

NATIONAL STANDARD METHOD

ELECTRON MICROSCOPY: DIFFERENTIAL CENTRIFUGATION (DOUBLE-SPIN METHOD)

VSOP 14

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









UK Clinical Virology Network

ELECTRON MICROSCOPY: DIFFERENTIAL CENTRIFUGATION (DOUBLE-SPIN METHOD)

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STATUS OF NATIONAL STANDARD METHODS

National Standard Methods, which include standard operating procedures (SOPs), algorithms and guidance notes, promote high quality practices and help to assure the comparability of diagnostic information obtained in different laboratories. This in turn facilitates standardisation of surveillance underpinned by research, development and audit and promotes public health and patient confidence in their healthcare services. The methods are well referenced and represent a good minimum standard for clinical and public health microbiology. However, in using National Standard Methods, laboratories should take account of local requirements and may need to undertake additional investigations. The methods also provide a reference point for method development.

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More details can be found on the website at www.evaluations-standards.org.uk. Contributions to the development of the documents can be made by contacting standards@hpa.org.uk.

The reader is informed that all taxonomy in this document was correct at time of issue.

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AMENDMENT PROCEDURE

Controlled document reference	VSOP 14
Controlled document title	Electron Microscopy: Differential Centrifugation (Double-Spin Method)

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

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

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
8/ 11.06.10	5	6	All 11	All 9 Notification to the HPA	Section renamed and reference inserted Document and references reviewed

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ELECTRON MICROSCOPY: DIFFERENTIAL CENTRIFUGATION (DOUBLE-SPIN METHOD)

SCOPE OF DOCUMENT

This procedure describes the concentration of virus using differential centrifugation before grid preparation.

This method has been found to be suitable for the examination of faecal specimens, urine specimens, tissue biopsy homogenates and the examination of cell cultures for the presence of viruses.

INTRODUCTION

Background

A homogenised specimen is centrifuged at low speed to remove large debris and bacteria. The clarified suspension is then subjected to one or more cycles of ultracentrifugation to recover any virus particles present. The procedure can be used to concentrate viruses from any type of specimen that can be homogenised in suspension. It is useful in any situation where there is sufficient extract to allow concentration to be performed (minimum volume 1 mL).

It is currently recommended that ultracentrifugation, ammonium sulphate precipitation ([VSOP 15 – Electron microscopy: Ammonium sulphate precipitation](#)), or immune capture ([VSOP 16 – Electron microscopy using solid phase immune electron microscopy](#)) is used for all faecal specimens from outbreaks of gastroenteritis.

1 SAFETY CONSIDERATIONS²⁻¹²

1.1 SPECIMEN COLLECTION

Appropriate hazard labelling according to local policy.

1.2 SPECIMEN TRANSPORT AND STORAGE

Sterile leakproof container in a sealed plastic bag.

1.3 SPECIMEN PROCESSING

- Bacteria, viruses, fungi or parasites, encountered unexpectedly, can cause severe and sometimes fatal disease. Laboratory acquired infections have been reported. Guidance on vaccination is given in the Health Protection Agency immunisation policy. Processing must be carried out by trained laboratory personnel in a properly equipped laboratory and under the supervision of a qualified microbiologist. Disposable gloves should be worn during all procedures
- Where the clinical features may indicate a Hazard Group 3 infection sample processing should only be carried out with appropriate containment conditions. It is recommended that faecal specimens from patients with bloody diarrhoea or haemolytic uraemic syndrome (*Escherichia coli* O157) are not processed until bacteriology results are available. Suspected Hazard Group 4 samples should only be processed for electron microscopy after inactivation
- Staff who are pregnant and susceptible to varicella/zoster, rubella or parvovirus should not handle any clinical material likely to contain these viruses
- Other samples may be handled as Hazard Group 2. Processing in a microbiological safety cabinet is not mandatory but is advisable. Some specimens may contain pathogenic bacteria or parasites
- Laboratory procedures that give rise to infectious aerosols must be conducted in a microbiological safety cabinet. Vortexing containers creates aerosols that can be released when the container is opened. Containers should be resistant to breakage (polypropylene, not glass or polystyrene) and containers that have been vortexed should only be opened within a laboratory safety cabinet
- Refer to current guidance on the safe handling of all Hazard Group 2 organisms documented in this SOP
- Prepared grids may be infectious prior to examination in the EM. Dispose of used grids by autoclaving
- It is recommended that any grids prepared from body fluids from patients who are HIV or HBV positive are inactivated with a drop of fixative (see appendix)
- Compliance with packing, postal and transport regulations is essential
- Arklone (1, 1, 2-Trichlorotrifluoroethane) has the capacity, like other fluorinated hydrocarbons, to cause ozone depletion and should be used in minimal quantities. It may be replaced with Vertrel® XF manufactured by Dupont™ which is not implicated in ozone depletion
- Use caution when handling forceps. Forceps should be decontaminated after use

1.3.1 ULTRACENTRIFUGATION

Ultracentrifugation presents particular safety hazards associated with the high rotational forces generated. Staff should be made aware of the dangers and instructed in their use. A service company or the manufacturer should regularly maintain the centrifuges and rotors.

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Laboratories should have their own maintenance protocols, in accordance with the manufacturer's recommendations.

1.3.2 CHEMICAL HANDLING

Phosphotungstic acid is corrosive. Gloves and eye protection should be worn when making the non-pH adjusted solution.

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

2 SPECIMEN COLLECTION

2.1 OPTIMAL TIME OF SPECIMEN COLLECTION

Faecal specimens

A minimum of 1 g of faeces is required which should be collected directly into a clean container.

Successful demonstration of virus particles by electron microscopy requires specimens that are taken during the acute phase of the illness. Best results are obtained with specimens collected within 48 hours of the onset of symptoms.

Urine

At least 5 mL of urine are required in a sterile leakproof container. The number of virus particles excreted in urine is directly related to the amount of urine produced. Experience indicates that the most consistent results are obtained from specimens of early-morning urine.

Biopsy samples

Biopsy material should be removed into a sterile clear bottle. The material must not be fixed. Separate specimens should be removed into appropriate fixative for histological investigation if required. Sufficient material should be removed to ensure a reasonable amount of the lesion is examined. Approximately 1 mm³ of lesion is required.

Cell cultures

Cell cultures that are suspected of containing viruses should be sent for examination if required. The specimen must contain both cell culture fluid and cells. Approximately 1 mL of cell/fluid suspension is required.

2.2 CORRECT SPECIMEN TYPE AND METHOD OF COLLECTION

As above

2.3 ADEQUATE QUANTITY AND APPROPRIATE NUMBER OF SPECIMENS

As above

3 SPECIMEN TRANSPORT AND STORAGE

3.1 TIME BETWEEN SPECIMEN COLLECTION AND PROCESSING

Specimens should be transported and processed as soon as possible.

3.2 SPECIAL CONSIDERATIONS TO MINIMISE DETERIORATION

Original samples should be stored at +4°C if processing cannot be performed within 24 hours.

Note: Faecal specimens must not be frozen

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4 EQUIPMENT AND REAGENTS

4.1 EQUIPMENT

- Bench centrifuge capable of producing 2,000 to 3,000x g
- Preparative ultracentrifuge and rotor capable of producing at least 25,000x g with a minimum sample volume of 1 mL
- Fine-point jeweller's forceps eg dumont forceps number 5. A separate pair of forceps is required for each sample processed. Forceps must be decontaminated after use
- Coated electron microscope grids (VSOP 12 – Preparation of coated grids for electron microscopy)
- Hydrophobic surface eg 'BenchKote', Parafilm or a Petri-Dish
- Strips of clean filter paper about 1 cm x 2.5 cm in size
- Clean 5 mL bottles or plastic tubes
- Clean glass microscope slides
- Disposable transfer pipettes
- Griffiths Tube glass homogenisers (optional)

4.2 REAGENTS

- Negative stain: The negative stain that is most widely used for viruses in electron microscopy is phosphotungstic acid. Alternatives are available
- Specimen diluent: deionised or glass distilled water, sterile phosphate-buffered-saline (PBS) and sterile single-strength minimal essential medium have all been used as a sample diluent. Broad-spectrum antibiotics and a fungicide may be incorporated into the sample diluent
- Deionised or glass distilled water
- Arklone (1,1,2-Trichlorotrifluoroethane) or Vertrel® XF

5 SPECIMEN PROCESSING

5.1 TEST SELECTION

N/A

5.2 CULTURE AND INVESTIGATION

N/A

5.3 PREPARATION OF CLINICAL SPECIMENS

Faeces

- Dilute faecal specimens to 10 - 20% weight/volume in the specimen diluent. The faecal extract must be thoroughly emulsified. If samples are mixed by shaking, or preferably vortexing, the emulsion must be enclosed within a sealed bottle which must be opened within a laboratory safety cabinet and remain unopened for at least 30 minutes to allow aerosols to settle
- Clarify the suspension by centrifuging (2,000 to 3,000x g for up to 30 minutes) in a sealed-bucket rotor

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- Alternatively, dilute the faecal specimen to 10 - 20% weight/volume in a 1:1 mixture of specimen diluent and Arklone or Vertrel® XF. This helps to release virus bound to membranes and remove lipids which interfere with attachment of the sample to the EM grid. Emulsify thoroughly, preferably by vortexing. Stand the sealed container at 4°C for 30 minutes. Centrifuge (2,000 to 3,000x g for up to 30 minutes) in a sealed-bucket rotor and harvest the upper aqueous layer

Urines

- Subject approximately 1 mL of the urine to two freeze/thaw cycles to disrupt any cellular material present in the specimen and to release virus particles into the suspension
- Clarify any cloudy urines by centrifuging (2,000 to 3,000x g for up to 30 minutes) in a sealed-bucket rotor

Biopsy material

- Disrupt biopsy material in about 1 mL of deionised water or glass distilled water in a suitable homogeniser (eg Griffiths tube)
- Clarify the suspension by centrifuging (2,000 to 3,000x g for up to 30 minutes) in a sealed-bucket rotor

Cell cultures

- Disruption of cell material to release virus may be achieved by freeze-thawing or by lysis in distilled water
- Clarify the suspension by centrifuging (2,000 to 3,000x g for up to 30 minutes) in a sealed-bucket rotor

5.4 CONCENTRATION BY CENTRIFUGATION

- Load from 1 to 10 mL (dependent on the type of rotor used) of clarified supernatant from the first spin into an ultracentrifuge tube. Forces in excess of 25,000x g should be used to pellet virus and the times needed to efficiently pellet virus should be established by trials or calculated from rotor k factors (see appendix). A sucrose layer can optionally be used to give a partial purification of the pellet. Up to one tenth of the tube volume of 20% to 30% weight/volume sucrose (dissolved in phosphate buffer or water) is carefully layered into the bottom of the tube before high-speed centrifugation
- Discard the entire supernatant, including any sucrose solution
- Resuspend the pellet in a small volume of sample diluent (30 to 150 µL depending on the starting volume). It can be beneficial at this stage to add a small quantity of Arklone or Vertrel® XF if not already used, and vortex. This helps to release virus bound to membranes. A third slow speed centrifugation (up to 2,500x g) may be necessary if the resuspended pellet is too turbid and is essential if Arklone or Vertrel® XF has been added in order to separate the solvent phase from the virus containing supernatant

5.5 PREPARATION AND STAINING OF GRIDS

- Spot the prepared specimen onto a hydrophobic surface pre-labelled with the specimen number. Specimen drops must be at least 1 cm apart to minimise the risk of sample-to-sample contamination
- Place a grid coated with a suitable support film, support side down, onto the surface of the drop using clean forceps and incubate at room temperature. The precise adsorption time varies depending on the number of virus particles expected in the preparation and the amount of 'debris' present in the sample and is best determined by experiment and experience

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- Pick up the grid and blot to remove excess fluid by touching a piece of filter paper to the edge of the grid. Transfer the grid to a drop of a suitable negative stain. After staining (staining time is variable and depends on experience, but should be a minimum of about 5 seconds), the grid is blotted to remove all but a thin film of stain and air dried, support film side up, on a clean piece of filter paper. An optional washing stage in which the inoculated grid is transferred onto a drop of deionised or glass distilled water for a few seconds may precede the staining if required
- Alternatively, equal volumes of stain and sample can be mixed and the grid applied as above
- Examine in the electron microscope using a screen magnification of approximately x50,000

5.6 IDENTIFICATION

Viruses are identified by their characteristic morphology.

6 QUALITY ASSURANCE

6.1 ASSESSMENT OF PREPARATION

Grids should be scanned for at least 5 minutes before being considered 'negative'. Grids with damaged support films should be repeated as insufficient grid surface will not permit the minimum required examination. Grids that are over-stained or have too much adherent material to be easily readable should be repeated with a more dilute or re-clarified extract.

6.2 INTERNAL AND EXTERNAL QUALITY ASSURANCE

Laboratories should participate in any external quality assurance schemes that may become available for electron microscopy¹³.

7 LIMITATIONS¹⁴

Successful detection of viruses depends on skill and experience of the microscopist, collection of specimens at the appropriate time, transport, storage and processing and the provision of adequate/suitable clinical information.

Viruses will not be detected unless there are sufficient numbers in the sample, which is usually considered to be 10^6 - 10^9 particles/mL sample.

The procedure(s) in these documents aim to describe good microbiological standard methods for the specimen types specified. Other procedures may be required and professional interpretation by qualified staff is essential. Please note that knowledge of infectious diseases changes constantly and although this NSM is regularly reviewed it may not include emerging pathogens.

8 REPORTING PROCEDURE

8.1 REPORTS

Negative samples should be reported as 'No virus particles seen'

Positive samples should be reported as eg 'Astrovirus particles present'

Some workers may wish to provide an estimate of the amount of virus present. Accurate quantitative assessment requires the application of specialist procedures but a well-tried scheme that gives consistent results is as follows:

+++	equates to one or more virus particles in each of four randomly selected fields.
++	equates to 10 or more virus particles seen in each of four grid-squares examined.
+	equates to between 1 and 10 virus particles seen in each of four grid-squares examined.
+/-	equates to between 1 and 10 virus particles seen in total.

8.2 REPORTING TIME

Urgent requests Telephone as soon as results are available

Written reports Normally available the next working day or in accordance with local reporting policy

9 NOTIFICATION TO THE HPA^{15,16}

Any positive result should be reported to the HPA or equivalent in accordance with published guidelines.

10 ACKNOWLEDGEMENTS AND CONTACTS

This National Standard Method has been developed, reviewed and revised by Dr Hazel Appleton of the Electron Microscopy Unit Centre for Infections and the Standard Methods Working Group for Virology (http://www.hpa-standardmethods.org.uk/wg_virology.asp). The contributions of many individuals in clinical virology laboratories and specialist organisations who have provided information and comment during the development of this document, and final editing by the Medical Editor are acknowledged.

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APPENDIX

Phosphotungstic Acid - this is used at a concentration of between 1% and 3% by weight in deionised water. The exact concentration used is a matter for personal preference. The pH is adjusted to approximately 6.5 (+/-0.1) by the addition of sodium hydroxide or potassium hydroxide. The ready-to-use stain is relatively stable at room temperature but the pH must be checked regularly.

Fixation of grids prior to negative staining - transfer the inoculated grid from the specimen to a drop of fixative (eg buffered formalin). Leave at room temperature for at least 2 min. Stain as above on a drop of phosphotungstic acid. This procedure may adversely affect the morphology of some common viruses.

Notes on centrifugation times - the time taken to pellet a given particle depend on the *k* factor of the rotor, the speed used and the sedimentation coefficient of the particle and can be calculated by the formula $t=k/s$ (*s*=the sedimentation coefficient in Svedberg units, *t*=time). The *k* factors for rotors are usually given by the manufacturer or can be calculated from a formula. The *k* factor varies widely from around 25 for a rotor running tubes with a short path length at very high maximum speeds to as much as 800 for tubes with a long path length at low maximum speeds. The time taken to pellet Poliovirus (*S* = 156) in the first example would be $t=25/156$, = 0.16 h in the second example would be $t=800/156$, = 5.12 h

Adherence of samples to grids - Some samples do not adhere well to the grid. This is a problem particularly for samples which have been inactivated prior to processing for electron microscopy. There are various methods for enhancing adherence. The simplest is to add bovine plasma albumin or bacitracin to the sample or stain at a final concentration of 0.05%. Alternatively grids may be pre-treated with 1% Alcian blue G800 or 1% poly-L-lysine, or submitted to glow discharge in a vacuum coating unit if suitable equipment is available.

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