

# Protocol of Water Test with AOT

## 1 Scope and Application

This standard is applicable to the enumeration of aerobic bacteria and Coliform bacteria in water.

This standard provides the MBS method for the enumeration in water samples of:

Aerobic bacteria or TVC (Total Viable Cells)

Total Coliforms

Thermotolerant Coliforms

*E. coli*

This standard is applicable to fresh water samples coming from rivers, natural or artificial lakes, civil and industrial sewages, potabilization plants.

This standard has a sensitivity of

From  $\leq 14$  CFU/100 ml up to  $1e9$  CFU/100ml when MBS method is used for counting bacteria eventually present on water samples of 100 ml.

Or

From  $\leq 1$  CFU/ml up to  $1e9$  CFU/1 ml when MBS method is used for counting bacteria eventually present on water samples of 1 ml.

## 2 Summary of the Method

This standard is based on MBS method which is represented by a colorimetric change occurring in disposable and single use vials. The color change is induced by the presence of bacteria.

The volume of the water samples to be analyzed could be either 1 ml or 100 ml.

The water samples have to be collected in the same way used for all microbiological methods of analysis.

No water pretreatment is required for MBS method other than the usual treatments required for all the microbiological methods for water analysis

The MBS method requires vial incubation at suitable temperatures into a thermostatic incubator. The incubation temperatures are identical to those used in microbiological analysis based on bacteria multiplication.

The MBS method is based on the presence of redox dyes that measure the redox state of the original medium contained into the disposable and single use vials. In the presence of bacteria, the redox state of the medium contained into the vials is modified and the redox dyes change color.

In the MBS method, the time required for color change in the presence of bacteria is inversely related to the number of bacteria present into the sample. Greater the number of bacteria present into the sample, lesser the time required for color change. If no bacteria are present into the sample, there is no color change.

The color change can be detected by a simple visual inspection of the color of the vial at different times after sample addition and incubation at a suitable temperature into a thermostatic incubator.

The color change can be also detected by a suitable instrument (the MBS Optical Reader) able to keep the vial at a suitable temperature, to look at the color change, to correlate the color change with the time after sample addition and to calculate directly the number of bacteria present into the sample.

### **3 Definitions of Method**

The following terms and definitions apply to this Standard:

Aerobic bacteria or TVC (Total Viable Cells): mesophilic aerobic bacteria or facultative anaerobic bacteria growing at and able to consume oxygen.

Total Coliforms: Gram-negative non-spore forming bacteria which can ferment lactose with

The production of acid and gas when incubated at 35–37 °C.

Thermotolerant Coliforms: Gram-negative non-spore forming bacteria which can ferment lactose with the production of acid and gas when incubated at. They are also known as Fecal Coliforms since are indicators of fecal contamination

*E. coli*: Gram-negative non-spore forming bacteria which can ferment lactose with the production of acid and can convert tryptophan into indole when incubated at.

MBS method: colorimetric method for bacterial enumeration based on the inverse correlation between bacteria concentrations and time required for color change.

MBS TVC VIALS: disposable, single use and ready to use vials prefilled with all the reagents required for the analysis of Total Viable Cells.

MBS TOTAL COLIFORMS VIALS: disposable, single use and ready to use vials prefilled with all the reagents required for the analysis of Total Coliforms,

MBS THERMOTOLERANT COLIFORMS VIALS: disposable, single use and ready to use vials prefilled with all the reagents required for the analysis of thermotolerant and fecal Coliforms,

MBS ESCHERICHIA COLI VIALS: disposable, single use and ready to use vials prefilled with all the reagents required for the analysis of *Escherichia coli*,

MBS AMPOULE: disposable, single use and ready to use ampoule filled with 10 ml of sterile water.

MBS OPTICAL READER(AOT): instrument able to incubate the MBS vials at the required temperatures, to detect the color change of the medium and to automatically

correlate the time required for color change with the number of bacteria present into the sample.

MPN: A Poisson distribution-based indirect counting method as MPN method in GBT 5750.12-2006.

Correlation table: table correlating the times required for color change with the bacterial concentrations present into the water samples. It is reported in the Quality Control Sheet

Present into each MBS vial pack.

Reference Colors: Pictures of the starting and final colors depicted in the Quality Control Sheet present into each MBS vial pack.

#### **4 Interferences**

As for all the microbiological methods of analysis, possible interference may arise by the presence in the water sample to be analyzed of high concentrations of antibacterial substances like detergents.

As for all the microbiological methods of analysis, in the presence of chlorine it is required to collect the water samples using bottles pre-filled with suitable amount of Sodium thiosulphate.

Specific interference with MBS method arises from the presence in the water sample of high concentrations of strong oxidants and reductants. Such possible interference is revealed by a change in the color of the vials immediately after sample addition. If the color of the vial immediately after sample addition is like the Reference starting color depicted in the Quality Control Sheet present into each MBS vial pack, there will be no interference.

#### **5 Safeties**

##### **HAZARDOUS COMPONENTS PRESENT INTO THE VIALS**

Name: 4-[(2R)-2-[(1S, 3S, 5S)-3, 5-dimethyl-2-oxocyclohexyl]-2-hydroxyethyl]piperidine-2, 6-dione

CAS number: 66-81-9

Hazard Symbols: T+, N

Risk Phrases: R28 - R51/53 - R68 - R61

Name: 4-[3-(4-hydroxyphenyl)-1, 1-dioxobenzo[c]oxathiol-3-yl] phenol

CAS number: 143-74-8

Hazard Symbols: Xi

Risk Phrases: R36/37/38

Name: sodium 1, 5-dichloro-4, 6-dioxo-1, 3, 5-triazin-2-olate dehydrate

CAS number: 51580-86-0

Risk phrases: R22-31-36/37

Classification and labeling:

Indications of danger: Xn: Harmful; N: Dangerous for the environment.

Risk phrases: R36/37/38: Irritating to eyes, respiratory system and skin.

Safety advice: S2-26-46-8-60-61: Keep out of reach of children. In case of contact with eyes, rinse immediately with plenty of water and consult a specialist. If swallowed, seek medical advice immediately inform him with the

packaging or label. Keep container dry. Avoid release into the environment. Refer to special instructions/safety data sheets.

Disclaimer: the above information is believed to be correct, but cannot be exhaustive and shall be used only as a guide. The producer can't be held responsible for any damage resulting from handling or from contact with the product.

## 6 Equipment and Supplies

Except conventional sterilization equipment's present in microbiology laboratories, other equipment's and materials are as follows:

**MBS vials and ampoule are provided by the producer.** This information is for the convenience of the user of the Standard, not necessarily indicate that the product is recognized. If any other equivalent product has the same effect, these equivalents shall be applied.

**Thermostatic incubator:** to incubate the vials at a constant temperature of  $\pm$  or  $\pm$ .

**Alternatively to the Thermostatic incubator, an optional Optical Reader device can be used.** This

Instrument should be able to incubate the vials at the required temperatures, to detect the color change of the medium and to automatically correlate the time required for color change with the number of bacteria present into the sample. The characteristics of the Optical Reader are reported in Appendix 3.

**Sterile pipettes:** 2 ml (with a scale  $\leq$  0.02 ml) or micropipette and tips.

## 7 Reagents and Standards

**No other reagent is required.**

## 8 Sample Collections, Preservation and Storage

The water samples should be collected, preserved, shipped and stored as for all the methods for water microbiological analysis.

## 9 Quality Control

A Quality Control Sheet is contained in each pack of vials. The Quality Control Sheet reports the analysis carried out for the specific production batch, the table correlating the time required for color change with the bacterial concentrations and the pictures of the starting and final colors.

Quality control of the vial starting color by consumers: look at the starting color immediately after sample addition.

Quality control of the vial final color by consumers: it is possible to look at the final color change 24 hours after addition of a 1:10'000 dilution of an overnight culture of *E.coli* ATCC 25922 and incubation at the temperature of or, accordingly with the vial to be checked. Compare the initial and final color with those provided in the Quality Control Sheet included in the vial pack

Quality control of the optional optical reader can be done by the consumers by incubating a

Vials filled with sterile water and another vial filled with a 1:10'000 dilution of an overnight culture of *E.coli* ATCC 25922

## 10 Calibrations and Standardization

Calibration experiments are usually done **only** if required by Authorities.

Calibration experiments can be done by comparing the results obtained by MBS method with the results obtained by a reference method.

For calibration experiments it is strictly required by Good Laboratory Practice to verify before the calibration that the reference method gives correct estimate of the bacterial concentrations in the water samples. For the verification of the correct use of the reference method, artificially and certified contaminated samples should be used. Certified samples containing different concentrations of *E. coli* can be obtained from Official Certification Authorities with microorganism concentrations certified according with ISO 30:1992. Run the reference method after diluting the samples with certified bacterial concentrations into sterilized water samples to be analyzed and check if the bacterial concentrations obtained by the reference method are equivalent to the certified bacterial concentrations.

The calibration experiments should be performed with naturally contaminated samples.

In the absence of water samples naturally contaminated, it is possible to artificially contaminate water samples using as reference microorganism *E. coli* ATCC 25922. This microorganisms should be added at different concentrations to previously sterilized water samples and let starved into the water samples for 48 hours at  $10 \pm$  . After 48 hours *E. coli* ATCC 25922 starvation into the water sample, these artificially contaminated water samples can be analyzed by both reference method and MBS method.

The calibration experiments should be run by simultaneous analyzing by both the reference method and MBS methods 4 samples with very different bacterial concentrations (e.g. no

Bacteria,  $1e2$  CFU/ml  $1e4$  CFU/ml,  $1e6$  CFU/ml). Each sample should be analyzed in quintuplicate.

Statistical analysis such as should be carried out to evaluate the differences between the reference method and MBS method.

The following parameters could be calculated:

**Accuracy** (using One-way analysis of variance and Two-way analysis of variance).

**Reliability** (using the statistical analysis of Coefficient of Variation (CV)).



**Uncertainty** (using the statistical analysis of chi-square test ( $\chi^2$ )).

In the presence of statistically significant differences between the results obtained by the reference method and the MBS method (possibly due to the presence of substances inhibiting bacterial metabolism in the water samples), it is possible to modify the parameters correlating bacterial concentrations into the water samples with the time required for color change. Ask the MBS method producer to obtain the new parameters suitable for the analysis of that water samples.

## 11 Analytical Procedures

For the analysis of Total Viable Cells, a single vial can be used (refer to Appendix 1: Operational procedures for the analysis of 1 ml of water).

For the analysis of Total Coliforms, Thermotolerant Coliforms and *E. coli*, a preliminary evaluation of the bacterial content of the water sample to be analyzed should be obtained. If the estimation of bacterial content of the water sample to be analyzed is  $\geq 1'000$  CFU/ml, a single vial can be used (refer to Appendix 1: Operational procedures for the analysis of 1 ml of water). If the estimation of bacterial content of the water sample to be analyzed is  $\leq 1'000$  CFU/ml, four vials should be used (refer to Appendix 2: Operational procedures for the analysis of 100 ml of water). In the absence of a preliminary evaluation of the bacterial content of the water sample to be analyzed, it is required to carry out the analysis according to Appendix 2: Operational procedures for the analysis of 100 ml of water.

### Appendix 1: Operational procedures for the analysis of 1 ml of water

#### 1.1 Total Viable Cells test

Rehydrate 1 MBS TVC vial with the ampoule filled with 10ml of sterile water and shake at 1400 ~ 1600r/min for 20 seconds until the reagent is completely dissolved and wait for 15 minutes before starting the analysis.

Using a sterile pipette, inoculate the vial with 1 ml of the water sample being sure to reach under the Vaseline layer and shake again at 1400 ~ 1600r/min for 20 seconds.

The starting color of the vial should correspond to the starting color indicated in the Quality Control Sheet. If the color is very different, interference could be present (see paragraph 4 Interferences)

Incubate the vials at in thermostat or in MBS Optical Reader.

### **1.2 Total Coliforms test**

Rehydrate 1 MBS COLI vial with the ampoule filled with 10ml of sterile water and shake at 1400 ~ 1600r/min for 20 seconds until the reagent is completely dissolved and wait for 15 minutes before starting the analysis.

Using a sterile pipette, inoculate the vial with 1 ml of the water sample being sure to reach under the Vaseline layer and shake again at 1400 ~ 1600r/min for 20 seconds.

The starting color of the vial should correspond to the starting color indicated in the Quality Control Sheet. If the color is very different, interference could be present (see paragraph 4 Interferences)

Incubate the vials at in thermostat or in MBS Optical Reader

### **1.3 Thermotolerant Coliforms test**

Rehydrate 1 MBS COLI vial with the ampoule filled with 10ml of sterile water and shake at 1400 ~ 1600r/min for 20 seconds until the reagent is completely dissolved and wait for 15 minutes before starting the analysis.

Using a sterile pipette, inoculate the vial with 1 ml of the water sample being sure to reach under the Vaseline layer and shake again at 1400 ~ 1600r/min for 20 seconds.

The starting color of the vial should correspond to the starting color indicated in the Quality Control Sheet. If the color is very different, interference could be present (see paragraph 4 Interferences)

Incubate the vial at in thermostat or in MBS Optical Reader

#### **1.4 *E. coli* test**

Rehydrate 1 MBS COLI vial with the ampoule filled with 10ml of sterile water and shake at 1400 ~ 1600r/min for 20 seconds until the reagent is completely dissolved and wait for 15 minutes before starting the analysis.

Using a sterile pipette, inoculate the vial with 1 ml of the water sample being sure to reach under the Vaseline layer and shake again at 1400 ~ 1600r/min for 20 seconds.

The starting color of the vial should correspond to the starting color indicated in the Quality Control Sheet. If the color is very different, interference could be present (see paragraph 4 Interferences)

Incubate the vial at in thermostat or in MBS Optical Reader

#### **1.5 Control of the analysis result**

When the optional MBS Optical Reader is used, it is not necessary to look at the vials at different times nor to calculate the bacterial concentration on the basis of the time required for color change. Simply look at the result indicated in the PDF file as bacterial concentration present into the water sample.

In the absence of the optional MBS Optical Reader, watch the color of the vial after a set number of hours depending on the type of analysis. Compare the color of the vial with those reported in the Quality Control Sheet. The analysis result is positive if, and only if, a complete

Color change of the vial content was reached. If the vial remained of the starting color after the maximal number of hours indicated in the Quality Control Sheet included in the vial pack, no bacteria have been detected into the analyzed water sample.

The number of hours required for color change does not depend only on the type of analysis, but includes the operational limit of acceptable bacterial load in the sample (i.e. the limits defined by law). The table correlating the time required for color change with the bacterial concentration is reported in the Quality Control Sheet.

## **1.6 Post-analysis sterilization**

In all the microbiological methods, the medium in which the analysis has been carried out may be dangerous after use for the possible growth of microorganisms and thus they cannot be safely disposed without a special sterilizing treatment.

**It is a unique and patented feature of the MBS method that the vials are self-sterilizing after use, not requiring special sterilizing treatment.**

To sterilize the MBS vials after use firmly press the top of the cap, without opening the vial, and shake for about 10 seconds. The cap contains a sterilizing agent (sodium 1, 5-dichloro-4, 6-dioxo-1, 3, 5-triazin-2-olate dehydrate). The cap is opened by the pressure on the top of the cap itself and releases the sterilizing agent into the vial. When the sterilizing agent is released into the vial, the sterilizing agent causes a color change of the vial.

**After 5-10 minutes the content of the vial is completely sterilized and the vial can be safely disposed as "Not Hazardous Special Waste", according to the Regulations of the Local Authorities**

## **Appendix 2: Operational procedures for the analysis of 100 ml of water**

### **2.1 Total Coliforms test**

**Rehydrate 4 MBS COLI vials** with the ampoule filled with 10ml of sterile water and shake at 1400 ~ 1600r/min for 20 seconds until the reagent is completely dissolved and wait for 5 minutes before starting the analysis.

Using a sterile pipette, **inoculate the 4 vials vial with 2 ml of the water sample** for each vial being sure to reach under the Vaseline layer and shake again at 1400 ~ 1600r/min for 20 seconds.

The starting color of the vial should correspond to the starting color indicated in the Quality Control Sheet. If the color is very different, interference could be present (see paragraph 4 Interferences)

**Incubate the vials at** in thermostat or in MBS Optical Reader

## **2.2 Thermotolerant Coliforms test**

**Rehydrate 4 MBS COLI vials** with the ampoule filled with 10ml of sterile water and shake at 1400 ~ 1600r/min for 20 seconds until the reagent is completely dissolved and wait for 5 minutes before starting the analysis.

Using a sterile pipette, **inoculate the 4 vials vial with 2 ml of the water sample** for each vial being sure to reach under the Vaseline layer and shake again at 1400 ~ 1600r/min for 20 seconds.

The starting color of the vial should correspond to the starting color indicated in the Quality Control Sheet. If the color is very different, interference could be present (see paragraph 4 Interferences)

**Incubate the vials at** in thermostat or in MBS Optical Reader

## **2.3 E.coli test**

**Rehydrate 4 MBS COLI vials** with the ampoule filled with 10ml of sterile water and shake at

1400 ~ 1600r/min for 20 seconds until the reagent is completely dissolved and wait for 15 minutes before starting the analysis.

Using a sterile pipette, **inoculate the 4 vials vial with 2 ml of the water sample** for each vial being sure to reach under the Vaseline layer and shake again at 1400 ~ 1600r/min for 20 seconds.

The starting color of the vial should correspond to the starting color indicated in the Quality Control Sheet. If the color is very different, interference could be present (see paragraph 4 Interferences)

**Incubate the vials at** in thermostat or in MBS Optical Reader

## 2.4 Control of the analysis result

When the optional MBS Optical Reader is used, it is not necessary to look at the vials at different times or to calculate the bacterial concentration on the basis of the MPN tables. Simply look at the result indicated in the PDF file as bacterial concentration present into the water sample.

In the absence of the optional MBS Optical Reader, look at the color of all the 4 vials after a set number of hours depending on the type of analysis, as indicated in the Quality Control Sheet. Compare the color of the vial with those reported in the Quality Control Sheet. The analysis result is positive if, and only if, a complete color change of the content of all the 4 vials was reached. If all the 4 vials remained of the starting color after the maximal number of hours indicated in the Quality Control Sheet included in the vial pack, no bacteria have been detected into the analyzed water sample and the bacteria present into 100 ml of the water samples are below the MPN number indicated in the following MPN tables.

If some of the 4 vials changed colors after the maximal number of hours indicated in the Quality Control Sheet, use MPN table to calculate the bacterial concentration (Poisson distribution-based indirect counting method)

### 1) MPN TABLE FOR TOTAL COLIFORMS AND *E.COLI* ANALYSIS

Number of vials with changed color	Number of vials without changed color	Min CFU/100 ml	Most Probable Number of CFU/100 ml	Max CFU/100 ml

0	4	0	< 14	14
1	3	2	14	100
2	2	8	35	140
3	1	20	69	240
4	0	20	> 69	> 240

## 2) MPN TABLE FOR THERMOTOLERANT COLIFORMS ANALYSIS

Number of vials with changed color	Number of vials without changed color	Min CFU/100 ml	Most Probable Number of CFU/100 ml	Max CFU/100 ml
0	4	0	< 28	28
1	3	4	28	200
2	2	16	70	280
3	1	40	140	420
4	0	40	> 140	> 420

If bacterial concentration is  $\geq 100$  CFU/100 ml, all the 4 vials will change the color before the maximal number of hours indicated in the Quality Control Sheet and you can calculate the number of bacteria present into the water sample on the basis of the time required for color change using the correlation table present into the Quality Control Sheet.

## 2.5 Post-analysis sterilization

In all the microbiological methods, the medium in which the analysis has been carried out may be dangerous after use for the possible growth of microorganisms and thus they cannot be safely disposed without a special sterilizing treatment.

It is a unique and patented feature of the MBS method that the vials are self-sterilizing after use, not requiring special sterilizing treatment.

To sterilize the MBS vials after use firmly press the top of the cap, without opening the vial, and shake for about 10 seconds. The cap contains a sterilizing agent (sodium 1, 5-dichloro-4, 6-dioxo-1, 3, 5-triazin-2-olate dehydrate). The cap is opened by the pressure on the top of the cap itself and releases the sterilizing agent into the vial. When the sterilizing agent is released into the vial, the sterilizing agent causes a color change of the vial. After 5-10 minutes the content of the vials is completely sterilized and the vial can be safely disposed as "Not Hazardous Special Waste", according to the Regulations of the Local Authorities

## Appendix 3.

### Specifications of the MBS Optical Reader (AOT):

Consumable format:	Vials (8 stations)
Sample capacity	1 ml /1 g in 10 ml water
Incubation:	Separated control each station – – 44°C (25- +/-)
Sensitivity:	1 CFU
Optical wavelength:	400~680 nm
Optical detector:	Phototransistor
Operation:	Automated scanning



Detecting time:	each 20 s
Interface:	Serial USB to PC or notebook (PC not included)
Software:	CD with user friendly software included
Data output:	PDF format report
Power requirement:	Universal 90~240V 50~60Hz
Color:	Black
Case dimension:	31x17, 5x8 cm (W x L x H) + Power Adapter
Weight:	+ Power Adapter
Storage:	Room Temp. (15- – Humidity 20-90% non-condensing)