Standing Committee of Analysts

The Microbiology of Recreational and Environmental Waters (2016) – Part 3 – Methods for the isolation and enumeration of *Escherichia coli* (including *E. coli* O157:H7)

Methods for the Examination of Waters and Associated Materials

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Methods for the Examination of Waters and Associated Materials

This booklet contains seven methods for the isolation and enumeration of *Escherichia coli* (including *E. coli* O157:H7).

A The enumeration of *Escherichia coli* by a single membrane filtration technique using membrane lauryl sulphate broth or agar incubated at 44°C

B The enumeration of *Escherichia coli* by a single membrane filtration technique using membrane lactose glucuronide agar incubated at 44°C

C The enumeration of *Escherichia coli* by a single membrane filtration technique using chromogenic tryptone bile glucuronide agar (TBX) incubated at 44°C

D The enumeration of *Escherichia coli* by a multiple tube most probable number technique using minerals modified glutamate medium incubated at 44°C

E The enumeration of *Escherichia coli* by a defined substrate most probable number technique incubated at 37°C

F The enumeration of *Escherichia coli* by a miniaturised most probable number technique incubated at 44°C

G The detection of *Escherichia coli* O157:H7 by selective enrichment and immuno-magnetic separation

This bluebook updates and replaces sections 7.2, 7.3 and 7.13 of the earlier version of The Microbiology of Recreational and Environmental Waters published in 2000.

Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products. They serve only as illustrative examples of the types of products available. Equivalent products may be available and it should be understood that the performance of the method might differ when other materials are used and all should be confirmed by validation of the method.

Contents

About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments, soils (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts (established 1972 by the Department of the Environment). At present, there are seven working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical, Inorganic and physical methods, Metals and metalloids
4 Solid substances
5 Organic impurities
6 Biological, biodegradability and inhibition methods
7 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the Agency's web-page (http://standingcommitteeofanalysts.co.uk/) or by post.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary. Users should ensure they are aware of the most recent version they seek.

Robert Carter
Secretary
June 2015

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.
Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such resources are; HSE website HSE: Information about health and safety at work ; RSC website http://www.rsc.org/learn-chemistry/collections/health-and-safety
"Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Biological Agents: Managing the Risks in Laboratories and Healthcare Premises", 2005 and "The Approved List of Biological Agents" 2013, produced by the Advisory Committee on Dangerous Pathogens of the Health and Safety Executive (HSE).

A The enumeration of *Escherichia coli* by a single membrane filtration technique using membrane lauryl sulphate broth or agar incubated at 44°C

This method has not been subjected to widespread use within the UK or verification of performance for recreational and environmental waters. Users of this method are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance. Information on the routine use of this method and similar methods, would be welcomed to assess their full capabilities.

A1 Introduction

Tests for *Escherichia coli* (*E. coli*) are used to determine the presence of faecal contamination in recreational and other waters as it is considered as the key indicator of faecal contamination. They provide the means of assessing the degree of faecal contamination and assessing the potential risk of infection for those who intend to use the water. These tests are also used for assessing the effectiveness of water treatment and disinfection in swimming pools, spa pools and hydrotherapy pools and for monitoring the reduction of micro-organisms in wastewater treatment systems and the quality of treated effluents. The significance of *E. coli* in recreational and other waters is described elsewhere[1] in this series.

A2 Scope

The method is suitable for the examination of surface and ground waters, saline waters, manmade recreational waters such as swimming pools, spa pools and hydrotherapy pools, and untreated and treated wastewaters. Water samples with higher turbidities should be analysed using an appropriate multiple tube most probable number (MPN) method (see method D).

Those wishing to use this method for swimming pools, spa pools and hydrotherapy pools should incubate filtered samples at 37°C as well as 44°C in order to detect and enumerate coliform bacteria. Confirmation methods for coliforms may be found elsewhere[2].

Users wishing to employ this method should verify its performance under their own laboratory conditions[3].

A3 Definitions

Organisms which produce acid from lactose and form all shades and sizes of yellow colonies on membrane filters (after incubation for 4 hours at 30°C followed by 14 hours at 44°C) on membrane lauryl sulphate broth (MLSB) or membrane lauryl sulphate agar (MLSA) are regarded as presumptive *E. coli*. In addition, colonies which, when sub-cultured to an appropriate medium containing a chromogenic substrate (for example membrane lactose glucuronide agar (MLGA) or tryptone bile glucuronide agar (TBX)), express the enzyme β-glucuronidase are regarded as confirmed *E. coli*.

For the purposes of water examination, *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44°C with the production of acid within 24 hours. Most strains produce β-glucuronidase.

A4 Principle

Aliquots of sample are filtered, and the membrane filters (with entrapped bacteria) placed on absorbent pads saturated with broth, or placed on agar, containing lactose and phenol red as an indicator of acidity. Isolation of presumptive colonies is followed by confirmatory tests for the production of β-glucuronidase.

A5 Limitations

The method is suitable for most types of aqueous samples except those with high turbidities, which tend to block the membrane filter. This will limit the volume of sample that can be filtered. Accumulated deposit on the membrane filter may mask or inhibit the growth of indicator organisms. The method when incubated ay 37°C also allows some species of non-coliform bacteria to grow, which when present in high numbers may inhibit growth of *E. coli*. The ideal number of colonies that should be counted from a single membrane filter is approximately 20 – 80 with a maximum of 100. Counts can be obtained from membrane filters containing more than 100 colonies provided that isolated colonies are present and that a hand lens or similar magnifying aid is used. Counts obtained in this way should be reported as an estimated count.

Where high numbers of organisms may be expected (for example, untreated wastewater) serial ten-fold dilutions should be made to obtain a countable number of colonies on a membrane filter. For sand, sediments, and waters with high turbidity, an appropriate MPN method should be used (see Sections D).

A6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[4] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[3] in this series.

A7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[3] in this series. Principally, appropriate membrane filtration apparatus and incubators (fan assisted, either static temperature or temperature cycling) are required. Other items include:

A7.1 Sterile sample containers of appropriate volume, made of suitable material. For swimming pools, spa and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l, for example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent.

A7.2 Incubators capable of maintaining temperatures of 30.0 ± 1.0°C and/or 44.0 ± 0.5°C, or cyclical incubators fitted with timers capable of attaining these temperatures and incubators capable of maintaining temperatures of 37.0 ± 1.0°C.

A7.3 Filtration apparatus, sterile filter funnels or filter funnels that can be sterilised, and a vacuum source.

A7.4 Sterile membrane filters, for example, white, 47 mm diameter, cellulose-based, 0.45 µm nominal pore size. Gridded membrane filters may facilitate the counting of colonies. If broth medium is used then appropriate absorbent pads are required.

A7.5 Smooth-tipped forceps.

A8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. Commercial formulations should be used and stored according to the manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in this method[3]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality[3]. Unless otherwise stated, chemical constituents should be added as the anhydrous salts. If the pH of the medium is not within the stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

A8.1 *Membrane lauryl sulphate broth (MLSB)*[5] *and agar (MLSA)*

Peptone	40 g
Yeast extract	6 g
Lactose	30 g
Phenol red (0.4% m/v aqueous solution)	50 ml
Sodium lauryl sulphate - specially pure	1 g
Water	1 litre

Add the ingredients to the water and mix gently to avoid the formation of froth. The final pH of the sterile medium should be 7.4 ± 0.2 and, to achieve this, it may be necessary to adjust the pH to about 7.6 before sterilisation. The detection of acid production is influenced by the pH of the medium, thus, it is important that the medium is of the correct pH value. Distribute the medium in screw-capped bottles, loosely seal and autoclave at 115°C for 10 minutes. The bottles may need to be removed from the autoclave as soon as possible after autoclaving in order to avoid possible breakdown of the lactose and reduction in the pH value. When cooled, the screw cap should be tightened.

The medium may be used in an agar form, as membrane lauryl sulphate agar (MLSA), by adding bacteriological agar (usually 10 - 13 g) in the above formulation before autoclaving begins. Allow the medium to cool, distribute it in Petri dishes and allow it to solidify. The pH of the medium after sterilisation should be 7.4 ± 0.2. The detection of acid production is influenced by the pH of the medium, thus, it is important that the medium is of the correct pH. To achieve this, it may be necessary to adjust the pH to about 7.6 before sterilisation. Petri dishes containing the agar medium may be stored at a temperature in the range 5 ± 3°C for up to one week, protected against dehydration. Storage beyond this time may result in a deterioration of performance of the medium.

The broth medium should be used as soon as possible but can be stored at a temperature in the range 5 ± 3°C for up to one month. During refrigerated storage, sodium lauryl sulphate may precipitate out of solution. Before use, allow the broth to reach room temperature and mix well before dispensing into Petri dishes.

A8.2 *Membrane lactose glucuronide agar (MLGA)*[6, 7]

Peptone	40 g
Yeast extract	6 g
Lactose	30 g
Phenol red (0.4% m/v solution)	50 ml
Sodium lauryl sulphate	1.0 g
Sodium pyruvate	0.5 g
Agar	10.0 g
5-bromo-4-chloro-3-indolyl- β-D-glucuronide (BCIG)	0.2 g
Water	1 litre

Suspend the ingredients, except BCIG, in the water and bring to the boil to dissolve. Dissolve the cyclohexylammonium salt of BCIG in 3 ml of solution consisting of 2.5 ml of 95 % v/v aqueous ethanol and 0.5 ml of 1M sodium hydroxide solution. Add this solution to the medium. The sodium salt of BCIG can be added directly to the medium. Mix the solution well and autoclave at 121°C for 15 minutes. Allow the medium to cool, distribute it in Petri dishes and allow it to solidify. Petri dishes containing the agar medium may be stored at a temperature in the range 5 ± 3°C for up to one week, protected against dehydration. Storage beyond this time may result in a deterioration of performance of the medium. The pH of the medium after sterilisation should be 7.4 ± 0.2. The detection of acid production is influenced by the pH of the medium, thus, it is important that the medium is of the correct pH. To achieve this, it may be necessary to adjust the pH to about 7.6 before sterilisation.

A8.3 *Tryptone bile glucuronide agar (TBX agar)*[8]

Tryptone	20 g
Bile salts No. 3	1.5 g
Agar	15 g
5-bromo-4-chloro-3-indolyl- β-D-glucuronide (BCIG)	75 mg
Water	1 litre

Suspend the ingredients, except BCIG, in the water and bring to the boil to dissolve. Dissolve the cyclohexylammonium salt of BCIG in 3 ml of a solution consisting of 2.5 ml of 95 % v/v aqueous ethanol and 0.5 ml of 1M sodium hydroxide solution. Add this solution to the medium. Alternatively, the sodium salt can be added directly to the medium. Mix the solution well and autoclave at 121°C for 15 minutes. Allow the medium to cool to 50°C, distribute in suitable volumes into Petri dishes, and allow the medium to solidify. Petri dishes containing the agar medium may be stored at temperatures between 5 ± 3°C for up to one week, protected against dehydration. Storage beyond this period may result in a deterioration of the medium. The pH of the medium after sterilisation should be 7.2 ± 0.2.

A8.4 *Other media*

Standard and commercial formulations of other media and reagents used in this method include Gram stain, quarter strength Ringer's solution and maximum recovery diluent.

A9 Analytical procedure

A9.1 *Sample preparation*

The volumes, and dilutions, of samples should be chosen so that the number of colonies to be counted on the MLSB or MLSA membrane filter lies, if possible, between 20 and 80. With some waters, it may be advantageous to filter a selection of different volumes of sample so that the number of colonies on at least one of the membrane filters is likely to fall within this range. For swimming pool, spa pool and hydrotherapy pool waters, filter 100 ml of the sample. For polluted waters either filter smaller volumes, or dilute the sample with quarter strength Ringer's solution or maximum recovery diluent before filtration. Guidance on the processing of sludges or solid materials can be found elsewhere[9]. The minimum volume of sample filtered should not be less than 1 ml. This is to ensure that colonies are evenly spread across the membrane filter and the volume filtered is still representative of the sample submitted. If smaller volumes are required to ensure the count of colonies lies within the above range, then appropriate dilutions should be made. A volume of each sample is filtered and incubated at 44°C.

A9.2 *Sample processing*

If membrane lauryl sulphate broth is used, for each sample, place a sterile absorbent pad into an empty sterile Petri dish. Aseptically, add sufficient membrane lauryl sulphate broth to saturate the pad, allow the medium to soak into the pad and discard any excess medium. If this is not carried out, confluent growth on the membrane filter may result.

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel. Holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter grid-side upwards onto the porous disc of the filter base. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample, or diluted sample, into the funnel. When the volume of sample to be filtered is less than 10 ml, add 10 - 20 ml of sterile diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) to the funnel before addition of the sample. This aids the dispersion of the bacteria over the entire surface of the membrane filter during the filtration process. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the sample slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and transfer the membrane filter carefully to a pad saturated with membrane lauryl sulphate broth or to a Petri dish containing membrane lauryl sulphate agar which should be well-dried (for example, the Petri dish should be left at room temperature for 2 hours or at 37°C for 30 minutes, prior to use). Ensure that no air bubbles are trapped between the membrane filter and the medium. 'Rolling' the membrane filter onto the medium minimises the likelihood of air bubbles becoming trapped. Cover the membrane filter with the lid of the Petri dish.

When the funnel is removed it can be placed in a boiling water bath if it is to be re-used. Alternatively, pre-sterilised filter funnels can be used for each sample. If different volumes of the same sample are to be examined, the funnel may be re-used without boiling provided that the smallest volume, or highest dilution of sample, is filtered first. For different samples, take a fresh pre-sterilised funnel or remove a funnel from the boiling water bath, allow the funnel to cool and repeat the filtration process. If funnels are re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling water for at least one minute. During the filtration of a series of samples, the filter base need not be sterilised unless it becomes contaminated or a membrane filter becomes damaged. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible, and no longer than 2 hours.

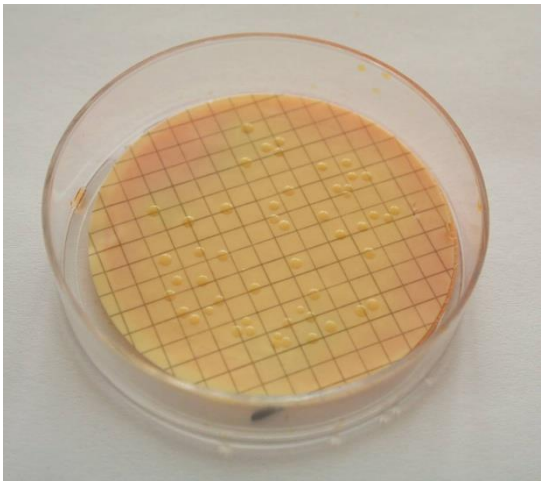
Where broth medium is used the dishes should be placed in a sealed container to prevent drying out of the medium. The Petri dishes of MLSA are inverted and incubated at 30°C for 4.00 ± 0.25 hours followed by 44°C for a minimum of 14 hours. Alternatively, cyclical temperature incubators can be used. Plates of MLSB should be incubated not inverted in sealed containers to avoid dehydration. Accurate temperature control and even temperature distribution are essential. False-positive results may be obtained if lower incubation temperatures are used and some organisms may fail to multiply at higher incubation temperatures. If, for any reason, an early indication of a result is required, membrane filters incubated at 44°C may be examined after approximately 8 hours but following this examination membrane filters must be returned to the incubator at 44°C for the full minimum incubation period of 14 hours prior to counting.

A9.3 *Reading of results*

After the total incubation period of 18 hours, examine the MLSB or MLSA membrane filters under good light, if necessary with a hand lens. Colours are liable to change on cooling and standing, hence, within 15 minutes of being removed from the incubator, count all yellow colonies (however faint) irrespective of size. The number of colonies counted on the MLSB or MLSA membrane filter incubated at 44°C is regarded as the number of presumptive *E. coli* (see Figure A1).

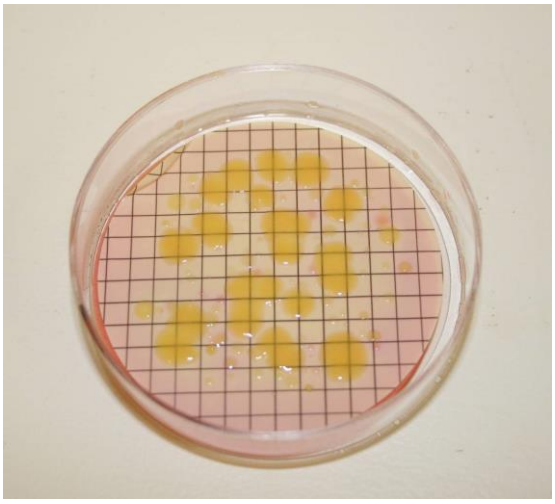
It is important to note whether pink colonies (from non-target organisms and non-lactose fermenting coliforms) are present in numbers that may interfere with the growth of presumptive *E. coli*. If the growth of pink colonies is considered to be such that they obscure lactose-fermenting colonies, a further sample should be taken and re-submitted for examination and this fact recorded. Alternatively, any portion of sample retained in a refrigerator may be re-examined using an appropriate dilution of the sample, to enable isolated colonies to develop. However, whilst a count of the number of colonies (of the re-examined sample) on the MLSB or MLSA membrane filter may be made, this value should not be reported and used to assess water quality, as this may not reflect the number of colonies in the original sample when first examined. In addition to appropriate dilution of the sample, MPN or presence-absence methods should also be considered.

Figure A1 Colonies of presumptive *E. coli* at 44°C on membrane lauryl sulphate broth



Tests for β-glucuronidase will assist in the confirmation of *E. coli* and may differentiate other species of coliform bacteria which exhibit the same presumptive profile as *E. coli* (for example *Klebsiella* species, see Figure A2).

Figure A2 Colonies of *Klebsiella* species isolated from surface water at 44°C on membrane lauryl sulphate broth



A9.4 *Confirmation tests*

Depending on the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of yellow colonies (however faint). If the aim of the examination is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be sub-cultured if fewer than ten are present or, at least ten colonies should be sub-cultured if more than ten are present. Colonies should be chosen at random, but to avoid any bias from, for example, unconscious choice of similar colonies, all the colonies in a randomly chosen segment of appropriate size should be sub-cultured. Where a number of colonies of different appearance are clearly distinguishable, a note of the number of each morphological type should also be made. Where one type of colonial type greatly exceeds another type, random choosing of colonies may result in the failure to culture the less

frequently occurring species. In these cases, additional consideration should be given to choosing all colonial species. The data and information obtained from the sub-cultured isolates are then used to calculate the confirmed counts of *E. coli.*

Colonies for confirmatory testing should be sub-cultured as soon as practicable, preferably within 60 minutes, as colony colours can fade after removal of the Petri dishes from the incubator. Petri dishes should be stored in the appropriate incubator prior to sub-culturing, if confirmation is not undertaken immediately after counting.

E. coli can be confirmed by sub-culture to an appropriate medium containing a chromogenic substrate for the enzyme β-glucuronidase (for example MLGA or TBX).

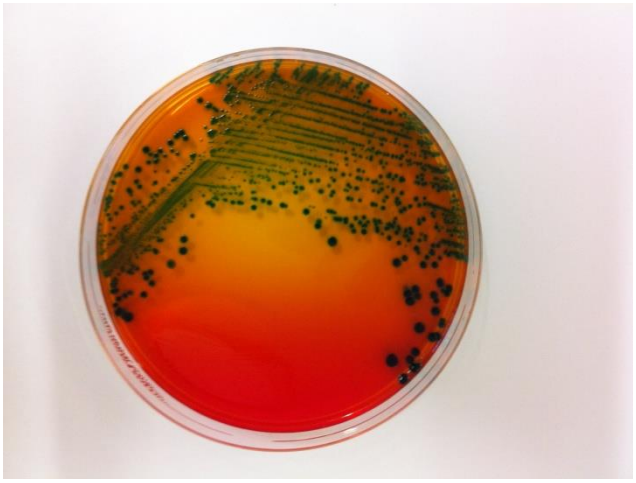
A9.4.1 Confirmation of presumptive *E. coli*

From the MLSB or MLSA membrane incubated at 44°C, sub-culture each colony to be tested to MLGA, TBX or Colilert reagent and incubate at 37°C for 21 ± 3 hours. Examine the sub-cultures for the expression of β-glucuronidase, green colonies on MLGA, blue colonies on TBX (see Figure A3) or fluorescent reaction with Colilert reagent.

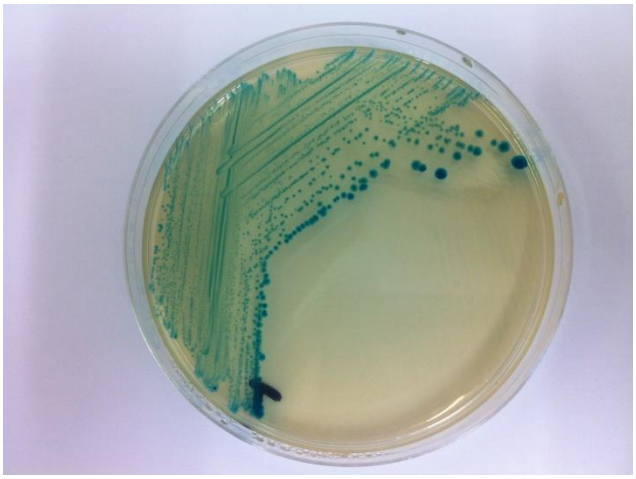
Note: If necessary, several different colonies may be inoculated onto a single agar plate.

Figure A3. Typical β-glucuronidase positive colonies on MLGA and TBX

E. coli on MLGA



E. coli on TBX



A10 Calculations

A10.1 *Presumptive E. coli*

The count from the MLSB or MLSA membrane filter for the 44°C incubation is regarded as the presumptive *E. coli* count. The number of presumptive *E. coli* is generally expressed as the number of colonies per 100 ml of sample. Taking into account any dilution (as necessary) the count is:

$$\text{Presumptive count per 100 ml} = \frac{N \times 100 \times DF}{\text{Volume of sample filtered (ml)}}$$

where N is the number of yellow colonies counted on the MLSB or MLSA membrane filter, and DF is the appropriate dilution, if required.

A10.2 *Confirmed E. coli*

The number of confirmed *E. coli* is calculated by multiplying the number of presumptive *E. coli* from the differing colony types by the proportion of the isolates that express β-glucuronidase on either MLGA or TXB. Numbers should be recorded to the nearest whole number

A11 Expression of results

Counts for presumptive and confirmed *E. coli* are expressed in colony forming units per volume of sample. For most samples, the volume is typically 100 ml.

A12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (*E. coli*) and non-target bacteria (for example *Klebsiella pneumoniae* as a coliform or *Pseudomonas aeruginosa*). Petri dishes should be incubated as appropriate to the test performed. Further details are given elsewhere[3] in this series.

For each batches of samples for monitoring of routine internal performance quality control it is appropriate to examine one (or more for batches) sample in duplicate[3]. A comparison of the counts can then be undertaken.

A13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2015) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2009) - Part 4 – Membrane filtration method for the isolation of coliforms and *Escherichia coli* (including *E. coli* O157). *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

3 Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

4. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

5. Membrane filtration media for the enumeration of coliform bacteria and *Escherichia coli* in water: comparison of Tergitol 7 and lauryl sulphate with Teepol 610, *Journal of Hygiene*, Joint Committee of the Public Health Laboratory Service and Standing Committee of Analysts, 1980, **85**, 181-191.

6. A medium detecting β-glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms from drinking water, *Letters in Applied Microbiology*, D P Sartory and L Howard, 1992, **15**, 273-276.

7. Standing Committee of Analysts, Evaluation trials for two media for the simultaneous detection and enumeration of *Escherichia coli* and coliform organisms 1998, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

8. ISO 16649-1: 2001. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli .* Part 1: Colony-count technique at 44°C using membranes and 5-bromo-4-chloro-3-indoyl-β-D-glucuronide. Geneva: International Organization for Standardization.

9. Standing Committee of Analysts, The Microbiology of Sewage Sludge (2003) - Part 2- Practices and procedures for sampling and sample preparation, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

B The enumeration of *Escherichia coli* by a single membrane filtration technique using membrane lactose glucuronide agar incubated at 44°C

B1 Introduction

Tests for *Escherichia coli* (*E. coli*) are used to determine the presence of faecal contamination in recreational and other waters as it is considered as the key indicator of faecal contamination. They provide the means of assessing the degree of faecal contamination and assessing the potential risk of infection for those who intend to use the water. The tests are also used for assessing the effectiveness of water treatment and disinfection in swimming pools, spa pools and hydrotherapy pools and for monitoring the reduction of micro-organisms in wastewater treatment systems and the quality of treated effluents. The significance of *E. coli* in recreational and other waters is described elsewhere[1] in this series.

B2 Scope

The method is suitable for the examination of surface and ground waters, saline waters, manmade recreational waters such as swimming pools, spa pools and hydrotherapy pools, and untreated and treated wastewaters. Water samples with higher turbidities should be analysed using an appropriate multiple tube most probable number (MPN) method (see method D).

Those wishing to use this method for swimming pools, spa pools and hydrotherapy pools should incubate filtered samples at 37°C in order to detect and enumerate coliform bacteria.

Users wishing to employ this method should verify its performance under their own laboratory conditions[2].

B3 Definitions

In the context of this method, organisms which produce acid from lactose and produce β-glucuronidase forming green colonies after incubation at 30°C for 4 hours followed by incubation at 44°C for 14 hours are regarded as *E. coli*. Most strains of *E. coli* express β-glucuronidase, as do some strains of *Shigella* and *Salmonella*.

For the purposes of water examination, *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44°C with the production of acid within 24 hours. Most strains produce β-glucuronidase.

B4 Principle

Organisms are entrapped on a membrane filter placed on an agar medium containing lactose, phenol red as an indicator of acidity, and the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) either as the cyclohexylammonium or sodium salt for the indication of the production of β-glucuronidase.

B5 Limitations

Enumeration of colonies by this method will exclude a proportion of strains of *E. coli* that are unable to grow at 44°C or that cannot express β-glucuronidase activity.

The method is suitable for most types of aqueous samples except those with high turbidities, which tend to block the membrane filter. This will limit the volume of sample that can be filtered. Accumulated deposit on the membrane filter may mask or inhibit the growth of indicator organisms. The method also allows some species of non-coliform bacteria to grow, which when present in high numbers may inhibit growth of *E. coli*. The ideal number of colonies that should be counted from a single membrane filter is approximately 20 – 80 with a maximum of 100. Counts can be obtained from membrane filters containing more than 100 colonies provided that isolated colonies are present and that a hand lens or similar magnifying aid is used. Counts obtained in this way should be reported as estimated counts.

Where high numbers of organisms may be expected (for example, untreated wastewater) serial ten-fold dilutions should be made to obtain a countable number of colonies on a membrane filter. For sand, sediments, and waters with high turbidities, an appropriate MPN method should be used (see Section D).

The growth of high numbers of coliform and non-coliform bacteria from environmental waters may inhibit the production of β-glucuronidase by *E. coli*.

B6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[3] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

B7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[2] in this series. Principally, appropriate membrane filtration apparatus and incubators (fan assisted, either static temperature or temperature cycling) are required. Other items include:

B7.1 Sterile sample containers of appropriate volume, made of suitable material. For swimming pools, spa pools and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l, for example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent.

B7.2 Incubators capable of maintaining temperatures of 30.0 ± 1.0°C and 44.0 ± 0.5°C, or cycling incubators, fitted with timers, capable of attaining these temperatures.

B7.3 Filtration apparatus, sterile filter funnels or funnels that can be sterilised, and a vacuum source.

B7.4 Sterile membrane filters, for example, white, 47 mm diameter, cellulose-based 0.45 µm nominal pore size. Gridded membrane filters may facilitate the counting of organisms.

B7.5 Smooth-tipped forceps.

B8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. Commercial formulations should be used and stored according to the manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in this method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar quality[2]. Unless otherwise stated chemical constituents should be added as the anhydrous salts. If the pH of the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator, they should be allowed to reach room temperature before use.

B8.1 *Membrane lactose glucuronide agar (MLGA)*[4, 5]

Peptone	40 g
Yeast extract	6 g
Lactose	30 g
Phenol red (0.4% m/v solution)	50 ml
Sodium lauryl sulphate	1.0 g
Sodium pyruvate	0.5 g
Agar	10.0 g
5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG)	0.2 g
Water	1 litre

Suspend the ingredients, except BCIG, in the water and bring to the boil to dissolve. Dissolve the cyclohexylammonium salt of BCIG in 3 ml of solution consisting of 2.5 ml of 95 % v/v aqueous ethanol and 0.5 ml of 1M sodium hydroxide solution. Add this solution to the medium. The sodium salt of BCIG can be added directly to the medium. Mix the solution well and autoclave at 121°C for 15 minutes. Allow the medium to cool, distribute it in Petri dishes and allow it to solidify. Petri dishes containing the agar medium may be stored at a temperature in the range 5 ± 3°C for up to 21 days (see Appendix 1, section 6), protected against dehydration. Storage beyond this time may result in a deterioration of performance of the medium. The pH of the medium after sterilisation should be 7.4 ± 0.2. The detection of acid production is influenced by the pH of the medium, thus, it is important that the medium is of the correct pH. To achieve this, it may be necessary to adjust the pH to about 7.6 before sterilisation.

B8.2 *Other media*

Standard and commercial formulations of other media and reagents used in this method include quarter strength Ringer's solution and maximum recovery diluent.

B9 Analytical procedure

B9.1 *Sample preparation*

The volumes, and dilutions, of samples should be chosen so that the number of colonies to be counted on the MLGA membrane filter lies, if possible, between 20 and 80. With some waters, it may be advantageous to filter a selection of different volumes of sample so that the number of colonies on at least one of the membrane filters is likely to fall within

this range. For swimming pool, spa pool and hydrotherapy pool waters, filter 100 ml of the sample. For polluted waters either filter smaller volumes, or dilute the sample with quarter strength Ringer's solution or maximum recovery diluent before filtration. Guidance on the processing of sludges or solid materials can be found elsewhere[7]. The minimum volume of sample filtered should not be less than 1 ml. This is to ensure that colonies are evenly spread across the membrane filter and the volume filtered is still representative of the sample submitted. If smaller volumes are required to ensure the count of colonies lies within the above range, then appropriate dilutions should be made. A single volume or dilution of each sample is filtered and incubated at 44°C.

B9.2 *Sample processing*

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel. Holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter grid-side upwards onto the porous disc of the filter base. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample, or diluted sample, into the funnel. When the volume of sample to be filtered is less than 10 ml, add 10 - 20 ml of sterile diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) to the funnel before addition of the sample. This aids the dispersion of the bacteria over the entire surface of the membrane filter during the filtration process. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the sample slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and transfer the membrane filter carefully to a Petri dish containing membrane lactose glucuronide agar, which should be well-dried (for example, the Petri dish should be left at room temperature for 2 hours or at 37°C for 30 minutes, prior to use). Ensure that no air bubbles are trapped between the membrane filter and the medium. 'Rolling' the membrane filter onto the medium minimises the likelihood of air bubbles becoming trapped. Cover the membrane filter with the lid of the Petri dish.

When the funnel is removed it can be placed in a boiling water bath if it is to be re-used. Alternatively, pre-sterilised filter funnels can be used for each sample. If different volumes of the same sample are to be examined, the funnel may be re-used without boiling provided that the smallest volume, or highest dilution of sample, is filtered first. For different samples, take a fresh pre-sterilised funnel or remove a funnel from the boiling water bath, allow the funnel to cool and repeat the filtration process. If funnels are re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling water for at least one minute. During the filtration of a series of samples, the filter base need not be sterilised unless it becomes contaminated or a membrane filter becomes damaged. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible, and no longer than 2 hours.

The Petri dishes are inverted and placed in an incubator at 30°C for 4.00 ± 0.25 hours then transferred to an incubator at 44°C for a minimum of 14 hours. Alternatively, a cycling temperature incubator can be used. Accurate temperature control and even temperature

distribution are essential. False positive results may be obtained if lower incubation temperatures are used and some organisms may fail to multiply at higher incubation temperatures. If, for any reason, an early indication of a result is required, the membrane filter at 44°C may be examined after an incubation time of approximately 8 hours but the membrane filter must be returned to the incubator at 44°C for the full minimum incubation period of 14 hours prior to counting.

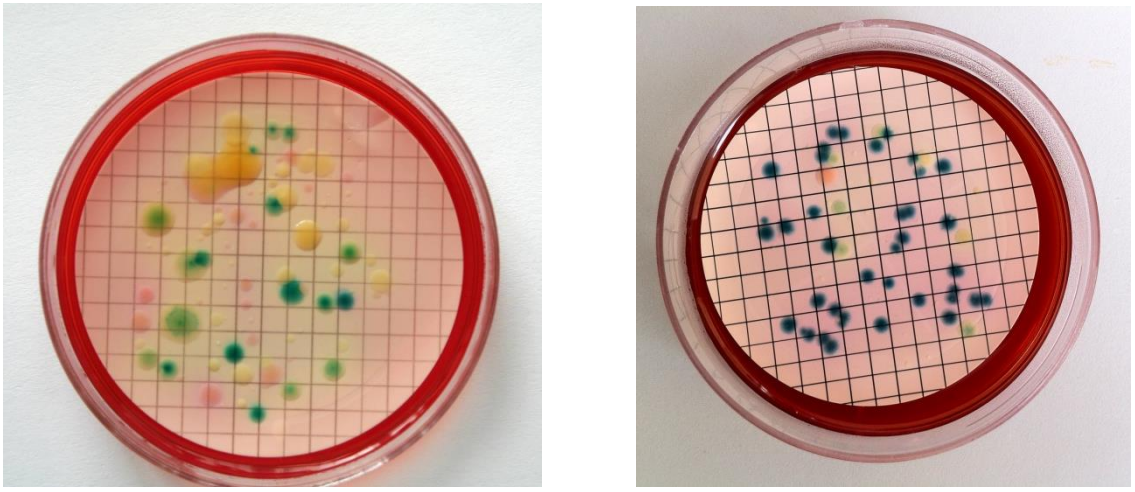
B9.3 *Reading of results*

After the total incubation period of 18 hours, examine the MLGA membrane filters under good light, if necessary with a hand lens. Count all green colonies (however faint the colour) irrespective of size. All green colonies are *E. coli* (see Figure B1). The total count of green colonies if present is regarded as the number of confirmed *E. coli*. Blue colonies should be regarded as presumptive *E. coli*.

It is important to note whether pink or yellow colonies (from non-target organisms) are present in numbers that may interfere with the growth of *E. coli*. If the growth of pink or yellow colonies is considered to be such that they may be obscuring *E. coli* colonies, a further sample should be taken and re-submitted for examination and this fact recorded. Alternatively, any portion of sample retained in the refrigerator may be re-examined using an appropriate dilution of the sample, to enable isolated colonies to develop. However, whilst a count of the number of colonies (of the re-examined sample) on the MLGA membrane filter may be made, this value should not be reported and used to assess water quality, as this may not reflect the number of colonies in the original sample when first examined. In addition to appropriate dilution of the sample, MPN or presence-absence methods should also be considered.

Occasionally, blue colonies may be noted and recorded. These are likely to be lactose-negative *E. coli*, but rarely, may be uncommon strains of *Aeromonas*. These should be counted as presumptive *E. coli*.

Figure B1 Green colonies of *E. coli* on membrane lactose glucuronide agar. The green colour can vary from pale green to dark green (almost blue) and different mixtures can be seen in environmental samples.



B9.4 *Confirmation tests*

The specificity of the green colonies on membrane lactose glucuronide agar being *E. coli* is very high, and, following suitable confirmation of performance within the laboratory, confirmation of green colonies should not be needed.

B10 Calculations

E. coli is generally expressed as the number of colonies per 100 ml of sample. Calculate the count as follows:

$$\text{Confirmed count per 100 ml} = \frac{N \times 100 \times DF}{\text{Volume of sample filtered (ml)}}$$

Where N is the number of green and blue colonies counted on the MLGA membrane filter at 44°C, and DF is the appropriate dilution if required.

The count of the total number of green and blue colonies on the MLGA membrane filter at 44°C is regarded as the confirmed *E. coli* count.

B11 Expression of results

Counts for confirmed *E. coli* are expressed in colony forming units per volume of sample. For most samples, the volume is typically100 ml.

B12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (*E. coli*) and non-target bacteria (for example *Klebsiella pneumoniae* as a coliform or *Pseudomonas aeruginosa*). Petri dishes should be incubated as appropriate. Further details are given elsewhere[(2)] in this series.

For each batches of samples for monitoring of routine internal performance quality control it is appropriate to examine one (or more for batches) sample in duplicate[(3)]. A comparison of the counts can then be undertaken.

B13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2015) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

3. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

4. A medium detecting β-glucuronidase for the simultaneous membrane filtration enumeration of *Escherichia coli* and coliforms from drinking water, *Letters in Applied Microbiology*, D P Sartory and L Howard, 1992, **15**, 273-276.

5. Standing Committee of Analysts, Evaluation trials for two media for the simultaneous detection and enumeration of *Escherichia coli* and coliform organisms 1998, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

6. Standing Committee of Analysts, The Microbiology of Sewage Sludge (2003) - Part 2 - Practices and Procedures for Sampling and Sample Preparation, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

C The enumeration of *Escherichia coli* by a single membrane filtration technique using chromogenic tryptone bile glucuronide agar (TBX) incubated at 44°C

This method has not been subjected to widespread use nor verification of performance. Users of this method are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance. Information on the routine use of this method and similar methods, would be welcomed to assess their full capabilities.

C1 Introduction

Tests for *Escherichia coli* (*E. coli*) are used to determine the presence of faecal contamination in recreational and other waters as it is considered as the key indicator of faecal contamination. They provide the means of assessing the degree of faecal contamination and assessing the potential risk of infection for those who intend to use the water. These tests are also used for assessing the effectiveness of water treatment and disinfection in swimming pools, spa pools and hydrotherapy pools, and for monitoring the reduction of micro-organisms in wastewater treatment systems and the quality of treated effluents. The significance of *E. coli* in recreational and other waters is described elsewhere[(1)] in this series.

C2 Scope

The method is suitable for the examination of surface and ground waters, saline waters, manmade recreational waters such as swimming pools, spa pools and hydrotherapy pools, and untreated and treated wastewaters. Water samples with higher turbidities should be analysed using an appropriate multiple tube most probable number (MPN) method.

Users wishing to employ this method should verify its performance under their own laboratory conditions[(2)].

C3 Definition

In the context of this method, organisms which produce β-glucuronidase forming blue colonies after incubation at 30°C for 4 hours followed by incubation at 44°C for 14 hours on tryptone bile glucuronide agar (TBX) are regarded as *E. coli*. Most strains of *E. coli* express β-glucuronidase, as do some strains of *Shigella* and *Salmonella*.

For the purposes of water examination, *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44°C with the production of acid within 24 hours. Most strains produce β-glucuronidase.

C4 Principle

Organisms are entrapped on a membrane filter placed on an agar medium containing tryptone and bile salts and the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) either as the cyclohexylammonium or sodium salt, for the indication of the production of β-glucuronidase.

C5 Limitations

Enumeration of colonies by this method will exclude a proportion of strains of *E. coli* that are unable to grow at 44°C or that cannot express β-glucuronidase activity.

The method is suitable for most types of aqueous samples except those with high turbidities, which tend to block the membrane filter. This will limit the volume of sample that can be filtered. Accumulated deposit on the membrane filter may mask or inhibit the growth of indicator organisms. The ideal number of colonies that should be counted from a single membrane filter is approximately 20 – 80 with a maximum of 100. Counts can be obtained from membrane filters containing more than 100 colonies provided that isolated colonies are present and that a hand lens or similar magnifying aid is used. Counts obtained in this way should be reported as an estimated count.

Where high numbers of organisms may be expected (for example, untreated wastewater) serial ten-fold dilutions should be made to obtain a countable number of colonies on a membrane filter. For sand, sediments, and waters with high turbidities, an appropriate most probable number (MPN) method should be used (see Section D).

C6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[3] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

C7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[2] in this series. Principally, appropriate membrane filtration apparatus and incubators (fan assisted, either static temperature or temperature cycling) are required. Other items include:

C7.1 Sterile sample containers of appropriate volume, made of suitable material. For swimming pools, spa pools and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l, for example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent.

C7.2 Incubators capable of maintaining temperatures of 30.0 ± 1.0°C and/or 44.0 ± 0.5°C, or cycling incubators, fitted with timers, capable of attaining these temperatures.

C7.3 Filtration apparatus, sterile filter funnels or funnels that can be sterilised, and a vacuum source.

C7.4 Sterile membrane filters, for example, white, 47 mm diameter, cellulose-based 0.45 µm nominal pore size. Gridded membrane filters may facilitate the counting of colonies.

C7.5 Smooth-tipped forceps.

C8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. Commercial formulations should be used and stored according to the manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in this method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality[2]. Unless otherwise stated chemical constituents should be added as the anhydrous salts. If the pH of the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator, they should be allowed to reach room temperature before use.

C8.1 *Tryptone bile glucuronide agar (TBX agar)*[4]

Tryptone	20 g
Bile salts No. 3	1.5 g
Agar	15 g
5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG)	75 mg
Water	1 litre

Suspend the ingredients, except BCIG, in the water and bring to the boil to dissolve. Dissolve the cyclohexylammonium salt of BCIG in 3 ml of a solution consisting of 2.5 ml of 95 % v/v aqueous ethanol and 0.5 ml of 1M sodium hydroxide solution. Add this solution to the medium. Alternatively, the sodium salt can be added directly to the medium. Mix the solution well and autoclave at 121°C for 15 minutes. Allow the medium to cool to 50°C, distribute in suitable volumes to Petri dishes, and allow the medium to solidify. The pH of the medium after sterilisation should be 7.2 $\pm$ 0.2. Petri dishes containing the agar medium may be stored at temperatures between 5 ± 3°C for up to one week, protected against dehydration. Storage beyond this period may result in a deterioration of the medium.

C8.2 *Other media*

Standard and commercial formulations of other media and reagents used in this method include quarter strength Ringer's solution and maximum recovery diluent.

C9 Analytical procedure

C9.1 *Sample preparation*

The volumes, and dilutions, of samples should be chosen so that the number of colonies to be counted on the TBX membrane filter lies, if possible, between 20 and 80. With some waters, it may be advantageous to filter a selection of different volumes of sample so that the number of colonies on at least one of the membrane filters is likely to fall within this range. Typically, for swimming pool, spa pool and hydrotherapy pool waters, filter 100 ml of the sample. For polluted waters either filter smaller volumes, or dilute the sample with quarter strength Ringer's solution or maximum recovery diluent before filtration. Guidance on the processing of sludges or solid materials can be found elsewhere[5]. The minimum volume of sample to be filtered should not be less than 1 ml. This is to ensure that colonies

are evenly spread across the membrane filter and the volume filtered is still representative of the sample submitted. If smaller volumes are required to ensure the count of colonies lies within the above range, then appropriate dilutions should be made. A single volume or dilution of each sample is filtered and incubated at 44°C.

C9.2 *Sample processing*

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel. Holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter grid-side upwards onto the porous disc of the filter base. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample, or diluted sample, into the funnel. When the volume of sample to be filtered is less than 10 ml, add 10 - 20 ml of sterile diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) to the funnel before addition of the sample. This aids the dispersion of the bacteria over the entire surface of the membrane filter during the filtration process. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the sample slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and transfer the membrane filter carefully to a Petri dish containing TBX agar, which should be well-dried (for example, the Petri dish should be left at room temperature for 2 hours or at 37°C for 30 minutes, prior to use). Ensure that no air bubbles are trapped between the membrane filter and the medium. 'Rolling' the membrane filter onto the medium minimises the likelihood of air bubbles becoming trapped. Cover the membrane filter with the lid of the Petri dish.

When the funnel is removed it can be placed in a boiling water bath if it is to be re-used. Alternatively, pre-sterilised filter funnels can be used for each sample. If different volumes of the same sample are to be examined, the funnel may be re-used without boiling provided that the smallest volume, or highest dilution of sample, is filtered first. For different samples, take a fresh pre-sterilised funnel or remove a funnel from the boiling water bath, allow the funnel to cool and repeat the filtration process. If funnels are re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling water for at least one minute. During the filtration of a series of samples, the filter base need not be sterilised unless it becomes contaminated or a membrane filter becomes damaged. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible, and no longer than 2 hours.

The Petri dishes are inverted and placed in an incubator at 30°C for 4.00 ± 0.25 hours then transferred to an incubator at 44.0 ± 0.5°C and incubated for 21 ± 3 hours. Alternatively, a cycling temperature incubator can be used. Accurate temperature control and even temperature distribution are essential. False-positive results may be obtained if lower incubation temperatures are used and some organisms may fail to multiply at higher incubation temperatures. If, for any reason, an early indication of a result is required, the membrane filter at 44°C may be examined after a total incubation time of approximately 14

hours but the membrane filter should be returned to the incubator at 44°C for the full minimum incubation period of 14 hours at the higher temperature prior to counting.

C9.3 *Reading of results*

After the total minimum incubation period of 18 hours, examine the TBX membrane filters under good light, if necessary with a hand lens. Count all blue colonies (i.e. that are β-glucuronidase-positive) (see Figures C1 and C2). These are regarded as *E. coli.*

Figure C1 Blue colonies of *E. coli* from a surface water on tryptone bile glucuronide agar

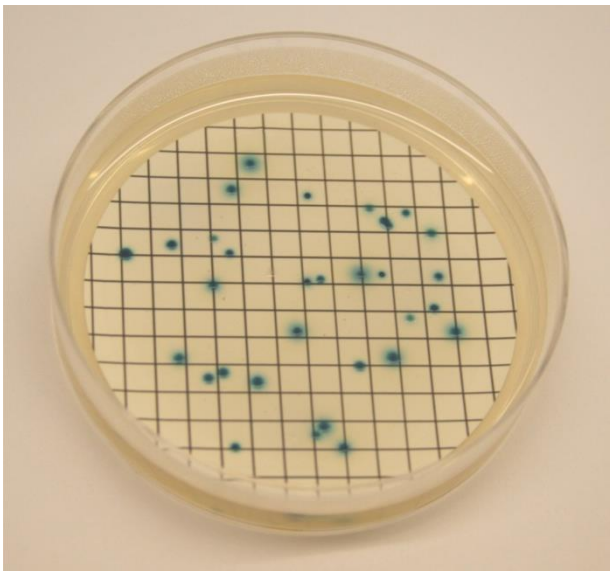
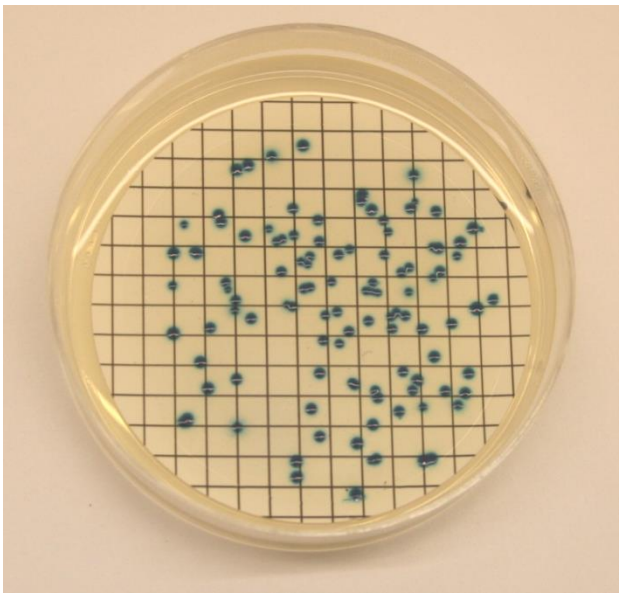


Figure C2 Blue colonies of *E. coli* from a reference culture (NCTC 09001) on tryptone bile glucuronide agar



C9.4 *Confirmation tests*

The specificity of tryptone bile glucuronide agar for *E. coli* is such that, following performance verification within the laboratory, confirmation of blue colonies as *E. coli* should not be required. The combination of media selectivity, incubation temperature and the specificity of β-glucuronidase are sufficient for most practical purposes.

C10 Calculations

The number of *E. coli* is generally expressed as the number of colonies per 100 ml of sample. Calculate the presumptive count as follows:

$$\text{Confirmed count per 100 ml} = \frac{N \times 100 \times DF}{\text{Volume of sample filtered (ml)}}$$

where N is the number of blue colonies counted on the TBX membrane filter, and DF is the appropriate dilution, if required.

The count of the total number of blue colonies on the TBX membrane filter is regarded as the number of *E. coli*.

C11 Expression of results

Counts for confirmed *E. coli* are expressed in colony forming units per volume of sample. For most samples, the volume is typically 100 ml.

C12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (*E. coli*) and non-target bacteria (for example *Klebsiella pneumonia* as a coliform or *Pseudomonas aeruginosa*). Further details are given elsewhere[2] in this series.

For each batches of samples for monitoring of routine internal performance quality control it is appropriate to examine one (or more for batches) sample in duplicate[3]. A comparison of the counts can then be undertaken.

C13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2015) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

3. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

4. ISO 16649-1: 2001. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli.* Part 1: Colony-count technique at 44°C using membranes and 5-bromo-4-chloro-3-indoyl-β-D-glucuronide. Geneva: International Organization for Standardization.

5. Standing Committee of Analysts, The Microbiology of Sewage Sludge (2003) - Part 2 - Practices and procedures for sampling and sample preparation, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

D The enumeration of *Escherichia coli* by a multiple tube most probable number technique using modified glutamate medium incubated at 44°C

This method has not been subjected to widespread use within the UK or verification of performance. Users of this method are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance. Information on the routine use of this method and similar methods, would be welcomed to assess their full capabilities.

D1 Introduction

Tests for *Escherichia coli* (*E. coli*) are used to determine the presence of faecal contamination in recreational and other waters as it is considered as the key indicator of faecal contamination. They provide the means of assessing the degree of faecal contamination and assessing the potential risk of infection for those who intend to use the water. The tests are also used for assessing the effectiveness of water treatment and disinfection in swimming pools, spa pools and hydrotherapy pools and for monitoring the reduction of micro-organisms in wastewater treatment systems and the quality of treated effluents. The significance of *E. coli* in recreational and other waters is described elsewhere[(1)] in this series.

D2 Scope

The method is suitable for the examination of surface and ground waters, saline waters, manmade recreational waters such as swimming pools, spa pools and hydrotherapy pools, and untreated and treated wastewaters, and water samples with higher turbidities.

Those wishing to use this method for swimming pools, spa pools and hydrotherapy pools should incubate processed samples at 37°C as well as 44°C in order to isolate coliform bacteria. Confirmation methods for coliforms may be found elsewhere[(2)].

Users wishing to employ this method should verify its performance under their own laboratory conditions[(3)].

D3 Definitions

In the context of the method, organisms which produce acid from lactose in minerals modified glutamate medium (MMGM) at 44°C are regarded as presumptive *E. coli*. Isolation of presumptive colonies is followed by confirmatory tests on MLGA or TBX for the demonstration of the production of β-glucuronidase.

For the purposes of water examination *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44°C with the production of acid within 24 hours, and which produce indole from tryptophan. Most strains produce β-glucuronidase.

D4 Principle

Bacteria are grown in a liquid medium containing lactose and bromocresol purple as an indicator of acidity. This is followed by confirmatory tests for the enzyme β-glucuronidase.

In this method, measured volumes of sample, or dilution of sample, are added to a series of tubes or bottles containing liquid differential medium. If, within the series, some of the tubes or bottles exhibit no characteristic growth in the medium following incubation, and other tubes or bottles exhibit some characteristic growth in the medium following incubation, then the most probable number of organisms in 100 ml of sample can be estimated from appropriate probability tables, see appendix D1. Confirmation that positive reactions (i.e. in those tubes or bottles showing characteristic growth) are due to a particular organism can be obtained by sub-culture to media containing a substrate for the enzyme β-glucuronidase (for example MLGA or TBX).

D5 Limitations

This method is labour intensive and may require the preparation of large numbers of tubes or bottles of media and appropriate sub-cultures.

D6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[4] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[3] in this series.

D7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [3] in this series. Other items include:

D7.1 Sterile sample containers of appropriate volume, made of suitable material. For swimming pools, spa and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l (for example, 0.1 ml of a 1.8 % m/v solution of $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

D7.2 Fan assisted incubators (or stirred water baths) capable of maintaining temperatures of 37°C ± 1.0°C and 44.0 ± 0.5°C.`

D7.3 Suitable bottle or test tube racks.

D8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. Commercial formulations should be used and stored according to the manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in this method[3]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar quality[3]. Unless otherwise stated, chemical constituents should be added as the anhydrous salts. If the pH of the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

Minerals modified glutamate medium[5, 6] is used for the isolation of *E. coli* and coliform bacteria from waters. The use of MacConkey broth may not be suitable because of variations in the inhibitory properties of different batches of bile salts.

D8.1 *Single-strength minerals modified glutamate medium (MMGM)*

Lactose	10.0 g
L (+) Glutamic acid sodium salt	6.35 g
L (+) Arginine monohydrochloride	20 mg
L (-) Aspartic acid	24 mg
L (-) Cystine	20 mg
Sodium formate	250 mg
Dipotassium hydrogen phosphate	0.9 g
Ammonium chloride	2.5 g
Magnesium sulphate heptahydrate	100 mg
Calcium chloride dihydrate	10 mg
Iron(III) citrate	10 mg
Thiamine (Aneurin hydrochloride)	1 mg
Nicotinic acid	1 mg
Pantothenic acid	1 mg
Bromocresol purple (1 % m/v ethanolic solution)	1 ml
Water	1 litre

Add the ingredients to the water and dissolve. Distribute (in 5 ml volumes) into suitable capped tubes or bottles. Sterilise the medium at 115°C for 10 minutes. Sterile media can be stored for up to one month at a temperature between 5 ± 3°C.

Double-strength medium is conveniently prepared in quantities of 10 (or more) litres. If the medium is not to be distributed in tubes immediately, the lactose and thiamine should be omitted and added before dispensing.

Several of the ingredients are more conveniently added as separate solutions and these may be prepared as follows:

SOLUTION 1	
L (+) Arginine monohydrochloride	400 mg
L (-) Aspartic acid	480 mg
Water	50 ml

Heat the water to about 50°C and dissolve the ingredients.

SOLUTION 2	
L (-) Cystine	400 mg
5M Sodium hydroxide	10 ml
Water	90 ml

Heat the water to about 50°C and dissolve the ingredients.

SOLUTION 3	
Nicotinic acid	20 mg
Pantothenic acid	20 mg
Water	5 ml

Dissolve the ingredients in the water without heating the solution.

SOLUTION 4

Iron(III) citrate	200 mg
Water	10 ml

Heat the water to about 50°C and dissolve the ingredients.

SOLUTION 5

Calcium chloride dihydrate	5 g
Water	100 ml
Concentrated hydrochloric acid	0.1 ml

Dissolve the ingredients in the water without heating the solution and sterilise the solution at 121°C for 20 minutes. Store as a stock solution.

SOLUTION 6

Thiamine	100 mg
Water	99 ml

Prepare a sterile 0.1 % m/v solution of thiamine in the water. This can be carried out by adding an ampoule of thiamine (100 mg) to 99 ml of sterile distilled water.

The above solutions may be stored at a temperature between 5 ± 3°C and any remaining unused solution should be discarded after 6 weeks.

To prepare 10 litres of double-strength medium, dissolve the appropriate quantities of L (+) glutamic acid sodium salt, sodium formate, dipotassium hydrogen phosphate, ammonium chloride and magnesium sulphate heptahydrate in 9 litres of hot distilled water at about 50°C. Add the whole of solutions 1, 2, 3 and 4, and 4 ml of solution 5. Adjust the pH of the medium to 6.9 ± 0.2 or higher if necessary, so that the final pH (when completely prepared and after sterilisation is 6.7 ± 0.2). After adjustment of the pH, add 20 ml of a 1 % m/v ethanolic solution of bromocresol purple. Dilute to a final volume of 10 litres.

If the medium is not required for immediate use, dispense the mixed solution (medium without lactose and thiamine) into suitable containers in 500 ml volumes. Autoclave the solutions at 115°C for 10 minutes. Solutions may be stored in the dark at room temperature for up to one month.

For use, add the necessary amounts of lactose and solution 6, (i.e. 10 g and 1 ml respectively). Allow the added lactose to dissolve and distribute into suitable tubes or bottles in 10 ml and 50 ml volumes. Cap the containers and sterilise at 115°C for 10 minutes.

D8.2 *Membrane lactose glucuronide agar (MLGA)*[7, 8]

Peptone	40 g
Yeast extract	6 g
Lactose	30 g
Phenol red (0.4% m/v solution)	50 ml
Sodium lauryl sulphate	1.0 g

Sodium pyruvate	0.5 g
Agar	10.0 g
5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG)	0.2 g
Water	1 litre

Suspend the ingredients, except BCIG, in the water and bring to the boil to dissolve. Dissolve the cyclohexylammonium salt of BCIG in 3 ml of solution consisting of 2.5 ml of 95 % v/v aqueous ethanol and 0.5 ml of 1M sodium hydroxide solution. Add this solution to the medium. The sodium salt of BCIG can be added directly to the medium. Mix the solution well and autoclave at 121°C for 15 minutes. Allow the medium to cool, distribute it in Petri dishes and allow it to solidify. Petri dishes containing the agar medium may be stored at a temperature in the range 5 ± 3°C for up to one week, protected against dehydration. Storage beyond this time may result in a deterioration of performance of the medium. The pH of the medium after sterilisation should be 7.4 ± 0.2. The detection of acid production is influenced by the pH of the medium, thus, it is important that the medium is of the correct pH. To achieve this, it may be necessary to adjust the pH to about 7.6 before sterilisation.

D8.3 *Tryptone bile glucuronide agar (TBX agar)*[9]

Tryptone	20 g
Bile salts No. 3	1.5 g
Agar	15 g
5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG)	75 mg
Water	1 litre

Suspend the ingredients, except BCIG, in the water and bring to the boil to dissolve. Dissolve the cyclohexylammonium salt of BCIG in 3 ml of a solution consisting of 2.5 ml of 95 % v/v aqueous ethanol and 0.5 ml of 1M sodium hydroxide solution. Add this solution to the medium. Alternatively, the sodium salt can be added directly to the medium. Mix the solution well and autoclave at 121°C for 15 minutes. Allow the medium to cool to 50°C, distribute in suitable volumes to Petri dishes, and allow the medium to solidify. Petri dishes containing the agar medium may be stored at temperatures between 5 ± 3°C for up to one week, protected against dehydration. Storage beyond this period may result in a deterioration of the medium. The pH of the medium after sterilisation should be 7.2 ± 0.2.

D8.4 *Other media*

Standard and commercial formulations of other media and reagents used in this method include quarter strength Ringer's solution and maximum recovery diluent.

D9 Analytical procedure

D9.1 *Volumes of sample for inoculation*

A series of different volumes of sample is inoculated into tubes or bottles of minerals modified glutamate medium.

For waters expected to be of good quality, for example, swimming pool waters, use 1 x 50 ml and 5 x 10 ml volumes of sample. Add the 50 ml and 10 ml volumes of sample to equal volumes of double-strength medium.

For waters expected to be of doubtful or unknown quality, use 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample. Add the 50 ml and 10 ml volumes of sample to equal volumes of double-strength medium and the 1 ml volumes of sample to 5 ml of single-strength medium.

For waters expected to be of a more polluted nature, use 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample. Add the 10 ml volumes of sample to equal volumes of double-strength medium and the 1 ml and 0.1 ml volumes of sample to 5 ml of single-strength medium.

For waters expected to be heavily polluted, dilutions (in a suitable diluent, for example, quarter-strength Ringer's solution or maximum recovery diluent) of a hundred-fold, or a thousand-fold or higher, may need to be prepared. Suitable volumes of these diluted samples should then be used and added to 5 ml of single-strength medium.

Samples of sediment and sand are analysed in a similar fashion with appropriate masses of wet weight being added to tubes of single-strength medium (for example, 1 x 1.0 g and 5 x 0.1 g for unpolluted sands or 5 x 1.0 g, 5 x 0.1 g and 5 x 0.01 g where counts are expected to be higher). Sand and sediment samples should be thoroughly mixed before being dispensed. Guidance on the processing of sludges or solid materials can be found elsewhere[10].

For all types of samples and in all cases, sufficient volumes of sample should be added to the medium so that, after incubation, some of the tubes or bottles exhibit characteristic growth within the medium and some of the tubes or bottles exhibit no growth within the medium. Sterile pipettes are used to transfer the sample to the tube or bottle containing the minerals modified glutamate medium. A spatula used to dispense solid material can be disinfected using an iso-propanol or ethanol wipe.

D9.2 *Sample processing*

After the tubes or bottles of minerals modified glutamate medium have been inoculated with the appropriate volume of sample, or diluted sample, each tube or bottle is capped or sealed and placed in an incubator at 44°C. After 21 ± 3 hours the tubes or bottles are examined for acid production (as demonstrated by the presence of a yellow coloration). All tubes or bottles that exhibit positive (characteristic) growth within the medium are retained for confirmatory testing. Some tubes or bottles may exhibit growth (i.e. a turbid reaction) without a colour change, these tubes are regarded as negative.

D9.3 *Reading of results*

The number of tubes or bottles for each series of volume of sample is recorded where a positive reaction is given, as demonstrated by growth within the medium and the production of a yellow coloration (see Figure D1). After this confirmation tests may be carried out.

Figure D1 A typical 11-tube MPN test with minerals modified glutamate broth

1 x 50 ml, plus equal volume of double-strength medium	5 x 10 ml, plus equal volumes of double-strength medium	5 x 1 ml, plus 5 ml of single-strength medium

Bottles that exhibit growth within the medium are indicated by turbidity and yellow colouration, regard these as positive.
Bottles that exhibit no growth within the medium are indicated by purple colouration, regard these as negative.

When dilutions of sample have been used, a consecutive series of volumes should be chosen whereby some of the tubes or bottles exhibit growth within the medium i.e. are positive, and some tubes or bottles exhibit no growth within the medium i.e. are negative. From the results, the MPN of bacteria in the sample is determined from probability tables, see Appendix D1.

D9.4 *Confirmation tests*

Any tubes or bottles showing a positive reaction are sub-cultured to media containing a substrate specific for the production of β-glucuronidase (for example MLGA or TBX, see method A).

D9.4.1 Confirmation of *E. coli*

For each tube or bottle showing characteristic growth within the MMGM medium sub-culture to MLGA or TBX and incubate at 37°C for 21 ± 3 hours. Examine the sub-cultures for the expression of β-glucuronidase, green colonies on MLGA or blue colonies on TBX (see Figure D2).

D10 Calculations

D10.1 *Presumptive E. coli*

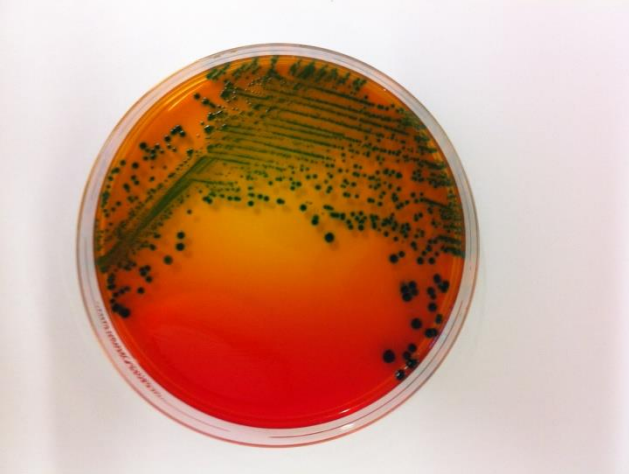
The number of minerals modified glutamate medium tubes or bottles of each volume of sample showing a positive reaction is counted, and then by reference to the appropriate tables in appendix D1, the MPN of *E. coli* present in 100 ml of sample is determined. For example, if in a 15-tube test comprising 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample, the number of tubes showing positive reactions in each consecutive series is 3, 2 and 0 respectively, then, from Table D3, the MPN is 13 organisms per 100 ml.

D10.2 *Confirmed E. coli*

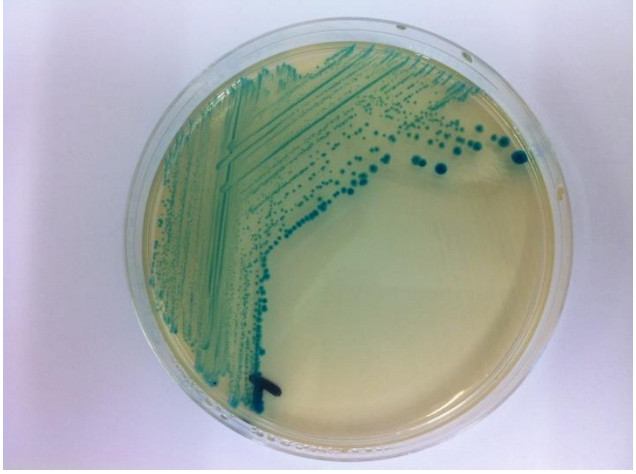
Confirmed *E. coli* are calculated by reference to the appropriate table in appendix D1 for the number of tubes or bottles that yield isolates that produce typical *E. coli* colonies on MLGA or TBX.

Figure D2 Typical β-glucuronidase positive colonies on MLGA and TBX

E. coli on MLGA



E. coli on TBX



D11 Expression of results

Presumptive and confirmed *E. coli* counts are expressed as MPN per volume of sample. For most samples, the volume is typically 100 ml. For sediment and sand samples the counts are expressed as MPN per weight of sample, usually adjusted to MPN count per gram wet weight. For inter-laboratory comparison purposes, a dry weight basis may be more appropriate.

D12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (*E. coli*) and non-target bacteria (for example *Klebsiella pneumoniae* as a coliform or *Pseudomonas aeruginosa*). Tubes or bottles should be incubated as appropriate. Further details are given elsewhere[(2)] in this series.

D13 References

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Appendix D1 Tables of most probable numbers

From the various combinations of positive and negative reactions for the different volumes examined, the following tables indicate the MPN of bacteria in 100 ml of sample. It is important to realise that the MPN is only an estimate, based on statistical probabilities and that the actual number may lie within a range of values. Approximate 95 % confidence intervals, which demonstrate the range of possible numbers (the MPR) which could yield the number of positive reactions, have been published[11]. A procedure for estimating these confidence intervals for other dilution series has also been published[12]. These confidence intervals are seldom of practical use when reporting results because they apply to the accuracy of the method and not the likely variability of organisms at the sampling source[13]. The MPR in tables D1 - D3 illustrates those situations where the method becomes relatively imprecise, particularly when nearly all the tubes show growth within the medium. In these situations, further dilutions should have been prepared and added to tubes of medium.

Table D1 gives the MPN (and where applicable the MPR) for a 6-tube series containing 1 x 50 ml and 5 x 10 ml volumes of sample. Similarly table D2 gives the MPN (and where applicable the MPR) for an 11-tube series comprising 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample. Table D3 shows data for a 15-tube series of 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of samples but gives only those values of the more likely combinations of positive and negative reactions. For example, positive reactions in the 0.1 ml tubes would not be expected if all of the 10 ml and 1 ml tubes were negative. Hence, MPN and MPR values for a combination of results like for instance 0, 0, 2 etc are not tabulated. If these unlikely combinations are observed in practice with greater than expected frequencies, then this might indicate that the statistical assumptions underlying the MPN estimation are not correct[11,14,15]. For example, the organisms may not have been uniformly distributed throughout the sample, or toxic substances may have been present.

Calculation of MPN

The number of positive reactions for each set of tubes is recorded and, from the relevant table, the MPN of organisms present in 100 ml of the sample is determined.

Where a series of dilutions of the sample is used, then the following rules should be applied, as illustrated by the numbers in bold, underlined, italic type in table D4.

(i) Use only three consecutive sets of dilutions for calculating the MPN.

(ii) Wherever possible, select three consecutive dilutions where the results are neither all positive nor all negative. The most efficient statistical estimate will result when about half the tubes are positive (see examples (a), (b) and (c) in table D4).

(iii) If less than three sets of dilutions give positive results, begin with the set containing the largest volume of sample (see example (d) in table D4).

(iv) If only one set of tubes gives a positive reaction, use this dilution and the one higher and one lower (see example (e) in table D4).

Table D1 MPN and MPR per 100 ml of sample for a 6-tube series containing 1 x 50 ml and 5 x 10 ml volumes of sample

Number of tubes giving a positive reaction		MPN per 100 ml	MPR* per 100 ml
1 x 50 ml	5 x 10 ml		
0	0	None found	
0	1	1	
0	2	2	
0	3	3	
0	4	4	4-5
0	5	6	
1	0	1	
1	1	2	
1	2	5	4-5
1	3	9	8-10
1	4	15	13-18
1	5	>18**	

* These numbers are at least 95 % as probable as the MPN.

** There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 18.

Table D2 MPN and MPR per 100 ml of sample for an 11-tube series of 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample

Number of tubes giving a positive reaction			MPN per 100 ml	MPR* per 100 ml
1 x 50 ml	5 x 10 ml	5 x 1 ml		
0	0	0	None found	
0	0	1	1	
0	1	0	1	
0	1	1	2	
0	2	0	2	
0	2	1	3	
0	3	0	3	
1	0	0	1	
1	0	1	2	
1	1	0	2	
1	1	1	4	
1	1	2	6	
1	2	0	4	4-5
1	2	1	7	6-7
1	2	2	9	9-10
1	3	0	8	7-9
1	3	1	10	10-11
1	3	2	13	12-13
1	3	3	17	15-18
1	4	0	12	11-14
1	4	1	16	15-19
1	4	2	21	19-24
1	4	3	27	24-30
1	4	4	33	30-38
1	5	0	23	20-27
1	5	1	33	29-40
1	5	2	53	44-65
1	5	3	91	75-110
1	5	4	160	134-190
1	5	5	>180**	

* These numbers are at least 95 % as probable as the MPN.

** There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 180.

Table D3 MPN and MPR per 100 ml of sample for a 15-tube series containing 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample

Number of tubes giving a positive reaction			MPN per 100 ml	MPR* per 100 ml
5 x 10 ml	5 x 1 ml	5 x 0.1 ml		
0	0	0	None found	
0	0	1	2	
0	1	0	2	
1	0	0	2	
1	0	1	4	
1	1	0	4	
2	2	0	4	
2	0	1	5	
2	1	0	5	
2	1	1	7	
2	2	0	7	7-9
2	3	0	11	
3	0	0	7	
3	0	1	9	
3	1	0	9	
3	1	1	13	
3	2	0	13	
3	2	1	16	14-16
3	3	0	16	14-16
4	0	0	11	11-13
4	0	1	14	14-16
4	1	0	16	14-16
4	1	1	20	18-20
4	2	0	20	18-22
4	2	1	25	23-27
4	3	0	25	23-27
4	3	1	31	29-34
4	4	0	32	29-34
4	4	1	38	34-41
5	0	0	22	20-23
5	0	1	29	25-34
5	0	2	41	36-50
5	1	0	31	27-36
5	1	1	43	36-50
5	1	2	60	50-70
5	1	3	85	70-95
5	2	0	50	40-55
5	2	1	70	60-80
5	2	2	95	80-110
5	2	3	120	105-135
5	3	0	75	65-90
5	3	1	110	90-125
5	3	2	140	120-160
5	3	3	175	155-200
5	3	4	210	185-240
5	4	0	130	110-150
5	4	1	170	150-200
5	4	2	220	190-250
5	4	3	280	240-320
5	4	4	345	300-390
5	5	0	240	200-280
5	5	1	350	290-420
5	5	2	540	450-600
5	5	3	910	750-1100
5	5	4	1600	1350-1900
5	5	5	>1800**	

* These numbers are at least 95 % as probable as the MPN.

** There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 1800.

Table D4 Examples of the derivation of the MPN from the numbers of positive reactions in a series of dilutions*

Example in text	Volume of sample (ml)					MPN per 100 ml
	10	1	0.1	0.01	0.001	
(a)	***5***	***3***	***2***	0		140
(b)	5	***5***	***3***	***2***	0	1400
(c)	5	***5***	***2***	***0***	0	500
(d)	***3***	***1***	***0***	0		9
(e)	***0***	***1***	***0***	0		2

* Numbers in bold, underlined, italic type indicate which results should be used in determining the MPN.

E The enumeration of *Escherichia coli* by a defined substrate most probable number technique incubated at 37°C

E1 Introduction

Tests for *Escherichia coli* (*E. coli*) are used to determine the presence of faecal contamination in recreational and other waters as it is considered as the key indicator of faecal contamination. They provide the means of assessing the degree of faecal contamination and assessing the potential risk of infection for those who intend to use the water. The tests are also used for assessing the effectiveness of water treatment and disinfection in swimming pools, spa pools and hydrotherapy pools and for monitoring the reduction of micro-organisms in wastewater treatment systems and the quality of treated effluents. The significance of *E. coli* in recreational and other waters is described elsewhere[1] in this series.

E2 Scope

The method is suitable for the examination of surface and ground waters, saline waters, manmade recreational waters such as swimming pools, spa pools and hydrotherapy pools, and untreated and treated wastewaters, and water samples with higher turbidities.

Whilst details of a specific commercial product are described in this document, this does not constitute an endorsement of the product but serves only as an illustrative example of the types of products available. Details of this method are included for information purposes only, as an example of the defined substrate techniques that are available.

Users wishing to employ this method, or similar methods from other manufacturers, should verify the performance under their own laboratory conditions[2]. Details of evaluation trials are reported elsewhere[3].

E3 Definitions

Defined substrate media are chemically defined formulations containing substrates for the specific detection of diagnostic enzymes associated with a particular group of organisms.

In the context of this method, organisms which produce β-galactosidase, as demonstrated by the production of a yellow colour through the enzymatic cleavage of ortho-nitrophenyl-β-D-galactopyranoside in a defined substrate medium, are regarded as coliform bacteria. In addition, organisms which produce β-glucuronidase, as demonstrated by the production of a yellow colour and blue-white fluorescence (under long wavelength ultra-violet illumination) through the enzymatic cleavage of 4-methylumbelliferyl-β-D-glucuronide in a defined substrate medium, are regarded as *E. coli*. This method is reported to be highly specific for coliform bacteria and *E. coli*, and confirmation tests including a negative oxidase test are not usually required.

Coliform bacteria are considered to be members of genera within the Family Enterobacteriaceae, capable of growth at 37°C, that possess β-galactosidase. This definition includes anaerogenic (i.e. non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Buttiauxella, Citrobacter, Enterobacter, Escherichia, Hafnia, Klebsiella, Leclercia, Pantoea, Raoultella, Serratia* and *Yersinia.*

For the purposes of water examination *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44°C with the production of acid within 24 hours, and which produce indole from tryptophan. Most strains produce β-glucuronidase.

Strains which possess these characteristics at 37°C but do not express them at 44°C may also be *E. coli.* When identified as *E. coli* they have the same sanitary and operational significance with regard to their faecal origin.

E4 Principle

Organisms are grown in a defined liquid medium containing substrates for the specific detection of the enzymes β-galactosidase and β-glucuronidase. The dehydrated medium is dissolved in 100 ml of sample, or 100 ml of diluted sample, which is then added to a 51-well reaction pouch. This is then sealed and incubated at 37°C for between 18 - 22 hours. If, within the pouch, some of the wells exhibit no growth in the medium after incubation, while other wells exhibit some growth in the medium after incubation, then the most probable number of organisms in 100 ml of sample can be estimated from appropriate probability tables, see Appendix E1.

E5 Limitations

This method is suitable for most types of aqueous samples, except those with high turbidities, which may mask or inhibit colour development. The presence of very high numbers of *Aeromonas* may result in false positive reactions. If high chlorine residuals are encountered after dechlorination a transient blue reaction may occur.

E6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[4] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

When ultra-violet lamps are used gloves and either goggles or a face shield suitable for use with appropriate ultra-violet-emitting sources should be worn.

E7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[2] in this series. Principally, fan assisted incubators are required. An example of the methodology for this type of method is presented and is based upon a commercially available system. Some of the equipment listed is specific to this system and alternative systems may be available for which other equipment may be required. Other items include:

E7.1 Sterile sample containers of appropriate volume, made of suitable material. For swimming pools, spa and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l, for example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent.

E7.2 Incubator capable of maintaining a temperature of 37.0 ± 1.0°C.

E7.3 Sterile 100 ml plastic bottles containing anti-foaming agent as supplied by the manufacturer of the test system or suitable equivalent.

E7.4 MPN reaction pouches as supplied by the manufacturer (for example, a 51-well system or a 97-well system if high counts are expected) and associated heat-sealing equipment.

E7.5 Ultra-violet long wavelength (365 - 366 nm) lamp, and viewer.

E7.6 Colour and fluorescence comparator as supplied by the manufacturer.

E8 Media and reagents

Commercial formulations of these media and reagents may be available. Formulations should be used and stored according to the manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the method[2].

E8.1 *Colilert® 18 medium*[5]

The medium is a commercially available formulation provided in sachets and is suitable for single samples. The medium is a chemically defined formulation with minimal nutrients and substrates for the specific detection of the enzymes β-galactosidase and β-glucuronidase. For MPN counts, the medium can be used in conjunction with Quanti-Tray® reaction pouches.

E9 Analytical procedure

E9.1 *Sample preparation*

The volume, or dilution, of samples should be chosen so that not all the wells show a positive response. For swimming pool and spa pool waters 100 ml of sample will generally be appropriate whilst for contaminated waters, appropriate dilutions should be prepared, and 100 ml of diluted sample used. When preparing dilutions use sterile distilled, deionised or similar grade water. Buffered solutions should not be used as they may adversely affect the performance.

E9.2 *Sample processing*

The sample or appropriate dilution (usually 100 ml) is decanted into a sterile bottle containing an anti-foam agent. Following the manufacturer's instructions, the contents of one sachet of medium is then aseptically added. After capping the bottle, the contents are gently agitated to ensure dissolution of the medium and then the bottle is left to stand for 10 minutes or until the medium is completely dissolved and dispersal of any air bubbles have been dispersed from the liquid. The contents of the bottle are then added to the MPN reaction pouch, which is then sealed in the apparatus provided by the manufacturer to produce a 51-well reaction pouch. Prolonged exposure of the inoculated reaction pouch to direct sunlight should be avoided as this may result in hydrolysis of the specific substrates causing false-positive reactions. The time between the inoculation of the reaction pouch and the beginning of the incubation stage should be as short as possible, and no longer than 2 hours.

Sealed MPN reaction pouches are then incubated, ‘well-side’ down, at 37°C for not less than 18 hours and not more than 22 hours. However, if the sample contains sediment or other particles the reaction pouches should be incubated ‘well-side’ up to avoid accumulation of material on the well wall that may interfere with the reading of positive wells.

E9.3 *Reading of results*

After incubation, the pouch is examined and the number of wells that have a sufficient yellow colour (see Figure E1) compared against the manufacturer’s comparator, is recorded. The pouch is then re-examined under a long wavelength ultra-violet lamp and the number of wells, that produce a blue-white fluorescence (see Figure E2) of sufficient intensity compared against the manufacturer’s comparator, is recorded. If the pouch is examined before the completion of 22 hours incubation and this examination reveals borderline responses, then it should be returned to the incubator for the remaining incubation period. After 22 hours incubation, the pouch is removed and re-examined as before.

Figure E1 Example of MPN reaction pouch with a defined substrate medium with 19 wells showing presence of coliform bacteria

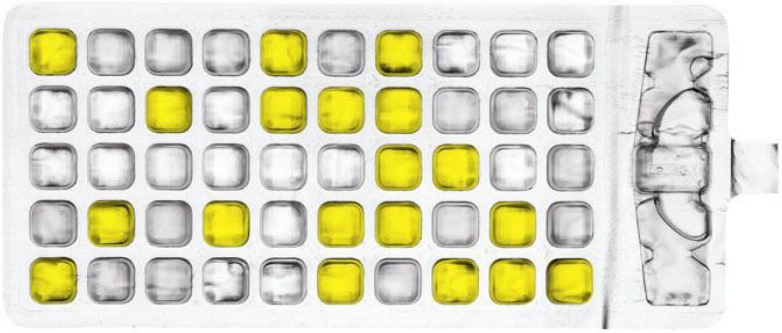
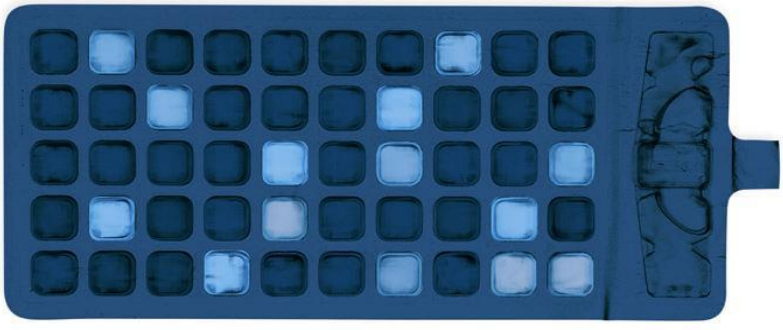


Figure E2 Example of MPN reaction pouch with a defined substrate medium with 14 fluorescing wells showing presence of *E. coli*



E9.4 *Confirmation tests*

This method is reported to be highly specific for coliform bacteria (yellow coloration in the wells) and *E. coli* (blue-white fluorescence in the wells). Hence, confirmation tests are not usually required. Should there be any doubt as to the type of organism and response detected, then wells showing a positive response should be sub-cultured and confirmatory tests undertaken. See methods B or C.

E10 Calculations

E10.1 *Confirmed coliform bacteria and E. coli*

The MPN of coliform bacteria is determined by reference to appropriate probability tables, see for example Appendix E1. This is derived from the number of wells showing a yellow coloration. For example, if there are 31 wells showing a yellow coloration in the reaction pouch, then from Appendix E1, the MPN of coliform bacteria is 48 per 100 ml of sample, or diluted sample, examined. Any dilution needs to be taken into account.

The MPN of *E. coli* is determined by reference to the same probability table. This is derived from the number of wells showing a blue-white fluorescence. For example, if there are 12 wells showing a blue-white fluorescence in the reaction pouch then, from Appendix E1, the MPN of *E. coli* is 14 per 100 ml of sample, or diluted sample examined. Any dilution needs to be taken into account.

E11 Expression of results

Confirmed coliform bacteria and *E. coli* counts are expressed as MPN counts per 100 ml volume of sample, or dilution of sample, after adjustment for any dilution. For most samples, the volume is typically 100 ml.

E12 Quality assurance

New batches of media should be tested with appropriate reference strains of target bacteria (for example *E. coli* and *Enterobacter aerogenes*) and non-target bacteria (for example *Aeromonas hydrophila* or *Pseudomonas aeruginosa*). Pouches should be incubated for 18 - 22 hours at 37°C. Further details are given elsewhere[2] in this series.

E13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2015) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

3. Standing Committee of Analysts, Evaluation trials for two media for the simultaneous detection and enumeration of *Escherichia coli* and coliform organisms 1998, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

4. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

5. IDEXX Laboratories, Milton Court, Churchfield Road, Chalfont St Peter, Buckinghamshire, SL9 9LW.

Appendix E1 MPN (and 95% confidence intervals) per 100 ml for a 51-well defined substrate medium reaction pouch

Number of wells showing a positive reaction	MPN per 100 ml	95 % confidence limits	Number of wells showing a positive reaction	MPN per 100 ml	95 % confidence limits
0	0	0-4	26	36	25-54
1	1	0-6	27	38	26-57
2	2	1-7	28	41	28-60
3	3	1-9	29	43	30-63
4	4	2-11	30	45	32-66
5	5	2-12	31	48	33-69
6	6	3-14	32	50	35-73
7	8	4-16	33	53	38-76
8	9	5-17	34	56	40-80
9	10	5-19	35	59	42-84
10	11	6-21	36	62	45-89
11	12	7-22	37	66	47-94
12	14	8-24	38	70	50-99
13	15	9-26	39	74	53-105
14	16	10-28	40	78	56-111
15	18	11-29	41	83	60-118
16	19	12-31	42	89	64-126
17	21	13-33	43	95	68-135
18	22	14-35	44	101	73-146
19	24	15-37	45	109	79-159
20	25	17-39	46	118	85-175
21	27	18-42	47	130	93-195
22	29	19-44	48	145	102-224
23	31	20-46	49	165	115-272
24	32	22-49	50	201	136-388
25	34	23-51	51	>201	

F The enumeration of *Escherichia coli* by a miniaturised most probable number technique incubated at 44°C

This method has not been subjected to widespread use within the UK or verification of performance. Users of this method are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance. Information on the routine use of this method and similar methods, would be welcomed to assess their full capabilities.

F1 Introduction

Tests for *Escherichia coli* (*E. coli*) are used to determine the presence of faecal contamination in recreational and other waters as it is considered as the key indicator of faecal contamination. They provide the means of assessing the degree of faecal contamination and assessing the potential risk of infection for those who intend to use the water. The tests are also used for assessing the effectiveness of water treatment and disinfection in swimming pools, spa pools and hydrotherapy pools and for monitoring the reduction of micro-organisms in wastewater treatment systems and the quality of treated effluents. The significance of *E. coli* and coliform bacteria in recreational and other waters is described elsewhere[(1)] in this series.

F2 Scope

The method is suitable for the examination of surface and ground waters, saline waters, manmade recreational waters such as swimming pools, spa pools and hydrotherapy pools, and untreated and treated wastewaters, and water samples with higher turbidities

Whilst details of a specific commercial product are described in this document, this does not constitute an endorsement of the product but serves only as an illustrative example of the type of product available. Details of this method are included for information purposes only, as an example of micro-titre plate methods that are available.

This method is based on ISO 9308-3:1998[(2)]. Users wishing to employ this method, or similar methods from other manufacturers, should verify the performance under their own laboratory conditions[(3)].

F3 Definitions

In the context of this method, organisms which are oxidase-negative and which produce β-glucuronidase, as demonstrated by the production blue-white fluorescence (under ultra-violet illumination at 366 nm) through the enzymatic cleavage of 4-methylumbelliferyl-β-D-glucuronide (MUG) in a selective medium at 44°C when incubated for 36 - 72 hours, are regarded as *E. coli*.

For the purposes of water examination *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44°C with the production of acid within 24 hours, and which produce indole from tryptophan. Most strains produce β-glucuronidase. Strains which possess these characteristics at 37°C but do not express them at 44°C may also be *E. coli* but will not be identified by this method.

F4 Principle

A micro-titre plate, comprising 96 wells, is used whereby portions of the diluted sample are inoculated into several rows of wells, each containing dehydrated culture medium. The plates are then incubated for between 36 and 72 hours at 44°C. If present, *E. coli* is indicated in each well by a blue fluorescence as seen under ultra-violet illumination due to the hydrolysis of 4-methylumbelliferyl-β-D-glucuronide. The most probable number of organisms in 100 ml of sample is then estimated from appropriate probability tables, see Appendix F1.

F5 Limitations

This method may not be suitable for samples where *E. coli* is likely to be absent, or present in low numbers. The method may only be applicable to samples containing *E. coli* in excess of 15 colony forming units in the volume examined.

F6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[4] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[3] in this series.

N,N-dimethylformamide, which is a component of one of the media, is toxic and carcinogenic. Inhalation, contact with skin or ingestion of this substance should be avoided, and it should only be used within a fume cupboard.

When ultra-violet lamps are used, gloves and either goggles or a face shield suitable for use with appropriate ultra-violet-emitting sources should be worn.

F7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[3] in this series. Principally, fan assisted incubators are required. Other items include:

F7.1 Sterile sample containers of appropriate volume, made of suitable material. For swimming pools, spa pools and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l, for example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent.

F7.2 Incubator capable of maintaining a temperature of 44.0 ± 0.5°C.

F7.3 Tunnel drier, fume cupboard or vertical laminar air flow cabinet.

F7.4 Ultra-violet long wavelength (365 - 366 nm) lamp and viewer.

F7.5 8-channel multi-pipette, with suitable sterile tips, adjustable or pre-set, capable of dispensing 200 µl of diluted sample into each well (or other equivalent system).

F7.6 Sterile 96-well micro-titre plates and sterile adhesive covering strips for sealing the wells.

F8 Media and reagents

Commercial formulations of some of these media and reagents are available. The performance of all media and reagents should be verified prior to their use. Variations in the preparation and storage of media should also be verified[3]. Unless otherwise stated, chemical constituents should be added as the anhydrous salts. Water should be distilled, deionised or of similar grade quality.

F8.1 *MUG/EC medium*

Tryptone	40 g
Salicin	1 g
Triton X 100®	1 g
MUG (4-methylumbelliferyl-β-D-glucuronide)	100 mg
Water	1 litre

Stir the ingredients (except MUG) in the water with gentle heating and then bring to the boil to dissolve completely. Allow the solution to cool. Add the MUG dissolved in 2 ml of N,N-dimethylformamide in a fume cupboard. Adjust the pH to 6.9 ± 0.2. Sterilise the solution by membrane filtration (0.2 µm nominal pore size). Dispense 100 µl of the sterile solution into each well contained in a 96-well micro-titre plate. Immediately dehydrate the medium using a tunnel drier or laminar air flow cabinet (F7.3). Dried micro-titre plates may be stored in the dark at 5 ± 3°C for up to one month in appropriate sterile packaging, prior to use. Prepared micro-titre plates are available commercially and should be used and stored according to manufacturer's instructions.

F8.2 *Special diluent*

Synthetic sea salt	22.5 g
Bromophenol blue (0.04 % m/v in 50 % aqueous ethanol)	10 ml
Water	1 litre

Dissolve the ingredients in the water and sterilise by autoclaving at 121°C for 15 - 20 minutes.

Special diluent may be stored at room temperature for up to two months.

Alternatively, a special diluent can be prepared as follows.

SOLUTION 1

Calcium chloride dihydrate	83.6 g
Potassium chloride	43.5 g
Strontium chloride hexahydrate	0.07 g
Water	1 litre

Heat the ingredients in the water to 50°C to dissolve.

SOLUTION 2

Sodium hydrogen carbonate	15.15 g
Sodium borate	3 g

Water	1 litre

Heat the ingredients in the water to 50°C to dissolve.

SOLUTION 3

Magnesium sulphate heptahydrate	190 g
Magnesium chloride	147 g
Water	1 litre

Heat the ingredients in the water to 50°C to dissolve.

Add 10 ml of solution 1, 10 ml of solution 2 and 20 ml of solution 3 to 950 ml of water. Add 14.9 g of sodium chloride, dissolve and adjust the pH to 7.5 $\pm$ 0.2. Add 10 ml of an 0.04 % m/v solution of bromophenol blue in 50 % aqueous ethanol and mix well. Dispense in suitable volumes in containers and sterilise by autoclaving at 121°C for 15 - 20 minutes.

F8.3 *Other media*

Standard and commercial formulations of other reagents used in this method include quarter strength Ringer's solution and maximum recovery diluent.

F9 Analytical procedure

F9.1 *Volumes of sample for inoculation*

Different dilutions of the same sample are inoculated into the wells of the micro-titre plate. The strength of the diluted samples will depend on the likely degree of faecal contamination of the sample.

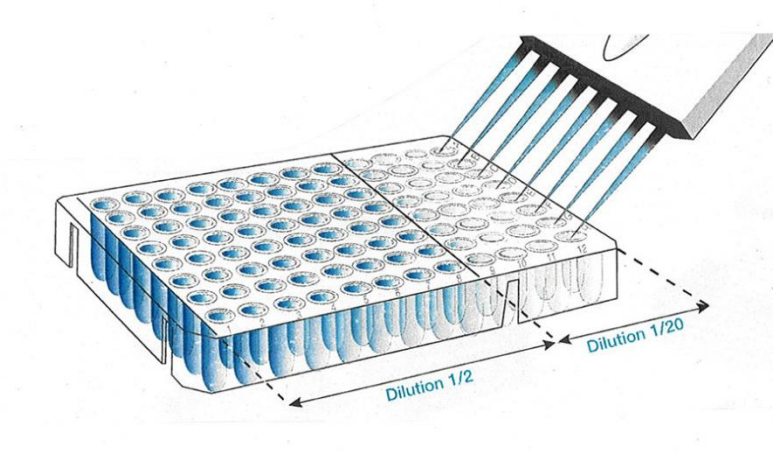
For saline waters, dilutions should be prepared using the special diluent (F8.2). For fresh surface waters and wastewaters, dilutions can be prepared in quarter strength Ringer's solution or maximum recovery diluent.

For example, for bathing waters and moderately polluted fresh surface waters prepare two dilutions of the sample, i.e.

1) dilute 20 ml of sample to 40 ml, i.e. a 1 in 2 dilution;
2) dilute 2 ml of sample to 40 ml, i.e. a 1 in 20 dilution.

Dispense 200 µl of the first diluted sample to each of the first 64 wells. See Figure F1. Identify each well. Dispense 200 µl of the second diluted sample to each of the remaining 32 wells. See Figure F1. Identify each well. This should enable counts to be obtained in the range 15 - 35000 *E. coli* per 100 ml of sample, i.e. a minimum of 15 up to 3.5 X 10^4.

Figure F1 Typical micro-titre plate with series of dilutions



For polluted fresh surface waters prepare 4 dilutions, i.e.

1) dilute 20 ml of sample to 40 ml, i.e. a 1 in 2 dilution.

From this solution prepare 3 further serial dilutions, namely

2) dilute 2 ml of solution 1) to 20 ml, i.e. a 1 in 20 dilution;
3) dilute 2 ml of solution 2) to 20 ml, i.e. a 1 in 200 dilution;
4) dilute 2 ml of solution 3) to 20 ml, i.e. a 1 in 2000 dilution.

Dispense 200 µl of each diluted sample to each of 24 wells. Identify each well. This should enable counts to be obtained in the range 40 - 3200000 *E. coli* per 100 ml of sample, i.e. a minimum of 40 up to 3.2 X 10^6.

For wastewater, prepare 6 dilutions, i.e.

1) dilute 20 ml of sample to 40 ml; i.e. a 1 in 2 dilution.

From this solution prepare 5 further serial dilutions, namely

2) dilute 2 ml of solution 1) to 20 ml, i.e. a 1 in 20 dilution;
3) dilute 2 ml of solution 2) to 20 ml, i.e. a 1 in 200 dilution;
4) dilute 2 ml of solution 3) to 20 ml, i.e. a 1 in 2000 dilution;
5) dilute 2 ml of solution 4) to 20 ml, i.e. a 1 in 20000 dilution;
6) dilute 2 ml of solution 5) to 20 ml, i.e. a 1 in 200000 dilution.

Dispense 200 µl of each diluted sample to each of 16 wells. Identify each well. This should enable counts to be obtained in the range 60 - 670000000 *E. coli* per 100 ml of sample, i.e. a minimum of 60 up to 6.7 X 10^8.

For all types of samples and in all cases, a sufficient number of diluted samples should be prepared and added to wells of the micro-titre plate. Following incubation, for at least one of the diluted samples within the series, some wells within the micro-titre plate should exhibit growth within the medium and some wells should show no growth within the medium.

F9.2 *Sample processing*

For each prepared diluted sample, label a sterile 90 mm diameter Petri dish. Decant an appropriate volume (typically 15 - 20 ml) of each diluted sample into their respective labelled Petri dishes.

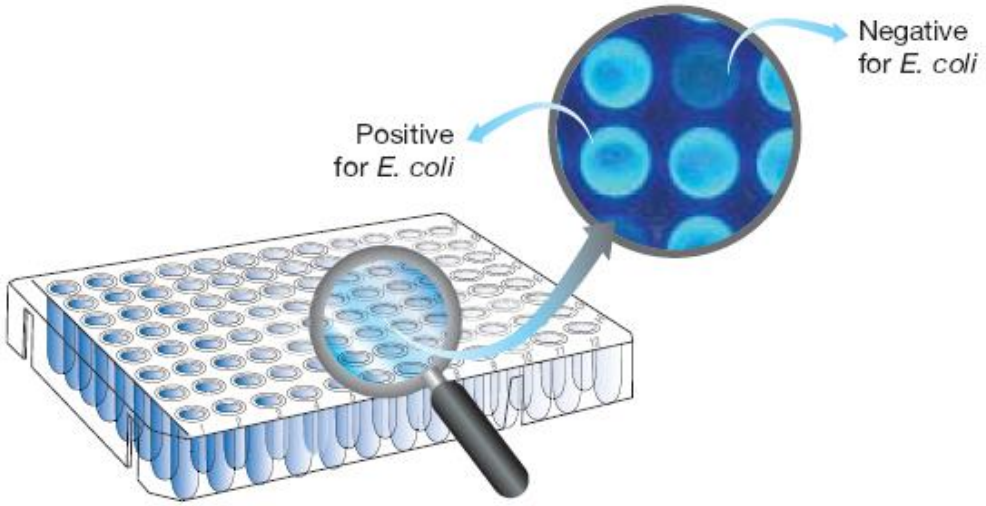
Use the multi-channel pipettor, set to deliver 200 µl from each channel, and eight sterile pipette tips to inoculate each diluted sample into the required number of wells in the micro-titre plate according to the type of sample and inoculation pattern described in section F9.1 using fresh pipettor tips for each dilution. After the wells of medium have been inoculated with the diluted samples, each well is covered with disposable sterile adhesive tape (F7.6).

Incubate the plates at 44.0 ± 0.5°C for not less than 36 hours and not more than 72 hours.

F9.3 *Reading of results*

After incubation, examine the wells under ultra-violet light. Wells showing a blue-white fluorescence indicate the presence of *E. coli* and are considered positive (see Figure F2). The number of positive wells for each dilution of the sample is recorded.

Figure F2 Example of MPN micro-titre plate with a defined substrate medium with fluorescing wells showing presence of *E. coli*



F9.4 *Confirmation tests*

This method is reported to be highly specific for *E. coli* (as demonstrated by blue-white fluorescence in the wells). Hence, confirmation tests are not usually required. Should there be any doubt as to the type of organism and response detected, then wells showing a positive response should be sub-cultured and confirmatory tests undertaken. See methods B or C.

F10 Calculations

F10.1 *Confirmed E. coli*

For a two-dilution series, the MPN of *E. coli* in the sample is determined using the tables in Appendix F1. Similar tables are available for 4- and 6-dilution series. A basic computer programme is available in Annexes A and B of ISO 9308-3:1998[(2)]. Statistical tables are supplied by the manufacturer[(5)] of the product and an example of how an MPN is derived is described below.

The number of wells exhibiting growth (i.e. positive wells) and number of wells inoculated in the micro-titre plate are used to generate a MPN.

Example of a two dilution series on a sea water sample:

Dilution	Number of positive wells	Number of wells inoculated
1/2	32	64
1/20	5	32

From these numbers and using the tables for two dilutions (see Appendix F1) then this corresponds to an MPN of 756 *E. coli* / 100 ml.

F11 Expression of results

Confirmed *E. coli* counts are expressed as MPN counts per volume of sample. For most samples, the volume is typically 100 ml.

F12 Quality assurance

The quality assurance in the preparation of the trays is described in ISO 9308-3:1998[(2)]. In addition, the standard also gives details of the preparation of calibration micro-titre plates. New batches of media should be tested by inoculating a suspension containing a known number of organisms of the appropriate reference bacteria (for example *E. coli* and *Enterobacter aerogenes*) and non-target bacteria (for example *Aeromonas hydrophila* or *Pseudomonas aeruginosa*). In addition, a suspension containing a known number of organisms of these reference organisms should be included with each batch of tests.

F13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2015) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. ISO 9308-3:1998 - Water Quality - detection and enumeration of *Escherichia coli* and coliform bacteria in surface and wastewater - Part 3 -Miniaturised method (most probable number) by inoculation in liquid medium, Geneva: International Organization for Standardization.

3. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

4. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

5. Bio-Rad Laboratories Limited, Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire, HP2 7DX.

Appendix F1 MPN tables based on the micro-titre technique

Based on a two-dilution series the following tables are used to determine the MPN of organisms in 100 ml of sample. With each MPN there would be associated lower and upper limit values. Similar tables are available for 4- and 6-dilution series.

Number of wells			Number of wells			Number of wells		
64	32		64	32		64	32	
Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml
0	1	15	9	0	144	16	0	272
0	2	30	9	1	160	16	1	289
0	3	45	9	2	176	16	2	307
0	4	60	9	3	192	16	3	324
			9	4	209	16	4	342
1	0	15	9	5	225	16	5	359
1	1	30	9	6	241	16	6	377
1	2	45				16	7	394
1	3	60	10	0	161			
1	4	75	10	1	177	17	0	292
1	5	90	10	2	194	17	1	309
			10	3	210	17	2	327
2	0	30	10	4	226	17	3	344
2	1	45	10	5	243	17	4	362
2	2	61	10	6	259	17	5	380
2	3	76				17	6	398
2	4	91	11	0	179	17	7	416
2	5	106	11	1	195	17	8	433
			11	2	212			
3	0	46	11	3	228	18	0	312
3	1	61	11	4	245	18	1	330
3	2	76	11	5	261	18	2	347
3	3	92	11	6	278	18	3	365
3	4	107	11	7	295	18	4	383
3	5	122				18	5	401
			12	0	197	18	6	419
4	0	61	12	1	213	18	7	437
4	1	77	12	2	230	18	8	455
4	2	92	12	3	247			
4	3	108	12	4	263	19	0	332
4	4	123	12	5	280	19	1	350
4	5	139	12	6	297	19	2	368
			12	7	314	19	3	386
5	0	77				19	4	405
5	1	93	13	0	215	19	5	423
5	2	108	13	1	232	19	6	441
5	3	124	13	2	249	19	7	459
5	4	140	13	3	266	19	8	478
5	5	155	13	4	282			
5	6	171	13	5	299	20	0	353
			13	6	316	20	1	371
6	0	94	13	7	333	20	2	390
6	1	109				20	3	408
6	2	125	14	0	234	20	4	426
6	3	141	14	1	251	20	5	445
6	4	156	14	2	268	20	6	463
6	5	172	14	3	285	20	7	482
6	6	188	14	4	302	20	8	500
			14	5	319			
7	0	110	14	6	336	21	0	375
7	1	126	14	7	353	21	1	393
7	2	142				21	2	412
7	3	158	15	0	253	21	3	430

Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml
64	32		64	32		64	32	
7	4	174	15	1	270	21	4	449
7	5	189	15	2	287	21	5	467
7	6	205	15	3	304	21	6	486
			15	4	322	21	7	505
8	0	127	15	5	339	21	8	524
8	1	143	15	6	356			
8	2	159	15	7	374			
8	3	175						
8	4	191						
8	5	207						
8	6	223						
22	0	397	28	0	539	34	0	705
22	1	415	28	1	559	34	1	728
22	2	434	28	2	580	34	2	750
22	3	453	28	3	600	34	3	773
22	4	472	28	4	621	34	4	796
22	5	490	28	5	641	34	5	818
22	6	509	28	6	662	34	6	841
22	7	528	28	7	683	34	7	865
22	8	547	28	8	704	34	8	888
			28	9	725	34	9	911
23	0	419	28	10	746	34	10	935
23	1	438				34	11	958
23	2	457	29	0	565			
23	3	476	29	1	585	35	0	736
23	4	495	29	2	606	35	1	759
23	5	514	29	3	627	35	2	782
23	6	533	29	4	648	35	3	805
23	7	553	29	5	669	35	4	828
23	8	572	29	6	690	35	5	851
23	9	591	29	7	711	35	6	875
			29	8	732	35	7	898
24	0	442	29	9	753	35	8	922
24	1	461	29	10	775	35	9	946
24	2	480				35	10	970
24	3	500	30	0	591	35	11	994
24	4	519	30	1	612			
24	5	538	30	2	633	36	0	767
24	6	558	30	3	654	36	1	791
24	7	577	30	4	676	36	2	814
24	8	597	30	5	697	36	3	838
24	9	617	30	6	718	36	4	861
			30	7	740	36	5	885
25	0	465	30	8	761	36	6	909
25	1	485	30	9	783	36	7	933
25	2	504	30	10	805	36	8	957
25	3	524				36	9	982
25	4	543	31	0	619	36	10	1006
25	5	563	31	1	640	36	11	1031
25	6	583	31	2	661			
25	7	603	31	3	683	37	0	800
25	8	623	31	4	704	37	1	824
25	9	643	31	5	726	37	2	848
			31	6	748	37	3	872
26	0	489	31	7	770	37	4	896
26	1	509	31	8	792	37	5	920
26	2	529	31	9	814	37	6	945
26	3	549	31	10	836	37	7	969
26	4	568				37	8	994
26	5	588	32	0	647	37	9	1019
26	6	609	32	1	668	37	10	1044

Number of wells			Number of wells			Number of wells		
64	32		64	32		64	32	
Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml
26	7	629	32	2	690	37	11	1069
26	8	649	32	3	712			
26	9	669	32	4	734	38	0	834
			32	5	756	38	1	858
27	0	514	32	6	778	38	2	882
27	1	534	32	7	800	38	3	907
27	2	554	32	8	823	38	4	931
27	3	574	32	9	845	38	5	956
27	4	594	32	10	868	38	6	981
27	5	615				38	7	1006
27	6	635	33	0	675	38	8	1032
27	7	655	33	1	697	38	9	1057
27	8	676	33	2	720	38	10	1083
27	9	696	33	3	742	38	11	1109
			33	4	764	38	12	1135
			33	5	787			
			33	6	809			
			33	7	832			
			33	8	855			
			33	9	878			
			33	10	901			
39	0	868	44	0	1064	48	0	1253
39	1	893	44	1	1092	48	1	1285
39	2	918	44	2	1120	48	2	1317
39	3	943	44	3	1148	48	3	1349
39	4	969	44	4	1177	48	4	1382
39	5	994	44	5	1206	48	5	1415
39	6	1020	44	6	1235	48	6	1448
39	7	1045	44	7	1265	48	7	1482
39	8	1071	44	8	1294	48	8	1516
39	9	1097	44	9	1324	48	9	1550
39	10	1123	44	10	1354	48	10	1585
39	11	1150	44	11	1385	48	11	1620
39	12	1176	44	12	1416	48	12	1655
			44	13	1446	48	13	1691
40	0	904				48	14	1727
40	1	930	45	0	1108			
40	2	955	45	1	1136	49	0	1306
40	3	981	45	2	1166	49	1	1339
40	4	1007	45	3	1195	49	2	1372
40	5	1033	45	4	1225	49	3	1406
40	6	1059	45	5	1254	49	4	1440
40	7	1086	45	6	1285	49	5	1474
40	8	1112	45	7	1315	49	6	1509
40	9	1139	45	8	1345	49	7	1544
40	10	1166	45	9	1376	49	8	1579
40	11	1193	45	10	1407	49	9	1615
40	12	1220	45	11	1439	49	10	1651
			45	12	1471	49	11	1688
41	0	942	45	13	1503	49	12	1725
41	1	968				49	13	1762
41	2	994	46	0	1154	49	14	1800
41	3	1020	46	1	1183	49	15	1838
41	4	1047	46	2	1213			
41	5	1074	46	3	1244	50	0	1363
41	6	1100	46	4	1274	50	1	1397
41	7	1128	46	5	1305	50	2	1431
41	8	1155	46	6	1336	50	3	1466
41	9	1182	46	7	1368	50	4	1502
41	10	1210	46	8	1399	50	5	1537
41	11	1238	46	9	1431	50	6	1573

Number of wells			Number of wells			Number of wells		
64	32		64	32		64	32	
Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml
41	11	1238	46	9	1431	50	6	1573
41	12	1266	46	10	1463	50	7	1610
			46	11	1496	50	8	1647
42	0	981	46	12	1529	50	9	1684
42	1	1007	46	13	1562	50	10	1722
42	2	1034	46	14	1595	50	11	1760
42	3	1061				50	12	1799
42	4	1089	47	0	1202	50	13	1838
42	5	1116	47	1	1233	50	14	1878
42	6	1143	47	2	1264	50	15	1918
42	7	1171	47	3	1295			
42	8	1199	47	4	1327	51	0	1423
42	9	1227	47	5	1358	51	1	1458
42	10	1256	47	6	1391	51	2	1494
42	11	1284	47	7	1423	51	3	1531
42	12	1313	47	8	1456	51	4	1567
			47	9	1489	51	5	1605
43	0	1021	47	10	1522	51	6	1642
43	1	1049	47	11	1556	51	7	1681
43	2	1076	47	12	1590	51	8	1719
43	3	1104	47	13	1625	51	9	1758
43	4	1132	47	14	1659	51	10	1798
43	5	1160				51	11	1838
43	6	1188				51	12	1879
43	7	1217				51	13	1920
43	8	1246				51	14	1962
43	9	1275				51	15	2004
43	10	1304						
43	11	1333						
43	12	1363						
43	13	1393						
52	0	1486	55	0	1706	58	0	1988
52	1	1523	55	1	1749	58	1	2041
52	2	1561	55	2	1793	58	2	2095
52	3	1599	55	3	1838	58	3	2150
52	4	1638	55	4	1883	58	4	2206
52	5	1677	55	5	1929	58	5	2263
52	6	1716	55	6	1976	58	6	2322
52	7	1756	55	7	2023	58	7	2383
52	8	1797	55	8	2072	58	8	2444
52	9	1838	55	9	2121	58	9	2508
52	10	1880	55	10	2172	58	10	2573
52	11	1922	55	11	2223	58	11	2640
52	12	1965	55	12	2275	58	12	2708
52	13	2009	55	13	2328	58	13	2779
52	14	2053	55	14	2382	58	14	2851
52	15	2098	55	15	2438	58	15	2926
52	16	2143	55	16	2494	58	16	3002
			55	17	2551	58	17	3082
53	0	1554				58	18	3163
53	1	1593	56	0	1792	58	19	3247
53	2	1633	56	1	1838			
53	3	1673	56	2	1884	59	0	2104
53	4	1713	56	3	1931	59	1	2161
53	5	1754	56	4	1980	59	2	2219
53	6	1796	56	5	2029	59	3	2279
53	7	1838	56	6	2079	59	4	2341
53	8	1881	56	7	2130	59	5	2404
53	9	1924	56	8	2182	59	6	2469
53	10	1969	56	9	2235	59	7	2536

Number of wells			Number of wells			Number of wells		
64	32		64	32		64	32	
Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml
53	11	2013	56	10	2290	59	8	2604
53	12	2059	56	11	2345	59	9	2675
53	13	2105	56	12	2401	59	10	2748
53	14	2152	56	13	2459	59	11	2823
53	15	2200	56	14	2518	59	12	2900
53	16	2249	56	15	2578	59	13	2980
			56	16	2640	59	14	3063
54	0	1627	56	17	2703	59	15	3148
54	1	1668	56	18	2767	59	16	3237
54	2	1710				59	17	3328
54	3	1752	57	0	1885	59	18	3423
54	4	1794	57	1	1934	59	19	3521
54	5	1838	57	2	1984	59	20	3623
54	6	1882	57	3	2035			
54	7	1927	57	4	2087	60	0	2234
54	8	1972	57	5	2140	60	1	2296
54	9	2018	57	6	2194	60	2	2361
54	10	2065	57	7	2249	60	3	2427
54	11	2113	57	8	2305	60	4	2496
54	12	2162	57	9	2363	60	5	2567
54	13	2211	57	10	2422	60	6	2639
54	14	2261	57	11	2482	60	7	2715
54	15	2312	57	12	2544	60	8	2792
54	16	2365	57	13	2608	60	9	2873
54	17	2418	57	14	2672	60	10	2956
			57	15	2739	60	11	3042
			57	16	2807	60	12	3132
			57	17	2877	60	13	3225
			57	18	2949	60	14	3322
						60	15	3422
						60	16	3527
						60	17	3636
						60	18	3750
						60	19	3870
						60	20	3994
						60	21	4124
61	0	2383	63	0	2773			
61	1	2453	63	1	2867			
61	2	2526	63	2	2966			
61	3	2601	63	3	3071			
61	4	2678	63	4	3181			
61	5	2759	63	5	3297			
61	6	2843	63	6	3421			
61	7	2929	63	7	3552			
61	8	3020	63	8	3693			
61	9	3114	63	9	3843			
61	10	3212	63	10	4005			
61	11	3315	63	11	4179			
61	12	3422	63	12	4368			
61	13	3534	63	13	4573			
61	14	3652	63	14	4797			
61	15	3776	63	15	5039			
61	16	3906	63	16	5306			
61	17	4044	63	17	5598			
61	18	4188	63	18	5918			
61	19	4341	63	19	6267			
61	20	4503	63	20	6648			
61	21	4674	63	21	7063			

Number of wells			Number of wells		
64	32		64	32	
Number of wells exhibiting growth		MPN per 100 ml	Number of wells exhibiting growth		MPN per 100 ml
61	22	4854	63	22	7511
			63	23	7994
62	0	2559	63	24	8513
62	1	2639	63	25	9070
62	2	2722	63	26	9666
62	3	2809	63	27	10305
62	4	2900	63	28	10991
62	5	2994	63	29	11730
62	6	3093			
62	7	3197	64	0	3045
62	8	3306	64	1	3162
62	9	3421	64	2	3286
62	10	3543	64	3	3420
62	11	3671	64	4	3564
62	12	3806	64	5	3720
62	13	3951	64	6	3889
62	14	4104	64	7	4074
62	15	4267	64	8	4277
62	16	4442	64	9	4502
62	17	4628	64	10	4753
62	18	4828	64	11	5035
62	19	5043	64	12	5352
62	20	5273	64	13	5712
62	21	5520	64	14	6119
62	22	5784	64	15	6581
62	23	6068	64	16	7101
62	24	6371	64	17	7683
62	25	6694	64	18	8329
			64	19	9043
			64	20	9826
			64	21	10687
			64	22	11636
			64	23	12687
			64	24	13864
			64	25	15199
			64	26	16740
			64	27	18563
			64	28	20795
			64	29	23671
			64	30	27726
			64	31	34659

G The detection of *Escherichia coli* O157:H7 by selective enrichment and immuno-magnetic separation

G1 Introduction

The recovery of *Escherichia coli* O157:H7 (*E coli* O157:H7) from environmental samples is often difficult because of the altered physiological state that bacteria sometimes develop in order to survive hostile environments. Infections involving *E. coli* O157:H7 have occasionally been implicated with contaminated water, but food-borne infections are more common. The significance of *E. coli* O157:H7 in wastewater and surface waters is described elsewhere[1] in this series.

E. coli O157:H7 is a recognised cause of haemorrhagic colitis, an illness characterised by bloody diarrhoea and severe abdominal pain but little or no fever. It is also one of the causes of haemolytic uraemic syndrome. Outbreaks involving *E. coli* O157:H7 have been associated with the consumption of food or contaminated water, and also contact with recreational water and by person-to-person contact. Symptoms can persist for up to 7 days. Strains of *E. coli* O157:H7 produce a toxin which is similar to that produced by *Shigella dysenteriae* Type 1 which is cytotoxic to Vero cells in cell culture.

This method may be subject to cross-contamination and aerosol production during the immuno-magnetic separation (IMS) procedure. Any laboratory that does not have access to automated IMS beads retrieval, for example a “Dynal Beadretriever™” or equivalent equipment, should consider whether to engage in the examination for *E. coli* O157:H7.

E. coli O157:H7 has been classified as a Hazard Group 3 organism[2]. Presumptive isolates and waters for which epidemiology strongly suggests that *E. coli* O157 may be present should be handled under containment level 3 conditions (as for *Salmonella* Typhi).

The automated IMS procedure should be performed in a class 1 safety cabinet. Access to the room during this procedure should be restricted. Presumptive colonies on agar plates should be transferred to category 3 facilities. All confirmatory tests should be performed in a containment level 3 laboratory.

G2 Scope

The method is suitable for the examination of surface and ground waters, saline waters, manmade recreational waters such as swimming pools, spa pools and hydrotherapy pools, and untreated and treated wastewaters. Water samples with higher turbidities may be analysed by adding the sample to an equal volume of the double-strength medium described in this method for the presence-absence test or using an appropriate most probable number technique.

Users wishing to employ this method should verify its performance under their own laboratory conditions[3].

G3 Definitions

In the context of this method, *E. coli* O157:H7 are strains of *E. coli* which do not ferment sorbitol, produce colourless to pale orange colonies on cefixime tellurite

sorbitol MacConkey agar, and which subsequently confirm by biochemical and serological tests.

G4 Principle

Organisms are isolated by membrane filtration or entrapment with filter-aid, then selective enrichment followed by an automated immuno-magnetic separation (AIMS), and inoculation onto a selective agar medium containing sorbitol as a fermentable carbohydrate and neutral red as an indicator of acidity. Alternatively, for more turbid samples, they may be analysed by adding an equal volume to double-strength medium as a presence absence test or as an MPN test. Isolation of colonies is followed by selection of typical non-sorbitol-fermenting colonies for identification by biochemical and serological tests.

G5 Limitations

This method does not identify atypical sorbitol-fermenting strains of *E. coli* O157:H7 nor other serotypes of *E. coli* that produce verocytotoxins.

This method is suitable for most types of aqueous samples except those with high turbidities, which tend to block the membrane filter. This will limit the volume of sample that can be filtered. In these instances, the use of several membrane filters or filter aid may be more appropriate. Alternatively samples can be inoculated directly into double or single strength medium.

When low numbers of *E. coli* O157:H7 are present, detection is improved when larger volumes of sample are examined. However, the presence of high numbers of competing organisms may inhibit the growth or detection of *E. coli* O157:H7.

G6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[4] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[3] in this series.

Strains of *E. coli* O157:H7 which produce verocytotoxin have been reclassified from “Hazard Group 2” to “Hazard Group 3”[2]. However, where samples are not expected to contain *E. coli* O157:H7, routine examination may be undertaken in “Hazard Group 2” containment facilities. Where substantial sub-culture work is required, this should be undertaken in “Hazard Group 3” containment facilities. In addition, those strains used as positive control strains should not produce verocytotoxin. Suitable strains are available commercially (for example, National Collection of Type Cultures 12900). Caution should be exercised in the disposal of contaminated materials, especially those containing *E. coli* O157:H7.

Disposable gloves and safety glasses should be worn throughout the IMS procedure.

G7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [3] in this series. Principally, appropriate membrane filtration apparatus, equipment for IMS and fan-assisted incubators are required. Other items include:

G7.1 Sterile sample containers of appropriate volume, made of suitable material. For swimming pools, spa and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l, for example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent.

G7.2 Incubators (or water baths) capable of maintaining temperatures of 37.0 ± 1°C and 42.0 ± 1°C.

G7.3 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.

G7.4 Sterile membrane filters, for example white, 47 mm diameter, cellulose-based, 0.45 µm nominal pore size.

G7.5 Smooth-tipped forceps.

G7.6 Automated IMS equipment, for example BeadRetriever™ (Dynal).

G7.7 Pipettors capable of delivering volumes of 10 and 1 ml and sterile pipette tips with barriers to prevent contamination of the pipettor barrel.

G7.8 Variable pipettors capable of delivering volumes between 10 and 100 µl and sterile pipette tips with barriers to prevent contamination of the pipettor barrel.

G7.9 Plastic sterile tube strips and sterile tube tip combs.

G8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in this method[3]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated, chemical constituents should be added as the anhydrous salts. If the pH of the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator, they should be allowed to reach room temperature before use.

G8.1 *Modified tryptone soya broth*[5]

Tryptone soya broth	30 g
Bile salts number 3	1.5 g
Dipotassium hydrogen phosphate	1.5 g
Novobiocin	20 mg
Water	1 litre

Dissolve the ingredients in the water and adjust the pH to 7.4 ± 0.2. Dispense the resulting solution in 90 ml volumes into suitable screw-capped containers and sterilise by autoclaving at 121°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. The sterilised medium may be stored at room temperature in the dark for up to one month, if protected from dehydration.

Double-strength modified tryptone soya broth can be prepared using double the amounts of ingredients in the 1000 ml of water.

G8.2 *Buffered peptone water*[6]

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	3.5 g
Potassium dihydrogen phosphate	1.5 g
Water	1 litre

Dissolve the ingredients in the water. Dispense the resulting solution in 90 ml volumes into suitable screw-capped tubes or bottles and sterilise by autoclaving at 121°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.2 ± 0.2. Autoclaved media may be stored in the dark at room temperature for up to one month, if protected from dehydration.

Double-strength buffered peptone water can be prepared using double the amounts of ingredients in the 1000 ml of water.

G8.3 *Cefixime tellurite sorbitol MacConkey agar*[7]

Peptone	20.0 g
Sorbitol	10.0 g
Bile salts number 3	1.5 g
Sodium chloride	5.0 g
Neutral red	30.0 mg
Crystal violet	1 mg
Potassium tellurite	2.5 mg
Cefixime	0.05 mg
Agar	15.0 g
Water	1 litre

Dissolve the ingredients, except cefixime and potassium tellurite, in the water. To achieve this, it will be necessary to heat to boiling. Dispense in appropriate volumes into suitable screw-capped bottles and sterilise by autoclaving at 121°C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.1 ± 0.2. Allow the medium to cool. This basal medium can be stored in the dark at room temperature, protected from dehydration, and used within one month. Prior to use, melt the basal medium if taken from store. Allow the molten medium to cool to approximately 50°C and add the following selective supplements which should be filter-sterilised.

(i) Cefixime solution: Dissolve 500 mg of cefixime in 100 ml of ethanol. This may be stored at between 5 ± 3°C and used within one month. Add 1 ml of this solution to 100 ml of ethanol and add 1 ml of the resulting solution to1 litre of the basal medium to give a final concentration of 0.05 mg/l.

(ii) Potassium tellurite solution: Dissolve 25 mg of potassium tellurite in 10 ml of water. The filter-sterilised solution may be stored at approximately -20°C and used

within one month. Add 1 ml of this solution to 1 litre of the basal medium to give a final concentration of 2.5 mg/l.

Mix the complete medium thoroughly and pour into sterile Petri dishes and allow the agar to solidify. Petri dishes may be stored at between 5 ± 3°C, protected against dehydration, and used within one month. Dishes should be dried in a suitable oven at 45 - 50°C for 30 minutes before use.

G8.4 *Modified phosphate buffered solution* (*PBST)*

Sodium chloride	8 g
Potassium chloride	20 mg
Disodium hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.2 g
Polyoxyethylene-sorbitan monolaurate (for example, Tween 20)	0.5 ml
Water	1 litre

Dissolve the ingredients in the water and check that the pH is 7.4 ± 0.2. Sterilise the resulting solution by autoclaving at 121°C for 15 minutes. After autoclaving, the pH of the solution should be checked to confirm a pH of 7.4 ± 0.2. Allow the solution to cool. This solution can be stored in the dark at room temperature for up to one month.

G8.5 *Filter-aid*[8]

Diatomaceous earth	1 g (approximately)
Water	15 ml

Weigh out appropriate amounts of filter-aid into suitable bottles and add the water. Sterilise by autoclaving at 121°C for 15 minutes. Filter-aid may be stored in the dark at room temperature for up to 12 months.

G8.6 M*agnetic beads*[9]

Para-magnetic beads coated with antibodies to *E. coli* O157:H7 antigen (for example, Dynabeads or equivalent)

G8.7 *Other media*

Standard and commercial formulations of other media and reagents used in this method include quarter strength Ringer's solution, maximum recovery diluent, nutrient agar (NA), MacConkey agar (MA) and *E. coli* antisera and latex agglutination kits.

G9 Analytical procedure

G9.1 *Sample preparation*

G9.1.1 *Surface waters and sea water*

Due to the likelihood that, if present, the numbers of *E. coli* O157:H7 in some surface

waters and sea water are likely to be low, for presence absence, a sample volume of at least 1000 ml should be examined.

For the membrane filtration multiple-tube technique, typically an 11-tube series can be used, i.e. the membrane filtration of 1 x 500 ml, 5 x 100 ml and 5 x 10 ml of sample. Alternatively, volumes of 500 ml and 100 ml can be filtered and the 10 ml volumes can be added directly to 10 ml volumes (or an equal volume) of double-strength modified tryptone soya broth or buffered peptone water. For a different series, smaller volumes of sample, for example 1 ml may be appropriate and these can be added directly to 9 ml of single strength modified tryptone soya broth or buffered peptone water. Turbid waters, unsuitable for membrane filtration, may be filtered using filter aid.

G9.1.2 T*reated wastewater*

Treated wastewater may be analysed as described in G9.1.1 although several membranes may be required for presence absence determinations. A sample volume of at least 100 ml may need to be examined.

The volumes may be reduced and volumes of 1 x 50 ml, 5 x 10 ml and 5 x 1 ml be used. The 50 ml and 10 ml volumes can be membrane filtered or added to equal volumes of double-strength modified tryptone soya broth or buffered peptone water. To represent smaller volumes of sample, a 1:10 dilution of the sample, for example 1.0 ml of sample diluted with quarter strength Ringer's solution or maximum recovery diluent, may be appropriate and 1 ml of these dilutions can be added directly to 9 ml of single strength modified tryptone soya broth or buffered peptone water.

G9.1.3 *Untreated wastewater*

For presence absence determinations, 100 ml of untreated wastewater may be required, as it may not be possible (owing to turbidity) to process larger volumes by membrane filtration. For an 11-tube most probable number series, the volumes of untreated wastewater are usually 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 50 ml and 10 ml volumes can be filtered or added to an equal volume of double-strength modified tryptone soya broth or buffered peptone water. The 1 ml volumes can be added to 9 ml of single-strength modified tryptone soya broth or buffered peptone water. To represent smaller volumes, for example 0.1 ml or 0.01 ml volumes of sample, a 1:10 and 1:100 dilution of the sample may be appropriate. The diluted samples (1 ml volumes) can be added to 9 ml of single-strength modified tryptone soya broth or buffered peptone water.

G9.1.4 *Sediment and sand*

Solid material can be dispensed as a single weight for presence absence by weighing, for example 10 g into an appropriate volume (typically 100 ml) of single strength modified tryptone soya broth or buffered peptone water. For the multiple-tube technique, weigh 1 x 50 g, 5 x 10 g and 5 x 1 g into appropriate volumes (typically 450 ml, 5 x 100 ml and 5 x 10 ml) of single strength modified tryptone soya broth or buffered peptone water. For smaller quantities, for example 100 mg, these can be added directly to 10 ml of single strength modified tryptone soya broth or buffered peptone water.

G9.2 Sample processing

G9.2.1 *Membrane presence absence or filtration-multiple tube technique*

Appropriate volumes of sample are filtered through membrane filters. Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter onto the porous disc of the filter base. If a gridded membrane filter is used, place grid-side upwards. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the water slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and carefully transfer the membrane filter to a tube or bottle containing, typically, 10 - 15 ml of single strength modified tryptone soya broth for the presence absence test or buffered peptone water for the multiple tube enumeration test, ensuring that the membrane filter is fully submerged. Record the volume filtered. Other volumes of sample should be similarly treated until all the filters are transferred to the corresponding tubes or bottles of single strength modified tryptone soya broth or buffered peptone water. The largest single volume of sample may require more than one membrane filter and, if so, all filters used for this volume should be transferred to the bottle or tube of single strength modified tryptone soya broth or buffered peptone water. Ensure that all membranes are fully submerged.

The funnel can be placed in a boiling water bath if it is to be re-used. Alternatively, pre-sterilised filter funnels can be used for each sample. If different volumes of the same sample are to be examined, the funnel may be re-used without boiling provided that the smallest volume of sample is filtered first. For different samples, a fresh pre-sterilised funnel should be taken or remove a funnel from the boiling water bath. Allow the funnel to cool and repeat the filtration process. If funnels are to be re-used, then after filtration of each sample, disinfect the funnel by immersing it in boiling distilled water for at least one minute. During the filtration of a series of samples, the filter base need not be sterilised unless it becomes contaminated or a membrane filter becomes damaged. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When disinfected funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible, and no longer than 2 hours.

G9.2.2 *Filter-aid*

The usual membrane filtration apparatus may be used but with a sterile absorbent pad in place of a membrane filter to act as a supporting base for the filter-aid. An aliquot of filter-aid (typically, 15 ml) should be filtered to form an initial layer on the absorbent pad. A second aliquot (typically, 15 ml) of filter-aid should be mixed with the volume of sample and then filtered. For turbid or dirty waters, additional aliquots of filter-aid may be required. When filtration is complete, remove the funnel carefully and transfer the absorbent pad and filter-aid to single strength modified tryptone soya broth or buffered peptone water. With the same medium, rinse any filter-aid adhering to the funnel into the culture vessel and make up to 100 ml.

For presence absence place the culture vessel into an incubator and incubate. For a most probable number test, re-suspend the filter aid in the single strength modified tryptone soya broth or buffered peptone water and dispense in a multiple-tube most probable number series using 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 1 ml volumes are inoculated into 9 ml of fresh sterile single strength modified tryptone soya broth or buffered peptone water.

G9.2.3 *Direct inoculation*

Where the numbers of *E. coli* O157:H7 in the sample are likely to be high, smaller volumes of sample, for example 50 ml and 10 ml can be inoculated directly into an equal volume of double strength modified tryptone soya broth or buffered peptone water. Volumes of 1 ml and subsequent dilutions of the sample can be inoculated directly into 9 ml of single strength modified tryptone soya broth or buffered peptone water.

G9.2.4 *Sediment and sand*

Samples of sediment and sand may be analysed by weighing appropriate amounts, for example, a single aliquot of 10 g for presence absence or 1 x 5 g, into 90 ml and 5 x 1 g and 5 x 0.1 g into 9 ml of single strength modified tryptone soya broth or buffered peptone water for a most probable number series. Larger weights of sample should be weighed into appropriately larger volumes of single strength modified tryptone soya broth or buffered peptone water.

G9.2.5 *Enrichment, automated immuno-magnetic separation (AIMS) and sub-culture to selective agar*

Care must be taken to avoid cross-contamination between enrichment samples.

Thoroughly mix the modified tryptone soya broth or buffered peptone water from sections G9.2.1 or G9.2.4. Incubate the modified tryptone soya broth at 42°C for 21 hours and the buffered peptone water at 37°C for 21 hours[10, 11]. Enrichment broths should be subjected to AIMS, firstly, after incubation for 6 - 7 hours, and then again at 21 hours. The AIMS should be performed according to the manufacturer's instructions.

Switch on the BeadRetriever™ and select the appropriate programme for *E. coli* O157:H7. Insert the sample tip comb into the BeadRetriever™ by holding the tip of the comb between the thumb and forefinger and sliding the tip comb into the tip comb slots as far as possible. Failure to do this will cause the machine to malfunction. One tip comb with five tips is used for processing five or less samples at any one time. One tube strip containing five tubes is placed into the tube strip tray and each tube strip is labelled with the appropriate sample number. A separate strip should be prepared for each sample to be tested by AIMS. Tubes 1 and 2 will contain sample and beads, tubes 3 and 4 are to rinse the beads and the beads are subsequently concentrated in tube 5.

Thoroughly mix the antibody-coated para-magnetic beads and transfer 10 µl of the suspension into sample tubes 1 and 2 of each tube strip. Pipette 0.5 ml of PBST (see G8.4) into sample tubes 1 and 2, 1 ml of PBST into sample tubes 3 and 4 and 100 µl of PBST into tube 5 of the tube strip. The prepared tray is designated Tray A.

Place a second sample tray without tubes in a class ll safety cabinet or on the bench

at least one metre away from Tray A. This is designated Tray B. Transfer sample tubes 1 and 2 for inoculation from Tray A to Tray B. Transfer 0.5 ml of enrichment broth into sample tubes 1 and 2 using a 1 ml sterile disposable pipette tip. The strip is returned to Tray A and fresh strips are inoculated with additional samples.

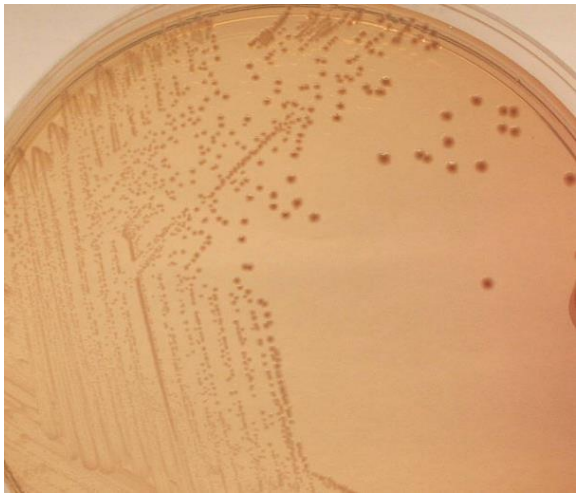
The prepared strip tray is slid into the machine, the top and front doors are closed and the machine started. Each run takes 23 minutes. Remove the sample tube tray from the BeadRetriever™. Tube 5 will contain 100 µl of bead/bacteria complex. Re-suspend the beads and inoculate them all onto cefixime tellurite sorbitol MacConkey agar, following manufacturer's instructions where provided, and incubate at 37°C for 21 hours.

G9.3 *Reading of results*

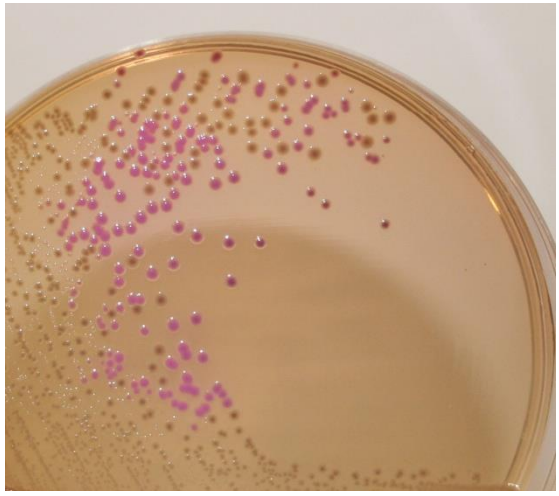
After incubation, examine the cefixime tellurite sorbitol MacConkey agar Petri dishes for typical non-sorbitol-fermenting colonies that are smooth and circular, 1 - 3 mm in diameter and colourless to pale orange in colour, usually with dark centres (see Figure G1). Strains of *E. coli* which ferment sorbitol are pink in colour (see Figure G1).

Figure G1 Colonies on cefixime tellurite sorbitol MacConkey agar

E. coli O157:H7



Pink colonies of sorbitol-fermenting *E. coli* and pale orange non-sorbitol-fermenting colonies of *E. coli* O157:H7



G9.4 *Confirmation tests*

Inoculate typical colonies onto nutrient agar or similar non-selective agar (and MacConkey agar if isolate purity needs to be checked) and incubate at 37°C for 21 hours. Isolates can then be subjected to serological identification using commercially available antisera or latex agglutination kits. Examine the slides for evidence of agglutination and carry out the tests with appropriate positive (non-verocytotoxin-producing strain of *E. coli* O157:H7) and negative (non-O157 strain of *E. coli*) controls, see Figure G2. Some isolates may require further identification by biochemical testing as some non-sorbitol-fermenting coliform bacteria (for example, *Escherichia hermanii*) can cross-react in the latex agglutination test. While chromogenic media can be used to demonstrate the lack of β-glucuronidase, some strains of *E. coli* O157:H7 may produce atypical biochemical profiles and results

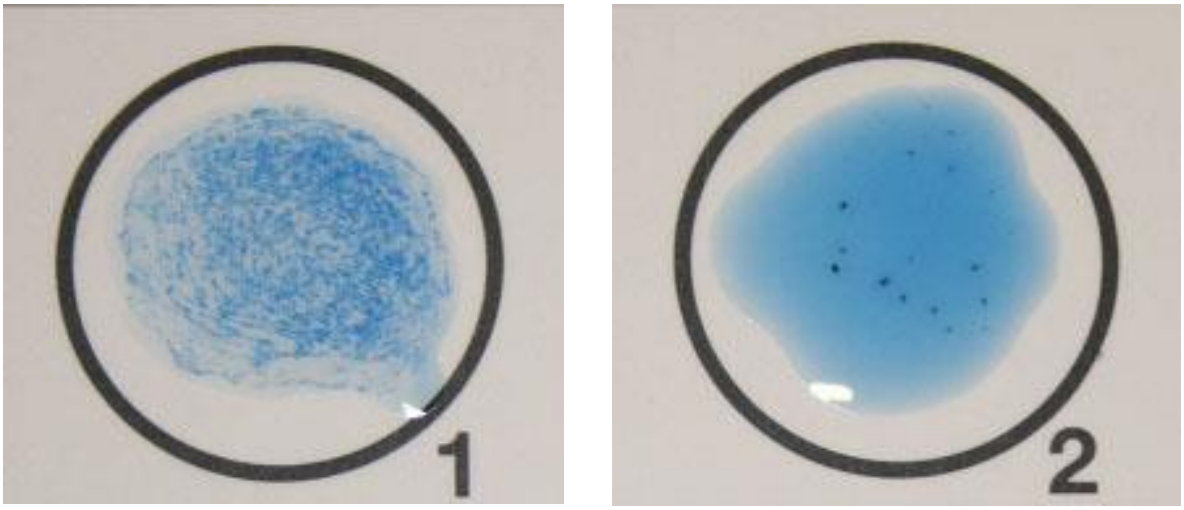
should be interpreted with caution.

If suspect colonies are isolated, the relevant authorities should be informed and arrangements made for further biochemical and toxin testing made without delay.

Figure G2 Agglutination test

reaction with *E. coli* O157:H7
regard as positive

reaction with another serotype of *E. coli*
regard as negative



G10 Calculations

The test indicates the presence or absence of *E. coli* O157:H7.

G11 Expression of results

E. coli O157:H7 are reported as being detected or not detected in the volume of sample examined.

G12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, non-verocytotoxin producing strain of *E. coli* O157:H7 NCTC 12900) and non-target bacteria (for example, other *E. coli*). Further details are given elsewhere[3] in this series.

G13 References

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Appendix 1 Evaluation of the storage time for prepared plates of membrane lactose glucuronide agar (MLGA)

1 Introduction

Membrane lactose glucuronide agar (MLGA) is widely used in the UK for the enumeration of *E. coli* and coliform bacteria from drinking water. In the original description of the medium it was stated that poured plates could be stored at 5 °C for up to one month (Sartory & Howard, 1992). Subsequent studies by Walter *et al.* (1994) indicated poorer recovery of *E. coli* and coliforms enumerated on MLGA stored aerobically for 14 days compared to recoveries on freshly prepared medium. They assumed that this may not have been noticed in the Sartory & Howard (1992) study as the number of samples analysed was very large and that it was unlikely that the MLGA used had been stored for an extended period. Further studies (Sartory, unpublished observations), however, also indicated poorer recovery with medium stored for one month compared to MLGA that was stored for less than seven days. As a result the method utilising MLGA as described in *The Microbiology of Drinking Water Part 4* (SCA, 2002) and its subsequent editions limit the storage time for MLGA prior to use to one week.

Over the intervening 13 years anecdotal evidence has indicated that a longer acceptable storage time may be appropriate. Additionally, one major manufacturer of MLGA has stated that the medium can be stored for up to six weeks. Consequently the SCA Working Group 2 decided to conduct a multi-laboratory study on the impact of storage on the performance of MLGA on the recovery of *E. coli* and coliform bacteria from water.

2 Study design

The aim of the multi-laboratory study was to investigate the shelf life of poured plates of MLGA in terms of:

its ability to isolate coliforms and *E. coli* from water at 37 °C
its ability to isolate *E. coli* at 44 °C from water and related materials

Additionally data on the following colony characteristics were gathered:

the typical size, colour and number of coliform colonies obtained at 37 °C
the typical size, colour and number of *E. coli* colonies at 37 °C
the typical size, colour and number of *E. coli* colonies at 44 °C

The participating laboratories were asked to prepare a single batch of plates of MLGA sufficient for the analysis of contaminated water samples for up to six weeks. These were stored at 5 ± 3 °C for the duration of the study. These were to be used to compare recoveries of *E. coli* and coliforms against “freshly prepared” MLGA (i.e. medium not more than seven days old).

For each sample four filtrations were performed and one membrane was placed on each of a “fresh” MLGA plate for incubation at 37 °C (for *E. coli* and coliforms), a “fresh” MLGA plate for incubation at 44 °C (for *E. coli*), a “stored” MLGA plate for incubation at 37 °C (for *E. coli* and coliforms) and a “stored” MLGA plate for incubation at 44 °C (for *E. coli*). All plates were incubated at 30 °C for four hours prior to incubation at their final temperature for 14 – 20 hours.

After incubation colonies were counted separately that were yellow or green and the number within these colours that were large or small and bright or pale in appearance

was recorded. The results were recorded on a supplied pro-forma Excel spreadsheet.

3 Data analysis

Data were received from eleven laboratories and analysed according to ISO 17994 (ISO, 2014) by assigning counts from stored MLGA medium as the Trial Method and corresponding counts from fresh MLGA medium as the Reference Method. Data from the three sets of comparisons (coliform bacteria incubated at 37 °C, *E. coli* incubated at 37 °C and *E. coli* incubated at 44°C) were censored by the removal of pairs of data where both counts were zero or where at least one count of a paired count was recorded as either > 100 or TNTC (too numerous to count). The paired count data were transferred to an Excel 2007 spreadsheet and analysed according to the mean relative difference approach of ISO 17994. Briefly, the relative difference (x) of each pair of counts was calculated using the equation x = 100(ln(a) - ln(b)), where ln(a) is the natural logarithm of the count by the trial method (stored MLGA), and ln(b) is the natural logarithm of the count by the reference method (fresh MLGA). Data with a zero count by one method had plus one (i.e. count +1) added to each pair of the counts prior to log-transformation. As the objective of the study was to show there was no difference between the trial method with an established reference method, it was considered that the 'two-sided' comparison according to ISO 17994 was appropriate. The percentage value of the upper and lower limits was set at +10% and -10% as suggested by ISO 17994. Initially the combined data for all the three data sets was analysed, not taking into account the length of storage time for the stored MLGA medium. The effect of length of storage time of MLGA on recoveries of coliform bacteria and *E. coli* was then studied for each parameter.

4 Coliform bacteria comparison

For this study the term coliform bacteria refers to those isolates that produced yellow colonies on MLGA after incubation at 30 °C for four hours followed by incubation at 37 °C to give a total incubation time of 21 ± 3 h (i.e. non-*E. coli* coliforms). There were 443 paired counts for these bacteria and the result of the ISO 17994 analysis of the data is presented in Table 1. Unfortunately, despite the large number of results, the outcome of the analysis is inconclusive (i.e. more samples are needed). Using the number of samples formula in ISO 17994 the total number of paired counts needed to achieve a conclusive outcome is 850 (i.e. 407 more). The "confidence limits" in Table 1 do, however, indicate a tendency for lower counts to be obtained on stored MLGA and further samples may well confirm this.

Table 1 Outcome of mean relative difference analysis of the paired counts of coliform bacteria on stored MLGA and on fresh MLGA incubated at 37 °C from 443 samples according to ISO 17994

Number of paired counts	Mean relative difference	Standard deviation	W *	X_L†	X_U‡	Outcome
443	- 4.46	65.03	6.18	- 10.64	1.72	Inconclusive

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

To ascertain the impact of storage time the data were simply plotted as the difference in counts against age of stored MLGA. The plot is presented in Figure 1. The visual data indicated little difference in counts up to about storage day 30 after which it appears that the stored MLGA tends to produce lower counts.

In order to assess the above observation the data were grouped into week lots for stored MLGA (i.e. 0 – 7 days, 8 – 14 days, 15 – 21 days, 22 – 28 days, 29 – 35 days

and 36 – 49 days) and reanalysed according to ISO 17994. The results are presented in Table 2. Although the individual data sets are small for ISO 17994 analysis the outcomes do indicate probable comparable performance for MLGA stored up to 28 days compared to fresh MLGA. After that there is an indication of deterioration of performance in recoveries for stored MLGA which becomes significant after 35 days storage.

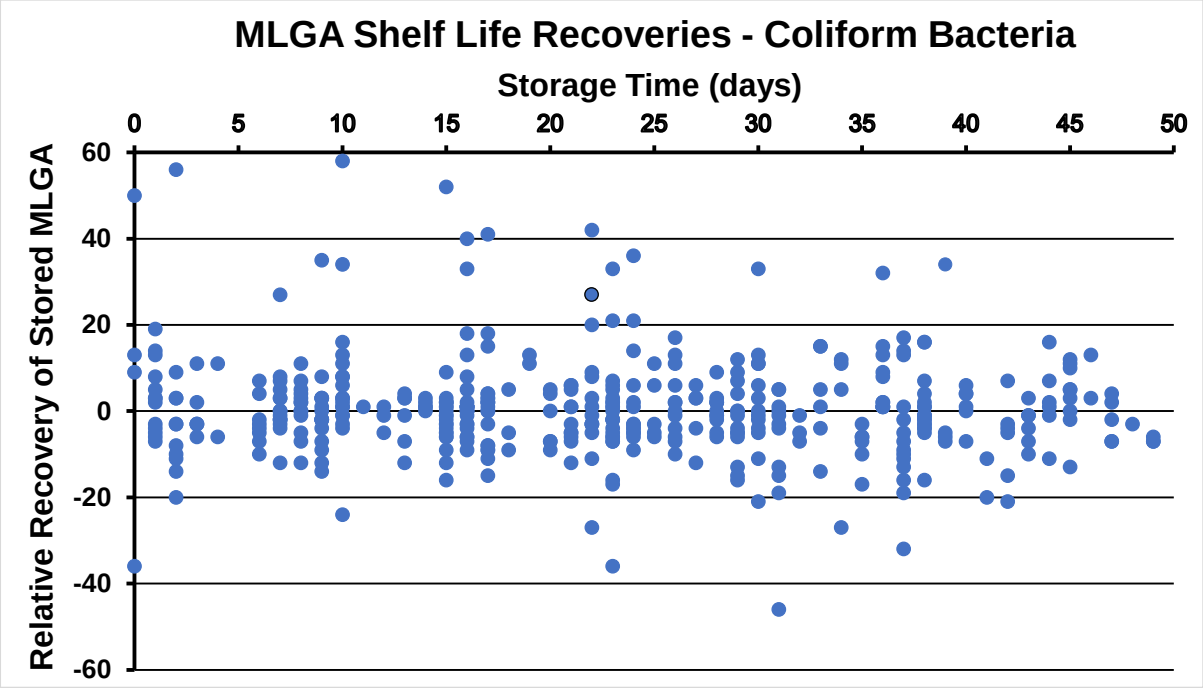

Figure 1 Plot of the difference in counts of non-*E. coli* coliforms on stored MLGA and fresh MLGA against storage time of stored MLGA before use

Table 2 Outcome of mean relative difference analysis of the paired counts of coliform bacteria on stored MLGA grouped into weekly lots compared to fresh MLGA incubated at 37 °C according to ISO 17994

Storage time (days)	Number of paired counts	Mean relative difference	Standard deviation	*W* *	X_L†	X_U‡	Outcome
0 – 7	59	4.08	61.62	16.05	- 11.97	20.12	Inconclusive
8 – 14	62	10.51	88.70	22.53	- 12.02	33.04	Inconclusive
15 – 21	88	0.57	46.49	9.93	- 9.37	10.50	Inconclusive
22 – 28	84	- 0.91	46.07	10.05	-10.96	9.14	Inconclusive
29 – 35	57	- 17.44	73.00	19.34	- 36.78	1.89	Inconclusive
36 – 49	93	- 19.85	70.13	14.54	- 34.39	- 5.30	Stored MLGA lower recovery

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

To verify the acceptability of MLGA plates stored for up to 28 days for the enumeration of coliform bacteria the data were reanalysed to compare four week storage data against longer storage (Table 3). Again, although the data sets are small the outcomes indicate equivalent performance for MLGA stored for up to 28 days, but there was a significantly lower recovery after that period.

The results of these analyses indicate that MLGA plates stored for up to 28 days

recover coliform bacteria to the same extent as MLGA stored for less than seven days.

Table 3 Outcome of mean relative difference analysis of the paired counts of coliform bacteria on MLGA stored for up to 28 days and for more than four weeks compared to fresh MLGA incubated at 37 °C according to ISO 17994

Storage time (days)	Number of paired counts	Mean relative difference	Standard deviation	*W* *	X_L†	X_U‡	Outcome
0 – 28	293	2.95	60.55	7.07	- 4.12	10.07	Inconclusive
29 – 49	150	- 18.93	71.00	11.59	- 30.53	- 7.34	Stored MLGA lower recovery

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

5 *E. coli* incubated at 37 °C comparison

There were 387 paired counts for *E. coli* enumerated after incubation at 37 °C and the result of the ISO 17994 analysis of the data is presented in Table 4. The statistical outcome for these samples is that there was no significant difference in recovery of *E. coli* between stored and fresh MLGA with the "confidence limits" of the analysis being well within those of +10% and -10% suggested by ISO 17994 for this type of comparison of performance.

Table 4 Outcome of mean relative difference analysis of the paired counts of *E. coli* on stored MLGA and on fresh MLGA incubated at 37 °C from 387 samples according to ISO 17994

Number of paired counts	Mean relative difference	Standard deviation	*W* *	X_L†	X_U‡	Outcome
387	- 1.07	75.11	7.64	- 8.70	6.57	Methods not different

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

To ascertain the impact of storage time the data was simply plotted as the difference in counts against age of stored MLGA. The plot is presented in Figure 2. The visual data indicate little difference in counts throughout the trial period.

Again, the data were grouped into week lots for stored MLGA (i.e. 0 – 7 days, 8 – 14 days, 15 – 21 days, 22 – 28 days, 29 – 35 days and 36 – 49 days) and reanalysed according to ISO 17994. The results are presented in Table 5. As previously the individual data sets are small for ISO 17994 analysis but the outcomes do indicate probable comparable performance for MLGA stored up to 28 days compared to fresh MLGA. However, the data for 29 – 35 days stored plates indicate under-recovery by stored MLGA, but this is not reflected in the data for plates stored for more than 35 days which indicate a tendency for equivalent performance with fresh MLGA plates.

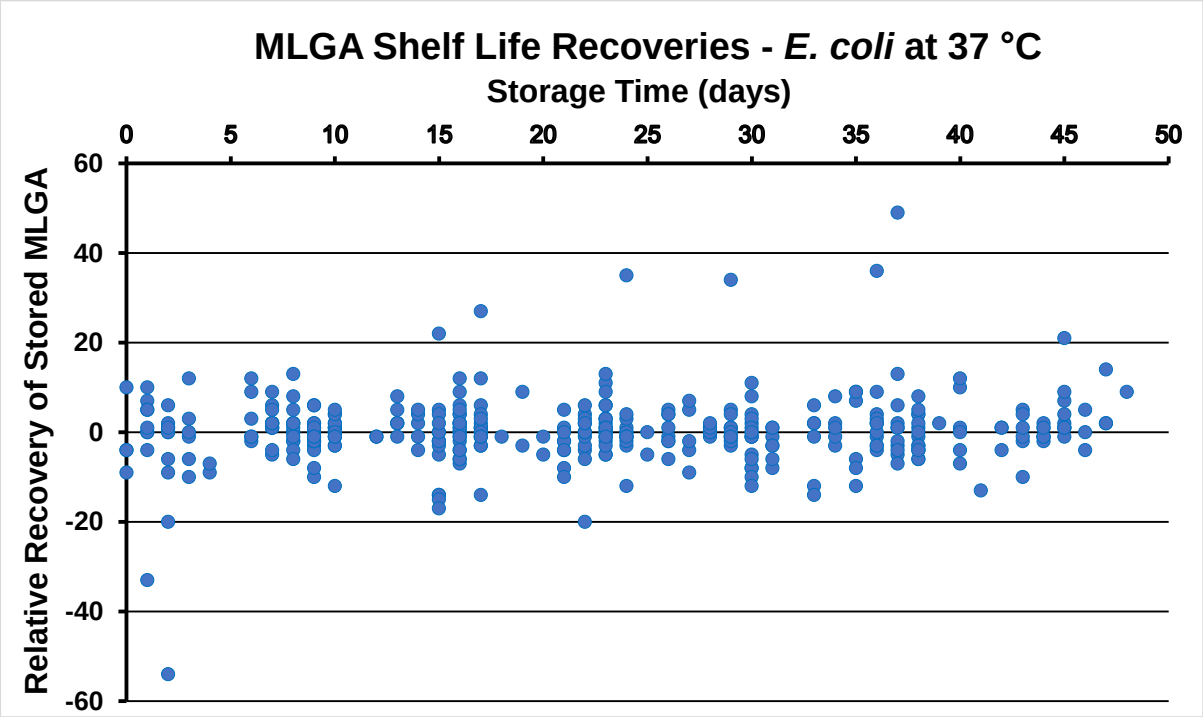

Figure 2 Plot of the difference in counts of *E. coli* incubated at 37 °C on stored MLGA and fresh MLGA against storage time of stored MLGA before use

Table 5 Outcome of mean relative difference analysis of the paired counts of *E. coli* on stored MLGA grouped into weekly lots compared to fresh MLGA incubated at 37 °C according to ISO 17994

Storage time (days)	Number of paired counts	Mean relative difference	Standard deviation	*W* *	X_L†	X_U‡	Outcome
0 – 7	53	8.84	56.26	15.45	- 6.61	24.30	Inconclusive
8 – 14	62	7.12	70.52	17.91	- 10.79	25.03	Inconclusive
15 – 21	69	0.48	56.68	13.65	- 13.17	14.13	Inconclusive
22 – 28	73	1.29	51.12	11.97	- 10.68	13.25	Inconclusive
29 – 35	52	- 32.95	111.41	30.90	- 63.85	- 2.05	Stored MLGA lower recovery
36 – 49	78	3.38	88.67	20.08	- 16.70	23.46	Inconclusive

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

To verify the acceptability of MLGA stored for up to 28 days for the enumeration of *E. coli* at 37 °C the data were reanalysed to compare four week storage data against longer storage (Table 6). Again, as the data sets may be small the outcomes indicate equivalent performance for MLGA stored for up to 28 days, but there is an indication of lower recovery after that period.

Table 6 Outcome of mean relative difference analysis of the paired counts of *E. coli* on MLGA stored for up to 28 days and for more than four weeks compared to fresh MLGA incubated at 37 °C according to ISO 17994

Storage time (days)	Number of paired counts	Mean relative difference	Standard deviation	*W* *	X_L†	X_U‡	Outcome
0 – 28	257	4.04	58.56	7.31	- 3.27	11.34	Inconclusive
29 – 49	130	-11.15	99.60	17.47	- 28.62	6.32	Inconclusive

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

The results of these analyses indicate that MLGA plates stored for up to 28 days and incubated at 37 °C recover *E. coli* to the same extent as MLGA stored for less than seven days.

6 *E. coli* incubated at 44 °C comparison

There were 367 paired counts for *E. coli* enumerated after incubation at 44 °C and the result of the ISO 17994 analysis of the data is presented in Table 7. The statistical outcome for these samples is that there was a significant under-recovery of *E. coli* by stored MLGA when incubated at 44 °C with the lower "confidence limit" being less than zero.

Table 7 Outcome of mean relative difference analysis of the paired counts of *E. coli* on stored MLGA and on fresh MLGA incubated at 44 °C from 367 samples according to ISO 17994

Number of paired counts	Mean relative difference	Standard deviation	*W* *	X_L†	X_U‡	Outcome
367	- 12.90	70.17	7.34	- 20.24	- 5.57	Stored MLGA lower recovery

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

To ascertain the impact of storage time the data were simply plotted as the difference in counts against age of stored MLGA. The plot is presented in Figure 3. The visual data indicate little difference in counts up to about storage day 25 after which it appears that the stored MLGA tends to produce lower counts.

To confirm the above observation the data were grouped into week lots for stored MLGA (i.e. 0 – 7 days, 8 – 14 days, 15 – 21 days, 22 – 28 days, 29 – 35 days and 36 – 49 days) and reanalysed according to ISO 17994. The results are presented in Table 8. As previously the individual data sets are small for ISO 17994 analysis but the outcomes do indicate probable comparable performance for MLGA stored up to 21 days compared to fresh MLGA. After that there is an indication of deterioration of performance in recoveries for stored MLGA which becomes significant after 28 days storage.

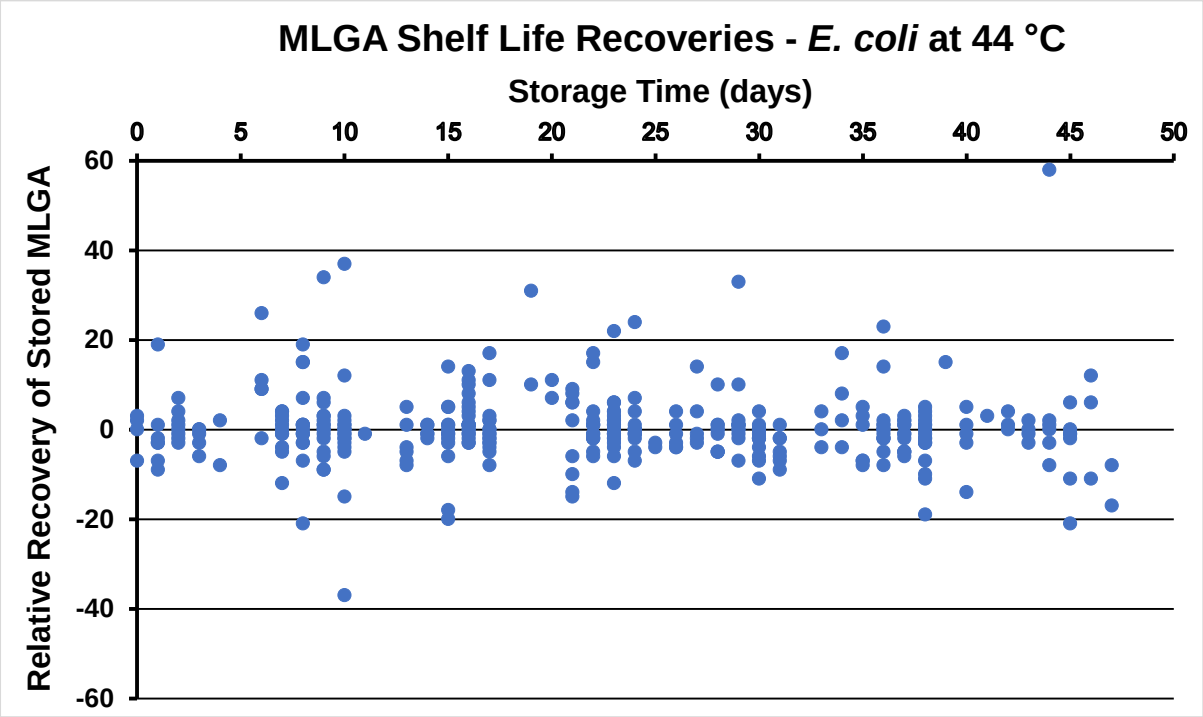

Figure 3 Plot of the difference in counts of *E. coli* incubated at 44 °C on stored MLGA and fresh MLGA against storage time of stored MLGA before use

Table 8 Outcome of mean relative difference analysis of the paired counts of *E. coli* on stored MLGA grouped into weekly lots compared to fresh MLGA incubated at 44 °C according to ISO 17994

Storage time (days)	Number of paired counts	Mean relative difference	Standard deviation	*W* *	X_L†	X_U‡	Outcome
0 – 7	43	0.24	41.47	12.65	- 12.41	12.89	Inconclusive
8 – 14	62	- 4.75	71.24	18.09	- 22.85	13.34	Inconclusive
15 – 21	64	- 1.63	58.29	14.57	- 16.20	12.95	Inconclusive
22 – 28	75	- 8.56	51.55	11.91	- 20.46	3.35	Inconclusive
29 – 35	47	- 41.66	90.41	26.38	- 68.03	- 15.28	Stored MLGA lower recovery
36 – 49	76	- 22.98	86.85	19.92	- 42.90	- 3.05	Stored MLGA lower recovery

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

To verify the acceptability of MLGA stored for up to 21 or 28 days for the enumeration of *E. coli* at 44 °C the data were reanalysed to compare three and four week storage data against longer storage (Table 9). Again, although the data sets may be small the outcomes indicate equivalent performance for MLGA stored for up to 21 days, but there is an indication of lower recovery after that period with significant under-recovery on MLGA stored for more than 28 days.

Table 9 Outcome of mean relative difference analysis of the paired counts of *E. coli* on MLGA stored for up to 21 days for up to 28 days and for more than four weeks compared to fresh MLGA incubated at 44 °C according to ISO 17994

Storage time (days)	Number of paired counts	Mean relative difference	Standard deviation	W *	X_L†	X_U‡	Outcome
0 – 21	169	- 2.30	59.59	9.17	- 11.47	6.87	Inconclusive
0 – 28	244	- 4.22	57.21	7.32	- 11.55	3.10	Inconclusive
29 – 49	123	- 30.21	88.33	15.93	- 46.04	- 14.19	Stored MLGA lower recovery

* Half width of the 'confidence interval' around the mean relative difference.
† Value of the relative difference at the lower 'confidence limit'.
‡ Value of the relative difference at the upper 'confidence limit'.

The results of these analyses indicate that MLGA plates stored for up to 21 days, and possibly 28 days, and incubated at 44 °C recover *E. coli* to the same extent as MLGA stored for less than seven days.

7 Impact of storage of MLGA on colony size and colouration

The data from the eleven laboratories contained information on the size and brightness of colouration of coliform and *E. coli* colonies on stored and fresh MLGA. The data, however, were not amenable for robust statistical analysis and so were examined visually. This did not reveal any obvious difference in the proportions of large or small colonies or in brightness of colonies for both coliforms and *E. coli* recovered on stored and fresh MLGA over the study period, regardless of the incubation temperature.

Two laboratories submitted observations on medium quality and performance for stored MLGA. One noted that stored MLGA became thinner in the Petri dish after three weeks storage with the Petri dish lids having excess condensation. This probably indicates loss of water during storage leading to shrinkage of the agar. Additionally, the laboratory reported reduction in "structural integrity" compared to fresh MLGA after approximately 17 – 18 days storage. The other laboratory reported an increase in the number of non-lactose fermenting "pink" colonies on MLGA stored for more than 40 days.

8 Conclusions and recommendations

This study assessed the recovery of coliform bacteria and *E. coli* on stored MLGA compared to recovery on fresh MLGA. The outcomes of ISO 17994 analyses of the data indicate that MLGA plates stored for up to 28 days provide equivalent recovery of coliforms and *E. coli* for plates incubated at 37 °C. For *E. coli* determinations at 44 °C the maximum storage time appears to be 21 days as the recovery of *E. coli* deteriorates after that storage time.

Thus, it is recommended that *The Microbiology of Drinking Water Part 4* is amended to allow storage of MLGA plates at 5 ± 3 °C for up to four weeks. Additionally, it is recommended that *The Microbiology of Recreational and Environmental Waters* is amended to allow storage of MLGA plates at 5 ± 3 °C for up to three weeks.

However, despite these findings, good practice would still be to use prepared media as fresh as practicable and that extended storage is dependent on correct storage of prepared media

9 Acknowledgements

SCA Working Group 2 is grateful to the management and staff of the following laboratories which participated in this study:-

ALcontrol Laboratories (Rotherham),
ALS Environmental (Coventry),
ALS Environmental (Wakefield),
CREH *Analytical* (Leeds),
Dŵr Cymru - Welsh Water (Newport),
Environment Agency (Exeter),
Northern Ireland Water (Belfast),
Severn Trent Water (Nottingham),
Severn Trent Water (Shrewsbury),
South West Water (Exeter) and
United Utilities (Warrington).

The investigation was organised by John Watkins (CREH *Analytical*) and data were analysed by David Sartory (SWM Consulting).

10 References

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Members assisting with this method

Without the good will and support given by these individuals and their respective organisations SCA would not be able to continue and produce the highly valued and respected blue book methods.

Zoe Bickel	South West Water
Peter Boyd	Formerly Public Health England
Simon Cole	Wessex Water
David Gaskell	United Utilities
Malcolm Morgan	SCA Strategic Board
David Sartory	SWM Consulting
Rhys Stephens	Welsh Water/Dŵr Cymru
Martin Walters	Environment Agency
John Watkins	CREH *Analytical* Limited
John Watson	South West Water

Grateful acknowledgement is made to John Watkins (CREH *Analytical*), IDEXX Laboratories and Bio-Rad Laboratories for providing colour photographs.