Methods for the isolation and identification of human enteric viruses from waters and associated materials 1995

Methods for the Examination of Waters and Associated Materials

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This booklet contains details of procedures for the isolation and identification of human enteric viruses. Although procedures have proven efficiency for certain viruses no performance data are available. Users should satisfy themselves that acceptable performance is achieved before methods are used routinely. Certain manufacturers' equipment and reagents are mentioned in the text. This does not form part of any endorsement by the Standing Committee of Analysts and other alternative suppliers may be suitable.

Contents

About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, groundwater, river and seawater, waste water and effluents as well as sewage sludges, sediments and biota. In addition, short reviews of the more important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods especially for chemical parameters should be fully evaluated with results from performance tests reported. 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 encompassing at least ten degrees of freedom 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. It is recognised that the performance criteria expected for chemical parameters will not be strictly applicable to microbiological methods. 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 in the series 'Methods for the Examination of Waters and Associated Materials' and their continuous revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is managed by the Drinking Water Inspectorate. At present there are nine working groups, each responsible for one section or aspect of water quality analysis. They are:

- 1.0 General principles of sampling and accuracy of results
- 2.0 Microbiological methods
- 3.0 Empirical and physical methods
- 4.0 Metals and metalloids
- 5.0 General non-metallic substances
- 6.0 Organic impurities
- 7.0 Biological monitoring
- 8.0 Sewage treatment and biodegradable methods
- 9.0 Radiochemical methods

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

Publication of new or revised methods will be notified to the technical press. An index of methods and the more important parameters and topics is available from HMSO (ISBN 0 11 752669 X).

Every effort is made to avoid errors appearing in the published text. If however, any are found, please notify the Secretary.

Dr D WESTWOOD
Secretary

11 June 1995

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 any regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 1988 SI 1988/1657. 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 and these should be consulted and be readily accessible to all analysts. Amongst such publications are those produced by the Royal Society of Chemistry, namely 'Safe Practices in Chemical Laboratories' and 'Hazards in the Chemical Laboratory', 5th edition, 1992; by Member Societies of the Microbiological Consultative Committee, 'Guidelines for Microbiological Safety', 1986, Portland Press, Colchester; and by the Public Health Laboratory Service 'Safety Precautions, Notes for Guidance'. Another useful publication is produced by the Department of Health entitled 'Good Laboratory Practice'.

1 Introduction

The occurrence of viruses in the aquatic environment has been extensively reviewed (1). Human enteric viruses occur in all types of water and wastewater and can be found in association with related particulate matter (soils, sediments and sludges), in aerosols and shellfish.

1.1 Virology

The main source of viral contamination in the aquatic environment is sewage. Sewage may contain any number of viruses shed in faeces. Table 1 lists the viruses most commonly associated with intestinal infection. The range of symptoms linked to each virus group is variable and dependent on the age and susceptibility of the host. Infection without clinical symptoms is common in many groups.

Table 1 Viruses associated with gastrointestinal infection

Virus	Associated major symptoms	Detectable in water
Enterovirus:		
polio, coxsackie A, coxsackie B, echo	paralysis (polio), fever, malaise, myalgia, meningitis or none	Yes
Rotavirus	diarrhoea	Yes
'Norwalk-like' viruses (SRSV)*	diarrhoea, vomiting	No
Astrovirus	diarrhoea	No
Calicivirus	diarrhoea	No
Adenovirus (types 1–39)	respiratory, eye, generalised, none	Yes
Adenovirus (types 40, 41)	diarrhoea	No
SRV, parvo	none	No
Reovirus	none	Yes
Coronavirus	none	No
Hepatitis A virus	hepatitis	No
Hepatitis E virus	hepatitis (mainly in Far East)	No

*SRSV = small round structured virus
SRV = small round virus

The detection and identification procedures described in this booklet are for those enteric viruses which grow in cell culture. The plaque assay methods favour the detection of poliovirus and coxsackievirus B; liquid assays will also isolate coxsackieviruses A7 and A9, echovirus, adenovirus and reovirus. Rotavirus is fastidious and cannot be detected using current conventional cell culture techniques. The assay involves immunochemical staining and therefore is more time-consuming than the enterovirus assays.

Hepatitis A is very fastidious and slow-growing. Highly specialised methods are required to detect the virus in any sample. Culture of the virus has been achieved using monkey kidney cells and detection using radioimmunofocus assay. This latter method is only a research technique at present, being particularly applied to the detection of hepatitis A virus in shellfish (2,3). Such studies have used cell-adapted virus; wild strains require up to 21 weeks incubation before antigen is detectable. No culture methods are currently available for the Norwalk-like viruses which appear to be the most important of the waterborne viruses causing gastroenteritis (4).

Advances in molecular biology will facilitate tests for these and other viruses. At present, these methods are used for research purposes and furthermore, do not indicate if the virus is infectious.

The major routes of enteric virus transmission are by person-to-person contact or from contaminated foodstuffs. Outbreaks of infection with Norwalk-like virus and rotavirus have resulted from the consumption of contaminated drinking water. Outbreaks of gastroenteritis and Hepatitis A have been associated with the consumption of shellfish that have been harvested from polluted waters, ineffectively depurated or inadequately cooked. Symptoms of gastroenteritis associated with immersion in poor quality recreational water have not, to date, been associated with any specific micro-organism including enteric viruses (5). The culturable enteroviruses have not been associated with transmission via the water route and, in the absence of systemic illness, they are not a cause of diarrhoea. Vaccine strains of poliovirus will not present a risk to fully vaccinated individuals.

1.2 Viruses in the water cycle

The numbers of viruses decrease from abstraction of raw water for treatment through to the delivery of potable water at the consumer's tap. Data suggest that drinking waters in the UK show evidence of virus contamination only infrequently, and this usually occurs when treatment processes have been inadequately applied. However, it should be noted that viruses have been detected even when residual disinfectant has been present and the water has satisfied current standards of bacteriological quality (6–10).

Similarly, groundwaters are only occasionally contaminated with viruses. However, outbreaks of viral gastroenteritis and hepatitis A infection, have been reported, particularly where treatment has been inadequate (11).

The microbiological quality of surface waters will generally reflect the level of sewage effluent loading they receive. The numbers and types of viruses present may vary widely and may fluctuate with season (12). There are three main areas of concern regarding viruses in surface waters:

(a) contaminated waters used for abstraction for potable water may overload the water treatment works and allow virus penetration into the water supply (13,14);

(b) there may be a risk to public health when such waters are used for recreational (5,15–17) and agricultural purposes (28); and

(c) contamination of nearshore coastal environments may pose a risk for bivalve shellfish culture.

Raw sewage contains very high numbers of viruses (18,19) while treated wastewater effluents usually have lower levels. In part, this is due to the effectiveness of wastewater treatment processes, although these may not always be reliable or effective (19). Some of the viruses may be removed by association with sludge solids and may be present in wastewater sludges (21–23). Nevertheless, levels of viruses in effluents may still be substantial (18,20).

The incidence and distribution of enteric viruses in certain areas of estuarine and marine waters reflect the virological quality of the rivers which flow into them, and the quality of any discharges of effluents. The occurrence of viruses in seawaters and sand has been documented (24–26) in many bathing areas and may be present in the absence of bacterial indicators.

The accumulation of viruses by filter-feeding shellfish is a cause for concern. Outbreaks of food-borne gastroenteritis and hepatitis A infection have been documented. These can be traced to the consumption of shellfish that have been harvested from polluted waters, inefficiently depurated or inadequately cooked (26). Shellfish are very difficult to process for the detection of culturable enteroviruses. Methods are of unproven efficiency and should therefore not be used for monitoring purposes.

Viruses will be present in wastewater and may also be present in aerosols produced during wastewater treatment processes and by spray-irrigation when effluents are used. Serological investigations have demonstrated the ability of viruses to infect susceptible persons

dealing with these materials although the risk from aerosol-borne virus is probably low (27,28).

The methodologies for the concentration of enteroviruses described in this booklet have proven efficiency for poliovirus and coxsackievirus B. Methodology may be less efficient for other enteric viruses. All the methods described should be properly evaluated in users' laboratories before being used routinely. The number of 10 litre samples that can be taken and processed for virus detection is constrained by laboratory practicalities. This low sampling frequency means that statistical analysis of results is of limited value.

2 Applications

2.1 Legislative requirements

2.1.1 Potable water

The European Community Directive (29) states that water intended for human consumption should not contain pathogenic organisms. The Water Act (1989) as consolidated in the Water Industry Act (1991) (30), and similar legislation in Scotland, places a duty on water undertakers to supply only water which is wholesome at the time of supply. The microbiological parameters which must be satisfied are contained in the Water Supply (Water Quality) Regulations 1989 (31) for England and Wales, and its Scottish equivalent. Whilst viruses are not specifically identified, the requirements are that water does not contain any element, organism or substance at a concentration or value which would be detrimental to public health. Similar requirements are contained in the Natural Mineral Waters Regulations (1985) (32).

2.1.2 Recreational water

A compliance level for enteroviruses is prescribed in the European Community Directive concerning the quality of bathing waters (33). This applies when there is reason to believe that the quality of the water has deteriorated as, for example, when bacteriological monitoring indicates a high level of faecal pollution, or there is proximity of a sewage discharge to the bathing area. Enteroviruses should be not detectable in 95% of the samples, with a minimum of two samples taken during the bathing season.

2.1.3 Wastewater and sludge

There are no European Community or UK regulatory requirements for the virological examination of effluents, wastewaters or sludges.

2.1.4 Shellfish

Bacteriological, but no virological testing is specified in the European Community Directive on shellfish (34).

2.2 Indications for virus testing

2.2.1 Potable water

Report 71 (35) includes a discussion of circumstances when testing for viruses may be considered. Briefly, tests for culturable enteroviruses may be worthwhile to:

(a) investigate post-treatment pollution of potable supply;

(b) investigate a process failure at a water treatment works, whether or not indicator bacteria have been detected;

(c) investigate an outbreak of gastrointestinal illness associated with a potable supply; tests on the source water may indicate the level of the virus presence; and

(d) evaluate a source water and the stages of water treatment.

2.2.2 Recreational water

Tests for culturable enteroviruses may provide data to assess the level of sewage contamination when a body of fresh water or seawater is used for any recreational activities involving direct contact with the water. A series of 10 litre samples taken at appropriate intervals throughout the bathing season may provide the necessary information. Tests for rotavirus may also be included.

2.2.3 Wastewater and sludge

Tests for viruses may be useful to assess the effectiveness of new treatment processes including disinfection. Two litres of effluent and 100 mL of sludge or raw sewage should provide sufficient material for testing. It should be noted that the methods used for materials containing large amounts of organic matter are of low efficiency and poor reproducibility. Tests for rotavirus may also be included.

2.2.4 Shellfish

Methods have been published for enterovirus assay (36) and others are in development. However, none has a standard of efficiency and reproducibility sufficient to warrant inclusion in this booklet.

3 Hazards

Many human enteric viruses are pathogenic and require handling in containment level 2 laboratories (37).

All cultures and stages of cultivation should be handled in specified areas with proper care by properly trained personnel.

All equipment should be sterilized before and after use, preferably by autoclaving at a minimum of 121°C for 20 minutes. Cultures should also be sterilized before disposal. Sodium hypochlorite solution (1000 mg/L available chlorine) is the most appropriate disinfectant for dealing with spillages or breakages and for surface swabbing.

Proper regard should be paid to the relevant codes of practice for safety in microbiology laboratories (38–39) and good general laboratory practice should be observed with particular regard for the handling of simian cell cultures.

4 Principles of methods

The methods described in this booklet are currently in use in UK water laboratories. Alternative methods using ultrafiltration, glass wool and magnetic beads are used in other parts of the world. No single method has been demonstrated to be superior. Procedures for the recovery, enumeration and identification of a range of enteric viruses (culturable enteroviruses, adenoviruses, reoviruses and rotaviruses) from a range of waters and associated materials are given. The detection of other potentially pathogenic enteric viruses known to occur in water and associated materials, including Norwalk-type viruses, caliciviruses and astroviruses, is possible from clinical samples but at present, the methods available are inappropriate for routine water sample testing.

4.1 Sample processing

When high levels of viruses are expected, for example in sewages, concentration of the sample may be unnecessary. When concentration is required this may be achieved by adsorption of the viruses onto a suitable matrix, followed by desorption into a small volume of eluant. A second concentration step may also be necessary in order to reduce the sample volume further.

4.2 Virus detection

Samples are inoculated onto cultured susceptible cells to demonstrate the presence of infectious virus. The cell death caused by culturable enterovirus, adenovirus or reovirus is apparent as a cytopathic effect in liquid assay. In agar assays cell death remains localised as viruses only spread directly from cell to cell. A vital stain in the agar will identify these light coloured areas as plaques.

Liquid assays

Many cell lines (including BGM, Vero, FL, HeLa and HEp-2) under liquid medium are sensitive to enteric virus infection. Using two or more cell lines may improve the isolation rate of different virus types. Cells under liquid media may support the growth of more enteric virus serotypes than cells suspended in agar but the procedure can take much longer. Virus multiplication produces cell degeneration, the characteristics of which may suggest the type of virus involved. BGM cells are commonly used for the detection of viruses, having been shown to be effective for many enteroviruses (40).

Agar assays

Agar assays can utilise a pre-formed confluent monolayer of cells or cells suspended throughout the medium. The suspended cell assay is capable of detecting low levels of enteroviruses, particularly poliovirus and coxsackievirus B (41, 42). The monolayer assay is more suited for slower growing enterovirus and reovirus. Substantially more cells are required for the suspended cell assay which may be produced using roller bottle cultures or "cell factories".

Rotavirus

Rotaviruses do not undergo a complete multiplication cycle in cell culture. Replication ceases when non-infectious particles are produced which lack the outer capsid. The virus antigen of the inner capsid, VP6, can be detected by serological staining using immunological probes labelled with enzymes or fluorescent molecules (43). The methods are applicable only to the Group A rotaviruses which possess the common group antigen.

4.3 Enumeration

In liquid assays, the number of virus infectious units can be calculated using 'most probable number' formulae. Plaques can be counted directly as can the stained cells of the rotavirus assay; it is assumed that a single infectious unit initiates each plaque or stained cell.

4.4 Confirmation

Confirmation is required where virus multiplication is monitored by the development of a cytopathic effect or plaque assay because cell death due to toxic effects of the sample needs to be excluded. The presumptive virus isolate is subcultured into fresh uninfected cell cultures. Serological identification can also be undertaken at this stage. It is not necessary to subculture viruses detected by immunocytoassay as such tests are usually virus-specific.

4.5 Factors affecting detection

The delay between time of sampling and processing should be kept to a minimum to avoid any possible inactivation of the viruses. Samples should be processed within 24 hours, and certainly within 36 hours of sampling. At all times it is desirable that samples should be kept cool (preferably below 10°C but not frozen) and out of direct sunlight before processing. It is essential that all chlorinated samples are dechlorinated at the time of sampling (section 5.3). Sample concentrates, if not tested immediately, should be frozen at -20°C or preferably lower. In transit, they should be stored at as low a temperature as practicable.

5 Sample collection

5.1 General principles

The sampling of waters and water-associated materials must be carried out with care. Good quality samples are essential if meaningful results are to be recorded. It is important to recognise that potential pathogens occur throughout the aquatic environment which could infect personnel. Samples should, therefore, be collected by trained and experienced persons. Sampling equipment should be sterilized before and after use. In the case of drinking water samples, guidance on bacteriological sampling is given in Report 71 (35).

5.2 Hazards

The provisions of the Health and Safety at Work etc Act (44) and the procedures given in Safety in Sewers and Sewage Works (45) should be observed. Other relevant national and local regulations should also be followed.

5.3 Potable waters

Samples should be collected aseptically into sterile containers of stainless steel or polypropylene containing sufficient sodium thiosulphate to provide a final concentration of 18 mg/litre to neutralise residual chlorine (35). Twenty litres is usually sufficient for routine virus monitoring purposes. If volumes in excess of 100 litres are to be sampled it is possible to carry out concentration of waters at the sampling location using special filters (see section 6.1).

5.4 Natural waters (fresh and marine)

Samples of untreated groundwaters can usually be obtained in the same way as for drinking waters when sampling taps are available. Where it may be necessary to pump from some depth, equipment which has been previously flushed with sodium hypochlorite solution at a final concentration of 20 mg/litre should be used. Samples from surface waters are usually taken in a clean, sterile, stainless steel or polypropylene bucket. The volumes sampled, 5—10 litres, often make it difficult to obtain a sub-surface sample. In any case, the sample should be taken from a spot which is representative of the whole body of the water. Rivers should be sampled in the middle of their flows. Where it is necessary to obtain samples from different depths a depth sampler should be used. Suitable devices are commercially available.

5.5 Sewage and effluents

Clean, sterile, containers should be used for dip sampling. The sample volume required is usually less than 1 litre. If pooled sampling (for example over a period of 24 hours) is possible then a subsample of the well-mixed composite can be used for virological analysis. Useful sample containers are those which have a wide mouth and are easily sterilized.

5.6 Sewage sludge

The means of obtaining samples of the different types of sewage sludge have been adequately described elsewhere (46). Briefly, pooled samples are taken into wide mouth, sterile containers from sampling outlets of digester tanks.

5.7 Sediments

Devices for the sampling of sediments are commercially available and can be readily made. A cheap, practical tool has been described (47).

5.8 Soils

Corers of suitable material and dimensions are available commercially or may be readily made. Sampling patterns (46) have been reported.

5.9 Information which should be supplied with samples

The following details, which are not exhaustive, should accompany any sample taken for virological determination;

(a) sample description;

(b) sampler's name or identification;

(c) date and time of sampling;

(d) type of material;

(e) location, preferably with map reference;

(f) for potable supplies:
  (i) disinfectant residual at time of sampling;
  (ii) whether bacteriological samples were also taken;
  (iii) if concentrated at the sampling location, the volume of water sampled;

(g) for samples from surface water:
  (i) the depth at which the sample was taken;
  (ii) whether from the side or middle of the water mass;
  (iii) whether the water level was above or below average (fresh waters);
  (iv) state of tide (marine and estuarine waters);
  (v) whether after heavy rainfall, flooding or during drought;

(h) for samples from sewage tanks, digesters or lagoons:
  (i) whether from the inlet or outlet pipe;
  (ii) the depth of sampling;
  (iii) whether a composite or sub-sample; and

(i) examination required and reason why sample was taken.

6 Virus concentration

It is essential to use good microbiological laboratory techniques when processing samples. When handling a range of sample types potable waters should be processed prior to material known or suspected of being contaminated to a greater extent.

6.1 Natural and drinking waters

The methods described in section 7 and outlined in figure 1 relate to the concentration of viruses from surface, ground and potable waters (48). The methods rely upon the adsorption of virus particles onto a suitable filter matrix. The virus particles are then displaced when an eluant of high protein content (at high pH) is passed through the filter. Further concentration of the virus is achieved by protein flocculation at low pH. Viruses adsorbed to the floc are recovered by centrifugation and dissolution of the pellet in a suitable buffer.

Occasionally, it may be necessary to concentrate large volumes (100—1000 litres) of water. This can be accomplished using electropositive filters which can be directly attached to the water supply and a known volume passed through. When sampling a chlorinated supply it is necessary to include an injection system for sodium thiosulphate before the filter stage in order to dechlorinate the water (49). Additionally, it may be advantageous to reduce the pH of the water to about pH 5.5 prior to filtration as this has been shown to improve virus adsorption (50). The filter can then be returned to the laboratory and processed.

6.2 Sewage and effluents

In many cases, it is unnecessary to concentrate viruses from such samples which may be inoculated directly into cell culture or other virus detection systems. This 'direct inoculation' method depends upon the adequate control of non-viral contamination by antibiotic and antifungal agents included in the assay medium. It is normally sufficient to assay 10 mL in this manner (51) although larger volumes can be assayed by using more cell cultures. If concentration is necessary, for instance where a wastewater has been disinfected, then the procedures described in section 7.2 and outlined in figure 1 can be applied to sample volumes up to 1 litre. In order to maintain a good flow rate through the filter, dilute the sample with sterile water to 5—10 litres prior to filtration. Where a comparison of samples before and after treatment is being undertaken, the same procedure for virus recovery must be followed.

6.3 Sludge

The method described in section 7.1.2 and outlined in figure 2 is a modification of that described by Hurst and Goyke (52). The particulate matter in the sample acts as the adsorptive matrix from which the viruses can be eluted.

6.4 Soil and sediment

These are treated as for sludges except where the sample is of a sandy or greater particulate size. Viruses tend not to adsorb to such material and the initial adsorption step in figure 2 can be omitted. Sandy samples should be suspended in the eluant and the desorbed viruses further concentrated by flocculation and centrifugation.

7 Detailed procedures

7.1 Sample processing

7.1.1 Concentration of viruses from water samples of up to 100 litres

This procedure can be used for the concentration of viruses in sample volumes of 1–100 litres. This includes drinking water, raw waters used for abstraction, estuarine waters and seawaters (figure 1). In addition, the procedure can be used to concentrate viruses in wastewater based on a one litre sample volume after prior dilution to 5–10 litres with sterilized drinking water.

7.1.1.1 Reagents and apparatus

Aluminium chloride ($AlCl_3.6H_2O$)—1 Molar;
Beakers—250mL;
Centrifuge—capable of at least 6000g with sterile centrifuge pots of 500 mL capacity and sealable caps;
Disodium hydrogen phosphate (Na_2HPO_4)—0.15 Molar, sterile;
Disposal bag;
Eluant solution—3 % m/V beef extract solution or 0.1—0.5% skimmed milk solution, sterile;
Filters in appropriate housings—glassfibre cartridges (8µm or 25µm), or cellulose nitrate disk membranes (0.45µm, 1.2µm) and a glassfibre pre-filter;
Ferric chloride—0.5 Molar;
Hose and connectors for use with pressure cans, sterile;
Hydrochloric acid (HCl)—0.1 Molar, 1 Molar and 5 Molar;
Measuring cylinder—100 mL;
Peristaltic pump or pressure vessels—10 or 20 litres capacity, stainless steel;
Pipettes—sterile, disposable 1mL, 10mL;
pH meter and buffers;
Sodium hydroxide (NaOH)—5 Molar; and
Universal containers—sterile, disposable.

7.1.1.2 Preparation of eluants

(i) Beef extract: prepare 5 litres of a 3% m/V solution in deionised water. Since many batches of beef extract do not flocculate at low pH it is essential that testing of batches is carried out prior to use. This can be achieved by preparing a solution and monitoring floc formation as the pH is lowered. A satisfactory batch should form a visible precipitate between pH 3.0 and 3.5, although some batches may flocculate at higher pH levels (but less than 4). The addition of ferric chloride may enhance adsorption.
Skimmed milk: prepare as a 0.1–0.5% m/V solution in deionised water. It is unnecessary to check that each batch flocculates at pH 4.0—4.5.

(ii) From the (beef extract) bulk eluant, take a subsample of 350 mL (for pH measurements).

(iii) Sterilize the bulk eluant preparation and the subsample by autoclaving. Allow to cool before use.

(iv) Using the subsample determine the amount of 5 Molar sodium hydroxide required to raise the pH to 9.5. Calculate the volume of sodium hydroxide required to raise the pH of the bulk eluant to 9.5 and add to the bulk, mixing well. Do not adjust the pH of the eluant prior to autoclaving since a pH of 9.5 will not be maintained during the sterilization process.

(v) Measure the volume of 5 Molar hydrochloric acid required to lower the pH of the subsample from 9.5 to 3.0—3.5 (depending upon batch) to achieve flocculation.

7.1.1.3 Conditioning of water sample

(i) Transfer the 10 litre sample under investigation from the sample bottle to a stainless steel pressure vessel taking extreme care not to contaminate the sample inadvertently. Take a subsample of 100 mL for pH adjustment measurements.

(ii) Using the 100 mL subsample, measure the volume of 1 Molar hydrochloric acid required to lower the pH to 3.5; calculate the volume of acid required to adjust the pH to 3.5 for the rest of the sample. Add the acid to the sample and mix well by rolling the pressure vessel several times.

(iii) If using a glassfibre filter as a virus adsorption matrix then add 5 mL of 1 Molar aluminium chloride per litre of water sample to give a final concentration of 0.0005 Molar.

7.1.1.4 Sample filtration

(i) Connect hose pipes to the filter apparatus and to the pressure vessel.

(ii) Apply pressure (air line or gas cylinder) to force the conditioned water out of the vessel and through the filter. Maintain a steady flow rate of 1–2 litres per minute. Excessive pressure may lead to rupture of the filter. If back pressure develops due to clogging of the filter then replace the clogged filter apparatus with a fresh unit. Continue filtration until all the water has passed through the filter(s) and allow air to pass through in order to expel any residual water in the system.

(iii) Connect the filter unit to a pressure vessel containing the chosen eluant.

(iv) Pass the eluant at pH 9.5 through the filter in the same direction as the original filtration taking care not to rupture the filter with excessive pressure.

(v) Collect the eluant into a sterilized centrifuge pot to give a final volume of about 350 mL. An alternative to the pressurised air system of filtration is to use a peristaltic pump to pass the water and eluant through the filter or to use a vacuum line.

(vi) Add sufficient 5 Molar hydrochloric acid, as determined in 7.1.1.2(v), to initiate flocculation. If the beef extract solution produces only a light floc this can be enhanced by the addition of 2–3 drops of ferric chloride solution.

(vii) Centrifuge at a minimum of 3500g for 20 minutes in a cooled centrifuge.

(viii) Discard the supernatant.

(ix) Resuspend the pellet in 0.15 Molar disodium hydrogen phosphate solution to a final volume of 5–10 mL.

(x) Assay immediately or store at -20°C or lower.

7.1.1.5 Clearing up

(i) All filters and filter holders used in the concentration of viruses from water should be considered potentially infectious and should be autoclaved before disposal or washing.

(ii) All apparatus should be cleaned with cold running water, brushing where necessary to remove any entrapped particulate matter. Before being used again in the concentration procedure all apparatus should be sterilized, preferably by autoclaving at 121°C for 20 minutes.

7.1.2 Concentration of viruses from sludges, soils and sediments

This suggested procedure can be used for the recovery of viruses from most water-associated particulate matter including wastewater sludges, water treatment sludges, soils, river and estuarine sediments and marine sediments (figure 2). This method may be affected by the type of material being processed and may result in variable recoveries.

7.1.2.1 *Materials*

Aluminium chloride $HCl_3.6H_2O$—1 Molar;
Centrifuge capable of 10000g, refrigerated;
Centrifuge pots—sterile, 50–100 mL;
Disodium hydrogen phosphate—0.15 Molar, sterile;
Eluant—sterile (3 %m/V beef extract or 0.5 %m/V skimmed milk), adjusted to pH 9.5;
Hydrochloric acid—5 Molar, 1 Molar;
Magnetic stirrer and followers;
Measuring cylinder—100mL;
Pipettes—sterile, disposable, 10 mL, 1 mL;
pH meter and buffers.

7.1.2.2 *Preparation of eluant*

See 7.1.1.2.

7.1.2.3 *Concentration procedure*

For sludges, it is useful to have some indication of the likely levels of dry solids prior to processing the sample.

(i) Weigh out 50 g of material, or if liquid, 50 mL, adjusted to approximately 4% m/V dry solids, into a sterile beaker.

(ii) Add an equal volume of sterile deionised water and mix thoroughly. Adjust to pH 3.5 with 5 Molar hydrochloric acid while gently mixing on a magnetic stirrer.

(iii) Add aluminium chloride to give a final concentration of 0.0005 Molar.

(iv) Gently stir for 30 minutes at room temperature avoiding frothing and aerosol production.

(v) Transfer to centrifuge pots and centrifuge at 10000g for 30 minutes.

(vi) Discard the supernatant and resuspend the pellet in eluant, mixing thoroughly.

(vii) Centrifuge at 10000g for 30 minutes.

(viii) Transfer the supernatant to a beaker and discard the pellet. Flocculate supernatant at low pH and recover precipitate as in 7.1.1.4.

7.1.2.4 *Detoxification*

Prepare an 0.05 Molar stock solution of dithizone (diphenylthiocarbazone) in chloroform (0.128 g in 10 mL). To 10 mL of sample concentrate add 1 mL dithizone solution and 9 mL chloroform. Mix well for 1 min. Centrifuge at 1500g for 15 mins. Carefully pipette the upper phase into a petri dish. Expose to air for approximately 15 mins to allow excess chloroform to evaporate. Discard the lower phase of contaminated chloroform according to appropriate local guidelines.

7.1.2.5 *Clearing up*

See 7.1.1.5.

7.2 Virus assay

It is assumed that, where facilities are available for the assay of viruses, personnel will be familiar with the basic principles of cell culture. Reference 53 gives a good basic guide to the handling of cell cultures. Appendix 1 lists some of the commonly used cell culture media.

7.2.1 Cytopathic enteric viruses—liquid assays

7.2.1.1 *Flask cultures*

Cell cultures are prepared in 75 cm^2 or 175 cm^2 flasks by standard procedures (53). Confluent cultures are drained and washed with serum-free medium before use. The sample (<1mL) is allowed to adsorb to the monolayer(s) for one hour at 37°C. The inoculum is removed, the cell sheet washed once again and maintenance medium added. The cultures are incubated for a period of up to 21 days at 37°C. Half of the medium is replaced with fresh maintenance medium every three to four days. If the culture shows no evidence of cytopathic effect after 21 days the sample is regarded as being free of virus. This procedure is useful for the detection of a range of viruses present at low levels in such samples as drinking and ground waters. If cultures show evidence of cytopathic effect they should be frozen (-20°C) and thawed once, to release intracellular virus, clarified by centrifugation to remove cell debris and identification carried out (see section 7.2.3).

7.2.1.2 *Tube cultures*

Confluent cell cultures in tubes are prepared by standard procedures (53) and changed to maintenance medium when confluent. The sample, or concentrate (100µL) is added to each tube and the cultures incubated at 37°C for 21 days. The maintenance medium may be replaced after 1 or 18 hours incubation if toxicity is observed. During the 21 day incubation period, the medium is changed every 3–4 days. The appearance of cytopathic effect is monitored by microscopical examination. If the culture shows no evidence of cytopathic effect after this period, the sample is regarded as being virus-free. This method is useful for the detection of a range of viruses particularly where numbers may be high. Positive cultures should be treated as for flasks.

7.1.2.3 *End-point titration*

Serial logarithmic (base 10) dilutions are made of the sample in serum-free medium. Three to five cell cultures are inoculated with a portion of each dilution (1 mL per 75 cm^2 flask; 0.1 mL per tube). The cultures are incubated at 37°C until cytopathic effect changes cease, usually between 5 and 10 days. The cultures are scored as being negative or positive for cytopathic effect. It is essential to include uninoculated cell cultures as negative controls. The end-point or titre is calculated as the highest dilution of virus producing cytopathic effect in 50% of the cultures (54) or may be calculated as most probable number using, for example, the tables of Chang et al (55).

7.2.2 Cytopathic enteroviruses—agar assays

7.2.2.1 *Suspended cell plaque assay (42, 56)*

A freshly prepared cell suspension is adjusted to 1 x 10^7 cells per mL in growth medium. For each sterile 90 mm diameter disposable plastic dish (triple-vented, bacteriological grade) 2 mL of cell suspension are mixed with 2 mL of sample and 10 mL of agar medium (appendix 1). Mixing may be in the plate or in a sterile, disposable, plastic, universal container. The mixture is then poured into the dish. The agar is allowed to set at room temperature in darkened surroundings. This is to minimise the photoinactivation of viruses by the neutral red stain in the medium. Once set, the plates are inverted and incubated at 37°C in an atmosphere of 5% carbon dioxide in air and greater than 95% relative humidity.

To ensure maximum sensitivity of this virus detection method, the whole sample concentrate should be assayed ie 10 mL of concentrate into 10 petri dishes. However, the total sample concentrate should not be tested on a single occasion. It is essential to include a positive virus control and a negative cell control petri dish with each assay.

Plaque development is monitored daily, usually for 2–5 days, until the cell culture degenerates and results expressed as plaque-forming units in the original sample volume.

7.2.2.2 Monolayer plaque assay (53)

Monolayers of cells in flasks (75 cm^2) are drained off and washed with serum-free medium. The drained monolayer is inoculated with a portion of sample (<1 mL) and allowed to adsorb for up to one hour at 37°C. The sample is washed off and agar overlay added (appendix 1). When the agar is set, the flasks are inverted and incubated at 37°C. Plaques are counted daily until cell degeneration occurs and results expressed as plaque-forming units in the original sample volume.

7.2.3 Confirmation and identification of enteric virus isolates (53)

Cell cultures showing cytopathic effect are freeze-thawed once and a portion of the lysate inoculated into a tube culture of the same cell line used for the initial isolation. Confirmation that plaques are of viral origin is obtained by removing a plug of agar from the edge of the plaque using a sterile pasteur pipette. The agar plug is inoculated into a tube culture of the same cell line used for the initial isolation. Tube cultures are incubated at 37°C as a rolling culture or as a static culture.

Development of cytopathic effect is monitored daily for seven days (21 days for adenoviruses). Those showing no cytopathic effect are scored as being negative. Positive cultures are frozen (-20°C or lower) and thawed once and the product used for identification purposes.

Identification of enterovirus, adenovirus and reovirus isolates is by a neutralisation assay outlined in figure 3. The cultures are incubated and monitored for cytopathic effect. Typical neutralisation patterns, caused by blocking of the viral replication by specific antibodies, are determined after the appropriate incubation period (53). Specific antisera for use in the neutralisation tests are commercially available.

While identification of isolates is desirable, it is not essential. However, virus isolates from potable waters should be identified.

7.2.4 Rotavirus—immunocytochemical detection

Assays are done in either 96-well microtitre plates or 24-well multi-dishes. Suitable cell lines are simian LLC-MK2 and MA-104B. After 4–24 hours incubation growth, medium is removed from the cell monolayer and washed with serum-free medium. Before inoculating on to cells, the sample can be pre-treated with trypsin by mixing equal volumes of sample and double strength serum-free medium containing trypsin to give a final concentration of 0.5 µg/mL and incubating at 37°C for 30 minutes (43). The sample is added to the cells and incubated at 37°C for 60 minutes.

Alternatively, the trypsin treatment can be omitted and the sample, diluted in serum-free medium containing Hepes buffer ($C_8H_{18}N_2O_4S$) at a final concentration of 20 mM is added to the cells and centrifuged for 60 minutes at 1500g.

Fresh serum-free medium or maintenance medium replaces the sample. Incubate overnight at 37°C in a 5% carbon dioxide in air atmosphere with greater than 95% relative humidity.

Cell monolayers are fixed with ice-cold methanol for 10 minutes and air dried. The procedure for labelling with conjugated antibody probes is outlined in figure 4. The optimal working dilutions of anti-rotavirus serum and anti-species conjugate (fluorescein isothiocnanate (FITC) or horse-radish peroxidase) should be determined before sample assays can be carried out. It is essential that adequate washing is achieved between the staining stages. Failure to do this will result in false-positive reactions.

8 Quality assurance

It is essential that adequate positive and negative controls are included in the above procedures in order to check the efficiency of concentration methods and to monitor the sensitivity of the cell lines. For example, for each plaque assay a positive control of known virus count of approximately 20 plaque-forming units per petri dish or flask should be included.

All concentration methods should be checked periodically using a known level of virus. For instance, an input of 30–50 plaque-forming units of poliovirus type 1 is an adequate control for methods used in the recovery and enumeration of the culturable enteroviruses. An adequate control of the rotavirus methodology is a preparation of bovine rotavirus which gives a count of 20–40 infective units per assay.

Cell cultures should be used only for defined periods. BGM cells, for instance, should be used between passages 75 and 120 because sensitivity of the cell line to enteroviruses has been reported to decrease beyond this level (40). The range of cell passages over which other cell lines, such as MA-104B and LLC-MK2, can be used without loss of sensitivity is unknown. It is good practice to define an in-house standard for the use of these cells. It is advisable to maintain a stock of low-passage cell lines in liquid nitrogen to ensure continuity of supply.

Whenever cell cultures show evidence of bacterial or fungal contamination they must be discarded and new stocks of cells prepared. All media used in the preparation of contaminated cell cultures should also be checked for sterility and, if contaminated, discarded after autoclaving.

Laboratories should be encouraged to participate in collaborative quality assurance exercises and to take part in any external microbiological quality assessment programmes.

It is essential that accurate record keeping of all aspects of quality assurance as well as of sample test details are maintained.

Appropriate quality checks of new batches of media, filters etc should be made before use.

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Figure 1 Concentration of viruses from natural and drinking waters

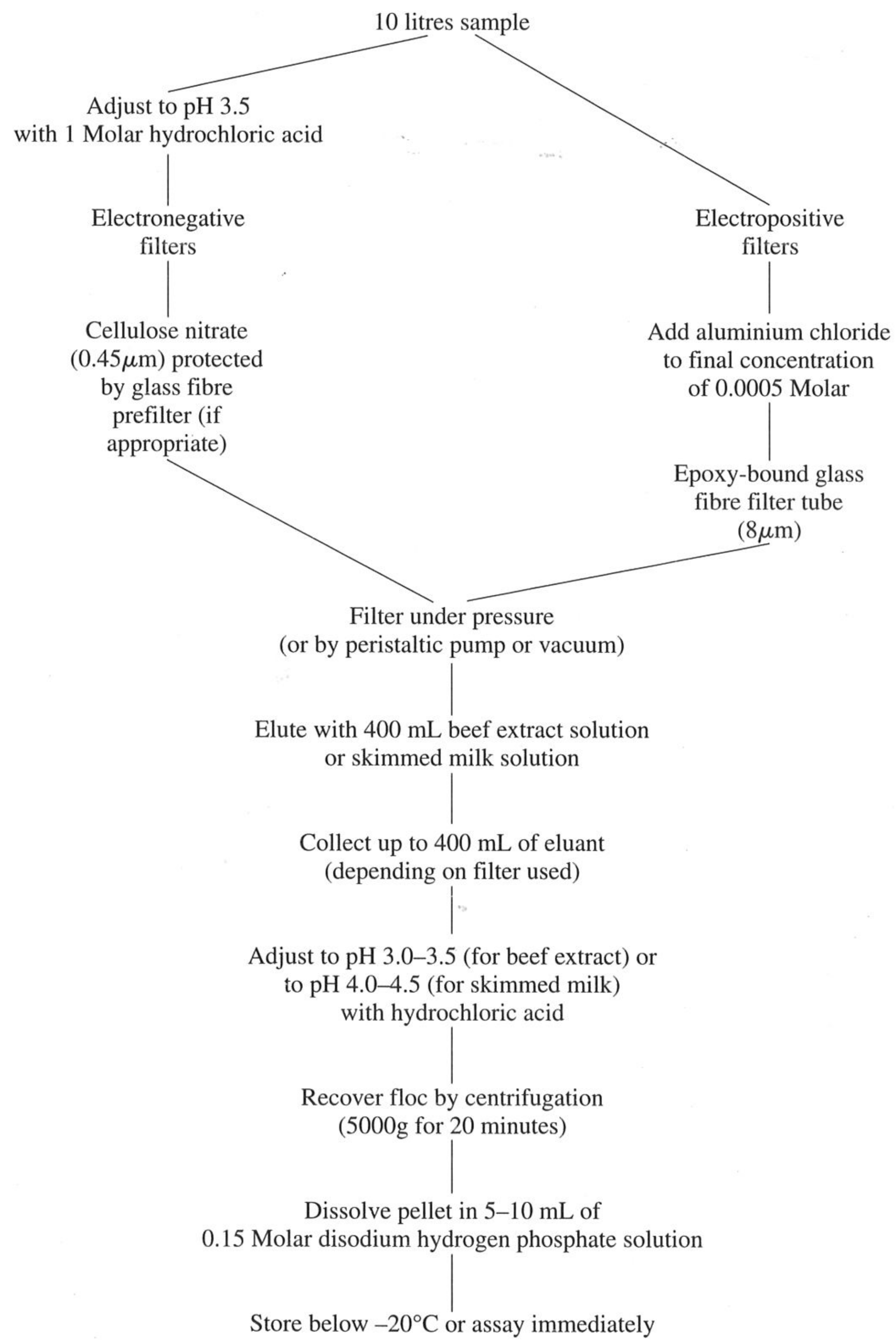


Figure 2 Concentration of viruses from sludges, soils and sediments

50 mL sample (about 4% dry weight solids)
or 50g of solid sample

Add equal volume of sterile deionised water

Adjust to pH 3.5 with 5 Molar hydrochloric acid

Add aluminium chloride to a final concentration of 0.0005 Molar

Stir at room temperature for 30 minutes

Centrifuge 10000g for 30 minutes

Resuspend pellet in
beef extract solution (3% m/V, pH 9.5) or
skimmed milk solution (0.1% m/V, pH 9.5)
and mix thoroughly

Centrifuge 10000g for 30 minutes

Flocculate supernatant at pH 3.0–3.5 (for beef extract)
or pH 4.0–4.5 (for skimmed milk)

Recover precipitate by centrifugation
(3500g for 20 minutes)

Dissolve pellet in 5–10 mL
0.15 Molar disodium hydrogen phosphate

Detoxify

Store below –20°C or assay immediately

Figure 3 Identification of enterovirus, reovirus and adenovirus isolates

96-well microtitre, flat-bottomed plate*

Add 25 μL of virus isolate
(usually diluted 10^{-3})

Add 25 μL of specific viral antiserum
(working dilution previously established)

Gently mix by tapping the plate and incubate
at 37°C for 30 minutes

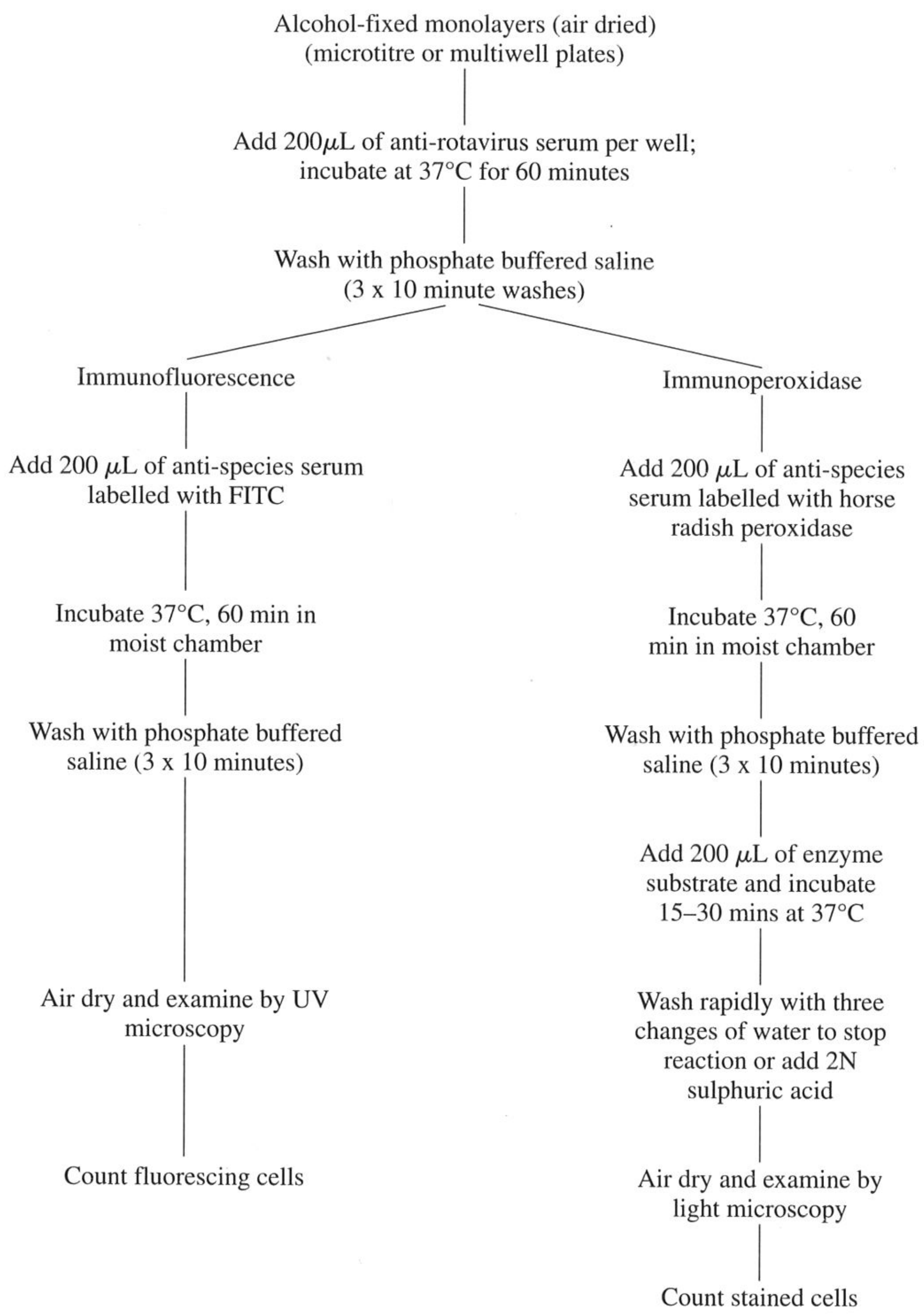
Add 100 μL cell suspension
(5×10^5 cells mL)

Incubate at 37°C in 5% carbon dioxide in air
atmosphere and greater than 95% relative humidity

Examine for cytopathic effect and neutralisation
from the second day of incubation

* Each well to contain antiserum to each virus type being screened. Include antiserum, virus and cell controls in all tests. If neutralisation fails to occur, repeat using higher dilutions of the virus isolate before screening against other virus types.

Figure 4 Immunolabelling of rotavirus-infected cells



Appendix 1 Some commonly used media

Cell culture media and plastics, virus reagents, water processing materials and chemicals may be obtained from reputable laboratory suppliers.

(a) BGM cell cultures—requirements (mL) for 1 litre working strength medium.

	Growth medium	Maintenance medium
Eagle's Minimal Essential Medium (MEM) (x10 conc)	100	100
Foetal bovine serum	50	20
Penicillin/Streptomycin (supplied at 5000 iu, μg/mL)	10	10
Glutamine (200mM)	10	10
Non-essential amino acids (x100)	10	10
(81.4 mg/L x 100)	10	10
Sodium bicarbonate (CO_2 gassed; 4.4 %m/V)	25	50
Sterile deionised water	to 1 litre	to 1 litre

[Serum-free medium for diluent and washing purposes is maintenance medium without serum, the deficit being made up with water].

(b) BGM agar medium for plaque assays (300mL)

Eagle's MEM	30
Foetal bovine serum	6
Penicillin/Streptomycin	3
4.4% Sodium bicarbonate	15
Glutamine	3
Non-essential amino acids	3
Gentamicin (4 mg/mL)	3
Fungizone (250μg/mL)	3
Neutral red stain (0.1% m/V)	9
Sterile deionised water	to 300mL

Warm the above mixture to 45°C then mix with 100 mLof 3.5% m/V Bacto agar which has been previously sterilized by autoclaving and held at 45°C. Sufficient for 30 petri dishes.

(c) LLC—MK2 cell culture—1 litre working strength medium

	Growth medium	Maintenance medium
Eagle's Medium 199 (x10)	100	100
Foetal bovine serum	50	10
Penicillin/Streptomycin	10	10
Sodium bicarbonate	25	40
Fungizone(250 μg/mL)	10	10
Sterile deionised water	to 1 litre	to 1 litre
Hepes buffer 1 Molar	2 mL/100mL growth medium	

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