
**Water quality — Detection and enumeration
of *Escherichia coli* and coliform bacteria
in surface and waste water —**

Part 3:

Miniaturized method (Most Probable Number)
by inoculation in liquid medium

*Qualité de l'eau — Recherche et dénombrement des Escherichia coli
et des bactéries coliformes dans les eaux de surface et résiduaires —*

*Partie 3: Méthode miniaturisée (nombre le plus probable) pour
ensemencement en milieu liquide*



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International Organization for Standardization
Case postale 56 • CH-1211 Genève 20 • Switzerland
Internet iso@iso.ch

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 9308-3 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

ISO 9308 consists of the following parts, under the general title *Water quality — Detection and enumeration of Escherichia coli and coliform bacteria in surface and waste water*:

- *Part 1: Membrane filtration method*
- *Part 2: Liquid enrichment method*
- *Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid media*

Annexes E and F form a normative part of this part of ISO 9308. Annexes A to D are for information only.

Water quality — Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and waste water —

Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium

1 Scope

This part of ISO 9308 specifies a miniaturized method for the detection and enumeration of *Escherichia coli* (*E. coli*) in surface and waste water by inoculation in a liquid medium. The method is applicable to all types of surface and waste waters, particularly those rich in suspended matter.

This method is not suitable for drinking water and any other type of water for which the guideline is less than 15 counts per 100 ml.

This method is not appropriate for enumeration and detection of coliform bacteria other than *E. coli*.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 3951, *Sampling procedures and charts for inspection by variables for percent nonconforming*.

ISO 5667-1, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes*.

ISO 5667-2, *Water quality — Sampling — Part 2: Guidance on sampling techniques*.

ISO 5667-3, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of samples*.

ISO 8199, *Water quality — General guide to the enumeration of microorganisms by culture*.

ISO/IEC Guide 2, *Standardization and related activities — Vocabulary*.

3 Terms and definitions

For the purposes of this part of ISO 9308, the terms and definitions given in ISO/IEC Guide 2 and the following apply.

3.1

Escherichia coli

E. coli

β -D-glucuronidase-positive microorganism growing at an incubation temperature of 44 °C in the specified liquid medium containing 4-methylumbelliferyl- β -D-glucuronide (MUG)

4 Principle

The diluted sample is inoculated in a row of microtitre plate wells containing dehydrated culture medium.

The microtitre plates are examined under ultraviolet light at 366 nm in the dark after an incubation period of 36 h minimum and 72 h maximum at 44 °C \pm 0,5 °C. The presence of *E. coli* is indicated by a blue fluorescence resulting from hydrolysis of MUG. The results are given as most probable number (MPN) per 100 ml.

5 Apparatus

With the exception of equipment supplied sterile, the glassware shall be sterilized in accordance with the instructions given in ISO 8199.

Usual microbiological laboratory equipment, and in particular:

5.1 Apparatus for sterilization by dry heat (oven) or steam (autoclave).

5.2 **Thermostatic incubator** regulated at 44 °C \pm 0,5 °C.

5.3 Tunnel drier or vertical laminar air flow cabinet (preferably class II).

5.4 **UV observation chamber** (Wood's Lamp, 366 nm).

WARNING: UV light causes irritation of eyes and skin. Use protective glasses and gloves.

5.5 Portable refractometer (optional).

5.6 **pH meter** with an accuracy of \pm 0,1

5.7 **Test tubes** of dimensions 16 mm \times 160 mm and 20 mm \times 200 mm, or flasks with similar capacity.

5.8 **8-channel multipipette**, adjustable or preset, or any other system suitable for measuring and distributing 200 μ l per well.

5.9 **Sterile tips** for multipipette.

5.10 **Equipment for membrane filtration** in accordance with ISO 8199, including membrane filters with a nominal pore size of 0,2 μ m, for sterilization of liquid media.

5.11 **Sterile microtitre plates**, 96-well, 350 μ l, flat bottomed, nonfluorescent.

5.12 Sterile adhesive covering strips for sealing microtitre plates.

5.13 **Sterile Petri dishes**, 90 mm in diameter.

6 Sampling

Take the samples and deliver them to the laboratory in accordance with ISO 8199 and ISO 5667-1, ISO 5667-2 and ISO 5667-3.

7 Culture media and diluent

7.1 General instructions

To ensure reproducible results, prepare culture medium and diluents, using either constituents of uniform quality and chemicals of recognized analytical grade, or a dehydrated diluent or complete medium prepared following the manufacturer's instructions. Prepare them with demineralized or distilled water free from substances capable of inhibiting growth under the test conditions. If the media are not used immediately, preserve them in the dark at $(5 \pm 3) ^\circ\text{C}$ for up to one month in conditions avoiding any alterations to their composition.

NOTE The use of chemicals of other grades is permissible providing they are shown to be of equivalent performance in the test.

7.2 Diluents

7.2.1 Special diluent (SD)

Synthetic sea salt ¹⁾	22,5 g
Bromophenol blue solution (optional)	10 ml
Demineralized or distilled water (7.2.2)	1000 ml

Sterilize in the autoclave (5.1) at $121 ^\circ\text{C} \pm 3 ^\circ\text{C}$ for 15 min to 20 min.

The bromophenol blue solution is prepared by adding 0,04 g in 100 ml of 50 % ethanol. It is only used to colour the SD blue and avoid confusing it with demineralized or distilled water.

7.2.2 Demineralized or distilled water, free from substances inhibiting growth under the test conditions.

Sterilize in the autoclave (5.1) at $121 ^\circ\text{C} \pm 3 ^\circ\text{C}$ for 15 min to 20 min.

7.3 Culture medium: MUG/EC medium

Composition

Tryptone	40 g
Salicin	1 g
Triton X 100®	1 g
MUG (4-methylumbelliferyl- β -D-glucuronide)	100 mg
Demineralized or distilled water (7.2.2)	1000 ml

¹⁾ A typical analysis of a commercially available and suitable synthetic sea salt is given in annex C. Other diluents, such as distilled water, can be used for *E. coli* enumeration, unless intestinal enterococci are to be enumerated from the same dilution tubes.

Successively add tryptone, salicin and Triton²⁾ to one litre of water, whilst maintaining a gentle heat and magnetic stirring, then bring to the boil until completely dissolved. Allow to cool and add the fluorogenic constituent MUG, dissolved in 2 ml of *N,N*-dimethylformamide.

WARNING: *N,N*-dimethylformamide is toxic and can cause cancer. Harmful by inhalation, in contact with skin and if swallowed. Use in a chemicals fume hood.

Adjust the pH to $6,9 \pm 0,2$.

Sterilize by filtration with membranes of average pore size $0,2 \mu\text{m}$ (5.10).

Distribute in 96-well microtitre plates (5.11) with a volume of 100 μl of media in each well (minimum capacity 350 μl) and dehydrate immediately in a tunnel drier or laminar air flow cabinet (5.3).

The manufacturing of the medium shall meet the quality criteria given in annex E.

8 Procedure

8.1 Choice of dilutions

The number of dilutions to inoculate varies according to the presumed level of contamination of the water to be tested. Table 1 gives examples.

Table 1

Origin of sample	No. of dilutions	No. of wells/dilution	Measurement limits, bacteria/100 ml
Bathing water	2	64 wells to 1/2 32 wells to 1/20	15 to $3,5 \times 10^4$
Fresh surface water	4	24 wells to 1/2 24 wells to 1/20 24 wells to 1/200 24 wells to 1/2 000	40 to $3,2 \times 10^6$
Waste water and treatment plants	6	16 wells to 1/2 Up to 16 wells to 1/200 000	60 to $6,7 \times 10^8$

8.2 Preparation of dilutions

NOTE These procedures should be performed in a biological safety cabinet, as aerosols may be created by the diluting and pipetting.

8.2.1 Fresh and brackish (waste) water (salinity < 30 g/kg, measured with a refractometer (5.5) or equivalent method)

Prepare the relevant number of sterile tubes (5.7) in a rack, according to the number of selected dilutions; add 9 ml of the special diluent (7.2.1) to each tube.

Vigorously stir the sample (clause 6) in order to obtain a homogeneous distribution of the microorganisms and using a sterile pipette, immediately transfer 9 ml of this homogenized sample to the first tube containing 9 ml of diluent (1/2 dilution).

²⁾ Triton X 100 is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 9308 and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

Using a fresh pipette, transfer 1 ml of this dilution (homogenized) to the second tube (1/20 dilution).

From the second tube (dilution 1/20 carefully homogenized) proceed, if necessary to another 1/10 dilution giving the dilution 1/200.

Continue as above until all the dilutions have been prepared.

8.2.2 Sea water (salinity ≥ 30 g/kg)

Prepare the relevant number of sterile tubes in a rack, according to the number of selected dilutions; add 9 ml of demineralized or distilled water (7.2.2) to the first tube and 9 ml of the special diluent (7.2.1) to the following tubes.

Vigorously stir the sample (clause 6) in order to obtain a homogeneous distribution of the microorganisms and using a sterile pipette, immediately transfer 9 ml of this homogenized sample to the first tube containing 9 ml of diluent (7.2.2) (1/2 dilution).

Using a fresh sterile pipette, transfer 1 ml of this dilution (homogenized) to the second tube (1/20 dilution).

From the second tube (dilution 1/20 carefully homogenized) proceed, if necessary to another 1/10 dilution, giving the dilution 1/200.

Continue as above until all the dilutions have been prepared.

8.3 Inoculation and incubation of microtitre plates

8.3.1 Inoculation

Transfer the contents of the first tube of dilution to an empty, sterile Petri dish of diameter 90 mm.

Using a multichannel pipette (5.8) with eight sterile tips (5.9), distribute 200 μ l into each well of microtitre plate (5.11) corresponding to this first dilution.

For subsequent dilutions (1/20, 1/200, etc.) operate in an identical manner, changing the Petri dish and the row of eight sterile tips between each dilution.

Alternatively, any other suitable system (5.8) may be used to distribute 200 μ l of each dilution per well according to Table 1.

CAUTION: Beware of contamination via overflow from one well to another.

8.3.2 Incubation

Once the microtitre plate is inoculated, cover with the disposable sterile adhesive tape (5.12) provided for this purpose.

Incubate the microtitre plate in an incubator (5.2) at $44 \text{ }^{\circ}\text{C} \pm 0,5 \text{ }^{\circ}\text{C}$ for a minimum of 36 h and a maximum of 72 h.

NOTE The microtitre plates should be handled with care, without tilting.

8.4 Reading of results

Place each microtitre plate, with the adhesive on, in the UV observation chamber (5.4).

Consider all wells in which a blue fluorescence is observed as being positive.

NOTE The reading may be carried out any time after 36 h, as the fluorescence does not alter with time.

9 Expression of results

9.1 Determination of characteristic number

For each chosen dilution note the number of positive (+) wells.

EXAMPLE 1: Bathing water

1/2 32 + out of 64

1/20 5 + out of 32

Record 32/5 as characteristic number

EXAMPLE 2: Surface water

1/2 24 + out of 24

1/20 18 + out of 24

1/200 5 + out of 24

1/2 000 1 + out of 24

Record 18/5/1 as characteristic number

EXAMPLE 3 : Waste water

1/2 16 + out of 16

1/20 16 + out of 16

1/200 12 + out of 16

1/2 000 5 + out of 16

1/20 000 0 + out of 16

1/200 000 0 + out of 16

Record 12/5/0 as characteristic number

Where three or more dilutions have been inoculated, a characteristic number of three figures, the last one 0 where possible, shall be recorded in accordance with ISO 8199.

9.2 Calculation of the MPN and its confidence interval

The MPN is a statistical estimation of the density of microorganisms, assumed to correspond to a Poisson distribution in the volumes inoculated. Confidence intervals are attached to this MPN.

Software shown in annex A or B enables the calculation of the MPN of *E. coli* per millilitre of water for each configuration of inoculations and the confidence interval at 95 %.

EXAMPLE 1: Assuming CN is the Characteristic Number, LO the Lower Limit and UP the Upper Limit:

If CN = 32/5, the software in annex A gives 7,56 *E. coli*/ml,

[LO = 5,42 – UP = 10,54],

i.e. 756/100 ml (542 to 1054).

EXAMPLE 2:

If CN = 18/5/1, the software in annex A gives 159,08/ml,

[LO = 101,99 – UP = 248,11]

EXAMPLE 3:

If CN = 12/5/0, the software in annex A gives 1724,61/ml

[LO = 1003,98 – UP = 2962,50]

If none of the wells is positive, express the result in the following form: < $n/100$ ml, n being the MPN for 1 positive well under the dilution conditions employed.

10 Test report

The test report shall include all details necessary for the complete identification of the sample, referring to the method used and the results.

The test report shall also mention any special phenomena observed during the test and any nonspecified or optional operations used in the method which may have altered the results.

11 Performance data

Information concerning the repeatability and reproducibility of the procedure, obtained from interlaboratory tests, is given in annex D.

Annex A
(informative)

Example of software for statistical analysis of MPNs

```

10  REM *****
20  REM GENERAL PURPOSE PROGRAM FOR MPN, ITS S.E., C.I.
30  REM AND HOMOGENEITY TEST STATISTICS
40  REM *****
50  REM
60  DIM A(10,6),X2(3,9)
70  REM SET PROGRAM LIMITS
80  D9=10
90  U9=50
100 L9=0
110 A1=.0005
120 E1=85
130 REM SET CHI-SQUARED SIGNIFICANCE LEVELS
140 GOSUB 1000
150 REM READ IN RESULTS OF A DILUTION SERIES
160 GOSUB 2000
170 REM CALC AND PRINT THE MPN
180 GOSUB 3000
190 REM CALC AND PRINT S.E. OF LOG10(MPN)
200 GOSUB 4000
210 REM CALC AND PRINT 95 PERCENT C.I. FOR MPN
220 GOSUB 5000
230 REM CALC AND PRINT DEVIANCE
240 GOSUB 6000
250 STOP
1000 REM SET CHI-SQUARED SIGNIFICANCE LEVELS
1010 FOR I=1 TO 3
1020 FOR J=1 TO 9
1030 READ X2(I,J)
1040 NEXT J
1050 NEXT I
1060 REM 5 PERCENT LEVELS DF=1...9
1070 DATA 3.84, 5.99, 7.81, 9.49, 11.07
1080 DATA 12.59, 14.07, 15.51, 16.92
1090 REM 1 PERCENT LEVELS
1100 DATA 6.63, 9.21, 11.34, 13.28, 15.09
1110 DATA 16.81, 18.48, 20.09, 21.67
1120 REM .1 PERCENT LEVELS
1130 DATA 10.83, 13.81, 16.27, 18.47, 20.52
1140 DATA 22.46, 24.32, 26.12, 27.88
1150 RETURN
2000 REM READ IN RESULTS OF A DILUTION SERIES
2010 PRINT "MPN GENERAL PURPOSE PROGRAM"
2020 PRINT "*****"
2030 PRINT " "
2040 PRINT "M.A. HURLEY AND M.E. ROSCOE"
2050 PRINT " "
2060 PRINT "NUMBER OF DILUTION LEVELS.....K=";

```

```

2070 INPUT N
2080 IF N<1 THEN GOTO 2060
2090 IF N<=D9 THEN GOTO 2120
2100 PRINT "ERROR *** LEVELS EXCEED MAXIMUM"
2110 STOP
2120 S1=0
2130 FOR I=1 TO N
2140 PRINT " "
2150 PRINT "LEVEL NUMBER .....I=";I
2160 PRINT "DILUTION FACTOR.....D=";
2170 INPUT A(I,2)
2180 PRINT "SUBSAMPLE VOLUME.....V=";
2190 INPUT A(I,1)
2200 PRINT "NUMBER OF SUBSAMPLES.....N=";
2210 INPUT A(I,3)
2220 PRINT "NUMBER OF POSITIVE SUBSAMPLES..P=";
2230 INPUT A(I,4)
2240 PRINT "IS THE DATA CORRECT FOR LEVEL ";I;"(Y OR N) ";
2250 INPUT R$
2260 IF R$="Y" THEN 2280
2270 GOTO 2140
2280 A(I,5)=A(I,1)*A(I,2)
2290 A(I,6)=A(I,5)*A(I,4)
2300 S1=S1+A(I,5)*A(I,3)
2310 NEXT I
2320 RETURN
3000 REM CALCULATES AND PRINTS MPN
3010 B1=0
3020 S3=0
3030 FOR J=1 TO N
3040 E2=A(J,5)*U9
3050 IF E2<E1 GOTO 3080
3060 E2=0
3070 GOTO 3090
3080 E2=EXP(-E2)
3090 S3=S3+A(J,6)/(1-E2)
3100 NEXT J
3110 IF S3-S1>=0 THEN 3130
3120 GOTO 3200
3130 FOR I=1 TO N
3140 A(I,5)=A(I,5)*2
3150 A(I,6)=A(I,6)*2
3160 NEXT I
3170 S1=S1*2
3180 B1=B1+1
3190 GOTO 3020
3200 X3=L9
3210 X4=U9
3220 X=(X3+X4)/2
3230 S=0
3240 FOR I=1 TO N
3250 E2=A(I,5)*X
3260 IF E2<E1 GOTO 3290
3270 E2=0
3280 GOTO 3300
3290 E2=EXP(-E2)
3300 S=S+A(I,6)/(1-E2)

```

```

3310 NEXT I
3320 IF ABS(S-S1)<A1 THEN 3380
3330 IF S-S1>0 THEN 3360
3340 X4=X
3350 GOTO 3220
3360 X3=X
3370 GOTO 3220
3380 X5=X*(2^B1)
3390 PRINT " "
3400 PRINT "MPN=";X5
3410 PRINT "FOR A SAMPLE WITH DILUTION FACTOR 1"
3420 PRINT "                                AND VOLUME 1"
3430 RETURN
4000 REM CALCS AND PRINTS S.E. OF LOG10 (MPN)
4010 S2=0
4020 FOR I=1 TO N
4030 X3=A(I,5)
4040 E2=X3*X
4050 IF E2<E1 GOTO 4080
4060 X4=0
4070 GOTO 4090
4080 X4=EXP(-E2)
4090 S3=X3*X3*A(I,3)*X4
4100 S3=S3/(1-X4)
4110 S2=S2+S3
4120 NEXT I
4130 V=1/(X*X*S2)
4140 S1=SQR(V)/LOG(10)
4150 PRINT " "
4160 PRINT "S.E. OF LOG10 (MPN)=";S1
4170 RETURN
5000 REM CALCS 95 PERCENT C.I. FOR MPN
5010 X3=LOG(X)+B1*LOG(2)
5020 S2=SQR(V)
5030 U=EXP(X3+1.96*S2)
5040 L=EXP(X3-1.96*S2)
5050 PRINT " "
5060 PRINT "95 PERCENT C.I. =" ;L;"TO";U
5070 RETURN
6000 REM CALCS. AND PRINTS DEVIANCE
6010 S3=0
6020 FOR I=1 TO N
6030 S4=0
6040 IF A(I,4)<=0 GOTO 6110
6050 E2=A(I,5)*X
6060 IF E2<E1 GOTO 6090
6070 E2=0
6080 GOTO 6100
6090 E2=EXP(-E2)
6100 S4=A(I,4)*LOG(A(I,4)/(A(I,3)*(1-E2)))
6110 S3=S3+S4
6120 S4=0
6130 IF A(I,4)>=A(I,3) GOTO 6160
6140 S4=A(I,3)-A(I,4)
6150 S4=S4*(LOG(S4/A(I,3))+A(I,5)*X)
6160 S3=S3+S4
6170 NEXT I

```

```
6180 D=2*S3
6190 REM CHI-SQUARED TEST OF DEVIANCE
6200 V=N-1
6210 PRINT " "
6220 PRINT "DEVIANCE =";D;"ON";V;" D.F."
6230 PRINT " "
6240 PRINT "CHI-SQUARED SIGNIFICANCE LEVELS FOR";V;" D.F."
6250 PRINT "    5 PERCENT    ";X2(1,V)
6260 PRINT "    1 PERCENT    ";X2(2,V)
6270 PRINT "    .1 PERCENT    ";X2(3,V)
6280 RETURN
7000 END
```

Annex B (informative)

Example of software for computation of MPN

```

10  DIM T (20)
20  DIM M (20)
30  DIM P (20)
40  L = 0
45  CLS: L = L + 1
50  PRINT "CALCULATION OF MPN N° ";L
60  PRINT " ....."
70  A = 1
80  PRINT
90  INPUT "NB OF DILUTIONS";DI: PRINT: PRINT
110 PRINT
120 P = 0: S =: U = 0
130 FOR I = 1 TO DI
140 PRINT "DILUTION"; I
150 INPUT "NB OF POSITIVE WELLS .."; P(I)
155 INPUT "NB OF WELLS .....";T(I)
160 INPUT "WATER VOLUME/WELL (ML)...";M(I): PRINT
170 P = P(I) + P
180 S = ((T(I) - P(I))*M(I)) + S
190 U = (M(I) * T(I)) + U
200 NEXT I
280 K = 1
290 NP = (P/U) * 2 ^ (K + K / 2 + K / 4 + K / 8 + K / 16 + K / 32 + K / 64 + K / 128 + K / 256 + K / 512)
300 PM = 0
310 FOR I = 1 TO DI
320 PM = PM + ((P(I) * M (I) * EXP ( - M(I) * NP)) / (1 - EXP (- M(I) * NP )))
330 NEXT I
340 IF PM < S THEN 370
350 K = K + A
360 GOTO 290
370 DT = S - PM
380 IF ABS (DT) <= .000005 GOTO 702
390 K = K - A
400 A = A / 10
410 GOTO 290
702 FOR I = 1 TO DI:ES = ES + P(I):NEXT I
710 FOR I = 1 TO DI
720 K (I) = (T(I) * (M(I)^2))
730 NEXT I
740 FOR I = 1 TO DI
745 IF (NP * M(I) > 88 THEN E(I) = 1.65E38:GOTO 760
750 E(I) = (EXP (M(I) * NP) - 1)
760 NEXT I
770 LL = 0
780 FOR I = 1 TO DI
790 LL = LL + (K (I) / E (I))
800 NEXT I
810 CL = (LOG (NP) - (1.96 * (1 / (NP * SQR (LL))))))

```



```

817  CU = (LOG (NP) + (1.96 * (1 / (NP * SQR (LL))))))
840  NP = INT (NP * 100 + .5) / 100
850  PRINT : PRINT " NPP = ";NP;" / ML"
860  PRINT : PRINT
870  PRINT "LIMITS INF=";INT ( EXP (CL)* 100 + .5) / 100;" / ML SUP=";INT (EXP (CU) * 100 + .5) / 100;" / ML"
880  PRINT : PRINT: INPUT "DO YOUWANT ANOTHER MPN (Y/N)?";RE$
890  IF RE$ = "N" THEN END
900  GOTO 45

```

Comment:

After display of the run number (L, line 50),

Input the number of dilutions (line 90).

For each dilution :

- display the dilution rank (line 140)
- input the number of positive wells or tubes (line 150),
- input the number of wells inoculated with this dilution (line 155),
- input the volume of water inoculated per well, in millilitres (line 160).

Calculation of the MPN in accordance with De Man [2] (lines 280-410)

Calculation of the lower and upper limits of confidence interval according to De Man [2] (lines 770-817).

Display of MPN (per millilitre) (line 850)

- the lower limit (per millilitre) (line 870)
- the upper limit (per millilitre) (line 870)

Question : "Another run ?"

If no : END.-

Annex C (informative)

Synthetic sea salt

C.1 Major ion composition of a convenient ocean synthetic sea salt

	Major ion	% Total mass	Ionic concentration at 34 g/kg salinity (mg/l)
Chloride	(Cl ⁻)	47,470	18 740
Sodium	(Na ⁺)	26,280	10 454
Sulfate	(SO ₄ ⁻²)	6,602	2 631
Magnesium	(Mg ⁺²)	3,230	1 256
Calcium	(Ca ⁺²)	1,013	400
Potassium	(K ⁺)	1,015	401
Bicarbonate	(HCO ₃ ⁻)	0,491	194
Borate	(B)	0,015	6,0
Strontium	(Sr ⁺²)	0,001	7,5
	TOTAL SOLIDS	86,11 %	34 089,50
Water	(H ₂ O)	13,88	
	TOTAL	99,99 %	

C.2 Example for preparation from defined substances

Three basic solutions shall be made as follows :

a) Solution A

CaCl ₂ ·2H ₂ O	83,6 g	
KCl	43,5 g	
SrCl ₂ ·6H ₂ O	0,07 g	
Distilled water	ad 1 000 ml	

b) Solution B

NaHCO ₃	15,15 g
Na ₂ B ₄ O ₇	3,0 g
Distilled water	ad 1 000 ml

c) Solution C

MgSO ₄ ·7H ₂ O	190,0 g
MgCl ₂ ·6H ₂ O	147,0 g
Distilled water	ad 1 000 ml

The diluent is made by adding to 960 ml distilled water 10 ml of solution A, 10 ml of solution B, 20 ml of solution C, and then 14,9 g sodium chloride, mixing until completely dissolved and setting the pH to $7,5 \pm 0,2$. The diluent is distributed to containers of desired volumes, and sterilized by autoclaving at $(121 \pm 3) ^\circ\text{C}$ for 15 min.

Annex D (informative)

Performance characteristics of the method

The performances of repeatability (r) and reproducibility (R) calculated in accordance with ISO 5725-2 as part of interlaboratory tests have shown:

a) On bathing waters

(spiked samples, with 100 French laboratories, on three occasions in 1995 and 1996) (one sea water and two fresh waters, without significant difference):

- | | |
|---|-----------------|
| i) At the level 100 <i>E. coli</i> / 100 ml: | $r \approx 3,6$ |
| | $R \approx 4,3$ |
| ii) At the level 2 000 <i>E. coli</i> / 100 ml: | $r \approx 1,7$ |
| | $R \approx 2,3$ |

and, for information only, during tests (in 1993 and 1994) limited to nine laboratories and four samples naturally contaminated:

b) On river waters containing

- | | |
|--|-----------------|
| between $1,2 \times 10^4$ and $5,7 \times 10^4$ <i>E. coli</i> / 100 ml: | $r \approx 1,9$ |
| | $R \approx 2,0$ |

c) On sewage waters containing

- | | |
|--|-----------------|
| between $1,2 \times 10^5$ and $1,9 \times 10^7$ <i>E. coli</i> / 100 ml: | $r \approx 2,1$ |
| | $R \approx 2,8$ |

Annex E (normative)

Quality criteria for manufacturing of the medium in microtitre plates

E.1 General

For each of the criteria which follow, a quality control shall be made on each batch of manufactured microtitre plates. The microtitre plates to be tested shall be taken at random or in a systematic way to constitute a sample in accordance with ISO 3951, respecting the general control level No. II of the normal control.

The threshold of positivity of a microtitre plate is defined as the fluorescence level leading to a positive reading, without ambiguity to the eye, under a Wood light (366 nm). It shall be measured under application of the protocol of annex F.

The quality criteria to be respected are given in E.2 to E.4. The batch is rejected if any of the criteria is not respected.

The strain to be tested is *E. coli* WR1, obtainable from RIVM, Bilthoven, the Netherlands, and the incubation period is 48 h at $(44 \pm 0,5)$ °C.

E.2 Background noise

Test for the absence of positive well in each microtitre plate of the sample, after inoculation with sterile special diluent and incubation of 48 h at $(44 \pm 0,5)$ °C. The medium background noise of the sample shall be inferior to 25 % of the positivity threshold defined above and the variation coefficient shall be inferior to 10 %.

E.3 Average level of fluorescence

This is the geometric mean of the fluorescence signal obtained from the 96 wells of a microtitre plate inoculated uniformly with 200 µl per well of a suspension of *E. coli* WR1 containing 500 microorganisms per ml of Special Diluent (7.2.1.), and incubated for 48 h at $(44 \pm 0,5)$ °C. The average signal so obtained shall be at least twice the threshold of positivity and the variation coefficient shall be inferior to 10 %.

E.4 Fertility

Fertility is calculated as the ratio of the number of microorganisms observed with the batch of microtitre plates under test to the number of microorganisms expected with a stable reference material (target value). The level of concentration should be brought close to the maximum of the precision of the method, i.e. about one microorganism per well (500 / 100 ml). The stability and the homogeneity (target value and confidence intervals) of the reference material should have been determined with one (or several) batch(es) of microtitre plates already accepted. The threshold of acceptance of the microtitre plates tested are: 0,66 to 1,5 of the target value. The variation coefficient should be inferior to 10 %.

Annex F (normative)

Preparation of calibration microtitre plates

F.1 Material and reagents

Current laboratory apparatus (precision balance, pH meter, glassware) and also:

F.1.1 4-Methylumbelliferone.

F.1.2 Absolute ethanol.

F.1.3 0,8 % Sodium chloride solution.

F.1.4 Glycine.

F.1.5 Sodium hydroxide.

F.1.6 Microtitre plates with 96 wells.

F.2 Preparation of the stock solution

Prepare a stock solution of 4-methylumbelliferone (F.1.1) in ethanol (F.1.2) by mixing the following:

4-Methylumbelliferone	0,044 g
Ethanol	10 ml

F.3 Preparation of the buffer solution

Prepare the following solutions:

a) Glycine solution

Dissolve 7,507 g of glycine and 5,84 g of sodium chloride in 1 000 ml distilled water.

b) Sodium hydroxide solution

Dissolve 4 g of sodium hydroxide in 1 000 ml distilled water.

In a beaker with agitator add 56,5 ml of the glycine solution a) to 43,5 ml of sodium hydroxide solution b). Check with the pH meter that the pH value is 10,3.

F.4 Preparation of the calibration plate

All the following operations shall take place at room temperature.

All the dilutions shall be made in a solution of 0,8 % sodium chloride (F.1.3.).

Prepare an intermediate solution at 1/100 of the stock solution (F.2). From the obtained solution, prepare daily the following solutions:

ST 1 =	100 µl of the intermediate solution	+ 0,9 ml of 0,8 % NaCl
ST 2 =	0,5 ml of ST 1	+ 0,5 ml of 0,8 % NaCl
ST 3 =	0,5 ml of ST2	+ 0,5 ml of 0,8 % NaCl
ST 4 =	0,5 ml of ST3	+ 0,5 ml of 0,8 % NaCl
ST 5 =	0,5 ml of ST4	+ 0,5 ml of 0,8 % NaCl

Display the different solutions in the uneven-numbered columns of the microtitre plate (F.1.6) following the instructions below (the even-numbered columns are not used):

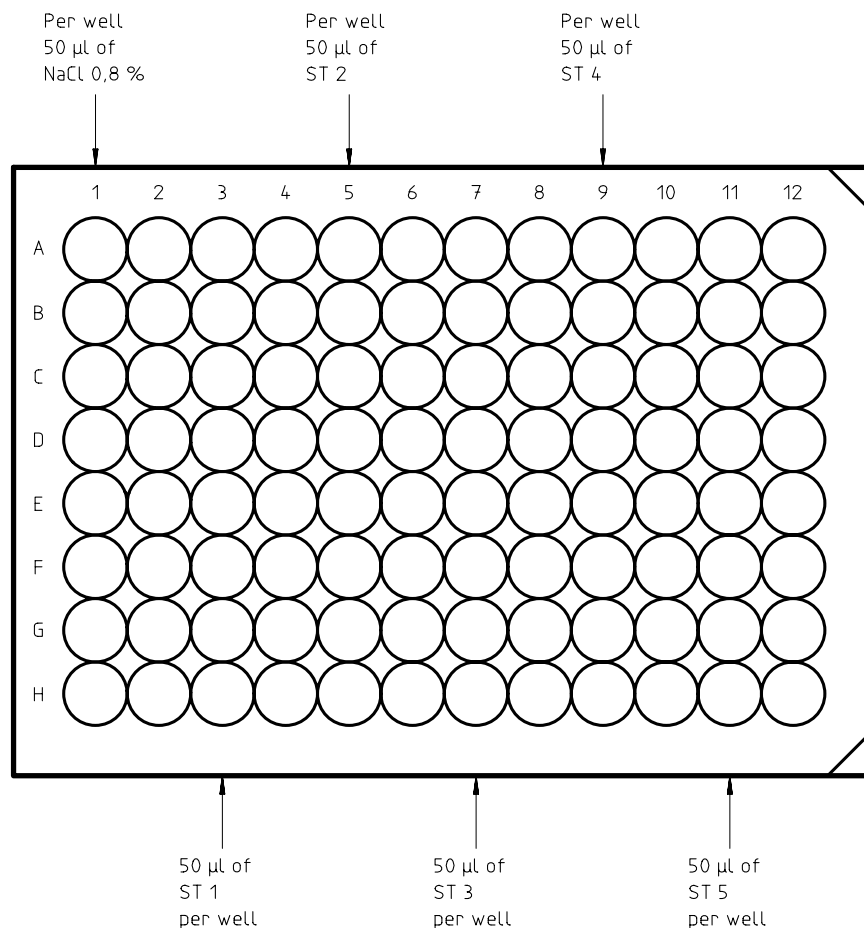


Figure F.1

Add in each well 100 µl of the pH 10,3 buffer solution (F.3). A range from 0 pg to about 1 500 pg of 4-methylumbelliferone/µl is thus obtained.

NOTE Experimentally it has been observed that the positivity threshold with 4-methylumbelliferone SIGMA (batch No. M-1381) and the transparent microtitre plates with flat bottom NUNC (ref. 1352) is 367 pg/µl when the microtitre plate is filled as described in annex F with the solution ST 3.

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