

HPA STANDARD METHOD

# ENUMERATION OF COLIFORM BACTERIA AND *ESCHERICHIA COLI* BY MEMBRANE FILTRATION

W 2

Issued by Standards Unit, Evaluations and Standards Laboratory  
Centre for Infections

**ENUMERATION OF COLIFORM BACTERIA AND *ESCHERICHIA COLI* BY MEMBRANE FILTRATION**

Issue no: 4.1 Issue date: 03.10.07 Issued by Standards Unit, Evaluations and Standards Laboratory in conjunction with the  
Regional Food, Water and Environmental Coordinators Forum Page 1 of 15

Reference no: W 2i4.1

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# AMENDMENT PROCEDURE

Controlled document reference	W 2
Controlled document title	Enumeration of coliform bacteria and <i>Escherichia coli</i> by membrane filtration

Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from [standards@hpa.org.uk](mailto:standards@hpa.org.uk).

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
7/ 04.10.07	4	4.1	9	<b>6.2 Filtration and incubation</b>	Corrected the temperature tolerance from 44°C ± 1°C to 44°C ± 0.5°C in line with MDW 2002.

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# ENUMERATION OF COLIFORM BACTERIA AND *ESCHERICHIA COLI* BY MEMBRANE FILTRATION

## SCOPE OF DOCUMENT

The method described is applicable to the enumeration of coliform bacteria and *Escherichia coli* in water samples intended for human consumption and in pool waters by the membrane filtration technique. It may be used for environmental and recreational water samples but not for designated bathing waters sampled under the bathing water regulations<sup>2</sup>. These samples are analysed using methods detailed by the Environment Agency.

## INTRODUCTION

### Background

Coliform bacteria are widely regarded as the most reliable microbiological indicator of water quality. They are found in the human and animal intestine and may serve as indicators of potential faecal pollution but may also be environmental in origin. 'Faecal coliform bacteria' and *E. coli* are only found in human and animal intestines and tests for their presence in water are necessary to confirm that pollution is of human and/or animal origin. The term 'faecal coliform' is not precise and is consequently not used in this National Standard Method (NSM). In the context of water microbiology in the United Kingdom faecal coliform bacteria are regarded as being *E. coli*.

Coliform bacteria and *E. coli* are sensitive to the disinfection processes used in water treatment and do not usually multiply in water particularly in temperate zones. These organisms should not be present in a 250 mL sample of mineral waters or drinking waters in bottles and containers or 100 mL for other waters of potable quality<sup>3-11</sup>.

This method is based on the method described in the Microbiology of Drinking Water 2002 document<sup>12</sup>.

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# 1 DEFINITIONS

In the context of this method the following definitions apply:

## *Presumptive coliform bacteria*

Organisms which produce acid from lactose and form all shades and sizes of yellow colonies on membranes soaked in membrane lauryl sulphate broth (MLSB) after incubation at 30°C for 4 hours followed by 37°C for 14 hours.

## *Presumptive E. coli*

Organisms which produce acid from lactose and form all shades and sizes of yellow colonies on membranes soaked in MLSB after incubation at 30°C for 4 hours followed by incubation at 44°C for 14 hours.

## *Coliform bacteria (total coliforms)*

Presumptive coliform bacteria which produce acid from lactose at 37°C within 48 hours, are oxidase negative and possess  $\beta$ -galactosidase.

## *E. coli*

Presumptive *E. coli* which also produce indole from tryptophan after incubation at 44°C for 24 hours. Most strains produce  $\beta$ -glucuronidase.

# 2 PRINCIPLE

A measured volume of the sample or a dilution of the sample is filtered through a membrane that is capable of retaining the organisms. The membrane is incubated on an absorbent pad saturated with a selective differential broth medium. Colonies of the target organisms are counted, confirmatory tests carried out and the result calculated as the colony count per 100 mL or 250 mL of sample.

# 3 SAFETY CONSIDERATIONS<sup>13-18</sup>

Normal microbiology laboratory precautions apply.

Care should be taken where manual lifting of large volumes of water is required.

## 3.1 SAMPLE TRANSPORT AND STORAGE

Compliance with current postal and transportation regulations as stated in the Advisory Committee on Dangerous Pathogens 2005<sup>19</sup> is essential.

## 3.2 SAMPLE PROCESSING

Care must be taken when using a boiling waterbath (National Standard Method: W1 Section 2)<sup>20</sup>.

- Amyl alcohol and concentrated hydrochloric acid used in the preparation of Kovacs reagent must be handled with care
- Acid protective gloves are to be worn when performing the indole test

***The above guidance should be supplemented with local COSHH and risk assessments***

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## 4 EQUIPMENT

Usual laboratory equipment and in addition:

- Membrane filtration manifold
- Filter funnels graduated to 50 mL and 100 mL
- Pyrex vacuum flask with protective jacket or equivalent: large volume eg 5 L
- Vacuum pump with moisture trap or protective filter, or alternative vacuum source
- Stainless steel flat tipped forceps or sterilisable equivalent
- Boiling waterbath (instrument steriliser)
- Cycling incubator: 30°C ± 1.0°C and 37°C ± 1.0°C
- Cycling incubator: 30°C ± 1.0°C and 44°C ± 0.5°C
- Incubator: 37°C ± 1.0°C
- Water bath: 44°C ± 0.5°C
- Petri dishes
- Sterile absorbent pads
- Cellulose ester 0.45 µm gridded filters
- Automatic pipettors and associated sterile pipette tips capable of delivering up to 10 mL and 1 mL volumes (optional)
- Pipettes / (sterile total delivery) 10 mL and 1 mL, graduated in 0.1 mL (optional)
- Pastettes

## 5 CULTURE MEDIA AND REAGENTS

Media with the following formulations should be used. Follow MDW 2002<sup>12</sup> for in-house preparation and shelf-life times.

Equivalent commercial media may be used; follow the manufacturer's instructions.

### *Peptone saline diluent (Maximum recovery diluent)*

Peptone	1.0 g
Sodium chloride	8.5 g
Water	1 L
pH	7.0 ± 0.2 at 25°C

### *Membrane lauryl sulphate broth (MLSB)*

Peptone	40.0 g
Yeast extract	6.0 g
Lactose	30.0 g
Phenol red	0.2 g
Sodium lauryl sulphate	1.0 g
Water	1 L
pH	7.4 ± 0.2 at 25°C

### *Lactose peptone water (LPW)*

Peptone	10.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Andrades indicator	10 mL
Water	1 L

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pH  $7.5 \pm 0.2$  at 25°C

*2% Tryptone water (TW)*

Tryptone	20.0 g
Sodium chloride	5.0 g
Water	1 L

pH  $7.5 \pm 0.2$  at 25°C

*MacConkey agar (MA)*

Bile Salts	5.0 g
Peptone	20.0 g
Lactose	10.0 g
Sodium chloride	5.0 g
Neutral red	0.05 g
Agar	12.0 g
Water	1 L

pH  $7.4 \pm 0.2$  at 25°C

*Nutrient agar (NA)*

Meat extract	10.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1 L

pH  $7.5 \pm 0.2$  at 25°C

*Kovacs reagent<sup>21</sup>*

<i>p</i> – Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol (analytical grade reagent, free from organic bases)	75 mL
Hydrochloric acid (concentrated)	25 mL

*Oxidase reagent* (prepare fresh or use commercially prepared strips etc)

Tetramethyl – <i>p</i> – phenylenediamine	
Hydrochloride	0.1 g
Water	10 mL

## 6 SAMPLE PROCESSING

### 6.1 SAMPLE PREPARATION AND DILUTIONS

The nature of the request and condition of the sample should be noted on arrival.

Water samples should be received and handled as described in National Standard Method: W 1 Section 5<sup>20</sup>.

The sample should be stored and transported at 2°C – 8°C. Samples should be analysed as soon as is practicable on the day of collection. In exceptional circumstances, if there is a delay, storage under the above conditions should not exceed 24 hours before the commencement of analysis.

Following the procedures laid down in National Standard Method: W 1 Section 6<sup>20</sup>, select suitable volumes for analysis and prepare any necessary dilutions.

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## 6.2 FILTRATION AND INCUBATION

Place an absorbent pad in each Petri dish and add sufficient MLSB to saturate the pad (usually 2.5 mL depending on the brand of pad) and soak for at least 5 minutes. If necessary, remove any excess medium from the saturated pad using, for example, a sterile pipette / pastette, before the membrane is placed on the pad. For each brand of pad it is important to check the amount of medium that is to be added and the time necessary for soaking in order to prevent drying out during incubation.

Following the procedures laid down in National Standard Method: W 1 Section 6<sup>20</sup>:

Filter a measured volume of sample through the membrane. For bottled mineral waters use 250 mL water, for other potable quality waters use volumes of 100 mL. For surface waters use 100 mL and, if expected to be highly polluted, use 10 mL and 1 mL volumes. Use two filters for each sample, one for coliform bacteria and one for *E. coli*. For mains drinking water and pool waters it is acceptable to use a single membrane where the result is likely or expected to be negative, incubated at 37°C, for both coliform bacteria and *E. coli*.

Place the membrane on a pad soaked in MLSB. Put the membranes and pads in a sealed container and place in an incubator as follows:

For coliform bacteria: 30°C ± 1°C for 4 hours ± 1 hour followed by 37°C ± 1°C for 14 hours ± 1 hour.

For *E. coli*: 30°C ± 1°C for 4 hours ± 1 hour followed by 44°C ± 0.5°C for 14 hours ± 1 hour.

## 6.3 COUNTING OF COLONIES

After a minimum total incubation period of 18 hours, enumerate the presumptive coliform bacteria and presumptive *E. coli* by counting and recording all yellow colonies irrespective of size. As colony colours are liable to change on cooling and standing, it is necessary to perform the count within 15 minutes of removing from the incubator<sup>12</sup>. It is important to note factors affecting the result eg when pink colonies are present in high numbers they may interfere with the growth or count of lactose fermenting colonies.

## 6.4 CONFIRMATORY TESTS

Select colonies for confirmation as described in National Standard Method: W 1 Section 6.3<sup>20</sup>.

Sub-culture colonies from both the 37°C and 44°C membranes for confirmation as coliform bacteria and *E. coli*. Presumptive coliform bacteria isolated at 37°C may confirm as *E. coli* and presumptive *E. coli* colonies isolated at 44°C may confirm as coliform bacteria but not *E. coli*. Also, colonies may be isolated on only one of the two membranes. When only one membrane has been used and incubated at 37°C, colonies should be confirmed for both coliform bacteria and *E. coli*. If the confirmed count for *E. coli* from the 44°C membrane filter is greater than the confirmed count for coliform bacteria from the 37°C membrane filter, then the higher count must be recorded as the confirmed count for coliform bacteria disregarding the colonies on the 37°C membrane. For example, if zero coliform bacteria have been isolated at 37°C but two *E. coli* isolated at 44°C, then the results should be reported as two coliform bacteria and two *E. coli*<sup>12</sup>.

### *Coliform bacteria*

Sub-culture selected yellow colonies to lactose peptone water (LPW), MacConkey agar (MA) and nutrient agar (NA). Spread the MA and NA plates in a manner to give isolated colonies.

Place the LPW in an incubator at 37°C. Examine for acid production after 20 ± 4 hours and if results are negative examine after further 20 ± 4 hours incubation. Place the MA and NA in an incubator at 37°C for 20 hours ± 4 hours. Coliform bacteria produce colonies on MA that are usually circular in shape, convex with a smooth surface and an entire edge. They are various

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shades of red in colour. If the growth on MA/NA is not pure then LPW should be repeated with a pure culture obtained from the MA/NA.

Perform an oxidase test on colonies from the NA plate. Pure cultures are essential for this test and it may be necessary to make further sub-cultures from characteristic colonies on the MA to NA. Moisten a piece of filter paper in a Petri dish with 2 – 3 drops of freshly prepared oxidase reagent or equivalent. Using a stick, glass rod or platinum/plastic (not nichrome) loop transfer a colony of the test organism to the filter paper and rub it on the moistened area. A positive reaction is indicated by the appearance of a dark purple colour within 10 seconds. No colour change or a purplish colour which develops later are both negative reactions.

Coliform bacteria are oxidase negative and produce acid from lactose.

#### *E. coli*

Sub-culture the same colonies selected for confirmation as coliform bacteria to LPW and TW. Place in a waterbath at 44°C for 20 hours ± 4 hours.

Perform an indole test on the TW culture as follows:

Add approximately 0.2 mL Kovacs reagent and shake gently. A deep red colour developing almost immediately in the upper layer indicates a positive result (indole production). *E. coli* is indole positive, produces acid in LPW and is oxidase negative.

## 7 CALCULATION OF RESULTS

Calculate the presumptive count of the test organisms as follows:

$$\text{Presumptive count / 100 mL} = \frac{\text{Number of colonies counted} \times 100}{\text{Volume tested}}$$

Following the procedures described in National Standard Method: W 1 Section 7<sup>20</sup>, calculate the number of confirmed coliform bacteria and *E. coli* present in the specified volume of the original sample.

## 8 REPORTING

Report the results using the procedure described in National Standard Method: W 1 Section 9<sup>20</sup>. Interpretation of results should follow QSOP 57 – The microbiological examination of water samples<sup>22</sup>.

If coliform bacteria or *E. coli* are not detected, report as:

**'Not detected in 100 mL'**

If the test organisms are detected report as:

**'a per 100 mL'**

where **a** is the confirmed count

When *E. coli* has been confirmed from both the 37°C and 44°C membrane, report the higher count. If *E. coli* or other coliform bacteria are detected at 44°C and when there are no colonies at 37°C be sure to report the colonies at 44°C as coliform bacteria. It is illogical to report for example:

*E. coli*: **"2 per 100 mL"** and

coliform bacteria: **"Not detected in 100 mL"** from the same sample.

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For natural mineral waters, bottled waters or waters in containers, the result is reported as the count per 250 mL.

## 9 QUALITY CONTROL

### 9.1 MEMBRANE FILTRATION

When the membrane filtration technique is used, internal quality control procedures must be carried out at least once daily depending on the workload of the laboratory. If more than one batch of media is used it is necessary to repeat the quality control for each batch (see National Standard Method W 1 Section 10)<sup>20</sup>.

The quantitative internal quality controls are performed using suspensions of positive and negative control organisms known to contain less than 100 colony forming units in the volume filtered.

#### Positive controls

*Escherichia coli* NCTC 9001 for incubation at 37°C (coliform bacteria positive) or 44°C (*E. coli* positive)

*Klebsiella aerogenes* NCTC 9528 for incubation at 37°C (coliform bacteria positive)

#### Negative controls

*Klebsiella aerogenes* NCTC 9528 for incubation at 44°C

*Pseudomonas aeruginosa* NCTC 10662

#### Blank control

Filter 100 mL of sterile distilled water or peptone saline diluent using the same funnel as used for the positive control following disinfection.

Incubate all quality control tests in parallel with routine tests, proceed with confirmatory tests and determine the counts.

### 9.2 CONFIRMATORY TESTS

For each batch of confirmatory tests inoculate known positive and negative controls as shown below:

#### *Lactose peptone water*

Test strains		37°C 24 or 48 hours	44°C 24 hours
<i>E. coli</i>	NCTC 9001	Not necessary	Acid
<i>Klebsiella aerogenes</i>	NCTC 9528	Acid	No growth
<i>Pseudomonas aeruginosa</i>	NCTC 10662	No acid	No acid

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### **Oxidase test**

#### **Test strains**

<i>Pseudomonas aeruginosa</i>	NCTC 10662	Positive (purple)
<i>E. coli</i>	NCTC 9001	Negative (colourless)

### **Indole production in tryptone water 44°C/24 hours**

#### **Test strains**

<i>E. coli</i>	NCTC 9001	Positive (red)
<i>Klebsiella aerogenes</i>	NCTC 9528	Negative (colourless)
<i>Pseudomonas aeruginosa</i>	NCTC 10662	Negative (colourless)

## **10 REFERENCE FACILITIES**

N/A

## **11 ACKNOWLEDGEMENTS AND CONTACTS**

This National Standard Method has been developed, reviewed and revised by the Water Working Group for Standard Methods ([http://www.hpa-standardmethods.org.uk/wg\\_water.asp](http://www.hpa-standardmethods.org.uk/wg_water.asp)). The contributions of many individuals in Food, Water and Environmental laboratories, reference laboratories and specialist organisations who have provided information and comment during the development of this document are acknowledged.

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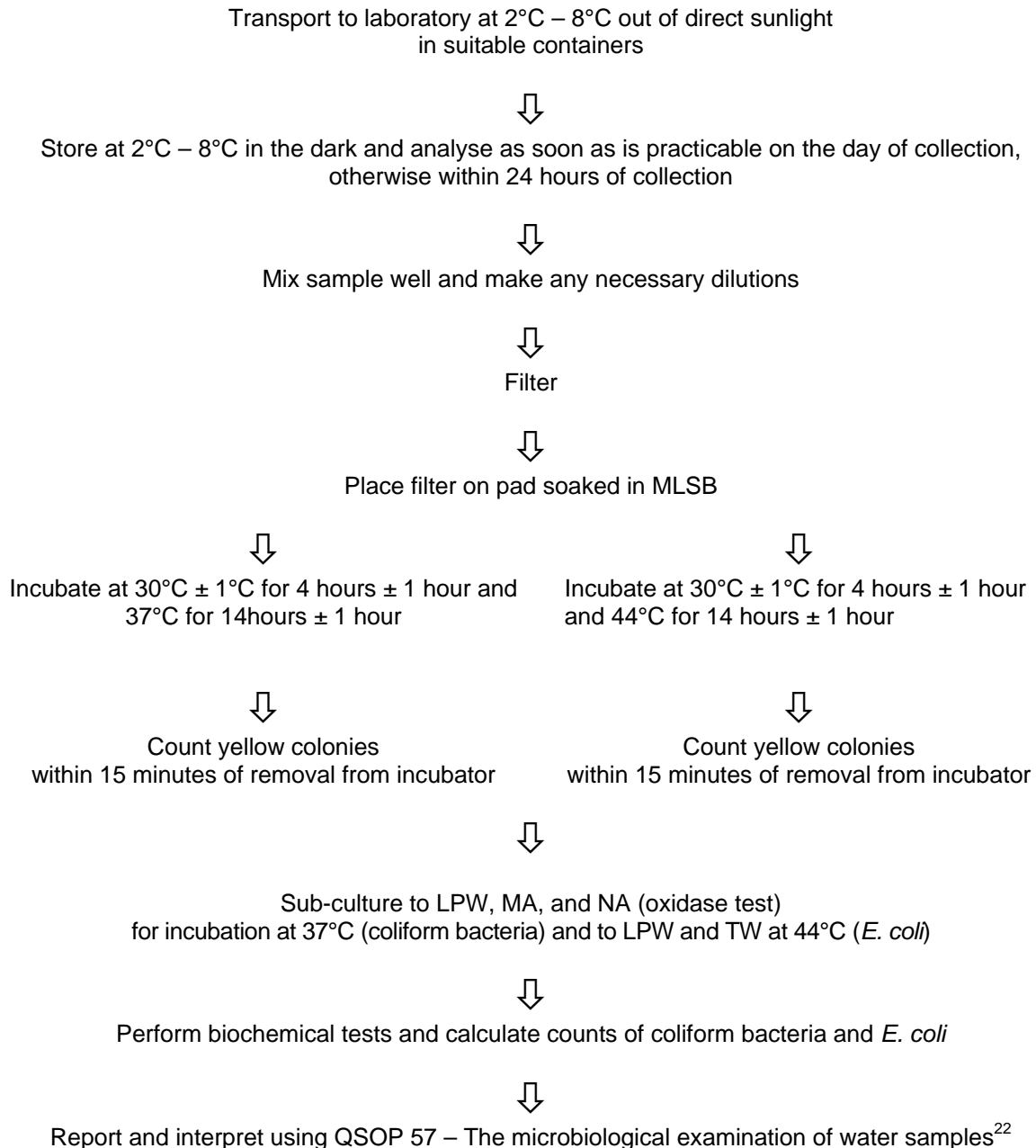
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# FLOWCHART SHOWING THE PROCESS FOR THE ENUMERATION OF COLIFORM BACTERIA AND *ESCHERICHIA COLI* BY MEMBRANE FILTRATION



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