

# Standing Committee of Analysts

The Microbiology of Recreational and Environmental Waters  
(2016) – Part 12 – Methods for the concentration of enteric  
viruses and the detection and enumeration of enteroviruses  
by suspended cell assay

Methods for the Examination of Waters and Associated Materials



**The Microbiology of Recreational and Environmental Waters (2016) – Part 12 – Methods for the concentration of enteric viruses and the detection and enumeration of enteroviruses by suspended cell assay**

**Methods for the Examination of Waters and Associated Materials**

This booklet contains four methods for the concentration of enteric viruses and one method for the detection and enumeration of enteroviruses.

- A The concentration of viruses from 10 l of water by membrane filtration
- B The concentration of viruses from 10 l of water by filtration using a glass fibre cartridge
- C The concentration of viruses from sand and sediments
- D The concentration of viruses from non filterable liquid samples
- E The detection and enumeration of enteroviruses by the suspended cell plaque assay.

This bluebook updates and replaces Methods for the Isolation and Identification of Human Enteric Viruses from Waters and Associated Materials 1995.

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.

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## 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. This committee was established in 1972 by the Department of the Environment and is now managed by the Standing Committee of Analysts. At present, there are eight 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
- 4 Metals and metalloids
- 5 General non-metallic substances
- 6 Organic impurities
- 7 Biological, biodegradability and inhibition methods
- 8 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 strategic 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 SCA 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

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## 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](http://www.hse.gov.uk/); 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 concentration of viruses from 10 L of water by membrane filtration**

### **A1 Introduction**

This concentration method has been used extensively for enteroviruses and can be used for other enteric viruses<sup>(1)</sup>. Other enteric groups such as adenovirus and norovirus may also be present in wastewater and recreational water<sup>(1, 2)</sup>. Many other methods have been used to concentrate these viruses from water<sup>(3, 4)</sup>. Each method should be optimised for each virus group under study.

Enteroviruses may be found in fresh and marine waters where there has been faecal contamination<sup>(2)</sup>. Poliovirus is now unlikely to be found in environmental water due to the introduction of an inactivated vaccine. Coxsackievirus B and echovirus will circulate in the population with the dominant serotype changing from year to year and are shed in large numbers in faeces particularly in the summer months. The use of enterovirus as a marker of human faecal pollution is well established. Although cultural detection methods remain important, the use of quantitative and qualitative molecular methods is widespread and important for an accurate assessment of the presence of all enteric viruses<sup>(5)</sup>. The significance of enteric viruses in recreational and other waters is described elsewhere<sup>(6)</sup> in this series.

### **A2 Scope**

The method is suitable for the examination of surface waters both fresh and saline, swimming pools, spa and hydrotherapy pools and primary and secondary wastewaters.

Users wishing to employ this method should verify its performance under their own laboratory conditions<sup>(7)</sup>. In particular, the recovery of each virus type from each sample matrix should be verified by this technique.

### **A3 Definitions**

The enteroviruses are members of the large *Enterovirus* genus in the Picornaviridae family and include at least 307 serotypes, of which 66 are human enterovirus serotypes. The taxonomy of the *Enterovirus* genus underwent substantial revision in 2013, and now consists of 12 species made up of 9 *Enterovirus* species and 3 *Rhinovirus* species. The three poliovirus serotypes are now included in the *Enterovirus C* species. Coxsackievirus A serotypes have been assigned to *Enterovirus A* and *C* species, and the echovirus serotypes to the *Enterovirus B* species.

Enteroviruses replicate and form plaques within seven days when grown in or under agar with a range of monkey kidney cells (see section E of this document).

### **A4 Principle**

The water sample is acidified to pH 3.5 to encourage the virus particles to become positively charged and so attach to the negatively charged cellulose nitrate membrane filter as the water is passed through it. The virus particles are then released when an eluent of high protein content and an alkaline pH of 9.5 is passed through the filter. The pH of the eluent is then lowered and a floc forms which contains proteins including viruses. The floc is centrifuged and forms a deposit which is resuspended in buffer<sup>(3)</sup>.

## **A5 Limitations**

The method is suitable for most types of water samples, except those with high turbidity, which tend to block the membrane filter. This will limit the volume of sample that can be filtered with one set of filters. It is not suitable for drinking water as the likely number of viruses is too low.

## **A6 Health and safety**

Media, reagents and viruses used in this method are covered by the Control of Substances Hazardous to Health Regulations<sup>(8)</sup> and appropriate assessments of risk should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere<sup>(7)</sup> in this series.

## **A7 Apparatus**

Standard virus laboratory equipment should be used which conforms to the performance criteria outlined elsewhere<sup>(7)</sup> in this series.

A7.1 Sterile sample bottles of appropriate volume (typically a 10 l plastic drum), made of suitable material, should be used. 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 ( $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ ) per 100 ml of sample, or equivalent may be suitable.

A7.2 Stainless steel pressure vessel (10 litre).

A7.3 PTFE coated metal tubing.

A7.4 Stainless steel connectors.

A7.5 Stainless steel filter holder, 142 mm.

A7.6 Reinforced plastic tubing (8.0 x 12.7 mm) 50-100 cm length.

A7.7 Jubilee clips (13-20 mm).

A7.8 Cordless kettle.

A7.9 Plastic tubing (8.0 x 12.0 mm) 12 cm length.

A7.10 Centrifuge (7,000 x g and 6 x 200 ml capacity).

A7.11 Refrigerator ( $5 \pm 3$  °C).

A7.12 Sterile graduated disposable pipettes (10 ml, 5 ml and 1 ml).

A7.13 Sterile plastic bijoux bottles.

A7.14 Glass beakers (500 ml).

A7.15 Centrifuge pots (300 ml to accommodate 200 ml of processed sample).



A7.16 Measuring cylinder (500 ml).

A7.17 Nitrocellulose filters (142 mm diameter, 0.45 µm).

A7.18 Fibre glass pre-filters (142 mm diameter).

A7.19 Measuring stick (e.g. a 10 ml pipette) with mark at 10 litre height for the sample container.

A7.20 pH meter and appropriate buffers for calibration.

A7.21 Orbital shaker.

## **A8 Reagents**

The performance of all reagents should be verified prior to their use in this method<sup>(7)</sup>. Variations in the preparation and storage of reagents 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 media are not within the stated range they should be adjusted accordingly. Skimmed milk powder and beef extract should be purchased from a commercial laboratory media supplier.

### *A 8.1 Skimmed milk in glycine buffer*

Skimmed milk powder	20 g
Glycine	7.5 g
Water	2 litre

Add the ingredients to the water and mix. It is important that the skim milk powder is fully mixed and this takes at least 2 hours. There is no need to adjust the pH during preparation as the reagents are pH adjusted before use. Distribute the medium in 400 ml volumes into loosely sealed or screw-capped bottles and autoclave at 115 °C for 10 minutes. Sterile media can be stored for one month providing it is kept in the dark at room temperature.

Before use, the skimmed milk buffer needs to be quality controlled. Aseptically remove a 10 ml portion to a sterile plastic universal. Lower the pH of the skim milk buffer with 0.1 M hydrochloric acid to pH 4.5 ± 0.1, checking the pH with a pH meter. A floc should be formed in the skim milk buffer. Should a floc fail to form, the skim milk buffer should be discarded and a fresh batch prepared.

### *A8.2 Beef extract in glycine buffer*

Beef extract	30 g
Glycine	7.5 g
Water	1 litre

Add the ingredients to the water and mix. There is no need to adjust the pH during preparation as the reagents are pH adjusted before use. Distribute the medium in loosely sealed or screw-capped bottles and autoclave at 115 °C for 10 minutes. Sterile media can be stored for one month providing it is kept in the dark at room temperature.

Before use, the beef extract buffer needs to be quality controlled. Aseptically remove a 10 ml portion to a sterile plastic universal. Lower the pH of the beef extract buffer with 0.1 M hydrochloric acid to pH  $3.3 \pm 0.2$ , checking the pH with a pH meter. A floc should be formed in the beef extract buffer. Should a floc fail to form, the beef extract buffer should be discarded and a fresh batch prepared.

#### A8.3 *Disodium hydrogen orthophosphate (0.15 M)*

Disodium hydrogen orthophosphate	21.3 g
Water	1 litre

Add the ingredients to the water and mix. Adjust the pH to  $7.0 \pm 0.2$ . Distribute the medium in loosely sealed or screw-capped bottles and autoclave at 121 °C for 15 minutes. Sterile media can be stored for one month if it is kept in the dark at room temperature.

#### A8.4 Hydrochloric acid (1 M and 0.1 M)

#### A8.5 Sodium hydroxide (1 M)

#### A8.6 Double-distilled or equivalent grade water

### A9 Analytical procedure

#### A9.1 *Preparation of reagents and filter stack*

For each sample, prepare 200 ml of either skimmed milk or beef extract in glycine buffer at pH 9.5 ( $\pm 0.2$ ) by the addition of 1 M sodium hydroxide. Add the measured 10 litre water sample to the pressure vessel. Place the underdrain support and the filter support on top of the filter holder. Prepare the 0.45  $\mu\text{m}$  filter by placing it in a dish of double distilled or equivalent grade water. Once soaked, put the filter on top of the filter support. Take care not to break the filter and to ensure there are no trapped air bubbles. Place the glass fibre pre filter on top of the nitrocellulose filter. Place the top of the filter holder on top and screw it down evenly, taking care to ensure that the top of the holder is level (see Figure A1).

**Figure A1** Membrane filtration adsorption elution equipment



## A9.2 *Conditioning of the water sample*

Adjust the pH of the water sample to pH 3.5 by the addition of 1 M hydrochloric acid. Stir the sample continuously with a measuring stick (A7.19) while adding the acid. Check the pH as acid is added to ensure the pH does not drop below pH 3.5. This may also be done in a collection bottle if more convenient for mixing well.

## A9.3 *Filtration of the water sample*

Place the lid on the pressure vessel and seal tightly. Open the side valve that can be found on the top-plate of the filter holder. Turn on the air line slowly if using compressed air. Alternatively a peristaltic pump may be used. As the pressure increases air will be forced out of the side valve. Once water starts to come out of the side valve close it immediately. Usually a pressure of two bar is sufficient to push the sample through the apparatus. Do not exceed a rate of one litre per minute. Once all the water has passed through to waste, release the pressure on the pressure vessel by opening the air hole. This will enable the lid to be opened.

## A9.4 *Elution*

Add 200 ml of skimmed milk or beef extract in glycine buffer at pH  $9.5 \pm 0.2$  to the pressure vessel and replace the lid. The skimmed milk or beef extract buffer also passes through the vessel under pressure by turning on the air-line or by pumping. As when filtering the 10 l volume, open the valve on the side of the top-plate. Once the buffer starts to elute close the valve on the side of the top-plate and turn the air-line off. Let the buffer trickle out slowly into a sterile glass beaker or centrifuge pot. Approximately 10 minutes or longer is suitable for elution.

## A9.5 *Flocculation of protein*

Once the 200 ml of chosen buffer is deposited in the glass beaker, lower the pH of the buffer solution very carefully to  $4.5 \pm 0.2$  for skimmed milk or  $3.3 \pm 0.2$  for beef extract by the addition of 1M HCl (usually 1.5 - 2 ml is required). Use 0.1 M HCl when the pH is close to pH 4.5 or 3.3. Place the eluent on an orbital shaker and shake for  $15 \pm 1$  minute.

The floc will be visible because the skimmed milk will turn very milky or the beef extract cloudy. Once the floc has formed the sample is transferred to a centrifuge bottle and stored at  $5\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$  until the complete set of samples of the same day are ready. All samples should be stored for at least 30 minutes before centrifugation.

## A9.6 *Centrifugation*

Balance the pots by the addition of flocculated 0.1% skimmed milk or beef extract buffer, load balanced (by weighing) pots opposite each other in the centrifuge. Centrifuge at approximately  $7000 \times g$  for 30 minutes. A visible pellet should be formed when centrifuged. As soon as the centrifuge stops carefully remove the centrifuge pots from the centrifuge.

## A9.7 *Suspension of the pellet*

After centrifugation, very gently pour off and discard the supernatant appropriately for infectious material. Add 8 ml of disodium hydrogen orthophosphate buffer ( $\text{Na}_2\text{HPO}_4$ ) at pH 7.0 to each centrifugation pot. Ensure that the buffer comes into contact with the deposit in the pot to obtain complete solution of the deposit. If the pellet is not dissolving then gently pipette the buffer up and down against the pellet.

Once the deposit has dissolved completely, measure and add buffer to a total volume of 10 ml. Transfer two 5 ml aliquots to sterile plastic bijoux bottles. Store at - 20 ± 5 °C until tested.

Test the processed concentrate by plaque assay or molecular detection if appropriate.

### A9.8 *Cleaning*

Disinfect the glass beakers, centrifugation pots, plastic tubing and measuring sticks by soaking them in a solution of sodium hypochlorite at a concentration of 10,000 mg/l (10% v/v) or by local guidelines appropriate for infectious material. Clean and prepare for use according to local procedures.

Between each filtration and after the last filtration the pressure vessel should be cleaned and sterilized. This may be done according to local procedures using an autoclave. An alternative method is to boil a kettle of water. Undo the top of the filter holder and place the top in a sink. Rinse the pressure vessel once with 2-3 L of hot tap water and discard water. Add the recently boiled very hot water to the pressure vessel, secure the lid and turn the air-line on. This will force the hot water through the pressure vessel, PTFE pressure hose and out through the top of the filter holder into the sink. (WARNING: The temperature of the hose will become very hot).

Discard the filter appropriately for infectious material and then rinse the filter holder and metal filters in generous amounts of hot water, making sure that water passes through the plastic tubing. The outside of the pressure vessel should then be washed with a 10% (v/v) solution of an appropriate detergent and then rinsed with tap water to remove all traces of the detergent. Dry the filter at room temperature.

### A10 **Quality assurance**

New batches of reagents should be tested for activity. In particular new batches of skimmed milk and beef extract must be tested for ability to form a floc at the correct pH. Batches which do not should be discarded.

A virus of known titre should be used to seed a water sample as a positive control and be processed with each batch of water samples. Ideally the recovery rate should be equal to or more than 30%. A negative control of 10 litres of tap water should be processed at the end of each batch of samples.

### A11 **References**

1. Surveillance of adenoviruses and noroviruses in European recreational waters, *Water Research*, A P Wyn-Jones, A Carducci *et al.*, 2011, **45**,1025-1038.
2. Enteric viruses in the aquatic environment, *Journal of Applied Microbiology*, A P Wyn-Jones and J Sellwood, 2001, **91**, 945-962.
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7. 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.
8. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677, The Stationery Office.

## **B The concentration of viruses from 10 L of water by glass fibre cartridge filtration**

### **B1 Introduction**

This concentration method has been used extensively for enteroviruses and can be used for other enteric viruses<sup>(1)</sup>. Other enteric groups such as adenovirus and norovirus may also be present in wastewater and recreational water<sup>(1,2)</sup>. Many other methods have been used to concentrate these viruses from water<sup>(3, 4)</sup>. Each method should be optimised for each virus group under study.

Enteroviruses may be found in fresh and marine waters where there has been faecal contamination<sup>(2)</sup>. Poliovirus is now unlikely to be found in environmental water due to the introduction of an inactivated vaccine. Coxsackievirus B and echovirus will circulate in the population with the dominant serotype changing from year to year and are shed in large numbers in faeces particularly in the summer months. The use of enterovirus as a marker of human faecal pollution is well established. Although cultural detection methods remain important, the use of quantitative and qualitative molecular methods is widespread and important for an accurate assessment of the presence of all enteric viruses<sup>(5)</sup>.

The significance of enteric viruses in recreational and other waters is described elsewhere<sup>(6)</sup> in this series.

### **B2 Scope**

The method is suitable for the examination of surface waters both fresh and saline, swimming pools, spa and hydrotherapy pools and primary and secondary wastewaters.

Users wishing to employ this method should verify its performance under their own laboratory conditions<sup>(7)</sup>. In particular, the recovery of each virus type from each sample matrix should be verified by this technique.

### **B3 Definitions**

The enteroviruses are members of the large *Enterovirus* genus in the Picornaviridae family and include at least 307 serotypes, of which at least 66 are human enterovirus serotypes. The taxonomy of the *Enterovirus* genus underwent substantial revision in 2013, and now consists of 12 species made up of 9 *Enterovirus* species and 3 *Rhinovirus* species. The three poliovirus serotypes are now included in the *Enterovirus C* species. Coxsackievirus A serotypes have been assigned to *Enterovirus A* and *C* species, and the echovirus serotypes to the *Enterovirus B* species. Enteroviruses replicate and form plaques within seven days when grown in or under agar with a range of monkey kidney cells (see section E of this document).

### **B4 Principle**

The water sample is acidified to pH 3.5 to encourage the virus particles to become positively charged and so attach to the negative charged filter cartridge as the water is passed through it. The virus particles are then released when an eluant of high protein content and an alkaline pH of 9.5 is passed through the cartridge. The pH of the eluant is then lowered and a floc forms which contains proteins including viruses. The floc is centrifuged and forms a deposit which is resuspended in buffer<sup>(3)</sup>.

## **B5 Limitations**

The method is suitable for most types of water samples, except those with high turbidity, which tend to block the cartridge filter. This will limit the volume of sample that can be filtered with one set of filters. It is not suitable for drinking water as the likely number of viruses is too low.

## **B6 Health and safety**

Media, reagents and viruses used in this method are covered by the Control of Substances Hazardous to Health Regulations<sup>(8)</sup> and appropriate assessments of risk should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere<sup>(7)</sup> in this series.

## **B7 Apparatus**

Standard virus laboratory equipment should be used which conforms to the performance criteria outlined elsewhere<sup>(7)</sup> in this series.

**B7.1** Sterile sample bottles of appropriate volume (typically a 10 l plastic drum), made of suitable material, should be used. 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 ( $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ ) per 100 ml of sample, or equivalent may be suitable.

**B7.2** Stainless steel pressure vessel (10 litre).

**B7.3** PTFE coated metal tubing.

**B7.4** Stainless steel connectors.

**B7.5** Reinforced plastic tubing (8.0 x 12.7 mm) 50-100 cm length.

**B7.6** Jubilee clips (13-20 mm).

**B7.7** Cordless kettle.

**B7.8** Plastic tubing (8.0 x 12.0 mm) 12 cm length.

**B7.9** Centrifuge (7,000 x g and 6 x 200 ml capacity).

**B7.10** Refrigerator ( $5 \pm 3$  °C).

**B7.11** Sterile graduated disposable pipettes (10 ml, 5 ml and 1 ml).

**B7.12** Sterile plastic bijoux bottles.

**B7.13** Glass beakers (500 ml).

**B7.14** Centrifuge pots (300 ml to accommodate 200 ml of processed sample).

**B7.15** Measuring cylinder (500 ml).

B7.16 Glass fibre filter cartridges (8 or 25 µm nominal pore size).

B7.17 Filter housing.

B7.18 Measuring stick (e.g. a 10 ml pipette) with mark at 10 litre height for the sample container.

B7.19 pH meter and appropriate buffers for calibration.

## **B8 Reagents**

The performance of all reagents should be verified prior to their use in this method<sup>(7)</sup>. Variations in the preparation and storage of reagents 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 media are not within the stated range they should be adjusted accordingly. Skimmed milk powder and beef extract should be purchased from a commercial supplier.

### *B8.1 Skimmed milk in glycine buffer*

Skimmed milk powder	20 g
Glycine	7.5 g
Water	2 litre

Add the ingredients to the water and mix. It is important that the skim milk powder is fully mixed and this takes at least 2 hours. There is no need to adjust the pH during preparation as the reagents are pH adjusted before use. Distribute the medium in 400 ml volumes into loosely sealed or screw-capped bottles and autoclave at 115 °C for 10 minutes. Sterile media can be stored for one month providing it is kept in the dark at room temperature.

Before use, the skimmed milk buffer needs to be quality controlled. Aseptically remove a 10 ml portion to a sterile plastic universal. Lower the pH of the skim milk buffer with 0.1 M HCl to  $4.5 \pm 0.1$ , checking the pH with a pH meter. A floc should be formed in the skim milk buffer. Should a floc fail to form, the skim milk buffer should be discarded and a fresh batch prepared.

### *B8.2 Beef extract in glycine buffer*

Beef extract	30 g
Glycine	7.5 g
Water	1 litre

Add the ingredients to the water and mix. There is no need to adjust the pH during preparation as the reagents are pH adjusted before use. Distribute the medium in loosely sealed or screw-capped bottles and autoclave at 115 °C for 10 minutes. Sterile media can be stored for one month providing it is kept in the dark at room temperature.

Before use, the beef extract buffer needs to be quality controlled. Aseptically remove a 10 ml portion to a sterile plastic universal. Lower the pH of the beef extract buffer with 0.1 M HCl to  $3.3 \pm 0.2$ , checking the pH with a pH meter. A floc should be formed in the beef extract buffer. Should a floc fail to form, the beef extract buffer should be discarded and a fresh batch prepared.



### B8.3 *Disodium hydrogen orthophosphate (0.15 M)*

Disodium hydrogen orthophosphate	21.3 g
Water	1 litre

Add the ingredients to the water and mix. Adjust the pH to  $7.0 \pm 0.2$ . Distribute the medium in loosely sealed or screw-capped bottles and autoclave at  $121\text{ }^{\circ}\text{C}$  for 15 minutes. Sterile media can be stored for one month if it is kept in the dark at room temperature.

### B8.4 Hydrochloric acid (1 M and 0.1 M)

### B8.5 Sodium hydroxide (1 M)

## **B9 Analytical procedure**

### B9.1 *Preparation of reagents and filter housing*

For each sample, prepare 200 ml of skimmed milk or beef extract in glycine buffer at pH 9.5 ( $\pm 0.2$ ), by the addition of 1 M sodium hydroxide. Add the measured 10 l water sample to the pressure vessel. Place the glass fibre cartridge in the housing and secure.

### B9.2 *Conditioning of the water sample*

Adjust the pH of the water sample to pH 3.5 by the addition of 1 M hydrochloric acid. Stir the sample continuously with a measuring stick (B7.18) while adding the acid. Check the pH as acid is added to ensure the pH does not drop below pH 3.5. This may also be done in a collection bottle if more convenient for mixing well.

### B9.3 *Filtration of the water sample*

Place the lid on the pressure vessel and seal tightly. Open the side valve that can be found on the top-plate of the filter holder. Turn on the air line slowly if using compressed air. Alternatively a peristaltic pump may be used. As the pressure increases air will be forced out of the side valve. Once water starts to come out of the side valve close it immediately. Usually a pressure of two bar is sufficient to push the sample through the apparatus. Do not exceed a rate of one litre per minute. Once all the water has passed through, release the pressure on the pressure vessel by opening the air hole. This will enable the lid to be opened.

### B9.4 *Elution*

Add 200 ml of skimmed milk or beef extract in glycine buffer at pH 9.5 ( $\pm 0.2$ ) to the pressure vessel and replace the lid. The skimmed milk or beef extract buffer also passes through the vessel under pressure by turning on the air-line or by pumping. Once the buffer starts to elute close the valve on the side of the top-plate and turn the air-line off. Let the buffer trickle out slowly into a sterile glass beaker or centrifuge pot. Approximately 10 minutes or longer is suitable for elution.

### B9.5 *Flocculation of protein*

Once the 200 ml of buffer is in the glass beaker, lower the pH of the buffer very carefully to  $4.5 \pm 0.2$  for skimmed milk or  $3.3 \pm 0.2$  for beef extract by the addition of 1 M HCl, usually 1.5 - 2 ml

is required. Use 0.1 M HCl when the pH is close to pH 4.5 or 3.3.

The floc will be visible because the skimmed milk will turn very milky or the beef extract turn cloudy. Once the floc has formed the sample is transferred to a centrifuge bottle and stored at  $5\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$  until the complete set of samples of the same day are ready. All samples should be stored for at least 30 minutes before centrifugation.

#### **B9.6** *Centrifugation*

Balance the pots by the addition of flocculated 0.1% skimmed milk or beef extract buffer, load balanced (by weighing) pots opposite each other in the centrifuge. Centrifuge at approximately  $7000 \times g$  for 30 minutes. A visible pellet should be formed when centrifuged. As soon as the centrifuge stops carefully remove the centrifuge pots from the centrifuge.

#### **B9.7** *Suspension of the pellet*

After centrifugation, very gently pour off and discard the supernatant appropriately for infectious material. Add 8 ml of disodium hydrogen orthophosphate buffer ( $\text{Na}_2\text{HPO}_4$ ) at pH 7.0 to each centrifugation pot. Ensure that the buffer comes into contact with the deposit in the pot to obtain complete solution of the deposit. If the pellet is not dissolving then gently pipette the buffer up and down against the pellet.

Once the deposit has dissolved completely, measure and add buffer to a total volume of 10 ml. Transfer two 5 ml aliquots to sterile plastic bijoux bottles. Store at  $-20 \pm 5\text{ }^{\circ}\text{C}$  until tested. Test the processed concentrate by plaque assay or molecular detection if appropriate.

#### **B9.8** *Cleaning*

Disinfect the glass beakers, centrifugation pots, plastic tubing and measuring sticks by soaking them in a solution of sodium hypochlorite at a concentration of 10,000 mg/l (10% v/v) or by local guidelines appropriate for infectious material. Clean and prepare for use according to local procedures. Between each filtration and after the last filtration the pressure vessel and the filter housing should be cleaned and sterilized. This may be done according to local procedures using an autoclave. Alternatively boil a kettle of water and rinse the pressure vessel once with 2 - 3 L of hot tap water and discard water. Add the recently boiled very hot water to the pressure vessel, secure the lid and turn the air-line on. This will force the hot water through the pressure vessel, PTFE pressure hose and out through the filter holder into the sink. (WARNING: The temperature of the hose will become very hot).

Discard the filter cartridge appropriately for infectious material and then rinse the filter holder in generous amounts of hot water, making sure that water passes through the plastic tubing. The outside of the pressure vessel should then be washed with a 10 % (v/v) solution of an appropriate detergent and then rinsed with tap water to remove all traces of the detergent. Dry at room temperature.

### **B10** *Quality assurance*

New batches of reagents should be tested for activity. In particular new batches of skimmed milk and beef extract must be tested for ability to form a floc at the correct pH. Batches which do not should be discarded.

A virus of known titre should be used to seed a water sample as a positive control and be processed with each batch of water samples. Ideally the recovery rate should be equal to or more than 30 %. A negative control of 10 litres of tap water should be processed at the end of each batch of samples.

## **B11 References**

1. Surveillance of adenoviruses and noroviruses in European recreational waters, *Water Research*, A P Wyn-Jones, A Carducci *et al.*, 2011, **45**,1025-1038.
2. Enteric viruses in the aquatic environment, *Journal of Applied Microbiology*, A P Wyn-Jones and J Sellwood, 2001, **91**, (6), 945-962.
3. Analytical methods for virus detection in water and food, *Food Analytical Methods*, A Bosch, G Sanchez *et al.*, 2011, **4**, 4-12.
4. VIROCLIME - Impact of climate change on the transport, fate and risk management of viral pathogens in water. European Commission Research Framework 7: 2013 ([www.viroclime.org](http://www.viroclime.org)).
5. International standardisation of a method for the detection of human pathogenic viruses in molluscan shellfish, *Food and Environmental Virology*, D Lees, 2010, **2**, 146-155.
6. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2014) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
7. 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*, in this series, Environment Agency. (currently under revision).
8. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677, The Stationery Office.

## **C The concentration of viruses from sand and sediment samples**

### **C1 Introduction**

This concentration method has been used extensively for enteroviruses and can be used for other enteric viruses<sup>(1)</sup>. Other enteric groups such as adenovirus and norovirus may also be present in wastewater and recreational water<sup>(1, 2)</sup>. Many other methods have been used to concentrate these viruses from water<sup>(3, 4)</sup>. Each method should be optimised for each virus group under study.

Enteroviruses may be found in fresh and marine waters where there has been faecal contamination<sup>(2)</sup>. Poliovirus is now unlikely to be found in environmental water due to the introduction of an inactivated vaccine. Coxsackievirus B and echovirus will circulate in the population with the dominant serotype changing from year to year and are shed in large numbers in faeces particularly in the summer months. The use of enterovirus as a marker of human faecal pollution is well established. Although cultural detection methods remain important, the use of quantitative and qualitative molecular methods is widespread and important for an accurate assessment of the presence of all enteric viruses<sup>(5)</sup>. The significance of enteric viruses in recreational and other waters is described elsewhere<sup>(6)</sup> in this series.

### **C2 Scope**

The method is suitable for the examination of sands and sediments.

Users wishing to employ this method should verify its performance under their own laboratory conditions<sup>(7)</sup>. In particular, the recovery of each virus type from each sample matrix should be verified by this technique.

### **C3 Definitions**

The enteroviruses are members of the large *Enterovirus* genus in the Picornaviridae family and include at least 307 serotypes, of which at least 66 are human enterovirus serotypes. The taxonomy of the *Enterovirus* genus underwent substantial revision in 2013, and now consists of 12 species made up of 9 *Enterovirus* species and 3 *Rhinovirus* species. The three poliovirus serotypes are now included in the *Enterovirus C* species. Coxsackievirus A serotypes have been assigned to *Enterovirus A* and *C* species, and the echovirus serotypes to the *Enterovirus B* species. Enteroviruses replicate and form plaques within seven days when grown in or under agar with a range of monkey kidney cells (see section E of this document).

### **C4 Principle**

The sample is acidified to pH 3.5 to encourage virus particles to attach to the matrix. The sample is mixed gently on a magnetic stirrer. The sample is centrifuged to deposit virus and matrix. The virus particles are then released when an eluant of high protein content alkaline pH of 9.5 is mixed with the deposit. The sample is centrifuged a second time to remove debris, leaving virus particles in the supernatant. The pH of the eluant is then lowered and a floc forms which contains proteins including viruses. The floc is centrifuged and forms a deposit which is resuspended in buffer<sup>(3)</sup>.

### **C5 Limitations**

The method is suitable sand and sediment which cannot be processed by filtration.

## C6 Health and safety

Media, reagents and viruses used in this method are covered by the Control of Substances Hazardous to Health Regulations<sup>(8)</sup> and appropriate assessments of risk should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere<sup>(7)</sup> in this series.

## C7 Apparatus

Standard virus laboratory equipment should be used which conforms to the performance criteria outlined elsewhere<sup>(7)</sup> in this series.

C7.1 Sterile sample containers of appropriate volume, made of suitable material, should be used.

C7.2 Centrifuge (10,000 x g and 6 x 200 ml capacity).

C7.3 Refrigerator ( $5 \pm 3$  °C).

C7.4 Sterile graduated disposable pipettes (10 ml, 5 ml and 1 ml).

C7.5 Sterile plastic bijoux bottles.

C7.6 Glass beakers (500 ml).

C7.7 Centrifuge pots (300 ml to accommodate 200 ml of processed sample).

C7.8 Measuring cylinder (500 ml).

C7.9 Magnetic stirrer.

C7.10 pH meter and appropriate buffers for calibration.

C7.11 Orbital shaker

## C8 Reagents

The performance of all reagents should be verified prior to their use in this method<sup>(7)</sup>. Variations in the preparation and storage of reagents 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 media are not within the stated range, then, they should be adjusted accordingly. Skimmed milk powder and beef extract should be purchased from a commercial supplier.

### C8.1 *Skimmed milk in glycine buffer*

Skimmed milk powder	20 g
Glycine	7.5 g
Water	2 litre

Add the ingredients to the water and mix. It is important that the skim milk powder is fully mixed and this takes at least 2 hours. There is no need to adjust the pH during preparation as the reagents are pH adjusted before use. Distribute the medium in 400 ml volumes into loosely sealed or screw-capped bottles and autoclave at 115 °C for 10 minutes. Sterile media can be stored for one month providing it is kept in the dark at room temperature.

Before use, the skimmed milk buffer needs to be quality controlled. Aseptically remove a 10 ml portion to a sterile plastic universal. Lower the pH of the skim milk buffer with 0.1 M HCl to  $4.5 \pm 0.1$ , checking the pH with a pH meter. A floc should be formed in the skim milk buffer. Should a floc fail to form, the skim milk buffer should be discarded and a fresh batch prepared.

#### C8.2 *Beef extract in glycine buffer*

Beef extract	30 g
Glycine	7.5 g
Water	1 litre

Add the ingredients to the water and mix. There is no need to adjust the pH during preparation as the reagents are pH adjusted before use. Distribute the medium in loosely sealed or screw-capped bottles and autoclave at 115 °C for 10 minutes. Sterile media can be stored for one month providing it is kept in the dark at room temperature.

Before use, the beef extract buffer needs to be quality controlled. Aseptically remove a 10 ml portion to a sterile plastic universal. Lower the pH of the beef extract buffer with 0.1 M HCl to  $3.3 \pm 0.2$ , checking the pH with a pH meter. A floc should be formed in the beef extract buffer. Should a floc fail to form, the beef extract buffer should be discarded and a fresh batch prepared.

#### C8.3 *Disodium hydrogen orthophosphate (0.15 M)*

Disodium hydrogen orthophosphate	21.3 g
Water	1 litre

Add the ingredients to the water and mix. Adjust the pH to  $7.0 \pm 0.2$ . Distribute the medium in loosely sealed or screw-capped bottles and autoclave at 121 °C for 15 minutes. Sterile media can be stored for one month if it is kept in the dark at room temperature.

#### C8.4 Hydrochloric acid (1 M and 0.1 M)

#### C8.5 Sodium hydroxide (1 M)

### C9 Analytical procedure

#### C9.1 *Preparation of reagents*

For each sample, prepare 50 ml skimmed milk or beef extract in glycine buffer at  $\text{pH } 9.5 \pm 0.2$  by the addition of 1 M sodium hydroxide.

#### C9.2 *Adsorption of viruses to particulate material*

Weigh out 50 g of sample into a sterile clean beaker. Add 50 ml of glycine buffer  $\text{pH } 9.5 \pm 0.2$ . Place on a magnetic stirrer and mix gently. Adjust the pH to 3.5 and continue stirring gently for

30 minutes at room temperature. Transfer to centrifuge pots and centrifuge at 10,000 x g for 30 minutes.

### C9.3 *Elution*

Discard the supernatant and add 50 ml of either skimmed milk or beef extract in glycine buffer at pH  $9.5 \pm 0.2$  to resuspend the pellet, mixing thoroughly on the magnetic stirrer for 30 minutes. Transfer to centrifuge pots and centrifuge at 10,000 x g for 30 minutes. Transfer the supernatant to a sterile beaker and discard the pellet.

### C9.4 *Flocculation of protein*

Once the 50 ml of buffer is in the glass beaker, lower the pH of the buffer very carefully to  $4.5 \pm 0.2$  for skimmed milk or  $3.3 \pm 0.2$  for beef extract by the addition of 1 M HCl, usually 1.5 - 2 ml is required. Use 0.1 M HCl when the pH is close to pH 4.5 or 3.3. Place the eluent on an orbital shaker and shake for  $15 \pm 1$  minute.

The floc will be visible because the skimmed milk will turn very milky or the beef extract cloudy. Once the floc has formed the sample is transferred to a centrifuge bottle and stored at  $5 \text{ }^\circ\text{C} \pm 3 \text{ }^\circ\text{C}$  until the complete set of samples of the same day are ready. All samples should be stored for at least 30 minutes before centrifugation.

### C9.5 *Centrifugation*

Balance the pots (by weighing) by the addition of flocculated 0.1% skimmed milk or beef extract buffer, load balanced pots opposite each other in the centrifuge. Centrifuge at approximately 7000 x g for 30 minutes. A visible pellet should be formed when centrifuged. As soon as the centrifuge stops carefully remove the centrifuge pots from the centrifuge.

### C9.6 *Suspension of the pellet*

After centrifugation, very gently pour off and discard the supernatant appropriately for infectious material. Add 8 ml of disodium hydrogen orthophosphate buffer ( $\text{Na}_2\text{HPO}_4$ ) at pH 7.0 to each centrifugation pot. Ensure that the buffer comes into contact with the deposit in the pot to obtain complete solution of the deposit. If the pellet is not dissolving then gently pipette the buffer up and down against the pellet.

Once the deposit has dissolved completely, measure and add buffer to a total volume of 10 ml. Transfer two 5 ml aliquots to sterile plastic bijoux bottles. Store at  $-20 \pm 5 \text{ }^\circ\text{C}$  freezer until tested.

Test the processed concentrate by plaque assay or molecular detection if appropriate.

### C9.8 *Cleaning*

Disinfect the glass beakers and centrifugation pots by soaking them in a solution of sodium hypochlorite at a concentration of 10,000 mg/l (10% v/v) or by local guidelines appropriate for infectious material. Clean and prepare for use according to local procedures.

Discard the sediment appropriately for infectious material.

## C10 Quality assurance

New batches of reagents should be tested for activity. In particular new batches of skimmed milk and beef extract must be tested for ability to form a floc at the correct pH. Batches which do not should be discarded.

A virus of known titre should be used to seed an equivalent matrix as a positive control and be processed with each batch of water samples.

## C11 References

1. Surveillance of adenoviruses and noroviruses in European recreational waters, *Water Research*, A P Wyn-Jones, A Carducci *et al.*, 2011, **45**,1025-1038.
2. Enteric viruses in the aquatic environment, *Journal of Applied Microbiology*, A P Wyn-Jones and J Sellwood, 2001, **91**, (6), 945-962.
3. Analytical methods for virus detection in water and food, *Food Analytical Methods*, A Bosch, G Sanchez *et al.*, 2011, **4**, 4-12.
4. VIROCLIME - Impact of climate change on the transport, fate and risk management of viral pathogens in water. European Commission Research Framework 7: 2013 ([www.viroclime.org](http://www.viroclime.org)).
5. International standardisation of a method for the detection of human pathogenic viruses in molluscan shellfish, *Food and Environmental Virology*, D Lees, 2010, **2**, 146-155.
6. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2014) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
7. 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
8. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677, The Stationery Office.



## **D The concentration of viruses from non filterable liquid samples**

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**

This concentration method has not been used extensively for enteroviruses but a similar method was evaluated using a limited number of samples in a European study<sup>(1)</sup>.

Enteroviruses may be found in fresh and marine waters where there has been faecal contamination. Poliovirus is now unlikely to be found in environmental water due to the introduction of an inactivated vaccine. Coxsackievirus B and echovirus will circulate in the population with the dominant serotype changing from year to year and are shed in large numbers in faeces particularly in the summer months. The use of enterovirus as a marker of human faecal pollution is well established. Although cultural detection methods remain important, the use of quantitative and qualitative molecular methods is widespread and important for an accurate assessment of the presence of all enteric viruses<sup>(2)</sup>. The significance of enteric viruses in recreational and other waters is described elsewhere<sup>(3)</sup> in this series.

### **D2 Scope**

The method is suitable for the examination of untreated sewage and sewage effluents.

Users wishing to employ this method should verify its performance under their own laboratory conditions<sup>(4)</sup>. In particular, the recovery of each virus type from each sample matrix should be verified by this technique.

### **D3 Definitions**

The enteroviruses are members of the large *Enterovirus* genus in the Picornaviridae family and include at least 307 serotypes, of which at least 66 are human enterovirus serotypes. The taxonomy of the *Enterovirus* genus underwent substantial revision in 2013, and now consists of 12 species made up of 9 *Enterovirus* species and 3 *Rhinovirus* species. The three poliovirus serotypes are now included in the *Enterovirus C* species. Coxsackievirus A serotypes have been assigned to *Enterovirus A* and *C* species, and the echovirus serotypes to the *Enterovirus B* species. Enteroviruses replicate and form plaques within seven days when grown in or under agar with a range of monkey kidney cells (see section E of this document).

### **D4 Principle**

The sample is mixed with an eluant of high protein content on an orbital shaker which releases any bound virus particles into suspension. The preparation is then ultrasonicated, and centrifuged and the supernatant collected. The pH of the supernatant is then lowered and a floc forms which contains proteins including virus. The floc is deposited by centrifugation and resuspended in neutral pH buffer<sup>(1)</sup>.

## D5 Limitations

The method is suitable for processing non-filterable samples (e.g. untreated sewage) which cannot be processed by filtration.

## D6 Health and safety

Media, reagents and viruses used in this method are covered by the Control of Substances Hazardous to Health Regulations<sup>(5)</sup> and appropriate assessments of risk should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere<sup>(4)</sup> in this series.

## D7 Apparatus

Standard virus laboratory equipment should be used which conforms to the performance criteria outlined elsewhere<sup>(4)</sup> in this series.

D7.1 Sterile sample bottles of appropriate volume, made of suitable material, should be used.

D7.2 Centrifuge (10,000 x g and 6 x 500 ml capacity).

D7.3 Refrigerator ( $5 \pm 3$  °C).

D7.4 Sterile graduated disposable pipettes (10 ml, 5 ml and 1 ml).

D7.5 Sterile plastic bijoux bottles.

D7.6 Lidded centrifuge pots (500 ml).

D7.7 Measuring cylinder (100 ml).

D7.8 Ultrasonic water bath (35-45kHz)

D7.9 pH meter and appropriate buffers for calibration.

## D8 Reagents

The performance of all reagents should be verified prior to their use in this method<sup>(4)</sup>. Variations in the preparation and storage of reagents 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 media are not within the stated range, then, they should be adjusted accordingly. Beef extract should be purchased from a commercial supplier.

### D8.1 *Beef extract (3 %) in glycine buffer*

Beef extract	30 g
Glycine	7.5 g
Water	1 litre

Add the ingredients to the water and mix. There is no need to adjust the pH during preparation as the reagents are pH adjusted before use. Distribute the medium in loosely sealed or screw-capped bottles and autoclave at  $115 \pm 3$  °C for  $10 \pm 1$  minutes. Sterile media can be stored for six months providing it is kept in the dark at room temperature.

Before use, the beef extract buffer needs to be quality controlled. Aseptically remove a 10 ml portion to a sterile plastic universal. Lower the pH of the beef extract buffer with 0.1 M HCl to  $3.3 \pm 0.2$ , checking the pH with a pH meter. A floc should be formed in the beef extract buffer. Should a floc fail to form, the beef extract buffer should be discarded and a fresh batch prepared.

#### D8.2 *Beef extract (30 %) in glycine buffer*

Beef extract	300 g
Glycine	7.5 g
Water	1 litre

Add the ingredients to the water and mix. There is no need to adjust the pH during preparation as the reagents are pH adjusted before use. Distribute the medium in loosely sealed or screw-capped bottles and autoclave at  $115 \pm 3$  °C for  $10 \pm 1$  minutes. Sterile media can be stored for six months providing it is kept in the dark at room temperature.

Before use, the beef extract buffer needs to be quality controlled. Aseptically remove a 10 ml portion to a sterile plastic universal. Lower the pH of the beef extract buffer with 0.1 M HCl to  $3.3 \pm 0.2$ , checking the pH with a pH meter. A floc should be formed in the beef extract buffer. Should a floc fail to form, the beef extract buffer should be discarded and a fresh batch prepared.

#### D8.3 *Disodium hydrogen orthophosphate (0.15 M)*

Disodium hydrogen orthophosphate	21.3 g
Water	1 litre

Add the ingredients to the water and mix. Adjust the pH to  $7.0 \pm 0.2$ . Distribute the medium in loosely sealed or screw-capped bottles and autoclave at  $121 \pm 3$  °C for  $15 \pm 1$  minutes. Sterile media can be stored for six months if it is kept in the dark at room temperature.

D8.4 Hydrochloric acid (1 M and 0.1 M).

D8.5 Sodium hydroxide solution (1 M).

D8.6 Double distilled or equivalent grade water.

### **D9 Analytical procedure**

#### D9.1 *Preparation of ultrasonic water bath*

De-gas an ultrasonic bath (filled to above the shoulder with chilled double distilled or equivalent grade water) by ultrasonication for  $30 \pm 1$  minutes.

#### D9.2 *Elution of viruses from non-filterable liquid samples*

Weigh / measure out  $200 \pm 1$  ml ( $100 \pm 1$  ml for highly turbid effluents or water samples) into a 500 ml centrifuge pot using a balance or a sterile pipette.

For 200 ml effluent samples add  $22.2 \text{ ml} \pm 1.0 \text{ ml}$  of 30 % beef extract solution (D8.2). For 100 ml highly turbid samples add  $11.1 \text{ ml} \pm 1.0 \text{ ml}$  of 30 % beef extract solution (D8.2).

Place the samples on a shaker (in the dark) and shake for  $15 \pm 1$  minutes.

Remove from shaker and place in an ultrasonic water bath filled with degassed water. Sonicate for  $30 \pm 1$  minute.

Accurately balance samples to within 0.1 g using sterile 3 % beef extract solution (D8.1).

Centrifuge samples at  $10,000 \times g$  at  $4 \text{ }^\circ\text{C}$  for 20 minutes.

Carefully collect the supernatant in another 500ml lidded centrifuge pot and discard the pellet. Store at  $5 \pm 3^\circ\text{C}$  until ready for flocculation.

### D9.3 *Flocculation of protein*

Balance the samples ( $\pm 1\text{g}$  for each centrifuge pair) using sterile 3 % beef extract.

Adjust the supernatant eluents to  $\text{pH } 3.3 \pm 0.2$  using 1N or 0.1N HCl. Accurately balance the samples to within 0.1 g using sterile double deionised water. The pH probe should be sterilised between samples by placing into a water bath held at  $70 \pm 2 \text{ }^\circ\text{C}$  for  $20 \pm 10$  seconds.

Place the samples on a shaker for  $10 \pm 1$  minutes.

### D9.4 *Centrifugation*

Load the balanced pots opposite each other in the centrifuge. Centrifuge at  $7000 \times g$  at  $4 \text{ }^\circ\text{C}$  for 20 minutes. A visible pellet should be formed when centrifuged. As soon as the centrifuge stops, remove the centrifuge pots from the centrifuge.

### D9.5 *Suspension of the pellet*

After centrifugation, carefully pour off supernatant and redissolve the pellet in 5 - 5.5 ml of 0.15 M disodium hydrogen orthophosphate solution. Ensure that the buffer comes into contact with the deposit in the pot to obtain complete solution of the deposit. If the pellet is not dissolving then gently pipette the buffer up and down against the pellet, but avoid generating bubbles. It may help the dissolution of the pellet if the disodium hydrogen orthophosphate solution is left in contact with the pellet at room temperature for 30 - 60 min, and then gently resuspended to dissolve. Mix well by placing on the shaker.

Transfer the concentrate into either a 7ml bijou bottle or a disposable universal bottle, recording the volume of concentrate on the bottle and store at  $-70 \text{ }^\circ\text{C} \pm 10 \text{ }^\circ\text{C}$  to await assay.

D9.6 Test the processed concentrate by plaque assay or molecular detection as appropriate.

### D9.7 *Cleaning*

Disinfect the glass beakers and centrifugation pots by soaking them in a solution of sodium hypochlorite at a concentration of 10,000 mg/l (10% v/v) or by local guidelines appropriate for infectious material. Clean and prepare for use according to local procedures.

Discard the sample appropriately for infectious material.

## D10 Quality assurance

New batches of reagents should be tested for activity. In particular new batches of beef extract must be tested for ability to form a floc at the correct pH. Batches which do not should be discarded.

A virus of known titre should be used to seed an equivalent matrix as a positive control and be processed with each batch of water samples.

## D11 References

1. VIROCLIME - Impact of climate change on the transport, fate and risk management of viral pathogens in water. European Commission Research Framework 7: 2013 ([www.viroclime.org](http://www.viroclime.org)).
2. International standardisation of a method for the detection of human pathogenic viruses in molluscan shellfish, *Food and Environmental Virology*, D Lees, 2010, **2**, 146-155.
3. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2014) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
4. 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
5. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677, The Stationery Office.

## **E The detection and enumeration of enteroviruses by suspended cell plaque assay**

### **E1 Introduction**

Enterovirus infection of man occurs all year round with a seasonal peak in the summer and autumn. The virus is species specific and replicates primarily in the gastro-intestinal tract. Most infections are asymptomatic but some may result in a flu-like illness that in a minority of cases leads to meningitis or paralysis. Gastro-enteritis may occur as part of wider systemic disease. The virus infects all ages but is particularly common in children. Many serotypes exist and the most common serotypes change from year to year.

Like all other viruses, enteroviruses are obligate intracellular parasites and will not replicate in the environment. Enteroviruses have been used as a marker for the presence of human faecal pollution for many years because the group was the first and still remains the most readily detectable human viruses in environmental samples.

BGM (Buffalo Green Monkey) cells have been shown to be sensitive to the infection of enterovirus including poliovirus and Coxsackievirus B<sup>(1, 2, 3)</sup> and are in widespread use throughout the world.

**WARNING — Enteroviruses are pathogens capable of causing illness. All samples and controls should be handled by trained staff in a laboratory with appropriate equipment. Staff should be fully vaccinated against poliovirus. Persons using this standard should be familiar with normal virology laboratory practice. This method does not purport to address fully all of the safety issues associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.**

### **E2 Scope**

This method is for the detection of those serotypes of enterovirus that replicate and form plaques in BGM cells under agar. These will largely be poliovirus and Coxsackievirus B serotypes. Most serotypes of echovirus, Coxsackievirus A and animal enteroviruses are unlikely to produce plaques under these conditions. Poliovirus is now unlikely to be found in environmental water due to the introduction of killed vaccine. Reoviruses are unlikely to be detectable within the specified seven day incubation period. It is applicable to any type of water and processed water sample although toxic elements may interfere with cell culture.

The method is suitable for the detection of enteroviruses in unprocessed wastewater or processed concentrates as described in sections A, B, C and D. Viruses can be detected at one plaque forming unit (pfu) per ml of sample.

Basic cell culture procedures including the preparation, maintenance and enumeration of BGM cell culture are not described but suggestions are made for media that may be used (see Appendix 1).

### **E3 Definitions**

The enteroviruses are members of the large *Enterovirus* genus in the Picornaviridae family and include at least 307 serotypes, of which at least 66 are human enterovirus serotypes. The taxonomy of the *Enterovirus* genus underwent substantial revision in 2013, and now consists of 12 species made up of 9 *Enterovirus* species and 3 *Rhinovirus* species. The three poliovirus

serotypes are now included in the *Enterovirus C* species. Coxsackievirus A serotypes have been assigned to *Enterovirus A* and C species, and the echovirus serotypes to the *Enterovirus B* species. Enteroviruses replicate and form plaques within seven days when grown in or under agar with a range of monkey kidney cells.

#### **E4 Principle**

A suspension of BGM cells is mixed with 1 ml of sample or sample concentrate. The mixture is added to warm agar in cell culture growth medium and poured into a Petri dish. The agar solidifies and the dishes are incubated in CO<sub>2</sub> atmosphere for up to seven days. The plaques are counted. The plaques which are areas of cell death can be visualised more easily by the addition of a vital stain such as neutral red to the agar medium. One infectious unit of virus particles is assumed to form one discreet plaque. Confirmation of the presence of virus particles in plaques can be undertaken by molecular methods.

#### **E5 Limitations**

Toxicity may be seen to be produced by some samples which may require dilution. Where high numbers of virus may be expected, serial ten-fold dilutions should be made to obtain a countable number of plaques. The maximum number of plaques that can be reliably counted in a dish is approximately 100.

#### **E6 Health and safety**

Media, reagents and viruses used in this method are covered by the Control of Substances Hazardous to Health Regulations<sup>(4)</sup> and appropriate assessments of risk should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere<sup>(5)</sup> in this series.

#### **E7 Apparatus**

Standard virus laboratory equipment should be used which conforms to the performance criteria outlined elsewhere<sup>(5)</sup> in this series. All plastic ware and glassware should be sterile.

Except for disposable glassware which is delivered sterile, glassware should be sterilised at  $170 \pm 10$  °C for at least 1 hour in a dry oven or at  $121 \pm 3$  °C for at least 15 minutes in an autoclave before use.

E7.1 Incubator capable of being maintained at  $37 \pm 1$  °C, with 5 % (volume fraction) CO<sub>2</sub> supply if Petri dishes are used.

E7.2 Water bath capable of maintaining  $45 \pm 2$  °C.

E7.3 Boiling water bath.

E7.4 Refrigerator capable of maintaining  $5 \pm 3$  °C.

E7.5 Freezer capable of maintaining  $-20 \pm 5$  °C.

E7.6 Petri dishes sterile, bacteriological grade, diameter 90 mm or 60 mm triple vented.

E7.7 Tissue culture flasks sterile, volume 25 cm<sup>2</sup>, 75 cm<sup>2</sup>, 80 cm<sup>2</sup>.

- E7.8 Pipettes sterile, graduated, volumes 1 ml and 10 ml.
- E7.9 Micropipette with sterile pipette tips 1 ml to 5 ml.
- E7.10 Measuring cylinder sterile, volume 50 ml.
- E7.11 Vertical laminar air-flow cabinet for cell culture procedures.
- E7.12 Liquid nitrogen optional for cell culture storage facility.
- E7.13 Inverted microscope for examining and counting cells.
- E7.14 Cell counting chamber, for example improved Neubauer.

## E8 Media and reagents

The performance of all reagents should be verified prior to their use in this method<sup>(5)</sup>. Variations in the preparation and storage of reagents 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 media is not within the stated range, then, they should be adjusted accordingly.

Examples of cell culture media are given in Appendix E1.

### E8.1 3 % Agar

Purified Agar Powder	15 g
Water	500 ml

Dissolve the agar in the water by boiling. Keep the molten agar in the  $45 \pm 2$  °C water bath and dispense in approximate 100 ml aliquots into clean glass bottles. Autoclave at  $115 \pm 3$  °C for 10 minutes. Store at room temperature and use within three months.

### E8.2 Overlay Medium - Overlay Medium (Double Strength)

Constituent	Volume of Overlay Media				Final Concentrations
	100 ml	200 ml	300 ml	400 ml	
Sterile water	60 ml	120 ml	180 ml	240 ml	
10x 199	20 ml	40 ml	60 ml	80 ml	2 x
FCS/NCS	6 ml	12 ml	18 ml	24 ml	6 %
Sodium bicarbonate	10 ml	20 ml	30 ml	40 ml	52 mM
Penicillin/ streptomycin	2 ml	4 ml	6 ml	8 ml	200 units/ml 20 mg/ml
Gentamicin	1 ml	2 ml	3 ml	4 ml	40 mg/ml
Nystatin	1 ml	2 ml	3 ml	4 ml	100 units/ml
Magnesium chloride	1 ml	2 ml	3 ml	4 ml	200 mg/ml
Neutral red	1 ml	2 ml	3 ml	4 ml	10 mg/ml

Store at  $5 \pm 3$  °C for up to one week.



### E8.3 *Other media*

Other media should be suitable for the culture and maintenance of BGM cells (see Appendix 1).

## **E9 Analytical procedure**

### E9.1 *Preparation of cell suspension*

Prepare a suspension of BGM cells in growth medium in a single container according to standard procedures<sup>(1)</sup>. Count the number of cells and adjust volume such that the cell numbers are  $1 \times 10^7$  cells per ml.

### E9.2 *Plaque assay*

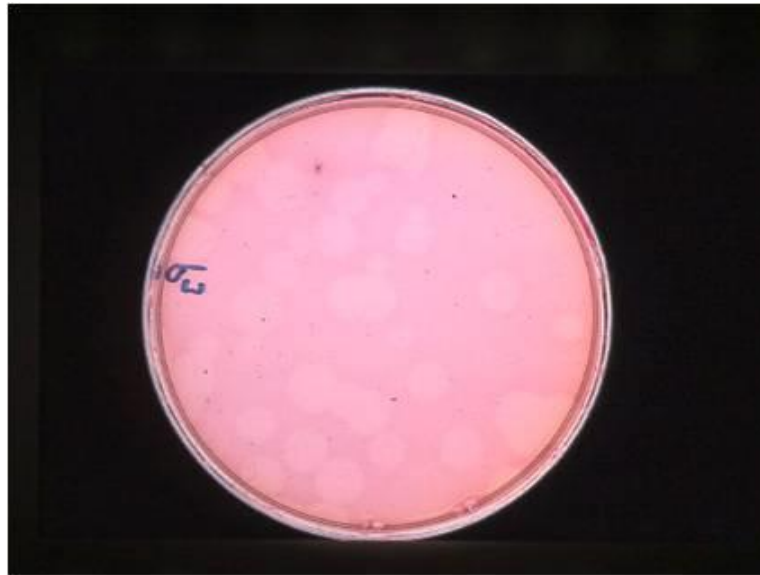
Melt enough agar for each sample (5 ml for each sample) and then place in a water bath at  $45 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$ . Prepare enough overlay medium for each sample (5 ml for each sample). Aliquot 5 ml volumes of overlay medium into sterile plastic universals and then place in the  $45 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$  water bath. Allow a minimum of 30 minutes for both the media and agar to equilibrate to the temperature of the water bath.

After samples have been defrosted place in the  $5 \pm 3 \text{ }^\circ\text{C}$  refrigerator until used. The maximum time that the samples should be kept in the refrigerator is one hour. Label sterile Petri dishes for each sample. Using a sterile graduated disposable pipette add 5 ml volumes of the molten agar into the 5 ml volumes of overlay medium, making sure that the agar is not cooled sufficiently for it to set. Leave in the  $45 \pm 2 \text{ }^\circ\text{C}$  water bath until needed.

For each sample mix 2 ml of cell suspension and 2 ml of the concentrate, the cell/virus mixture may be allowed to stand at room temperature for 30 minutes to allow the virus to enter the cells. In dim light, to reduce photo-inactivation of virus by neutral red, add the cell/virus mixture to the media and agar and gently mix. After adding the cells and virus to the media and agar and mixing, immediately pour the contents of the universal into the appropriate labelled Petri dish.

After the plate has been poured place in a dark area to dry. Repeat for each Petri dish. Once dry, the Petri dishes are placed upside down, to prevent formation of condensation on the agar, in a humidified incubator at  $37 \pm 1 \text{ }^\circ\text{C}$  with  $5 \pm 2 \text{ } \%$   $\text{CO}_2$ . Plates are incubated for up to 7 days and plaques can be counted from day 2 to day 7. Clear areas in the agar are marked and counted as plaques (see Figure E1). Record the number of new plaques for each day. Confirmation of virus particles in plaques can be undertaken by molecular methods

**Figure E1** Plaques of enterovirus in a suspended cell assay using BGM cells



### **E10 Calculation**

After day seven the total number of plaque forming units (pfu) seen per plate and therefore per 2 ml of concentrate is calculated. The total volume of concentrate produced by processing 10 L of sample should be assayed to estimate as accurately as possible the total number of infectious enterovirus plaque forming units in the original sample.

### **E11 Expression of results**

The number of enteroviruses are expressed in plaque forming units per volume of sample. For most samples the volume is typically 10 L.

### **E12 Quality assurance**

A positive control sample should be included in every assay. This consists of a suspended cell assay inoculated with a known amount of virus plaque forming units. The tolerances for the QC sample is  $\pm 30\%$  of the plaque count obtained after the virus has been dispensed into vials and frozen. This tolerance should be revised to reflect actual laboratory performance once sufficient data has been obtained.

### **E13 References**

1. Optimization of the BGM cell line and viral assay procedures for monitoring viruses in the environment, *Applied and Environmental Microbiology*, D R Dahling and B A Wright, 1986, **51**, 790-812.
2. Optimisation of suspended cell method and comparison with cell monolayer technique for virus assays, *Journal of Virological Methods*, D R Dahling and B A Wright, 1988, **20**, 169-179.
3. Detection of enteroviruses: an assessment of ten cell lines, *Water Science and Technology*, R Morris, 1985 **17**, 81-88.
4. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677, The Stationery Office.

5. 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*, in this series, Environment Agency. (currently under revision)

## Appendix E1 Suggested media for cell culture components

### 1 BGM Growth Medium

Total volume	100 ml	500 ml	Final concentration
Sterile distilled, deionised or of similar grade water	79.5	397.5	
MEM (10x)	10	50	1 x
Foetal Calf Serum (FCS)	5	25	5 %
L-glutamine (0,2 M)	1.5	7.5	
Sodium Bicarbonate (NaHCO <sub>3</sub> ) (4,4 %)	2.5	12.5	
Penicillin / Streptomycin (10 000 units ml <sup>-1</sup> )	1	5	100 units ml <sup>-1</sup>
Penicillin / Streptomycin (10 mg ml <sup>-1</sup> )			100 µg ml <sup>-1</sup>
Nystatin (10 000 units ml <sup>-1</sup> )	0.5	2.5	50 units ml <sup>-1</sup>

Store at 5 ± 3 °C for up to one week.

### 2 BGM Maintenance Medium

Total volume	100 ml	500 ml	Final concentration
Sterile distilled, deionised or of similar grade water	81	405	
MEM (10x)	10	50	1 x
Foetal Calf Serum (FCS)	2	10	2 %
L-glutamine (0,2 M)	1.5	7.5	
Sodium Bicarbonate (NaHCO <sub>3</sub> ) (4,4 %)	4	20	
Penicillin / Streptomycin (10 000 units ml <sup>-1</sup> )	1	5	100 units ml <sup>-1</sup>
Penicillin / Streptomycin (10 mg ml <sup>-1</sup> )			100 µg ml <sup>-1</sup>
Nystatin (10 000 units ml <sup>-1</sup> )	0.5	2.5	50 units ml <sup>-1</sup>

Store at 5 ± 3 °C for up to one week.

## Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users wish to receive advance notice of forthcoming publications, please contact the Secretary.

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