

NATIONAL STANDARD METHOD

ENUMERATION OF *CLOSTRIDIUM PERFRINGENS* BY MEMBRANE FILTRATION

W 5

Issued by Standards Unit, Evaluations and Standards Laboratory
Specialist and Reference Microbiology Division

ENUMERATION OF *CLOSTRIDIUM PERFRINGENS* BY MEMBRANE FILTRATION

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AMENDMENT PROCEDURE

Controlled document reference	W 5
Controlled document title	Standard Operating Procedure for Enumeration of <i>Clostridium perfringens</i> by membrane filtration

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

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
4/ 03.05.05	3	3.1	1	Front page	Redesigned
			2	Status of document	Reworded
			4	Amendment page	Redesigned

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STANDARD OPERATING PROCEDURE FOR ENUMERATION OF *CLOSTRIDIUM PERFRINGENS* BY MEMBRANE FILTRATION

INTRODUCTION

Scope

The method gives general guidelines for the enumeration of *Clostridium perfringens* in water samples intended for human consumption and environmental water samples, by the membrane filtration method.

Background

C. perfringens plays a subsidiary role in water examination². These organisms form spores, which are resistant to heating when compared with vegetative cells and advantage is taken of this for the detection of clostridia in water. *C. perfringens* is the most important of the sulphite reducing clostridia and is normally present in human and animal faeces. Clostridial spores survive longer than coliforms, *Escherichia coli* or enterococci and are consequently used as an indicator of past faecal pollution. The spores are not always inactivated by chlorination but are not a hazard to health in potable water. Although the resistance of spores of *C. perfringens* gives them similarities to *Cryptosporidium* and *Giardia* they are not reliable indicators of the presence of these parasites in drinking or recreational waters. Spores of *C. perfringens* have been used to test the efficiency of filtration processes but are present in smaller numbers than aerobic spore bearers and are therefore less useful.

In environmental samples such as surface waters a wide range of *Clostridium* species may be present. Most strains of *C. perfringens* grow at 44°C, whereas some other species do not. Incubation at 44°C can therefore improve the specificity for and recovery of *C. perfringens* in such samples and it has been adopted for drinking water in ISO CD 6461 part 2² and by the Standing Committee of Analysts 2004³

Standards for *C. perfringens* are given for drinking water and water sampled from abattoirs⁴, and drinking water in containers. This method is based on the draft method described in ISO 6461 CD part 2².

The method described in the Drinking Water Directive 98/83/EC based on M-CP agar gives very poor recovery of *C. perfringens* and was rejected by ISO in favour of methods based on TSC agar as described here.

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1.0 DEFINITIONS

For the purpose of this method the following definitions apply:

Presumptive *Clostridium perfringens*

Gram positive, anaerobic spore forming rods that grow as black or grey to yellow brown colonies on sulphite containing media at 44°C.

Confirmed *Clostridium perfringens*

Gram positive, anaerobic spore forming rods that produce characteristic colonies on sulphite containing media at 44°C and are non-motile, reduce nitrate, ferment lactose and liquefy gelatine.

2.0 PRINCIPLE

A measured volume of the sample or a dilution of it is filtered through a membrane that is capable of retaining spores of clostridia. The membrane is incubated on a selective/differential agar and characteristic colonies are counted. Confirmatory tests are then carried out and the result is calculated as the colony count per 100 mL of sample volume⁴. If a count of spores alone is required and for non-potable waters with high background counts, the sample is first pre-heated at 60°C to kill vegetative bacteria.

3.0 SAFETY CONSIDERATIONS⁵⁻¹¹

Normal microbiology laboratory precautions apply. In addition care should be taken with placing samples in the waterbath at 60°C and also if a boiling waterbath is used to melt agar (Section 6). Use heat and water resistant gloves and do not put face or hands over the bath when removing objects.

3.1 Sample collection

N/A

3.2 Sample transport and storage

Compliance with current postal and transportation regulations is essential.

3.3 Sample processing

- Some *Clostridium* species are pathogenic, isolation and identification must be performed by trained laboratory personnel in a properly equipped laboratory and under the supervision of a qualified microbiologist
- Care must be taken in the disposal and sterilisation of all test materials

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

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4.0 EQUIPMENT

Usual laboratory equipment and in addition:

- Waterbaths: 45°C ± 1°C and 60°C ± 2°C
- Boiling waterbath maintained at 100°C ± 2°C
- Membrane filtration manifold
- Sterile filter funnels graduated to 100 mL (must be autoclaved before use)
- Sterile stainless steel flat tipped forceps
- Boiling waterbath (instrument steriliser)
- Pyrex vacuum flask with protective jacket: large volume eg 5 L or equivalent
- Vacuum pump with moisture trap or protective filter, or alternative vacuum source
- Incubators: 44°C ± 1.0°C and 36°C ± 2°C
- Facilities for anaerobic incubation
- Petri dishes
- Cellulose ester 0.45 µm pore size gridded filters
- Automatic pipettors and associated sterile pipette tips capable of delivering up to 10 mL and 1 mL volumes (optional)
- Pipettes (sterile total delivery) 10 mL and 1 mL graduated in 0.1 mL volumes (optional)

5.0 CULTURE MEDIA AND REAGENTS

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

Unless otherwise stated chemical constituents are added as anhydrous salts

Peptone saline diluent (Maximum recovery diluent)

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

Quarter strength Ringer's solution

Sodium chloride	2.25 g
Potassium chloride	0.11 g
Calcium chloride, anhydrous	0.12 g
Sodium bicarbonate	0.05 g
Water	1 L

Tryptose sulphite cycloserine agar

Tryptose	15.0 g
Soya peptone	5.0 g
Yeast extract	5.0 g
Sodium metabisulphite	1.0 g
Ferric ammonium citrate	1.0 g
D – cycloserine	0.4 g
Agar	12.0 g
Water	1 L
pH	7.6 ± 0.2 at 25°C

Note. The performance of the medium deteriorates during storage due to exposure to oxygen resulting in reduced growth and colony blackening. Ideally the complete tryptose sulphite cycloserine agar medium (TSCA) should be prepared on the day of use by adding

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cycloserine supplement to melted and cooled (45°C) perfringens agar base. If this is not practicable prepared media should be stored in a refrigerator under anaerobic conditions at 2 – 8°C for not more than 7 days. Petri dishes once removed from the refrigerator should be discarded if not used.

Buffered Nitrate-Motility medium:

Beef extract	3.0 g
Peptone	5.0 g
Potassium nitrate	5.0 g
D-Galactose	5.0 g
Glycerol	5.0 g
Disodium hydrogen phosphate (Na ₂ HPO ₄)	2.5 g
Agar	3.0 g to 5.0 g*
Water	1 L

* Depending on the gel strength of the agar

Dissolve the solid ingredients in 950mL water by heating to boiling point whilst stirring continuously. Dissolve the glycerol in 50 mL water in a separate container and add this to the base medium and mix thoroughly before dispensing and autoclaving.

Nitrate reagent A

Sulphanilic acid	0.8 g
Acetic acid (15% by volume)	100 mL

Nitrate reagent B

5-amino-2-naphthalene-sulfonic acid	0.6 g
Acetic acid (15% by volume)	100 mL

Lactose-gelatine medium:

Enzymatic digest of casein	15 g
Yeast extract	10 g
Gelatine	120 g
Water	1 L
Lactose	10 g
Phenol red (0.4 % w/v solution)	12.5 mL

Blood agar

Columbia agar or any other suitable base with 5% horse blood.

6.0 SAMPLE PROCESSING

6.1 Sample Preparation and dilutions

Water samples should be received and handled as described in SOP: W 1 Section 5.

Protect the samples from direct sunlight and transport in an insulated container or refrigerator at 2°C – 10°C. The nature of the request and condition of the specimen must be noted on arrival. 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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For non-potable waters where spores alone are sought heat a volume of the sample to be tested and maintain at $60^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in a water bath for 15 ± 1 minutes. The time needed to reach this temperature can be determined with a similar bottle containing the same volume of water and a thermometer.

Note: This must **not** be performed for drinking waters (to comply with the “Drinking Water Directive”).

Following the procedures laid down in SOP: W 1 Section 5, select suitable volumes for analysis and prepare any necessary dilutions.

6.2 Filtration and incubation

Prior to filtration the filter funnel should be autoclaved to inactivate any spores remaining after filtration of other samples and which boiling would not have inactivated.

Following the procedures laid down in SOP W1 Section 5; filter a measured volume of sample through the membrane. For potable waters (including bottled waters) it is usual to use volumes of 100 mL.

Place the membrane, grid face upwards, onto the base of a 50mm Petri dish, containing TSCA, ensuring that no air bubbles are trapped under the filter.

For all water samples incubate anaerobically at $44^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

Examine the plates at 21 hours \pm 3 hours. The time taken between placing the membrane on the TSCA and starting incubation should be as short as possible and not exceed 2 hours.

6.3 Counting of colonies

Count all colonies that show a black or grey to yellow brown staining of the TSCA medium when viewed either from above or below the filter. Some colonies may exhibit very faint staining of the medium but should still be counted.

6.4 Confirmatory tests

If more than one volume or dilution has been filtered proceed if possible with the membrane yielding between 20 and 80 colonies.

Select colonies for confirmation as described in SOP W1 Section 5. Subculture each colony to two blood agar (BA) plates. Place one plate in an incubator for aerobic incubation at $36^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and the other in an incubator for anaerobic incubation at the same temperature. Examine the plates after 21 \pm 3 hours for presence or absence of growth and for purity. Perform confirmatory tests on subcultures that only grow anaerobically. Colonies of *C. perfringens* characteristically produce clear zones of haemolysis on blood agar.

Inoculate the following media:

Buffered Nitrate-Motility medium

Immediately prior to use heat the medium in boiling water for 10 - 15 minutes and cool rapidly to set. Inoculate by stabbing into the medium and incubate under anaerobic conditions at $36^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 21 \pm 3 hours. After incubation examine the medium for growth along the line of the stab. Motility is evident as diffuse growth out into the medium away from the stab line.

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Mix equal volumes of nitrate reagents A and B immediately before use and test for the presence of nitrate by adding 0.2 mL to 0.5 mL of this mixture to each tube of Buffered Nitrate-Motility medium. The formation of a red colour confirms the presence of nitrite produced by the reduction of nitrate. If a red colour does not develop within 15 minutes add a small amount of zinc dust and allow to stand for 10 minutes.

If a red colour develops no reduction of nitrate has taken place and the test is considered negative.

If no red colour develops following addition of zinc dust this means no nitrate remains and has been completely converted to nitrogen gas and the test is recorded as positive.

Lactose gelatine medium

Immediately prior to use heat the medium in boiling water for 10 - 15 minutes and then cool rapidly. Inoculate the medium and incubate anaerobically at $36^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 21 ± 3 hours. Examine the tubes for a yellow colour indicating the production of acid. Chill the tubes for 1 - 2 hours at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and check for gelatine liquefaction. The tubes can be examined after 1 hour but any that have not solidified should be returned to the refrigerator for a further hour. If the medium has solidified reincubate at $36^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for an additional 21 ± 3 hours and again check for liquefaction of gelatine.

C. perfringens produces black or grey to yellow brown colonies in TSCA agar, is non-motile, reduces nitrate to nitrite, produces acid from lactose and liquefies gelatine within 44 ± 4 hours.

7.0 CALCULATION OF RESULTS

Calculate the presumptive counts of the test organisms as follows: -

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

where A = the volume as specified to be reported in the appropriate regulations.

Following the procedure described in SOP: W 1 Section 7; calculate the numbers of *C. perfringens* present in the original sample.

8.0 REPORTING

Report the results using the procedure described in SOP: W 1 Section 9

If *C. perfringens* is not detected, report as:

Not detected in 100 mL²

If the test organisms are detected, report as:

a in 100 mL

where a is the confirmed count.

9.0 QUALITY CONTROL

Membrane filtration

When the membrane filtration technique is used internal quality control procedures must be carried out at least once daily depending on the workload of the laboratory. If more than

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one batch of media is used in a session it is necessary to repeat the quality control test for each batch.

The quantitative internal quality controls are to be carried out using suspensions of positive and negative control organisms known to contain between 20 - 80 colony forming units in the volume filtered.

Positive control
C. perfringens NCTC 8237

Negative control
E. coli NCTC 9001

Incubate all tests in parallel with routine tests.

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Flowchart showing the enumeration of *Clostridium perfringens* by membrane filtration

Transport to laboratory at 2°C – 10°C out of direct sunlight in suitable containers



Store at 2°C – 10°C in the dark and analyse as soon as is practicable on the day of collection, otherwise within 24 hours of collection



If spores are sought, heat sample in waterbath at 60°C for 15 minutes
(NOT POTABLE WATER SAMPLES)



Allow to cool



Mix sample well and make any necessary dilutions



Filter



Place membrane on tryptose sulphite cycloserine agar



Incubate anaerobically at 44°C



After 24 hours count black / grey to yellow brown colonies



Subculture to blood agar for confirmation



Incubate aerobically and anaerobically



Perform confirmatory tests



Calculate confirmed counts for *Clostridium perfringens*

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