to a **level of agreement almost perfect** according to the interpretation of Kappa coefficient.
- For enumeration, **MICA Advance Total Coliforms** shows **good correlation** compared to the standard method ISO 9308-1.
- The **tolerance intervals** β -ETI of MICA Advance Total coliforms **are within acceptable limits** ($AL \pm 0.5 \log$).
- The repeatability factor $u^2_r = 0.029$ which corresponds to a **weak uncertainty for repeatability**.
- The MICA Advance solution demonstrates **robust performance regarding incubation time variability**.

Annex I: bibliographic references

1. Blanch AR, Galofré B, Lucena F, Terradillos A, Vilanova X, Ribas F. Characterization of bacterial coliform occurrences in different zones of a drinking water distribution system. *J Appl Microbiol.* 2007 Mar;102(3):711-21. doi: 10.1111/j.1365-2672.2006.03141.x. PMID: 17309620.
2. Vinita Rawat, Sanjay Kumar Jha, Arundhati Bag, Monil Singhai, Chandra Mohan Singh Rawat; The bacteriological quality of drinking water in Haldwani Block of Nainital District, Uttarakhand, India. *J Water Health* 1 September 2012; 10 (3): 465–470. doi: <https://doi.org/10.2166/wh.2012.035>
3. Botsaris, G., Kanetis, L., Slaný, M. *et al.* Microbial quality and molecular identification of cultivable microorganisms isolated from an urban drinking water distribution system (Limassol, Cyprus). *Environ Monit Assess* **187**, 739 (2015). <https://doi.org/10.1007/s10661-015-4957-9>
4. Peter Kämpfer, Anita Nienhüser, Gabriele Packroff, Frank Wernicke, Arnd Mehling, Katja Nixdorf, Stefanie Fiedler, Claudia Kolauch, Michael Esser, Molecular identification of coliform bacteria isolated from drinking water reservoirs with traditional methods and the Colilert-18 system, *International Journal of Hygiene and Environmental Health*, Volume 211, Issues 3–4, 2008, Pages 374-384, <https://doi.org/10.1016/j.ijheh.2007.07.021>.
5. Carolin Reitter, Heike Petzoldt, Andreas Korth, Felix Schwab, Claudia Stange, Beate Hambsch, Andreas Tiehm, Ilias Lagkouvardos, Johannes Gescher, Michael Hügler, Seasonal dynamics in the number and composition of coliform bacteria in drinking water reservoirs, *Science of The Total Environment*, Volume 787, 2021, <https://doi.org/10.1016/j.scitotenv.2021.147539>.
6. Tokajian S, Hashwa F. Incidence of antibiotic resistance in coliforms from drinking water and their identification using the Biolog and the API identification systems. *J Chemother.* 2004 Feb;16(1):45-50. doi: 10.1179/joc.2004.16.1.45. PMID: 15077998.
7. Afnor validation Certificate BKR 23/08- 06/12. For enumeration of *Escherichia coli* and coliforms. Protocol for water human consumption.
8. Afnor validation Certificate IDX 33/01-11/09 for the enumeration of *Escherichia coli* and coliforms in drinking water (except bottled waters).
9. McHugh ML. Interrater reliability: the kappa statistic. *Biochem Med (Zagreb).* 2012;22(3):276-82. PMID: 23092060
10. Report of AGLAE (accredited interlaboratory comparison organization NF EN ISO/CEI 17043).

Appendix I: Strains tested – comparison enumeration MICA Advance Total Coliforms vs ISO 9308-1

Strains	Origin
<i>Escherichia coli</i>	ATCC 25922
<i>Escherichia coli</i>	ATCC 11775
<i>Klebsiella variicola</i>	ATCC 31488
<i>Citrobacter freundii</i>	ATCC 8090
<i>Citrobacter koseri</i>	ATCC 27028
<i>Enterobacter aerogenes</i>	ATCC 35028
<i>Klebsiella oxytoca</i>	ATCC 13182
<i>Klebsiella pneumoniae</i>	ATCC 13883
<i>Raoultella planticola</i>	CIP 100751T
<i>Escherichia coli</i>	ATCC 8739
<i>Escherichia coli</i>	ATCC 13706
<i>Escherichia coli</i>	ATCC 23631
<i>Serratia marcescens</i>	Drinking water
<i>Enterobacter cloacae</i> cplx	Drinking water
<i>Raoultella (klebsiella) ornithinolytica</i>	Drinking water
<i>Enterobacter hormaechei</i>	Environmental
<i>Kluyvera</i> sp	DSM 30112
<i>Leclercia</i> sp	DSM 22903
<i>Klebsiella aerogenes</i>	ATCC 13048
<i>Citrobacter freundii</i>	ATCC 43864

Appendix II: Raw data – accuracy profile

Level	Reference method result					Alternative method result				
	rep 1	rep 2	rep 3	rep 4	rep 5	rep 1	rep 2	rep 3	rep 4	rep 5
Low	5	15	2	9	2	4	3	6	10	7
Low	12	17	15	17	24	23	13	12	10	11
Intermediate	24	20	24	28	40	25	21	21	22	28
Intermediate	51	45	42	39	48	42	41	32	46	48
High	41	53	48	41	50	37	27	25	37	43
High	84	106	64	114	113	96	104	60	63	71



Technical support

Email : **support@diamidex.com**

Tel : +33 7 61 90 45 95

Monday to Friday – 9.00 to 18.00
Central European Time (UTC+1)

