Soils, sludges and treated bio-wastes — Detection and enumeration of *Escherichia coli* in sludges, soils and organic fertilisers of similar consistency to the matrices validated **—** Part 2: Miniaturised method (Most Probable Number) by inoculation in liquid medium

Boden, Schlamm und behandelle Bio-abfälle — Quantitativer Nachweis von Escherichia coli aus Schlämmen, Böden, Dügemitels und Bodenverbesserern, Kultursubstraten sowie Bioabfällen — Teil 2: Miniaturisiertes Verfahren durch Animpfen in Flüssigmedium (MPN-Verfahren)

Sols, boues et bio-déchets traités — Détection et dénombrement de Escherichia coli dans les boues, les sols et les fertilisants organiques de consistance similaire aux matrices validées — Partie 2 : Méthode miniaturisée (Nombre le Plus Probable) par ensemencement en milieu liquide

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Contents

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Foreword

This document has been prepared in the framework of the project Horizontal.

This document is a working document.

The following TC's have been involved in the preparation of the standard: TC 308.

The standard is divided into three parts:

- part 1 describes a membrane filtration method for quantification,
- part 2 describes a miniaturised semi-quantitative MPN method,
- part 3 describes a semi-quantitative macromethod.

This standard is applicable and validated for several types of matrices. The table below indicates which ones.

Material	Validated	Document
Mesophilic anaerobic digested sewage sludge		
Anaerobic treated biowaste		
Pelletised air dried sludge		
Digested sewage sludge presscake		
Composted sewage sludge		
Composted green waste		
Composted biowaste		

Introduction

This document is developed in the framework of the project “Horizontal”. It is the result of a desk study “Hygienic Parameters Feasibility of Horizontal Standards for *Escherichia coli* and *Salmonella* spp. in sludge, soils, soil improvers, growing media and biowastes” and aims at evaluation of the latest developments in assessing *E. coli* in sludge, soil and organic fertilisers. After discussion with all parties concerned in CEN and selection of a number of test methods described in this study the standard has been developed further as a modular horizontal method and has been validated within the project “Horizontal”.

Sludge, soils and organic fertilisers can contain pathogenic micro-organisms such as *Salmonella* spp. which occur mainly in the intestinal tract of humans and animals and are transmitted through faecal contamination. The use of such contaminated materials in agriculture may cause outbreaks of infection due to the production of contaminated food and animal feedstocks and may also be transmitted to wild animals. There is a need to monitor the efficacy of the storage and treatment processes to control pathogens such as *Salmonella* spp., and application rates to land.

Escherichia coli is a non-pathogenic, Gram negative bacterium with a faecal origin. Consequently, it can be used as an indicator of faecal contamination. It can also be used to monitor the effectiveness of pasteurisation or disinfection treatments but it is comparatively sensitive (to heat, high pH) and cannot therefore reflect the behaviour of all pathogens in these materials.

Suitable quality control procedures, at least those described in ISO 8199:2005, have to be applied.

WARNING — "Waste and sludge samples may contain hazardous and inflammable substances. They may contain pathogens and be liable to biological action. Consequently it is recommended that these samples should be handled with special care. The gases which may be produced by microbiological activity are potentially inflammable and will pressurise sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided wherever possible. National regulations should be followed with respect to microbiological hazards associated with this method".

The texts of the chapters 1 to 12 are normative; annexes are normative or informative, as stated in the top lines of the annexes.

1 Scope

This part of the European standard describes a miniaturized most probable number (MPN) method for the semi-quantitative detection of *Escherichia coli* in sludge, soils and organic fertilisers of similar consistency to the matrices validated. It is suitable to evaluate the Log reduction of *E. coli* through treatment as well as the quality of the end product.

This method has a limit of detection (5 %) of approximately 67 *E. coli* MPN per g of wet weight, and a quantification range of 6 Log [ENV ISO 13843].

2 Normative references

These normative references are cited at appropriate places in the text and the publications are listed hereafter. For dated references, only the edition cited applies. For undated references the latest edition of the publication referred to applies (including any amendments).

EN ISO 5667-13:1997, *Water Quality — Sampling — Part 13: Guidance on sampling of sludge from sewage and water treatment works.*

EN 12880:2000, *Characterisation of Sludge — Determination of dry residue and water content.*

ISO 8199:2005, *Water quality — General guidance on the enumeration of micro-organisms by culture.*

EN ISO 9308-3:1998, *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.*

NF EN 12176:1998, Characterization of sludge — Determination of pH value.

ENV ISO 13843:2001, *Water quality — Guidance on validation of microbiological methods*.

ISO 5725:1994, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests*.

3 Definitions

For the purposes of this European Standard, the following terms and definitions apply.

3.1
Escherichia coli
Escherichia coli belongs to the family of Enterobacteriaceae, is Gram-negative, non-sporulating, rod-shaped bacteria, able to ferment lactose and to grow at 44 °C. Most *E. coli* strains are able to produce indole from tryptophan and are *β*-glucuronidase-positive

3.2
method definition
β-glucuronidase-positive able to hydrolyse 4-methylumbelliferyl-β-D-glucuronide (MUG) when growing at the incubation temperature of 44 °C in the specified liquid medium. In addition, indole shall be produced from tryptophan

3.3
Most probable number (MPN)
Every well whose inoculum contains even one viable organism will produce detectable growth or change. The individual wells of the sample are independent. The essence of the MPN method is to dilute the sample to

such a degree that inocula in the wells will sometimes but not always contain viable organisms. The "outcome", *i.e.*, the number of wells and the number of wells with growth at each dilution, will imply an estimate of the original, undiluted concentration of bacteria in the sample. In order to obtain estimates over a broad range of possible concentrations, microbiologists use serial dilutions incubating tubes at several dilutions. The MPN is the number which makes the observed outcome most probable

3.4
dry residue
the dry mass portion of the material obtained after the specified drying process. It is expressed as percent or in grams per kilogram [EN 12880:2000]

4 Symbols and abbreviations

E. coli: *Escherichia coli*

MUG: 4-methylumbelliferyl-β-D-glucuronide

SMD: Special Microplate Diluent

MPN: Most Probable Number

CN: characteristic number

5 Principle

This method is based on the EN ISO 9308-3:1998: “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”.

The following text describes the sample preparation to prepare a liquid suspension, then the analysis is performed following the EN ISO 9308-3, reaching Most Probable Number results in 100 mL. The final result is calculated to express the *E. coli* MPN per g of sample. The whole procedure described in the EN ISO 9308-3 is adapted to perform the analysis of *E. coli* in sludge, soils and organic fertilisers of similar consistency to the matrices validated.

The detection and enumeration of *E. coli* from sludge, soils and organic fertilisers requires the following stages:

a) preparation of a homogenised sample suspension in peptone saline solution;

b) inoculation of the diluted homogenised sample in a row of microtiter plate wells containing dehydrated culture medium;

c) examination of the microtiter plate under ultraviolet light at 366 nm in the dark after an incubation period at (44 ± 1) °C during 48 h. The presence of *E. coli* is indicated by a blue fluorescence resulting from hydrolysis of MUG;

d) confirmation of the presence of *E. coli* by addition of Kovacs reagent, in each blue fluorescent well;

e) results are given as the *E. coli* Most Probable Number per g of sample (wet weight).

6 Reagents, diluents and culture media

6.1 General instructions

To ensure reproducible results, prepare culture media and diluents using either constituents of uniform quality and chemicals of recognised analytical grade, or a dehydrated diluent or complete medium prepared following the manufacturer's instructions. Prepare them with demineralised or distilled water free from substances capable of inhibiting growth under the test conditions [ISO 8199:2005].

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

6.2 Peptone saline solution

Casein peptone	1 g
Sodium chloride (NaCl)	8.5 g
Distilled water	1 000 mL

Dissolve the compounds in the water, if necessary by heating. Adjust the pH (7.8) by adding sodium hydroxide solution or hydrochloric acid so that, after sterilisation, it will correspond to (7.0 ± 0.5) at 25°C.

Sterilise the solution in a steam steriliser (7.1) at (121 ± 3) °C for (15 ± 1) min.

6.3 Special Microplate Diluent (SMD)

6.3.1 Bromophenol blue solution (optional)

Bromophenol blue	0.04 g
50 % ethanol	100 mL

Dissolve bromophenol blue in 100 mL ethanol.

It is only used to colour the SMD in blue and to avoid confusing with demineralised or distilled water.

6.3.2 SMD

Synthetic sea salt	22.5 g
Bromophenol blue solution (optional)	10 mL
Distilled water	1 000 mL

Dissolve the ingredients in the water. Pour 18 mL fraction into sterile tubes.

Sterilize in the autoclave (7.1) at (121 ± 3) °C for (15 ± 1) min.

NOTE A typical analysis of a commercially available and suitable synthetic sea salt is given in informative Annex C.

6.4 Culture medium: MUG/EC medium

6.4.1 MUG solution

MUG* (4-methyl-umbelliferyl β-D-glucuronide)	100 mg
N-N-dimethylformamide	2 mL

Dissolve MUG, the fluorogenic constituent, in N-N-dimethylformamide.

WARNING — N-N dimethylformamide is toxic. Harmful by inhalation, in contact with skin and if swallowed. May cause cancer. Use in a chemical fume hood.

6.4.2 MUG/EC medium

Tryptone	40 g
Salicin	1 g
Triton x100	1 g
MUG solution	2 mL
Distilled water	1 000 mL

Successively add Tryptone, Salicin and Triton to one litre of distilled water while maintaining a gentle heat and magnetic stirring. Bring to the boil until complete dissolution. Allow to cool and add the MUG solution.

Adjust the pH to (6,9 ± 0,2).

Sterilize by filtration with membranes of average pore size 0,2 µm (7.14).

Distribute in 96-well microtitre plates (7.15) with a volume of 100 µL of media in each well (maximum capacity 350 µL) and dehydrate immediately in a tunnel drier or laminar airflow cabinet (7.3).

6.5 Kovac's reagent

4-di-methylaminobenzaldehyde, $C_9H_{11}NO$	5.0 g
Isoamyl alcohol, $C_5H_{12}O$	75.0 mL
Hydrochloric acid (p=1,18 $g.mL^{-1}$)	25.0 mL

Dissolve 4-di-methylaminobenzaldehyde in isoamyl alcohol and heat in a water bath at 60°C for 5 min. Then add slowly 25 mL of hydrochloric acid.

The reagent will be ready for use after 6 to 7 h (indicated by a yellow colour). Store in the refrigerator and protect from light.

WARNING — Kovacs reagent is harmful if swallowed, irritating to the respiratory system and to the skin. It is recommended to use it in a flow cabinet.

7 Apparatus

With the exception of equipment supplied sterile, the glassware shall be sterilised in accordance with the instructions given in ISO 8199:2005.

Usual microbiological laboratory equipment and in particular:

7.1 Apparatus for sterilisation by dry heat (oven) or steam (autoclave)

7.2 Thermostatic incubator regulated at (44 ± 1) °C

7.3 Tunnel drier or vertical laminar airflow cabinet (preferably class II)

7.4 Homogeniser

7.5 Sterile homogeniser bags, 250 mL volume, with or without integrated mesh to exclude large particulate matter

7.6 Ultraviolet observation chamber (Wood's Lamp, 366 nm)

WARNING — UV light causes irritation of eye and skin. Use protective glasses and gloves.

7.7 Portable refractometer (optional)

7.8 pH meter with an accuracy of ± 0.1

7.9 Sterile test tubes of 40 mL volume, or flasks with similar capacity

7.10 Sterile flasks, of nominal capacities e.g. 250 mL

7.11 Sterile graduated pipettes, glass or disposable plastic ware, capable of dispersing 2 mL and 18 mL

7.12 Adjustable or pre-set 8-channel multi-pipette or any other suitable system used for measuring and distributing 200 µL per well

7.13 Sterile tips for multi-pipette

7.14 Equipment for membrane filtration according to ISO 8199:2005, including membrane filters with a nominal pore size of 0,2 µm, for sterilization of liquid media

7.15 Sterile microtiter plates – 96 wells, 350 µL, flat-bottomed, non-fluorescent

7.16 Sterile adhesive covering strips for sealing microtitre plates

7.17 Sterile Petri dishes, 90 mm in diameter

7.18 Analytical balance

7.19 Laboratory spatula

7.20 Vortex mixer

7.21 Stirrer and magnetic bars

8 Sampling and hazards

Take samples of at least 100 g wet weight and deliver them to the laboratory as quickly as possible (within 24 h). In order to prevent propagation or inactivation of *E. coli* during transport to the laboratory and subsequent storage, refrigerate the sample at (5 ± 3) °C.

8.1 General

Samples are liable to ferment and can contain pathogenic micro-organisms. It is essential to keep them away from any food or drink, and to protect any cuts. When transporting and handling samples, it is essential that national and international regulations relating to bio-hazardous samples are followed.

Bursting glass bottles containing sludge can produce micro-organism contaminated shrapnel. Plastic bottles can also burst and produce a hazardous spray and aerosol.

See also the Warning note in the introduction.

8.2 Storage

It is not advisable to store samples in the open laboratory. If samples are to be stored, store them at (5 ± 3) °C for no more than 72 h after receipt.

8.3 Handling

Cleanliness when working is essential. When handling sludge samples, it is necessary to wear gloves, face and eye protection, and sufficient body protection to guard against bottles bursting. The gas evolved is usually flammable, so all equipment used in the vicinity shall be flame proof to avoid any source of ignition.

See also the Warning note in Introduction.

9 Procedure

9.1 Sample preparation

Weigh a representative sub-sample of 25 g (wet weight) into a homogeniser bag (7.5) with an integrated mesh if large debris is to be excluded.

Add an appropriate volume of peptone saline suspension (6.1) so that the final weight is 100 g. Place the homogeniser bag (7.5) in a homogeniser (7.4) and homogenize for 1 min.

For lime-treated materials :

adjust the pH to (7.0 ± 0.5) with 1 mol L^{-1} hydrochloric acid. The sample is mixed by shaking between each addition of hydrochloric acid to ensure the correct pH is achieved. The sample is transferred to a sterile 250 mL container and tested using a pH meter (7.10).

NOTE 1 If the pH drops below 4,5 during the neutralisation process, start a new analysis with a fresh test portion.

NOTE 2 For other relevant sludge treatment chemicals (e.g. peracetic acid), a suitable neutralisation procedure must be used (see for example EN 1040:1997).

9.2 Analysis

9.2.1 Preparation of dilutions

Prepare the 1/2 dilution in SMD (6.2) and then serial ten fold dilutions from 1/20 to 1/2 000 000 also in SMD as follow:

Vigorously stir the primary suspension (9.1.2) in order to obtain a homogeneous suspension and, using a sterile pipette, immediately transfer 18 mL of this homogenised suspension to a first tube (7.9) containing 18 mL of SMD to obtain the 1/2 dilution.

Using a fresh pipette (7.11), transfer 2 mL of this first dilution (homogenised by handle shaking) to a second 18 mL SMD tube to obtain the 1/20 dilution.

From this second tube (1/20 dilution carefully homogenised by handle shaking) proceed to another 1/10 dilution giving the following dilution 1/200, while adding 2 mL of 1/20 dilution in 18 mL of SMD.

Continue as above until all the dilutions to 1/2 000 000 have been prepared.

NOTE Appropriate precautions should be taken as aerosols may be created by the diluting and pipetting.

9.2.2 Inoculation and incubation of microtitre plates

Inoculate a microtitre plate (7.15) containing the MUG/EC medium in each well (6.3) while distributing each dilution from 1/20 to 1/2 000 000, each in 16 consecutive wells (the ½ dilution is only used for the preparation of the serial dilutions):

Transfer the contents of the last dilution tube (1/2 000 000) to an empty sterile Petri dish of 90 mm diameter (7.17).

Using a multi-channel pipette (7.12) with 8 sterile tips (7.13), distribute 200 µL per well into 16 wells of the microtitre plate (7.15) corresponding to this last dilution (use the 2 last columns on the left side of the microtitre plate corresponding to the 11A to 11H and 12A to 12H columns).

For subsequent dilutions (1/200, 1/2 000, etc…), operate in an identical manner using for each successive dilution the 2 following 8 wells columns of the microtitre plate. The two last 8 wells columns on the left of the microtitre plate should correspond to the 1/20 dilution.

Alternatively, any other suitable system (7.12) may be used to distribute 200 µL of each dilution per well.

WARNING — Beware of contamination via an overflow from one well to another.

Once the microtitre plate is inoculated, cover with the disposable adhesive tape (7.16) provided for this purpose. Incubate the microtitre plate in an incubator (7.2) at (44 ± 1) °C during (48 ± 4) h.

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

9.2.3 Reading

Place each microtitre plate with the adhesive on, in the UV observation chamber (7.6).
Note the number of positive blue fluorescent wells for each dilution.

Then, using a adjustable pipette with a sterile tip, distribute 15 µL of Kovac's reagent in each blue fluorescent well. Wait 1-2 min for the colour change.
Note the number of positive red-top wells for each dilution.

Consider all wells that are blue fluorescent and red topped positive.

9.3 Determination of the dry residue content

The numbers of *E. coli* may be calculated per wet weight or dry weight. For the latter, it is necessary to determine the dry residue of the sample using the method described in EN 12880:2000. This shall be performed in parallel with the microbiological analysis.

10 Expression of results

10.1 Determination of the characteristic number

For each of the 6 inoculated dilutions, note the number of positive wells and identify the corresponding characteristic number (CN) according to the instructions given in ISO 8199:2005 for MPN calculation:

The CN corresponds to the number of positive wells of the 3 last dilutions giving a number of positive wells > 0 (Table 1).

When it is possible, choose 3 serial dilutions for which results are neither totally positive nor totally negative. If it is not possible, it is better to choose the 3 serial dilutions with positive results than negative results (Table 1, examples **a** and **b**).

If less than 3 serial dilutions give positive results, start from the dilution containing the higher concentration in sample and follow with the 2 next dilutions (Table 1, example **c**).

If there are only positive wells for one of the serial dilutions, start from this dilution and select the previous and the next dilutions (Table 1, example **d**).

Table 1 : Examples for determination of the characteristic number

	1/20	1/200	1/2 000	1/20 000	1/200 000	1/2 000 000	CN
a	16	16	9	3	0	0	16/9/3
b	16	16	9	7	1	0	9/7/1
c	12	5	0	0	0	0	12/5/0
d	0	1	0	0	0	0	0/1/0
e	16	16	12	5	0	0	16/12/5

In the example **e** (Table 1), the CN will be 16/12/5 for dilution 1/200, 1/2 000, 1/20 000.

10.2 Calculation of the MPN and its confidence interval

The Most Probable Number (MPN) is a statistical estimation of the density of micro-organisms, assumed to correspond to a Poisson distribution in the volumes inoculated. Confidence intervals are attached to this MPN.

The MPN corresponding to the identified CN can be obtained from the MPN_i table given in Annex A (informative). The confidence limits at 95 % are attached to this MPN_i.

Proceed then as follows:

Identify the intermediary MPN (MPN_i) from the CN directly in the table in Annex A. The final MPN per mL of suspension (9.1.2), taking into account the dilution steps corresponding to the CN, is calculated from MPN_i as follows:

* If CN correspond to dilutions 1/20, 1/200 and 1/2 000, MPN.mL $^{-1}$ suspension = MPN_i

* If CN correspond to dilutions 1/200, 1/2 000 and 1/20 000, MPN.mL $^{-1}$ suspension = 10 x MPN_i

* If CN correspond to dilutions 1/2 000, 1/20 000 and 1/200 000, MPN.mL $^{-1}$ suspension = 100 x MPN_i

* If CN correspond to dilutions 1/20 000, 1/200 000 and 1/2 000 000, MPN.mL $^{-1}$ suspension = 1 000 x MPN_i

The result per g of wet weight is then calculated as follows:

E. coli MPN g $^{-1}$ wet weight = MPN_{ww} = MPN x 100 mL of total suspension / 25 g

The result may be converted in MPN g^{-1} of dry matter as follows:

E. coli MPN g $^{-1}$ dry matter = $MPN_{dw} = MPN_{ww} \times 100$ / % of dry matter

In the example **e**, Table 1:

If CN = 16/12/5 for dilutions 1/200, 1/2 000 and 1/20 000, the MPN_i statistical table in Annex A gives 1 758.2 mL^{-1}, with a lower limit of 1 018.2 mL^{-1} and an upper limit of 3 036.2 mL^{-1}.

The MPN is then 17 582 mL^{-1}suspension (Lower limit = 10 182 mL^{-1}; Upper limit = 30 362 mL^{-1}).

The result per g wet weight is then:

70 328 *E. coli* MPN g^{-1} wet weight (Lower limit = 40 728 MPN g^{-1} wet weight; Upper limit = 121 448 MPN g^{-1} wet weight).

If none of the wells is positive, express the result in the following form: **< n mL^{-1}**,
n being the MPN for 1 positive well under the dilution conditions employed.

If all the wells are positive, express the result in the following form: **> n mL^{-1},**
n being the MPN lower limit for all positive wells under the dilution conditions employed.

Note **Low and upper limits should always be given.**

11 Performance data

Performance data in terms of repeatability and reproducibility of the procedure, obtained by interlaboratory tests of the validation study (European scale Interlaboratory trial) performed during the FP6 EU Horizontal-Hyg project is given in Annex B (informative).

12 Test report

The test report shall contain the following information:

a) A reference to this European Standard including its date of publication;

b) Sampling report including precise identification of the sample

c) specific reporting on the considered quantification standard

d) expression of results according to clause 10

e) any deviation from this standard, and any facts which may have influenced the result.

Annex A
(informative)

MPN Statistical Table
For Microtiter plate 96 wells
6 dilutions (1/20 to 1/2000000) / 16 wells seeded per dilution

Table A.1

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
0/16	0/16	1/16	5,60	0,80	41,00
0/16	0/16	2/16	11,30	2,70	46,70
0/16	1/16	0/16	5,60	0,80	41,00
0/16	1/16	1/16	11,30	2,70	46,70
0/16	1/16	2/16	16,90	5,20	54,90
0/16	2/16	0/16	11,30	2,70	46,80
0/16	2/16	1/16	17,00	5,30	54,90
0/16	3/16	0/16	17,00	5,30	55,00
0/16	3/16	1/16	22,70	8,10	63,50
0/16	4/16	0/16	22,80	8,20	63,60
1/16	0/16	0/16	5,80	0,80	41,00
1/16	0/16	1/16	11,60	2,90	47,10
1/16	0/16	2/16	17,40	5,50	55,50
1/16	1/16	0/16	11,70	2,90	47,20
1/16	1/16	1/16	17,50	5,50	55,60
1/16	1/16	2/16	23,30	8,40	64,40
1/16	2/16	0/16	17,50	5,50	55,70
1/16	2/16	1/16	23,30	8,50	64,50
1/16	2/16	2/16	29,20	11,60	73,30
1/16	3/16	0/16	23,40	8,50	64,60
1/16	3/16	1/16	29,30	11,70	73,40
1/16	4/16	0/16	29,40	11,70	73,60
1/16	4/16	1/16	35,20	15,10	82,50
1/16	5/16	0/16	35,30	15,10	82,60
2/16	0/16	0/16	12,00	3,00	47,60
2/16	0/16	1/16	17,90	5,70	56,30
2/16	0/16	2/16	23,90	8,80	65,40

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
2/16	1/16	0/16	18,00	5,70	56,40
2/16	1/16	1/16	24,00	8,80	65,50
2/16	1/16	2/16	30,00	12,10	74,60
2/16	2/16	0/16	24,10	8,80	65,60
2/16	2/16	1/16	30,10	12,10	74,70
2/16	2/16	2/16	36,20	15,60	83,90
2/16	3/16	0/16	30,20	12,20	74,80
2/16	3/16	1/16	36,30	15,70	84,00
2/16	4/16	0/16	36,40	15,70	84,20
2/16	4/16	1/16	42,50	19,30	93,40
2/16	5/16	0/16	42,60	19,40	93,60
3/16	0/16	0/16	18,5	6	57,2
3/16	0/16	1/16	24,7	9,2	66,5
3/16	0/16	2/16	30,9	12,6	75,9
3/16	0/16	3/16	37,1	16,2	85,4
3/16	1/16	0/16	24,8	9,2	66,6
3/16	1/16	1/16	31	12,6	76,1
3/16	1/16	1/16	37,3	16,2	85,5
3/16	2/16	0/16	31,1	12,7	76,2
3/16	2/16	1/16	37,4	16,3	85,7
3/16	2/16	2/16	43,6	20	95,2
3/16	3/16	0/16	37,5	16,4	85,8
3/16	3/16	1/16	43,8	20,1	95,3
3/16	3/16	2/16	50,1	23,9	104,9
3/16	4/16	0/16	43,9	20,2	95,5
3/16	4/16	1/16	50,2	24	105,1
3/16	5/16	0/16	50,4	24,1	105,3
3/16	5/16	1/16	56,7	28	114,9
3/16	6/16	0/16	56,9	28,1	115,1

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
4/16	0/16	0/16	25,5	9,6	67,8
4/16	0/16	1/16	32	13,2	77,5
4/16	0/16	2/16	38,4	16,9	87,3
4/16	0/16	3/16	44,9	20,8	97
4/16	1/16	0/16	32,1	13,2	77,6
4/16	1/16	1/16	38,5	17	87,4
4/16	1/16	2/16	45	20,8	97,2
4/16	1/16	3/16	51,5	24,8	107,1
4/16	2/16	0/16	38,6	17	87,6
4/16	2/16	1/16	45,1	20,9	97,4
4/16	2/16	2/16	51,7	24,9	107,3
4/16	3/16	0/16	45,3	21	97,6
4/16	3/16	1/16	51,8	25	107,5
4/16	3/16	2/16	58,4	29,1	117,4
4/16	4/16	0/16	52	25,1	107,7
4/16	4/16	1/16	58,6	29,2	117,7
4/16	4/16	2/16	65,2	33,3	127,7
4/16	5/16	0/16	58,8	29,3	118
4/16	5/16	1/16	65,4	33,4	128
4/16	6/16	0/16	65,6	33,6	128,3
5/16	0/16	0/16	33,1	13,8	79,2
5/16	0/16	1/16	39,8	17,7	89,3
5/16	0/16	2/16	46,5	21,7	99,4
5/16	0/16	3/16	53,2	25,9	109,6
5/16	1/16	0/16	39,9	17,8	89,5
5/16	1/16	1/16	46,6	21,8	99,7
5/16	1/16	2/16	53,4	26	109,9
5/16	1/16	3/16	60,2	30,2	120,1
5/16	2/16	0/16	46,8	21,9	99,9
5/16	2/16	1/16	53,6	26,1	110,1
5/16	2/16	2/16	60,4	30,3	120,4
5/16	2/16	3/16	67,3	34,6	130,8
5/16	3/16	0/16	53,7	26,2	110,4
5/16	3/16	1/16	60,6	30,4	120,7
5/16	3/16	2/16	67,5	34,7	131,1

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
5/16	4/16	0/16	60,8	30,5	121
5/16	4/16	1/16	67,7	34,9	131,4
5/16	4/16	2/16	74,6	39,2	141,9
5/16	5/16	0/16	67,9	35	131,7
5/16	5/16	1/16	74,9	39,4	142,3
5/16	6/16	0/16	75,1	39,6	142,6
5/16	6/16	1/16	82,1	44	153,3
5/16	7/16	0/16	82,4	44,2	153,7
6/16	0/16	0/16	41,2	18,6	91,5
6/16	0/16	1/16	48,2	22,8	102,1
6/16	0/16	2/16	55,3	27,1	112,7
6/16	0/16	3/16	62,2	31,5	123,3
6/16	1/16	0/16	48,4	22,9	102,3
6/16	1/16	1/16	55,4	27,2	112,9
6/16	1/16	2/16	62,2	31,6	123,6
6/16	1/16	3/16	69,7	36,1	134,4
6/16	2/16	0/16	55,6	27,3	113,2
6/16	2/16	1/16	62,8	31,8	124
6/16	2/16	2/16	69,9	36,3	134,8
6/16	2/16	3/16	77,1	40,8	145,7
6/16	3/16	0/16	63	31,9	124,3
6/16	3/16	1/16	70,1	36,4	135,1
6/16	3/16	2/16	77,4	41	146,1
6/16	4/16	0/16	70,4	36,6	135,5
6/16	4/16	1/16	77,6	41,2	146,5
6/16	4/16	2/16	84,9	45,8	157,6
6/16	5/16	0/16	77,9	41,3	146,9
6/16	5/16	1/16	85,2	46	158
6/16	5/16	2/16	92,6	50,7	169,2
6/16	6/16	0/16	85,5	46,2	158,4
6/16	6/16	1/16	92,9	50,9	169,7
6/16	7/16	0/16	93,2	51,1	170,2

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
7/16	0/16	0/16	50,1	23,9	104,9
7/16	0/16	1/16	57,5	28,5	116
7/16	0/16	2/16	64,9	33,1	127,1
7/16	0/16	3/16	72,3	37,8	138,4
7/16	1/16	0/16	57,6	28,6	116,3
7/16	1/16	1/16	65,1	33,2	127,5
7/16	1/16	2/16	72,6	37,9	138,8
7/16	1/16	3/16	80,1	42,7	150,2
7/16	2/16	0/16	65,3	33,4	127,8
7/16	2/16	1/16	72,8	38,1	139,2
7/16	2/16	2/16	80,4	42,9	150,6
7/16	2/16	3/16	88	47,7	162,2
7/16	3/16	0/16	73,1	38,3	139,5
7/16	3/16	1/16	80,7	43,1	151
7/16	3/16	2/16	88,3	47,9	162,7
7/16	3/16	3/16	96	52,8	174,5
7/16	4/16	0/16	80,9	43,3	151,5
7/16	4/16	1/16	88,6	48,1	163,2
7/16	4/16	2/16	96,3	53	175
7/16	5/16	0/16	88,9	48,3	163,6
7/16	5/16	1/16	96,7	53,3	175,5
7/16	5/16	2/16	104,5	58,2	187,6
7/16	6/16	0/16	97	53,5	176
7/16	6/16	1/16	104,9	58,5	188,1
7/16	7/16	0/16	105,3	58,7	188,7
8/16	0/16	0/16	59,8	30	119,6
8/16	0/16	1/16	67,8	34,8	131,3
8/16	0/16	2/16	75,5	39,8	143,2
8/16	0/16	3/16	83,4	44,8	155,2
8/16	1/16	0/16	67,9	35	131,7
8/16	1/16	1/16	75,9	39,9	143,6
8/16	1/16	2/16	83,7	45	155,6
8/16	1/16	3/16	91,7	50,1	167,8
8/16	2/16	0/16	76	40,1	144
8/16	2/16	1/16	84	45,2	156,1
8/16	2/16	2/16	92	50,3	168,3
8/16	2/16	3/16	100,1	55,4	180,8
8/16	3/16	0/16	84,3	45,4	156,5
8/16	3/16	1/16	92,3	50,5	168,9
8/16	3/16	2/16	100,5	55,7	181,3
8/16	3/16	3/16	108,7	60,9	194
8/16	4/16	0/16	92,7	50,7	169,4
8/16	4/16	1/16	100,9	55,9	181,9
8/16	4/16	2/16	109,1	61,1	194,7
8/16	4/16	3/16	117,4	66,4	207,6

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
8/16	5/16	0/16	101,3	56,1	182,5
8/16	5/16	1/16	109,5	61,4	195,3
8/16	5/16	2/16	117,9	66,7	208,3
8/16	6/16	0/16	109,9	61,7	195,9
8/16	6/16	1/16	118,3	67	209
8/16	6/16	2/16	126,8	72,3	222,3
8/16	7/16	0/16	118,8	67,3	209,7
8/16	7/16	1/16	127,3	72,6	223,1
8/16	8/16	0/16	127,8	72,9	223,9
9/16	0/16	0/16	70,7	36,8	135,9
9/16	0/16	1/16	78,9	42	148,4
9/16	0/16	2/16	87,3	47,3	161,1
9/16	0/16	3/16	95,7	52,6	174
9/16	1/16	0/16	79,7	42,2	148,9
9/16	1/16	1/16	87,6	47,5	161,6
9/16	1/16	2/16	96,1	52,9	174,6
9/16	1/16	3/16	104,6	58,3	187,8
9/16	2/16	0/16	87,9	47,7	162,1
9/16	2/16	1/16	96,5	53,1	175,2
9/16	2/16	2/16	105,1	58,6	188,4
9/16	2/16	3/16	113,7	64,1	201,9
9/16	3/16	0/16	96,8	53,4	175,7
9/16	3/16	1/16	105,5	58,8	189,1
9/16	3/16	2/16	114,2	64,4	202,6
9/16	3/16	3/16	123	69,9	216,4
9/16	4/16	0/16	105,9	59,1	189,7
9/16	4/16	1/16	114,7	64,7	203,3
9/16	4/16	2/16	123,5	70,3	217,2
9/16	4/16	3/16	132,5	75,9	231,4
9/16	5/16	0/16	115,1	65	204
9/16	5/16	1/16	124	70,6	218
9/16	5/16	2/16	133,1	76,2	232,3
9/16	5/16	3/16	142,2	81,9	246,8
9/16	6/16	0/16	124,6	70,9	218,8
9/16	6/16	1/16	133,6	76,6	233,1
9/16	6/16	2/16	142,8	82,3	247,8
9/16	7/16	0/16	134,5	76,9	234
9/16	7/16	1/16	143,4	82,6	248,7
9/16	7/16	2/16	152,7	88,4	263,8
9/16	8/16	0/16	144	83	249,7
9/16	8/16	1/16	153,4	88,8	264,9
9/16	9/16	0/16	154	89,2	265,9

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
10/16	0/16	0/16	82,8	44,4	154,3
10/16	0/16	1/16	91,7	50,1	167,8
10/16	0/16	2/16	100,6	55,8	181,6
10/16	0/16	3/16	109,7	61,5	195,6
10/16	1/16	0/16	92	50,3	168,4
10/16	1/16	1/16	101	56	182,2
10/16	1/16	2/16	110,2	61,8	196,3
10/16	1/16	3/16	119,4	67,7	210,8
10/16	2/16	0/16	101,5	56,3	182,9
10/16	2/16	1/16	110,6	62,1	197,1
10/16	2/16	2/16	119,9	68	211,6
10/16	2/16	3/16	129,4	73,9	226,4
10/16	3/16	0/16	111,1	62,4	197,8
10/16	3/16	1/16	120,5	68,3	212,4
10/16	3/16	2/16	129,9	74,3	227,3
10/16	3/16	3/16	139,6	80,3	242,6
10/16	4/16	0/16	121	68,7	213,2
10/16	4/16	1/16	130,5	74,6	228,2
10/16	4/16	2/16	140,2	80,7	243,6
10/16	4/16	3/16	150	86,7	259,4
10/16	5/16	0/16	131,1	75	229,1
10/16	5/16	1/16	140,8	81,1	244,6
10/16	5/16	2/16	150,7	87,1	260,5
10/16	5/16	3/16	160,7	93,3	276,8
10/16	6/16	0/16	141,4	81,4	245,6
10/16	6/16	1/16	151,4	87,6	261,6
10/16	6/16	2/16	161,4	93,7	278
10/16	6/16	3/16	171,6	99,9	294,8
10/16	7/16	0/16	152,1	88	262,7
10/16	7/16	1/16	162,2	94,2	279,2
10/16	7/16	2/16	172,4	100,4	296,2
10/16	8/16	0/16	162,9	94,6	280,5
10/16	8/16	1/16	173,2	100,9	297,6
10/16	9/16	0/16	174,1	101,4	298,9
11/16	0/16	0/16	96,6	53,2	175,4
11/16	0/16	1/16	106,2	59,3	190,2
11/16	0/16	2/16	116	65,5	205,4
11/16	0/16	3/16	125,9	71,7	220,9

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
11/16	1/16	0/16	106,7	59,6	190,9
11/16	1/16	1/16	116,5	65,8	206,2
11/16	1/16	2/16	126,5	72,1	221,8
11/16	1/16	3/16	136,6	78,5	237,9
11/16	1/16	4/16	147	84,9	254,5
11/16	2/16	0/16	117	66,2	207
11/16	2/16	1/16	127,1	72,5	222,8
11/16	2/16	2/16	137,3	78,9	239
11/16	2/16	3/16	147,7	85,3	255,6
11/16	2/16	4/16	158,2	91,8	272,8
11/16	3/16	0/16	127,7	72,8	223,7
11/16	3/16	1/16	137,9	79,3	240
11/16	3/16	2/16	148,4	85,7	256,8
11/16	3/16	3/16	159	92,3	274,1
11/16	3/16	4/16	169,8	98,8	291,9
11/16	4/16	0/16	138,6	79,7	241,1
11/16	4/16	1/16	149,1	86,2	257,9
11/16	4/16	2/16	159,8	92,7	275,4
11/16	4/16	3/16	170,7	99,3	293,3
11/16	5/16	0/16	149,8	86,6	259,1
11/16	5/16	1/16	160,6	93,2	276,7
11/16	5/16	2/16	171,6	99,9	294,7
11/16	5/16	3/16	182,7	106,5	313,4
11/16	6/16	0/16	161,4	93,7	278
11/16	6/16	1/16	172,4	100,4	296,2
11/16	6/16	2/16	183,7	107,1	315
11/16	6/16	3/16	195,1	113,8	334,4
11/16	7/16	0/16	173,3	100,9	297,7
11/16	7/16	1/16	184,6	107,7	316,6
11/16	7/16	2/16	196,2	114,4	336,2
11/16	8/16	0/16	185,6	108,2	318,3
11/16	8/16	1/16	197,2	115	338,1
11/16	8/16	2/16	209	121,9	358,5
11/16	9/16	0/16	198,3	115,7	339,9
11/16	9/16	1/16	210,2	122,5	360,5
11/16	10/16	0/16	211,4	123,2	362,6

Characteristic Number			MPN in 1 ml	Lower limit	Upper limit
12/16	0/16	0/16	112,6	63,4	200,1
12/16	0/16	1/16	123,2	70	216,7
12/16	0/16	2/16	134	76,8	233,7
12/16	0/16	3/16	145	83,7	251,4
12/16	1/16	0/16	123,8	70,4	217,6
12/16	1/16	1/16	134,7	77,2	234,8
12/16	1/16	2/16	145,8	84,1	252,6
12/16	1/16	3/16	157,1	91,1	271
12/16	1/16	4/16	168,7	98,1	290
12/16	2/16	0/16	135,3	77,7	235,9
12/16	2/16	1/16	146,5	84,6	253,8
12/16	2/16	2/16	157,9	91,6	272,3
12/16	2/16	3/16	169,6	98,7	291,5
12/16	2/16	4/16	181,6	105,8	311,5
12/16	3/16	0/16	147,3	85,1	255
12/16	3/16	1/16	158,8	92,1	273,7
12/16	3/16	2/16	170,6	99,3	293,1
12/16	3/16	3/16	182,6	106,4	313,2
12/16	3/16	4/16	194,9	113,7	334,1
12/16	4/16	0/16	159,6	92,6	275,1
12/16	4/16	1/16	171,5	99,8	294,6
12/16	4/16	2/16	183,6	107,1	314,9
12/16	4/16	3/16	196	114,3	336
12/16	4/16	4/16	208,7	121,7	357,9
12/16	5/16	0/16	172,4	100,4	296,2
12/16	5/16	1/16	184,7	107,7	316,7
12/16	5/16	2/16	197,2	115	338
12/16	5/16	3/16	209,9	122,4	360,1
12/16	5/16	4/16	223	129,8	383,1
12/16	6/16	0/16	185,7	108,3	318,5
12/16	6/16	1/16	198,3	115,7	339,9
12/16	6/16	2/16	211,2	123,1	362,3
12/16	6/16	3/16	224,4	130,6	385,6
12/16	7/16	0/16	199,5	116,4	342
12/16	7/16	1/16	212,5	123,9	364,5
12/16	7/16	2/16	225,8	131,4	388,1
12/16	7/16	3/16	239,4	139	412,6
12/16	8/16	0/16	213,8	124,6	366,8
12/16	8/16	1/16	227,2	132,2	390,6
12/16	8/16	2/16	241	139,8	415,4
12/16	9/16	0/16	228,7	133	393,2
12/16	9/16	1/16	242,6	140,7	418,2
12/16	9/16	2/16	256,8	148,4	444,4

Characteristic Number			MPN in 1 ml	Lower limit	Upper limit
12/16	10/16	0/16	244,2	141,5	421,1
12/16	10/16	1/16	258,5	149,3	447,6
13/16	0/16	0/16	131,7	75,4	230,1
13/16	0/16	1/16	143,6	82,8	249,1
13/16	0/16	2/16	155,8	90,3	268,9
13/16	0/16	3/16	168,4	97,9	289,5
13/16	0/16	4/16	181,3	105,7	311,1
13/16	1/16	0/16	144,4	83,3	250,4
13/16	1/16	1/16	156,7	90,9	270,3
13/16	1/16	2/16	169,4	98,5	291,2
13/16	1/16	3/16	182,4	106,4	312,9
13/16	1/16	4/16	195,8	114,3	335,7
13/16	2/16	0/16	157,6	91,4	271,8
13/16	2/16	1/16	170,4	99,2	292,8
13/16	2/16	2/16	183,5	107	314,8
13/16	2/16	3/16	197,1	115	337,8
13/16	2/16	4/16	211	123	362
13/16	3/16	0/16	171,4	99,8	294,5
13/16	3/16	1/16	184,7	107,7	316,7
13/16	3/16	2/16	198,3	115,7	340
13/16	3/16	3/16	212,4	123,8	364,4
13/16	3/16	4/16	226,9	132	390
13/16	4/16	0/16	185,8	108,4	318,7
13/16	4/16	1/16	199,6	116,5	342,2
13/16	4/16	2/16	213,8	124,6	366,9
13/16	4/16	3/16	228,5	132,9	392,8
13/16	4/16	4/16	243,6	141,2	420,1
13/16	5/16	0/16	200,9	117,2	344,4
13/16	5/16	1/16	215,3	125,4	369,4
13/16	5/16	2/16	230,1	133,8	395,6
13/16	5/16	3/16	245,3	142,2	423,3
13/16	5/16	4/16	261	150,7	452,3
13/16	6/16	0/16	216,7	126,3	372
13/16	6/16	1/16	231,7	134,7	398,5
13/16	6/16	2/16	247,1	143,2	426,5
13/16	6/16	3/16	263	151,7	455,9
13/16	6/16	4/16	279,4	160,3	486,9
13/16	7/16	0/16	233,3	135,6	401,5
13/16	7/16	1/16	248,9	144,1	429,8
13/16	7/16	2/16	265	152,8	459,7
13/16	7/16	3/16	281,6	161,5	491,1
13/16	7/16	4/16	298,7	170,3	524,1

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
13/16	8/16	0/16	250,8	145,1	433,2
13/16	8/16	1/16	267	153,9	463,5
13/16	8/16	2/16	283,8	162,6	495,4
13/16	8/16	3/16	301,2	171,5	528,9
13/16	9/16	0/16	269,1	154,9	467,4
13/16	9/16	1/16	286,1	163,8	499,8
13/16	9/16	2/16	303,7	172,8	533,8
13/16	9/16	3/16	321,8	181,8	569,7
13/16	10/16	0/16	288,5	165	504,2
13/16	10/16	1/16	306,3	174,1	538,9
13/16	10/16	2/16	324,6	183,2	575,3
13/16	11/16	0/16	308,9	175,4	544
13/16	11/16	1/16	327,5	184,6	581
14/16	0/16	0/16	155,3	90	267,9
14/16	0/16	1/16	169,1	98,4	290,7
14/16	0/16	2/16	183,5	107	314,7
14/16	0/16	3/16	198,4	115,7	340,1
14/16	0/16	4/16	213,9	124,6	366,9
14/16	1/16	0/16	170,2	99	292,5
14/16	1/16	1/16	184,7	107,7	316,8
14/16	1/16	2/16	199,8	116,5	342,5
14/16	1/16	3/16	215,4	125,5	369,7
14/16	1/16	4/16	231,7	134,7	398,7
14/16	2/16	0/16	186	108,5	318,9
14/16	2/16	1/16	201,2	117,4	344,9
14/16	2/16	2/16	217,1	126,5	372,5
14/16	2/16	3/16	233,5	135,7	401,9
14/16	2/16	4/16	250,7	145,1	433,1
14/16	3/16	0/16	202,7	118,2	347,5
14/16	3/16	1/16	218,7	127,4	375,4
14/16	3/16	2/16	235,4	136,7	405,2
14/16	3/16	3/16	252,8	146,2	436,9
14/16	3/16	4/16	270,9	155,9	470,7
14/16	3/16	5/16	289,8	165,7	506,8
14/16	4/16	0/16	220,4	128,3	378,4
14/16	4/16	1/16	237,3	137,8	408,6
14/16	4/16	2/16	254,9	147,4	440,8
14/16	4/16	3/16	273,3	157,1	475,2
14/16	4/16	4/16	292,4	167,1	511,9
14/16	4/16	5/16	312,4	177,2	551

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
14/16	5/16	0/16	239,2	138,8	412,1
14/16	5/16	1/16	257	148,5	444,8
14/16	5/16	2/16	275,7	158,4	479,8
14/16	5/16	3/16	295,1	168,4	517,2
14/16	5/16	4/16	315,5	178,7	557
14/16	5/16	5/16	336,7	189,1	599,4
14/16	6/16	0/16	259,2	149,7	448,9
14/16	6/16	1/16	278,2	159,7	484,5
14/16	6/16	2/16	297,9	169,9	522,6
14/16	6/16	3/16	318,6	180,2	563,2
14/16	6/16	4/16	340,1	190,8	606,4
14/16	7/16	0/16	280,7	161	489,4
14/16	7/16	1/16	300,8	171,3	528,1
14/16	7/16	2/16	321,8	181,8	569,5
14/16	7/16	3/16	343,7	192,5	613,7
14/16	7/16	4/16	366,6	203,4	660,5
14/16	8/16	0/16	303,7	172,8	533,8
14/16	8/16	1/16	325	183,4	576,1
14/16	8/16	2/16	347,4	194,2	621,2
14/16	8/16	3/16	370,7	205,4	669
14/16	8/16	4/16	394,9	216,8	719,5
14/16	9/16	0/16	328,4	185,1	582,9
14/16	9/16	1/16	351,1	196,1	628,9
14/16	9/16	2/16	374,9	207,4	677,8
14/16	9/16	3/16	399,6	219	729,4
14/16	9/16	4/16	425,4	231	783,4
14/16	10/16	0/16	355	197,9	636,9
14/16	10/16	1/16	379,2	209,4	686,8
14/16	10/16	2/16	404,5	221,2	739,5
14/16	10/16	3/16	430,8	233,5	794,7
14/16	11/16	0/16	383,7	211,5	696,2
14/16	11/16	1/16	409,5	223,6	750,1
14/16	11/16	2/16	436,3	236,1	806,5
14/16	11/16	3/16	464,2	249,1	864,9
14/16	12/16	0/16	414,7	226	761
14/16	12/16	1/16	442,1	238,8	818,6
14/16	12/16	2/16	470,6	252,1	878,3

Characteristic Number			MPN in 1 ml	Lower limit	Upper limit
15/16	0/16	0/16	186,2	108,6	319,2
15/16	0/16	1/16	203,2	118,5	348,4
15/16	0/16	2/16	221,1	128,8	379,8
15/16	0/16	3/16	240,1	139,3	413,7
15/16	0/16	4/16	260,1	150,2	450,5
15/16	1/16	0/16	204,9	119,5	351,2
15/16	1/16	1/16	223	129,8	383,1
15/16	1/16	2/16	242,3	140,5	417,7
15/16	1/16	3/16	262,6	151,5	455,3
15/16	1/16	4/16	284,2	162,8	496,1
15/16	2/16	0/16	225	130,9	386,6
15/16	2/16	1/16	244,5	141,8	421,8
15/16	2/16	2/16	265,2	152,9	460,1
15/16	2/16	3/16	287,2	164,4	501,9
15/16	2/16	4/16	310,6	176,2	547,4
15/16	2/16	5/16	335,5	188,5	597,1
15/16	3/16	0/16	246,8	143	426
15/16	3/16	1/16	267,9	154,3	465,1
15/16	3/16	2/16	290,3	166	507,8
15/16	3/16	3/16	314,2	178	554,5
15/16	3/16	4/16	339,6	190,5	605,4
15/16	3/16	5/16	366,6	203,4	660,7
15/16	4/16	0/16	270,7	155,8	470,3
15/16	4/16	1/16	293,5	167,6	514
15/16	4/16	2/16	317,9	179,9	561,8
15/16	4/16	3/16	343,8	192,6	614
15/16	4/16	4/16	371,5	205,8	670,8
15/16	4/16	5/16	401	219,6	732,2
15/16	5/16	0/16	296,8	169,3	520,4
15/16	5/16	1/16	321,7	181,7	569,4
15/16	5/16	2/16	348,3	194,7	623
15/16	5/16	3/16	376,6	208,2	681,4
15/16	5/16	4/16	406,9	222,3	744,5
15/16	5/16	5/16	439	237,3	812
15/16	6/16	0/16	325,6	183,7	577,3
15/16	6/16	1/16	353,3	196,9	632,4
15/16	6/16	2/16	382	210,7	692,5
15/16	6/16	3/16	413	225,2	757,4
15/16	6/16	4/16	446	240,6	826,8
15/16	6/16	5/16	480,9	257	899,8
15/16	7/16	0/16	359,4	199,2	642,1
15/16	7/16	1/16	387,5	213,3	704
15/16	7/16	2/16	419,4	228,2	770,9
15/16	7/16	3/16	453,4	244	842,3
15/16	7/16	4/16	489,3	261	917,3
15/16	7/16	5/16	527,1	279,3	994,8

Characteristic Number			MPN in 1 ml	Lower limit	Upper limit
15/16	8/16	0/16	393,3	216	716,1
15/16	8/16	1/16	426,1	231,3	785
15/16	8/16	2/16	461,1	247,7	858,5
15/16	8/16	3/16	498,2	265,2	935,6
15/16	8/16	4/16	531,3	284,2	1015
15/16	8/16	5/16	576,2	304,8	1095,5
15/16	9/16	0/16	433,2	234,6	799,8
15/16	9/16	1/16	469,2	251,5	875,5
15/16	9/16	2/16	507,5	269,7	954,8
15/16	9/16	3/16	547,7	289,5	1036,2
15/16	9/16	4/16	589,7	310,9	1118,6
15/16	9/16	5/16	633,2	334	1200,7
15/16	10/16	0/16	477,8	255,5	893,3
15/16	10/16	1/16	516,6	274,5	974,8
15/16	10/16	2/16	558,9	295,1	1058,4
15/16	10/16	3/16	602,3	317,5	1142,6
15/16	10/16	4/16	647,3	341,6	1226,5
15/16	10/16	5/16	693,6	367,3	1309,5
15/16	11/16	0/16	527,6	279,5	995,9
15/16	11/16	1/16	570,6	301,1	1081,6
15/16	11/16	2/16	615,6	324,5	1167,7
15/16	11/16	3/16	662,7	349,7	1253,4
15/16	11/16	4/16	710	376,7	1338,1
15/16	11/16	5/16	758,9	405,1	1421,8
15/16	12/16	0/16	583,1	307,5	1105,9
15/16	12/16	1/16	629,6	332	1194
15/16	12/16	2/16	677,8	358,5	1281,6
15/16	12/16	3/16	727,4	386,7	1368,2
15/16	12/16	4/16	778,1	416,4	1453,8
15/16	13/16	0/16	644,6	340,1	1221,6
15/16	13/16	1/16	694,5	367,9	1311,1
15/16	13/16	2/16	745,8	397,4	1399,7
15/16	13/16	3/16	798,4	428,5	1487,5
15/16	13/16	4/16	851,9	460,9	1574,8
15/16	14/16	0/16	712,3	378	1342,2
15/16	14/16	1/16	765,6	409	1432,9
15/16	14/16	2/16	829,6	441,5	1523
15/16	14/16	3/16	875,6	475,3	1612,9
16/16	0/16	0/16	231,2	134,4	397,6
16/16	0/16	1/16	254,5	147,2	440,2
16/16	0/16	2/16	280	160,7	488,1
16/16	0/16	3/16	308	174,9	542,3
16/16	0/16	4/16	338,8	190,1	603,8

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
16/16	1/16	0/16	257,5	148,8	445,6
16/16	1/16	1/16	283,6	162,5	494,9
16/16	1/16	2/16	312,3	177,1	550,8
16/16	1/16	3/16	344,1	192,7	614,5
16/16	1/16	4/16	379,3	209,5	687
16/16	1/16	5/16	418,4	227,7	768,8
16/16	2/16	0/16	287,3	164,4	501,9
16/16	2/16	1/16	316,8	179,3	559,7
16/16	2/16	2/16	349,6	195,3	625,8
16/16	2/16	3/16	386,2	212,7	701,3
16/16	2/16	4/16	426,9	231,7	786,6
16/16	2/16	5/16	472	252,8	881,3
16/16	3/16	0/16	321,6	181,7	569,1
16/16	3/16	1/16	355,5	198,1	637,8
16/16	3/16	2/16	393,4	216,1	716,4
16/16	3/16	3/16	435,9	235,8	805,5
16/16	3/16	4/16	483,1	258	904,3
16/16	3/16	5/16	535,1	283,2	1010,9
16/16	3/16	6/16	591,5	311,8	1121,9
16/16	4/16	0/16	361,6	201,1	650,4
16/16	4/16	1/16	406,3	219,7	732,5
16/16	4/16	2/16	445,5	240,3	825,7
16/16	4/16	3/16	495	263,7	929
16/16	4/16	4/16	549,6	290,4	1040
16/16	4/16	5/16	608,9	320,9	1155,1
16/16	4/16	6/16	672	355,2	1271,2
16/16	5/16	0/16	409,3	223,5	749,7
16/16	5/16	1/16	455,8	245,2	847,3
16/16	5/16	2/16	503,1	269,9	955,5
16/16	5/16	3/16	565,3	298,4	1071,1
16/16	5/16	4/16	627,7	331	1190,5
16/16	5/16	5/16	694,2	367,7	1310,6
16/16	5/16	6/16	763,7	407,9	1429,8
16/16	5/16	7/16	835,5	450,9	1548,2
16/16	6/16	0/16	462,8	250,4	870,6
16/16	6/16	1/16	521,7	276,6	983,9
16/16	6/16	2/16	582,5	307,1	1104,5
16/16	6/16	3/16	648,3	342,2	1228,5
16/16	6/16	4/16	713,2	381,5	1352,7
16/16	6/16	5/16	794,9	424,4	1476,3
16/16	6/16	6/16	867,1	470,1	1599,2
16/16	6/16	7/16	944,3	517,7	1722,4

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
16/16	7/16	0/16	536,8	284,1	1014,5
16/16	7/16	1/16	601,2	316,9	1140,5
16/16	7/16	2/16	670,9	354,6	1269,3
16/16	7/16	3/16	744,9	396,9	1398,1
16/16	7/16	4/16	822,1	442,8	1526,5
16/16	7/16	5/16	901,8	491,4	1654,8
16/16	7/16	6/16	983,2	541,9	1783,9
16/16	7/16	7/16	1065,4	593,8	1914,7
16/16	7/16	8/16	1150,9	646,7	2048
16/16	8/16	0/16	621,7	327,8	1179,2
16/16	8/16	1/16	695,7	368,5	1313,2
16/16	8/16	2/16	774,1	414,1	1447,2
16/16	8/16	3/16	855,8	463,2	1581,1
16/16	8/16	4/16	940	515,1	1715,6
16/16	8/16	5/16	1026,2	568,8	1851,7
16/16	8/16	6/16	1114,3	623,9	1990,3
16/16	8/16	7/16	1204	680	2132
16/16	8/16	8/16	1295,5	736,9	2277,5
16/16	9/16	0/16	723,1	384,2	1360,9
16/16	9/16	1/16	806,4	433,3	1500,7
16/16	9/16	2/16	893,1	486,1	1641
16/16	9/16	3/16	982,5	541,5	1782,8
16/16	9/16	4/16	1074,2	598,7	1927,1
16/16	9/16	5/16	1167,9	657,4	2074,9
16/16	9/16	6/16	1263,8	717,2	2226,8
16/16	9/16	7/16	1361,6	777,9	2383,3
16/16	9/16	8/16	1461,6	839,5	2545
16/16	9/16	9/16	1563,8	901,7	2712,1
16/16	10/16	0/16	842,4	455,1	1559,4
16/16	10/16	1/16	934,8	511,8	1707,3
16/16	10/16	2/16	1030,1	571,2	1857,8
16/16	10/16	3/16	1128	632,5	2012
16/16	10/16	4/16	1228,5	695,2	2170,8
16/16	10/16	5/16	1331,4	759,2	2334,9
16/16	10/16	6/16	1437	824,3	2505
16/16	10/16	7/16	1545,2	890,4	2681,5
16/16	10/16	8/16	1656,3	957,4	2865,2
16/16	10/16	9/16	1770,2	1025,3	3056,4

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
16/16	11/16	0/16	981,7	541	1781,4
16/16	11/16	1/16	1083,9	604,8	1942,4
16/16	11/16	2/16	1189,2	670,7	2108,6
16/16	11/16	3/16	1297,7	738,3	2280,9
16/16	11/16	4/16	1409,3	807,3	2460,2
16/16	11/16	5/16	1524,1	877,6	2647
16/16	11/16	6/16	1642,6	949,2	2842,4
16/16	11/16	7/16	1764,7	1022	3047,1
16/16	11/16	8/16	1890,7	1095,9	3261,7
16/16	11/16	9/16	2020,7	1170,9	3487,3
16/16	11/16	10/16	2155,1	1246,9	3724,8
16/16	12/16	0/16	1145,4	643,3	2039,3
16/16	12/16	1/16	1259,6	714,6	2220,3
16/16	12/16	2/16	1377,8	787,9	2409,3
16/16	12/16	3/16	1500,1	863	2607,7
16/16	12/16	4/16	1626,8	939,7	2816,2
16/16	12/16	5/16	1758,2	1018,2	3036,2
16/16	12/16	6/16	1894,7	1098,3	3268,9
16/16	12/16	7/16	2036,5	1179,9	3515
16/16	12/16	8/16	2184	1263,1	3776,5
16/16	12/16	9/16	2337,8	1347,8	4055,1
16/16	12/16	10/16	2497,8	1433,8	4351,6
16/16	12/16	11/16	2664,9	1521,2	4668,4
16/16	13/16	0/16	1341,9	765,7	2351,8
16/16	13/16	1/16	1472,4	846	2562,5
16/16	13/16	2/16	1608,6	928,8	2786
16/16	13/16	3/16	1750,7	1013,7	3023,4
16/16	13/16	4/16	1899,5	1101,1	3276,9
16/16	13/16	5/16	2055,5	1190,7	3548,3
16/16	13/16	6/16	2219,3	1282,6	3839,8
16/16	13/16	7/16	2391,6	1376,9	4154
16/16	13/16	8/16	2573,3	1473,6	4493,8
16/16	13/16	9/16	2764,9	1572,5	4861,5
16/16	13/16	10/16	2967,2	1673,7	5260,3
16/16	13/16	11/16	3181,5	1777,7	5693,9
16/16	13/16	12/16	3408,3	1884,4	6164,7

Characteristic Number			MPNi in 1 ml	Lower limit	Upper limit
16/16	14/16	0/16	1587	915,7	2750,3
16/16	14/16	1/16	1741,7	1008,4	3008,2
16/16	14/16	2/16	1905,3	1104,4	3286,8
16/16	14/16	3/16	2078,8	1203,9	3589,3
16/16	14/16	4/16	2263,4	1307	3919,5
16/16	14/16	5/16	2460,3	1413,8	4281,5
16/16	14/16	6/16	2671,1	1524,5	4680,4
16/16	14/16	7/16	2897,6	1639,2	5121,9
16/16	14/16	8/16	3141,5	1758,5	5612,1
16/16	14/16	9/16	3405	1882,8	6157,7
16/16	14/16	10/16	3689,7	2012,9	6763,3
16/16	14/16	11/16	3997,9	2150	7434
16/16	14/16	12/16	4331,7	2296,2	8171,4
16/16	14/16	13/16	4693,3	2454,4	8974,6
16/16	15/16	0/16	1912,4	1108,6	3299,2
16/16	15/16	1/16	2108,2	1220,6	3641,5
16/16	15/16	2/16	2320,4	1338,3	4023,2
16/16	15/16	3/16	2551,8	1462,3	4453,1
16/16	15/16	4/16	2806	1593,3	4941,6
16/16	15/16	5/16	3087	1732,2	5501,3
16/16	15/16	6/16	3399,7	1880,4	6146,7
16/16	15/16	7/16	3749,8	2039,9	6893
16/16	15/16	8/16	4143,8	2214,1	7755,4
16/16	15/16	9/16	4588,4	2408,4	8741,7
16/16	15/16	10/16	5090,1	2630,4	9849,9
16/16	15/16	11/16	5654,3	2889,9	11063,3
16/16	15/16	12/16	6284,4	3196,4	12355,9
16/16	15/16	13/16	6985,8	3559,1	13712
16/16	15/16	14/16	7762,6	3982,9	15129
16/16	15/16	15/16	8616,7	4467,7	16618,6
16/16	16/16	0/16	2398,1	1380,4	4165,9
16/16	16/16	1/16	2682,4	1530,3	4702,1
16/16	16/16	2/16	3009,4	1694,5	5344,8
16/16	16/16	3/16	3392,1	1876,9	6130,6
16/16	16/16	4/16	3849,5	2084,4	7109,5
16/16	16/16	5/16	4407,8	2329,5	8340,4
16/16	16/16	6/16	5100,2	2635	9872,1
16/16	16/16	7/16	5963,9	3038,1	11707,4
16/16	16/16	8/16	7022,5	3578,6	13780,7
16/16	16/16	9/16	8299,46	4285,9	16071,4

Annex B
(informative)

Performance data of the interlaboratory comparison

B.1 Objective of the interlaboratory comparison

In a European wide laboratory comparison study according to ISO 5725-2, the performance characteristics of the standard “Detection and enumeration of *Escherichia coli* in sludges, soils and treated biowaste. Part 2: Miniaturised method (MPN) by inoculation in liquid medium filtration” were established.

B.2 Material used in the interlaboratory comparison study

The interlaboratory comparison of the miniaturised method for semi-quantification of *E. coli* in soil, sludge and treated biowaste took place from May to July 2007. It was carried out with 14 European laboratories on 7 different matrices. The matrices selected for the interlaboratory comparison were chosen to represent soil, sludge and biowaste as broad as possible, because the standard will find general application across different types of soil and soil related materials (detailed information can be found in the final report on the interlaboratory comparison study [2]).

Table B.2-1 provides a list of the type of matrices chosen for *E. coli* detection.

Table B.2-1 Matrices types tested in the interlaboratory comparison trial

Mesophilic anaerobic digested sewage sludge
Anaerobic treated biowaste
Pelletised air-dried sludge
Digested sewage sludge presscake
Composted sewage sludge
Composted green waste
Composted biowaste

In the interlaboratory comparison study the following starting points were used:

The laboratory samples were all taken from a large batch of the different matrices according to the normal practice. The choice was made to analysed only spiked samples so as to obtain positive results. The spiking, the mixing and the sub-sampling were carried out as needed to prepare representative laboratory samples of approximately 150 g from the large batch sample. These were sent out by courier to each of the participating laboratories.

The experimental plan designed by project Horizontal-Hygiene on the basis of each laboratory being given 3 laboratory samples of each of the 2 batches of the seven matrices to be tested.

B.3 First assessment of the precision of the method

The statistical evaluation was conducted according to ISO 13843. The limit of detection, the upper limit of quantification, the range of quantification and the results of dispersion U^2 were obtained (Table B.3-1).

The limit of detection corresponds to the number of particles (germs per test portion) when the probability of a negative result is 5% (superior limit of the confidence interval of the null result).

Poisson distribution corresponds to the random distribution of the number of particles at the moment of sampling a perfectly homogenised suspension.

The relative variance U^2 corresponds to the relative standard deviation squared ratio of the standard deviation squared and the mean squared as:

$$U^2 = s^2/m^2$$

NOTE this statistic is commonly used to express dispersion or uncertainty of microbiological test results.

Table B.3-1 — Summary of components of the *E. coli* Miniaturised method (MPN) precision

Limit of detection (5%) *E. coli* /g wet weight	**Upper limit of quantification (5%)** *E. coli* /g wet weight	**Range of quantification** Log10 unit	**Results of dispersion U^2**
67.40	$7.07\ 10^7$	6.0	0.1

NOTE In judging the results it is important to consider that they do not depend on the experimental data but only on the design of the measurement protocol (random variation).

B.4 Interlaboratory comparison results

The statistical evaluation was conducted according to ISO 5725-2. The average values, the repeatability (r) and the reproducibility (R) were obtained (Table B.4-1).

The repeatability corresponds to the maximum difference that can be expected (with a 95% statistical confidence) between one test result and another, both test results being obtained under the following conditions: the tests are performed in accordance with all the requirements of the present standard by the same laboratory using its own facilities and testing laboratories samples obtained from the same primary field sample and prepared under identical procedures.

The repeatability limit was calculated using the relationship: $r_{test} = f.\sqrt{2}.s_{r.test}$ with the critical range factor f = 2.

NOTE The above relationship refers to the difference that may be found between two measurement results performed each on two laboratory samples obtained under the same conditions. The value f = 2 used in the factor $f.\sqrt{2}$ corresponds to the theoretical factor of 1.96 for a pure normal distribution at 95% statistical confidence. Also, this value f = 2 corresponds to the usual value k = 2 of the coverage factor recommended in the Guide to the expression of Uncertainty in Measurement (GUM). However, it may be necessary to a use a larger value for f in situations as described clause 12.

The reproducibility, like repeatability corresponds to the maximum difference that can be expected (with a 95% statistical confidence) between one test result and another test result obtained by another laboratory, both test results being obtained under the following conditions: the tests are performed in accordance with all the requirements of the present standard by two different laboratories using their own facilities and testing laboratory samples obtained from the same primary field sample and prepared under identical procedures.

This reproducibility limit was calculated using the relationship: $R = f.\sqrt{2}.s_R$ with the critical range factor f = 2.

NOTE The above relationship refers to the difference that may be found between two measurement results performed each on two laboratory samples obtained under the same conditions. The value f = 2 used in the factor $f.\sqrt{2}$ corresponds to the theoretical factor of 1.96 for a pure normal distribution at 95% statistical confidence. Also, this value f = 2 corresponds to the usual value k = 2 of the coverage factor recommended in the Guide to the expression of Uncertainty in Measurement (GUM). In the case when reference is made to the dispersion of the values that could reasonably be attributed to the parameter being measured, the dispersion limit is equal to $k.s_R$ with the usual value k = 2, resulting in a dispersion limit lower than the reproducibility limit (i.e. a ration of $\sqrt{2}$). However it may be necessary to use a larger value $f.\sqrt{2}$ (or k) in situation as described in clause 9.

In case of relatively heterogeneous materials, the repeatability and the reproducibility limits may be larger than the values given in Tables B.4-1 (this means that the value chosen for the critical range factor f is larger than 2 as well as for the coverage factor k for dispersion). This is because the extreme results may have been obtained in accordance with the present standard and/or be caused by the variability within, or in between, the laboratory samples.

For the calculations, as the test results were expressed on a log scale, the standard deviations in repeatability and Reproducibility conditions, respectively s_r and s_R were also expressed on a log scale. The expression of repeatability and Reproducibility in terms of maximum difference that can be expected between one test and another is given then by the limit of repeatability and Reproducibility respectively $r = 2\sqrt{2}.s_r$ and $R = 2\sqrt{2}.s_R$.

In order to make easier the interpretation, the values of repeatability and reproducibility are expressed in terms of maximal difference between two independent measurements on log scale, with a confidence level of 95%:

Example

Assuming r_1 and r_2 two independent measurements observed for a given method in repeatability conditions with $r_1 > r_2$:

$log(r_1) - log(r_2) \leq 0.9$ (95% of the cases), corresponding to almost 1 log of difference between results

The deviations between test results obtained under repeatability and reproducibility conditions can also be expressed by the maximal ratio between two independent measurements on natural scale (number of germs), with a confidence level of 95%

Example

$log(r_1) - log(r_2) \leq 0.9$ (95% of the cases)

then $r_1/r_2 \leq 10^{0.9}$

then $r_1/r_2 \leq 7.9$

thus *r_1 is significantly higher than r_2 if $r_1/r_2 > 7.9$.*

Table B.4-1 — Summary of *E. coli* Miniaturised method (MPN) results of inter-laboratory comparison

Matrix	Overall mean (*E. coli* /g wet weight)	Repeatability (ratio)	Reproducibility (ratio)	Discarded outliers (statistical fitness)	Removed data (other reasons)	Total number of data	Total number of labs
Mesophilic anaerobic digested sewage sludge	< 67.40[(1)]	-	-	-	-	0	14
	< 67.40[(1)]	-	-	-	-	3	14
Anaerobic treated biowaste	1378339	2.4	10.5	1	-	30	13
	2470799	5.3	38.1	-	-	36	13
Pelletised air dried sludge	97	25.0	74.9	-	-	24	13
	724659	10.5	56115.6	-	-	33	14
Digested sewage sludge presscake	6205	23.8	37364.2	-	1	30	14
	2099	4.4	4718.9	-	-	39	11
Composted sewage sludge	30307023	11.9	11.9	1	-	18	13
	2036471	7.2	21.5	-	-	36	13
Composted green waste	1809954	4.5	4.5	1	-	36	13
	396173	4.2	11.0	1	-	36	13
Composted biowaste	16868[(2)]	1863.6[(2)]	1863.6[(2)]	-	-	21	13
	14915052	18.1		1	-	24	13

[(1)] Theoretical limit of detection with a probability of 95% calculated for the method

[(2)] Few data were finally available for data processing. The observed variance was only random variation (no significant laboratory bias). Estimation to be considered carefully

NOTE In judging the results it is important to consider the concentrations levels, at which measurements have been carried out.

Annex C
(informative)

Synthetic Sea Salt

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

MAJOR ION		% TOTAL WEIGHT	IONIC CONCENTRATIONS AT 34 $^{0}/_{00}$ SALINITY (mg/l)
Chloride	(Cl^-)	47.470	18 740
Sodium	(Na^+)	26.280	10 454
Sulfate	($SO4^=$)	6.602	2 631
Magnesium	(Mg^{++})	3.230	1 256
Calcium	(Ca^{++})	1.013	400
Potassium	(K^+)	1.015	401
Bicarbonate	(HCO_3^-)	0.491	194
Borate	(B)	0.015	6.0
Strontium	(Sr^{++})	0.001	7.5
	SOLIDS TOTAL	86.11%	34 089.50
Water	(H_2O)	13.88	
	TOTAL	99.99%	

C. 2 Example for preparation from defined substances:

Three basal solutions are to be made as follows:

Solution A

$CaCl_2$, $2H_2O$	86.6 g
KCl	43.5 g
$SrCl_2.6H_2O$	0.07 g
Distilled Water	Make up to 1 000 mL

Solution B

$NaHCO_3$	5.15 g
$Na_2B_4O_7$	3.0 g
Distilled Water	Make up to 1 000 mL

Solution C

$MgSO_4,7H_2O$	190.0 g
$MgCl_2.6H_2O$	147.0 g
Distilled Water	Make up to 1 000 mL

The diluent is made by adding to 960 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 ± 0.2).

The diluent is distributed into containers of desired volumes, and sterilised by autoclaving at (121 ± 3)°C for (15 ± 1) min.

Annex D
(normative)

Quality criteria for the manufacturing of the medium in microtitre plates (*E. coli*)

For each of the criteria which follow, a quality control has to be made on each batch of manufactured microtitre plates. The microtitre plates to be tested are taken at random or in a systematic way to constitute a sample in accordance with ISO 3951, respecting the general control level n° II of the normal control.

The threshold of positivity of a microtitre plate is defined as being the fluorescence level leading to a positive reading without ambiguity, to the eye, under a Wood light (366 nm).

The quality criteria to be respected are the following:

1) the background noise: absence of positive well in each microtitre plate of the sample, after inoculation with sterile special diluent and incubation of 48 h at 44 °C. The medium background noise of the sample has to be inferior to 25 % of the positivity threshold defined above and the variation coefficient has to be inferior to 10 %;

2) 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 germs per mL of Special Microplate Diluent (7.2), and incubation for 48 h at 44 °C. The average signal so obtained has to be at least twice the threshold of positivity and variation coefficient has to be inferior to 10 %;

3) fertility: is calculated as the ratio of the number of germs observed with the batch of microtitre plates under test to the number of germs expected with a stable reference material (target value). The level of concentration should be brought up to around the maximum of the precision of the method, that is to say about one germ 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 % (in logarithmic units).

The strain to be tested is *E. coli* WR1 and the incubation is 48h at 44°C.

The batch is rejected if any of the criteria is not respected.

* *E. coli* WR1 can be obtained NCTC, UK (NCTC number 13167).

Bibliography

[1] CEN/TC 308 – doc525:2001, *Revision of Directive 86/278/EEC (3rd Draft)*

[2] Maux, M., Molinier, O. and Guarini, P. 2007. Validation Study Report - Interlaboratory trial to evaluate the performances of the 6 draft *E. coli* and *Salmonella* spp. Horizontal-Hygiene standards (DL 2/1.10), EC-FP6-project Horizontal-Hyg contract n° SSPI-CT-2003-502411.