

HPA STANDARD METHOD

GENERAL TECHNIQUE FOR THE DETECTION AND ENUMERATION OF BACTERIA BY NEGATIVE PRESSURE MEMBRANE FILTRATION

W 1

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

**GENERAL TECHNIQUE FOR THE DETECTION AND ENUMERATION OF BACTERIA BY NEGATIVE PRESSURE
MEMBRANE FILTRATION**

Issue no: 4.1 Issue date: 05.04.07 Issued by Standards Unit, Evaluations and Standards Laboratory in conjunction with the
Regional Food, Water and Environmental Coordinators Forum Page 1 of 15

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AMENDMENT PROCEDURE

Controlled document reference	W 1
Controlled document title	General technique for the detection and enumeration of bacteria by negative pressure membrane filtration

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

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

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
7/ 05.04.07	4	4.1	5	Background	Coliforms changed to coliform bacteria
			6	Equipment	Coliforms changed to coliform bacteria
			10	6.3 Confirmatory tests	Coliforms changed to coliform bacteria Information on private water supplies added. Enterococci not required under current regulations but to be determined on a retest following a failure

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SCOPE OF DOCUMENT

This method is applicable to waters from a wide range of sources including potable waters, pool waters and surface waters. It is not applicable to the isolation of *Legionella* species by positive pressure filtration for which an alternative method is available.

INTRODUCTION

Background

The negative pressure membrane filtration method is used for the enumeration of coliform bacteria, *Escherichia coli*, enterococci, *Clostridium perfringens*, sulphite-reducing *Clostridia*, *Pseudomonas aeruginosa* and other bacteria as may be necessary in various water samples. The method is based on the Microbiology of Drinking Water 2002 document².

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1 PRINCIPLE

The sample of water is drawn through a 47 mm diameter hydrophilic membrane filter under negative pressure. The effective pore size of the membrane (0.2 µm or 0.45 µm) is such that the bacteria in the water are retained on the membrane. The membrane is then placed on an agar growth medium or a filter pad soaked in a liquid growth medium and, after appropriate incubation, a count is made of the organisms forming presumptive target colonies on the membrane.

For the presence/absence detection of specific pathogens by culture, the membrane may be placed in an appropriate enrichment medium.

2 SAFETY CONSIDERATIONS³⁻⁸

Normal microbiology laboratory precautions apply.

2.1 SAMPLE TRANSPORT AND STORAGE

Compliance with current postal and transportation regulations as stated in the Advisory Committee on Dangerous Pathogens 2005⁹ is essential.

2.2 SAMPLE PROCESSING

Care must be taken when using boiling water or a boiling waterbath for disinfection of the filtration apparatus.

Use heat and water resistant protective gloves and do not put the face or hands over the bath when opening to remove objects.

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

3 EQUIPMENT

Usual laboratory equipment and in addition:

- Membrane filtration manifold
- Filter funnels
- Vacuum pump, with moisture trap or protective filter to prevent carryover of water vapour, or alternative vacuum source
- Pyrex vacuum flask with protective jacket or equivalent: large volume eg 5 L
- Stainless steel flat tipped forceps or sterilisable equivalent
- Boiling waterbath (instrument steriliser)
- Petri dishes
- Membranes, sterile, as detailed in the method for the organism sought (usually cellulose ester 47 mm diameter 0.45 µm pore size, gridded or polyamide 0.2 µm pore size for Legionella). These must be from suppliers accredited to ISO 9001 or equivalent and each batch must have been subjected to quality control procedures to ensure that they give satisfactory recovery of the organisms under test. See section 10.1 below
- Sterile absorbent pads (coliform bacteria and *E. coli*). See section 10.1 below
- Automatic pipettors and associated sterile pipette tips capable of delivering up to 10 mL and 1 mL volumes (optional)
- Pipettes (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)

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The membrane filtration apparatus consists of a base supporting a porous disc. The filter funnel, which should be graduated at 50 mL and 100 mL for most examinations, is secured to the base by means of screw-threads, clamps or by magnetic action. The materials of construction must be known not to be detrimental to the microorganisms and may be glass, plastics or metal. The filtration apparatus is connected to a vacuum source via a receiving vessel. The receiver must be capable of collecting the filtrate from at least one sample, and the vacuum source must be protected from carry over of water vapour by an appropriate hydrophobic air filter. For the examination of large numbers of water samples, multiple filtration units may be used. If necessary, the filtration apparatus, including spare funnels and funnel stands, should be sterilised in the autoclave. Sufficient funnels are required for one apparatus so that one funnel can be disinfected by immersion in boiling water for at least one minute between samples. Alternatively, boiling water can be added to the funnels *in situ*. After disinfection, each funnel and base should be allowed to cool before use.

In some locations it may be necessary to use distilled or de-ionised water to prevent the accumulation of scale.

4 CULTURE MEDIA AND REAGENTS

As specified in the individual test method for the organism sought.

5 SAMPLE REQUIREMENTS

5.1 SAMPLE COLLECTION

Samples should be collected in accordance with MDW 2002 part 2¹⁰ or for *Legionella*, National Standard Methods W12-15¹¹⁻¹⁴.

5.2 BIOCIDES NEUTRALISATION

It is important that the sample is representative and has not been damaged or changed during transport or storage. For chlorinated, brominated waters, sodium thiosulphate pentahydrate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) should be added to the collecting bottles before sterilisation to ensure the presence of sufficient sodium thiosulphate to neutralise the anticipated amount of chlorine in the sample. Thiosulphate can also be used to neutralise other oxidising biocides including bromine, iodine, chlorine dioxide, ozone and hydrogen peroxide. Approximately 18 mg of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ / L will neutralise up to 5 mg of chlorine/litre and should be sufficient for potable and swimming pool water samples. Samples collected for *Legionella* detection from cooling towers or after disinfection of water systems may have much higher chlorine concentrations and should therefore have $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ at 180 mg/litre which will neutralise up to 50 mg chlorine per litre.

Where non-oxidising biocides have been used eg quaternary ammonium compounds this should have been noted on the sample submission form and the sample should be processed without delay.

Routine samples should be protected from direct sunlight and transported in an insulated container or refrigerator at 2°C – 8°C. For pathogens such as *Legionella* see the appropriate National Standard Methods for transport and storage conditions (eg W12-15)¹¹⁻¹⁴. Samples should be analysed as soon as is practicable on the day of collection. In exceptional circumstances, if there is a delay, storage under the above conditions should not exceed 24 hours before the commencement of analysis.

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6 SAMPLE PROCESSING

6.1 SAMPLE PREPARATION

6.1.1 MIXING

Invert the sample bottle rapidly several times in order to mix the sample thoroughly. If the bottle is full, remove the stopper or cap and retain in the hand. Pour off some of the contents, replace the stopper or cap and again shake the bottle in order to distribute any organisms uniformly through the water.

6.1.2 DILUTION

The volumes are to be chosen so that the number of colonies to be counted on the membrane lies, if possible, between 20 and 80. With some waters, it may be advantageous to filter different volumes so that the number of colonies on one of the membranes is likely to fall within this range. For bottled mineral waters, other bottled waters and waters in containers^{15,16}, filter 250 mL; for treated waters filter 100 mL of the sample; for polluted waters either filter smaller volumes or dilute the sample before filtration. When the volume to be filtered is less than 10 mL, add sufficient sterile diluent to the funnel prior to addition of the sample to give a minimum volume of 10 mL.

Note: A minimum of one litre samples are filtered for the enumeration of *Legionella* and pathogen detection procedures. For some pathogens filter aid (diatomaceous earth) may be added to the sample which is then filtered through a sterile absorbent pad rather than a membrane (see the individual test method for the organism sought).

6.2 MEMBRANE FILTRATION PROCEDURE

6.2.1 PREPARATION

Prior to use, assemble the sterile filtration apparatus in a level position and connect to the empty receiver and source of vacuum.

Where liquid media are used place an absorbent pad in a Petri dish and add enough of the relevant medium (usually 2.5 mL depending on the brand of pad) and leave to soak for at least 5 minutes. For each brand of pad it is important to check the amount of medium that is to be added and the time necessary for soaking in order to prevent drying out during incubation.

If necessary, remove any excess medium from the saturated pad using, for example, a sterile dropping pipette, before the membrane is placed in position. If this is not done, confluent growth may result.

Do not alternate the filtration of known polluted samples or positive control samples with those of treated water samples through the same apparatus.

Filter all of the samples of chlorinated water, firstly those expected to give negative results and then those that are known to be polluted. Alternatively a separate membrane filtration apparatus can be reserved for all chlorinated samples and another for polluted samples. Where large numbers of samples are regularly processed then it is preferable to process clean and contaminated samples on separate benches.

6.2.2 PROCEDURE

Remove the funnel and place a sterile membrane, gridded side upwards, on the porous disc of the filter base. Grasp only the outer part of the membrane filter with flat-ended sterile forceps.

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Replace the sterile funnel securely on the filter base and ensure that it is in a level position.

With the vacuum turned off, pour or pipette the required volume of freshly mixed sample into the funnel and cover the funnel.

Open the stopcock and apply a vacuum of about 500 mm of mercury to filter the water slowly through the membrane.

Turn off the vacuum as soon as the sample has been filtered so that the membrane is not excessively dried.

Remove the funnel and transfer the membrane face up carefully either to a pad saturated with the medium or to the surface of an agar plate. Ensure that no air bubbles are trapped between the membrane and the medium.

For different volumes of the same sample, the funnel may be re-used without boiling provided that the smallest volumes or greatest dilution are filtered first.

For different samples, remove a funnel from the boiling water bath, allow to cool and repeat the filtration process.

After filtration of each sample, disinfect the funnel either by immersing it in boiling water, or by adding boiling water to it *in situ*. The contact time should be at least one minute. Check that the receiving vessel does not need emptying. During the filtration of a series of samples the filter base need not be disinfected unless it is contaminated or a membrane is damaged. When funnels are not in use they should be covered with a funnel lid, sterile Petri dish or equivalent.

At the end of each day empty the contents and either disinfect or sterilise the receiver.

6.2.3 INCUBATION

Place the Petri dishes containing the membranes inside a sealed container or a polythene bag to prevent drying out if broth-soaked pads are utilised. For anaerobic or microaerophilic organisms, special incubation conditions are required.

Incubate the membranes at the temperature and for the time specified for the organism sought.

6.2.4 COUNTING OF COLONIES

After incubation, count the characteristic colonies on the membrane in good light. If necessary, use a hand lens or low power binocular microscope. As colours of colonies are liable to change on cooling and standing, count the colonies within 30 minutes of removal from the incubator.

Express the result as the number of the test organisms per volume of water sample as specified in the appropriate test method.

6.3 CONFIRMATORY TESTS

If confirmation is required select the colonies for confirmation as follows: if more than one volume or dilution has been filtered, proceed if possible with the membrane yielding between 20 and 80 colonies.

Where colonies are sub-cultured to agar media the plates should be spread to give discrete colonies.

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Potable waters

In all potable waters, except currently private supplies it is required that there should be no coliform bacteria, including *E. coli*, or enterococci in a specified volume. Until the regulations are updated, enterococci are not required for initial samples from private water supplies but should be determined on retest samples after a failure. Some types of potable water also require that *Clostridium perfringens*, and for mineral waters, sulphite-reducing clostridia, are not detected in a specified volume. In practice it is considered sufficient to select colonies for testing as follows:

Membranes containing 10 or less colonies:

Sub-culture all colonies.

Membranes containing more than 10 colonies:

If only one colony type is present then record the number of presumptive colonies. Select a segment of the membrane containing more than 10 colonies and sub-culture 10 colonies.

When two or more presumptive colony types are present record the number of each morphological type. Sub-culture at least four of each type or as many as are available, ensuring that a minimum of 10 colonies are sub-cultured from the segment of the membrane.

Non-potable waters (waters, including pool waters, which are not sampled on a regulatory basis)

Membranes containing 5 or less colonies:

Sub-culture all colonies.

Membranes containing more than 5 colonies:

If only one colony type is present then record the number of presumptive colonies. Select a segment of the membrane containing more than 5 colonies. This will avoid bias that can occur when sub-culturing colonies from the membrane. Sub-culture a minimum of 5 isolated colonies.

When two or more presumptive positive colony types are present then record the number of each morphological type. Sub-culture at least two of each type or of as many types as are available, ensuring a minimum of 5 colonies is sub-cultured from the membrane.

6.4 RECORDING OF RESULTS

For membranes containing presumptive colonies of one morphological type record the following information on the laboratory worksheet:

The number of presumptive positives on the membrane (A)

The number of colonies sub-cultured from the chosen segment (B)

The number of sub-cultures confirmed positive (C)

For membranes containing presumptive colonies of more than one morphological type record the following information on the laboratory worksheet:

The number of presumptive positives on the membrane (A)

The total number of presumptive colonies in the chosen segment (N)

The number of each morphological type in the chosen segment (S), (L) etc

The number of each morphological type sub-cultured (BS) (BL) etc

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The number of each of the sub-cultures confirmed positive (CS) (CL) etc

7 CALCULATION OF RESULTS

Membranes containing presumptive colonies of one morphological type:

The number of confirmed colonies (**X**) is calculated by

$$X = \frac{C \times A}{B}$$

Example - a membrane containing 80 presumptive colonies of one morphological type, 10 colonies are sub-cultured from a chosen segment and of these 3 colonies are confirmed.

Therefore if: **A** = 80 **B** = 10 **C** = 3

The confirmed count per membrane

$$X = \frac{3 \times 80}{10} = 24$$

Note: Ensure the result is multiplied by the reciprocal of the sample dilution and/or the volume factor so that the result may be expressed as the number of confirmed colony forming units per specified volume of sample.

Membranes containing presumptive colonies of two (or more) morphological types:

The number of confirmed colonies per segment:

$$Y = \frac{(C_s \times S)}{B_s} + \frac{(C_L \times L)}{B_L}$$

The number of confirmed colonies per membrane:

$$X = \frac{Y \times A}{N}$$

Example - a membrane from a private water supply containing 80 presumptive colonies of two morphological types, the segment chosen contains 15 small colonies and 5 large colonies, making a total of 20 colonies. Of the 5 small colonies sub-cultured, 3 are confirmed and of the 5 large colonies 2 colonies are confirmed.

Therefore:

A = 80	N = 20
S = 15	L = 5
B_s = 5	B_L = 5
C_s = 3	C_L = 2

The confirmed count per segment is:

$$Y = \frac{3 \times 15}{5} + \frac{2 \times 5}{5}$$

$$Y = 9 + 2$$

$$Y = 11$$

Therefore the confirmed count per membrane is:

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$$X = \frac{11 \times 80}{20}$$

$$X = 44$$

Note: Ensure the result is multiplied by the reciprocal of the sample dilution and/or the volume factor so that the result is expressed as the number of confirmed colony forming units per specified volume of sample.

8 UNCERTAINTY OF MEASUREMENT

Laboratories need to produce data on uncertainty of measurement (UM) for the tests that they perform and may be required to report relevant information in certain circumstances (see BS DD260¹⁷). Variation in results comes about through errors in pipetting, incubation conditions etc but in a well controlled laboratory these are normally much less than the natural variation between the numbers of organisms in samples from the same body of water or sub-samples of the same sample.

Random distribution of microorganisms in a sample causes imprecision in the results. Counts on membranes are subject to statistical variation and replicate tests on the same sample are unlikely to give the same number of colonies².

9 REPORTING

9.1 FACTORS THAT MAY AFFECT THE RESULT

A note should be made on the report of any factors that may adversely affect the result in particular the presence of biocides in the sample that could not be inactivated.

9.2 ENUMERATION

The test report specifies the method used, all information necessary for the complete identification of the sample and will also mention details of any incidents that may have influenced the result. The result is expressed as a colony count per specified volume of sample.

If the test organisms are not detected then report as:

'Not detected in v'

where **v** is the specified volume

Results may be reported as 0 / specified volume with an explanatory note stating that 0 = Not Detected.

If there are more than 100 colonies on the membrane and dilution is not performed report as:

'Greater than 100 in v'

where **v** is the specified volume

If there are more than 100 colonies on all membranes where dilution is used report as:

'Greater than 100 x d in v'

where **d** is the dilution factor and **v** is the specified volume

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The report should also have reference to appropriate legislation or guidelines.

9.3 PRESENCE / ABSENCE TESTS

If the test organisms are not detected then report as:

'Not detected in v'

where **v** is the specified volume

If the test organisms are detected then report as:

'Detected in v'

where **v** is the specified volume

10 QUALITY CONTROL

10.1 MEMBRANES AND PADS

Each new batch of membranes and absorbent pads, where these are used, should be subjected to quality control tests to confirm that the quantitative recovery is adequate and that the colony appearance, size, and colour is typical. The gridlines on some batches of membranes can affect the colony shape or even inhibit growth. Some colour changes, particularly with modern chromogenic and fluorogenic substrates can differ significantly with manufacturer and batch of membrane.

10.2 GENERAL

Refer to the quality control procedures specified for the individual test method.

11 REFERENCE FACILITIES

N/A

12 ACKNOWLEDGEMENTS AND CONTACTS

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

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APPENDIX: FLOWCHART SHOWING THE PROCESS FOR THE ENUMERATION OF BACTERIA BY NEGATIVE PRESSURE MEMBRANE FILTRATION

Transport to laboratory at 2°C – 8°C out of direct sunlight
in suitable containers or as appropriate



Store at 2°C – 8°C in the dark and analyse as soon as is practicable on the day of collection,
otherwise within 24 hours of collection



Mix sample well and make any necessary dilutions



Filter



Place filter on medium and incubate at appropriate temperature(s)



After incubation count characteristic colonies



Carry out confirmatory tests



Calculate count of test organism



Report as count/volume quoting the appropriate legislation/guidelines

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