

NATIONAL STANDARD METHOD

PROCEDURE FOR THE CARE AND PROPAGATION OF CELL CULTURES FOR VIRUS ISOLATION

VSOP 39

Issued by Standards Unit, Department for Evaluations, Standards and Training
Centre for Infections



UK Clinical Virology Network

PROCEDURE FOR THE CARE AND PROPAGATION OF CELL CULTURES FOR VIRUS ISOLATION

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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.

National Standard Methods are developed, reviewed and updated through an open and wide consultation process where the views of all participants are considered and the resulting documents reflect the majority agreement of contributors.

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The performance of standard methods depends on the quality of reagents, equipment, commercial and in-house test procedures. Laboratories should ensure that these have been validated and shown to be fit for purpose. Internal and external quality assurance procedures should also be in place.

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The HPA aims to be a fully Caldicott compliant organisation. It seeks to take every possible precaution to prevent unauthorised disclosure of patient details and to ensure that patient-related records are kept under secure conditions¹.

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 39
Controlled document title	Procedure for the care and propagation of cell cultures for virus isolation

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
1/ 11/12/09	1	2	1 20	Title page References	SMF logo added Assessed and updated

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PROCEDURE FOR THE CARE AND PROPAGATION OF CELL CULTURES FOR VIRUS ISOLATION

SCOPE OF DOCUMENT

This National Standard Method describes the production of cell cultures for virus isolation/detection.

The cell lines included in the Standard Method are by no means exclusive, or exhaustive, but are among those most commonly used for virus isolation/detection. The most important consideration when selecting cell lines for this purpose is their susceptibility to the virus(es) under investigation.

The cell lines most commonly used for virus isolation and their individual requirements².

CELL LINE	DISSOCIATING AGENTS	VIRUS SENSITIVITY*
<p>RhMK Uncharacterised primary or secondary cells.</p> <p>Origin Rhesus monkey kidney.</p> <p>Mixed cell types present.</p> <p>No longer available in the UK from April 2006 for ethical reasons.</p>	Trypsin/Versene	<p>Influenza viruses Parainfluenza viruses Enteroviruses. Polioviruses Adenoviruses Mumps virus Measles virus</p>
<p>MRC-5 Well characterised semi-continuous cell line.</p> <p>Origin human foetal lung.</p> <p>Fibroblastic in character</p>	Trypsin/Versene	<p>Herpes simplex virus Adenoviruses Respiratory syncytial virus Varicella Zoster virus Cytomegalovirus Enteroviruses Polioviruses Rhinoviruses</p>
<p>HEp-2 Well characterised continuous cell line.</p> <p>Origin human carcinoma of larynx.</p> <p>Epithelial in character.</p>	Versene	<p>Adenoviruses Respiratory syncytial virus Herpes simplex Enteroviruses Polioviruses</p>
<p>VERO Well characterised continuous cell line.</p> <p>Origin African green monkey lung.</p> <p>Epithelial in character</p>	Trypsin/Versene	<p>Herpes simplex virus Enteroviruses Polioviruses Adenoviruses Measles virus</p>

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<p>PLC-PRF5 Well-characterised continuous cell line.</p> <p>Origin human hepatoma.</p> <p>Hepatitis B virus surface antigen expressed.</p>	<p>Trypsin/Versene</p>	<p>Influenza viruses Parainfluenza viruses Adenoviruses Enteroviruses Polioviruses Respiratory syncytial virus</p>
<p>MDCK Well-characterised continuous cell line.</p> <p>Origin – Dog Kidney</p> <p>Epithelial in Character</p>	<p>Trypsin/Versene NB Cells may require prolonged treatment for dissociation</p>	<p>Influenza viruses Parainfluenza viruses</p>

* The cell line has been shown to grow the listed viruses but may not be the cell line of choice for those viruses.

INTRODUCTION

Background

The process by which mammalian cells are removed from tissues and grown under controlled conditions in a laboratory is referred to as cell culture. Once established a cell culture is capable of self sustenance through growth and replication until limited by a variable such as nutrient depletion. Therefore all cultures will need periodic medium changes (“feeding”) followed eventually by subculture when the cells are proliferating. Intervals between sub-cultures can vary from one cell line to another depending on the rate of growth and metabolism. When all the available substrate is used or when the cell concentration exceeds the capacity of the medium either the frequency of medium changing must increase or the culture must be divided.

The usual practice in sub-culturing an adherent cell line involves removal of the medium and dissociation of the cells in the monolayer with the proteolytic enzyme trypsin, the chelating agent versene (EDTA) or a mixture of both³.

Exceptionally, some cell monolayers cannot be dissociated with trypsin and require the action of alternative proteases such as acutase, pronase, dispase or collagenase⁴⁻⁶.

A major problem in cell culture production is the maintenance of sterility of components and freedom of cell lines from contamination. Bacteria especially mycoplasma, viruses, fungi or other cell types can contaminate cell cultures. The contamination can originate in the tissue used to initiate the cell culture, the components of media, the operator or from the environment.

TECHNICAL INFORMATION/LIMITATIONS

N/A

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1 SAFETY CONSIDERATIONS⁷⁻¹⁶

1.1 SPECIMEN COLLECTION

N/A

1.2 SPECIMEN TRANSPORT AND STORAGE

N/A

1.3 SPECIMEN PROCESSING

Local COSHH and general risk assessments (GRA) must be carried out.

Some cell cultures and the chemicals used during their culture are potentially hazardous and must be handled with caution. When there is doubt in regard to the risks implicated in handling a reagent or cell line, refer to the appropriate local COSHH assessment for further information. All staff involved in handling cell cultures must be made aware of the hazards and risks involved.

Although carefully selected, controlled, and monitored animals are used, cell lines derived from them may contain viruses that are pathogenic to humans. Cells from animals of unknown or uncertain origin must not be used for virus isolation.

Continuous cell lines may have been derived from carcinomas or transformed by a viral agent. Although the risk to laboratory staff may be statistically small, they should be handled with the same precautions as animal derived cells.

Cell lines which may be hazardous (see appropriate COSHH references) should be processed in a class II safety cabinet and the operator should wear gloves and a gown at all times.

Cell lines must not be disposed of as general waste but using the same methods as for pathogenic microorganisms. This involves either autoclaving and/or incineration of all containers and instruments used. All liquid waste should be disposed of into Hypochlorite solution (2,500ppm Chlorine) or other suitable disinfectant solution.

In attempting to isolate viruses such as herpes simplex from HIV-positive patients there is the danger that if cells such as MRC 5 are used which have CD4 receptors HIV might also replicate if incubation exceeds three days. Hence any manipulations should take place in Containment Level 3, or tubes not opened after inoculation.

2 SPECIMEN COLLECTION

2.1 OPTIMAL TIME OF SPECIMEN COLLECTION

N/A

2.2 CORRECT SPECIMEN TYPE AND METHOD OF COLLECTION

NA

2.3 ADEQUATE QUANTITY AND APPROPRIATE NUMBER OF SPECIMENS

NA

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3 SPECIMEN TRANSPORT AND STORAGE

3.1 TIME BETWEEN SPECIMEN COLLECTION AND PROCESSING

3.2 SPECIAL CONSIDERATIONS TO MINIMISE DETERIORATION

Compliance with current postal and transportation regulations is essential.

For transport by road or rail, cell cultures should be wrapped in absorbent protective materials and packed in strong containers filled with polystyrene chips or other appropriate shock-absorbing material. For transport by air, see the current appropriate regulations.

Special consideration must be made to minimise deterioration. Prolonged exposure to temperatures below 10°C and above 35°C must be avoided. The delivery system used should guarantee delivery within 24 hrs.

4 EQUIPMENT AND REAGENTS

4.1 EQUIPMENT

4.1.1 INCUBATORS

Incubation can be performed in sealed flasks in a regular dry incubator or hot room capable of maintaining temperatures within the range 30 – 37°C some vessels eg dishes or multiwell plates require a controlled atmosphere with high humidity and increased CO₂ levels.

CO₂ levels should be checked daily and the incubator recalibrated every six months. The temperature should be checked daily using a calibrated thermometer or thermocouple.

The interiors of incubators should be cleaned at regular intervals to prevent a build-up of microorganisms, using alcohol or a mild phenolic disinfectant followed by cleaning with a mild detergent solution.

4.1.2 CABINETS

Class II safety cabinets should be used for manipulation of hazardous cell lines. Laminar flow cabinets (vertical flow) may be used for manipulating reagents and media but only after local risk assessments have been carried out. All cabinets should meet all the relevant current standards.

To prevent cross contamination between cell lines and build-up of microorganisms, cabinet interiors should be cleaned when the cell line being manipulated changes and at the end of each work period. Alcohol (Industrial methylated spirit) or a phenolic disinfectant should be used.

The airflow should be checked weekly using a calibrated anemometer. Readings should be taken at each corner and the centre of the front opening; airflows must comply with the manufacturer's and national safety recommendations¹⁶.

Biological tests may be carried out to check the performance of the filters on a monthly basis. These tests should consist of exposing bacterial and fungal culture plates at several positions across the work surface of the cabinet for at least an hour whilst the cabinet is operating, to detect the presence of any bacterial or fungal contamination due to a failure of the filters.

Cabinets should be checked at 6 monthly intervals by an appropriately qualified engineer and a certificate of satisfactory performance issued. Records of any failure of cabinets and the reason must be maintained.

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4.1.3 CULTURE VESSELS

Disposable plastic tissue culture grade vessels are preferred but suitable neutral glass alternatives may be used.

Glass vessels must be cleaned using a cell culture compatible cleaning agent, followed by thorough rinsing in tap water (x6) and deionised water (x3). Alternatively, a suitable automatic washer may be used which has a rinse programme compatible with cell culture requirements, a cleaning agent compatible with cell culture must be used.

4.1.4 OTHER EQUIPMENT

Pipettes - in order to prevent contamination, use only those which have an incorporated membrane filter.

Centrifuges - these must comply with the current relevant safety standards.

Microscopes - both inverted and standard microscopes are required.

Racks for cell culture tubes; these should be designed to hold the tubes in a slightly inclined position, which permits attachment and growth of cells towards the bottom of the tubes and ensures covering of the monolayer with the nutrient medium. Preferably, they should be capable of withstanding sterilisation by autoclaving.

4.2 REAGENTS

Unless otherwise stated, all reagents are sterile and all procedures/manipulations must be performed aseptically.

Reagents and media must only be obtained from reputable sources.

Samples of every batch of reagents, solutions, and media produced "in house", or commercial reagents aliquoted prior to use, must be tested for sterility after being dispensed into their containers.

4.2.1 BASAL MEDIA

A wide range of media may be used but those stated below have been found to produce satisfactory growth in the cell lines listed in this National Standard Method.

To ensure that a more consistent, quality controlled product is used, basal media should be purchased ready for use from an accredited supplier (appropriate ISO or similar). Batches should be tested for suitability prior to use and large volumes of approved batches reserved.

Minimal essential medium-Earles salt buffered (EMEM)

In order to reduce the number of reagents to be added, it is suggested that medium is purchased ready for use, thereby reducing the risk of contamination. Medium should be purchased which already contains sodium bicarbonate, L-glutamine, and a pH indicator.

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4.2.2 COMPLETE MEDIA

For propagation and maintenance of cells, the following complete media may be produced by making the listed additions aseptically to the basal medium:

EMEM growth medium

To EMEM add: -

Penicillin	final concentration	50 iu/mL
Streptomycin	final concentration	100 µg/mL
Amphotericin B	final concentration	2.5 µg/mL
Newborn calf serum ³	final concentration	10%

EMEM maintenance medium

To EMEM add: -

Penicillin	final concentration	50 iu/mL
Streptomycin	final concentration	100 µg/mL
Amphotericin B	final concentration	2.5 µg/mL
Foetal calf serum ³	final concentration	2%

4.2.3 SERA

In routine use both newborn and foetal bovine sera have been found suitable for cultivation of the cells listed in this Standard Method. However, enhanced sera, offering reduced batch to batch variation, and offering equal or improved performance. All serum used must be tested for suitability prior to purchase. It is advisable to reserve a large batch and test for suitability prior to confirming purchase. If serum is received part frozen it is advisable to allow it to thaw completely before refreezing.

Do not use heat inactivated serum as it is not suitable for cultivation of some cell lines.

4.2.4 SUPPLEMENTARY REAGENTS

Antibiotics

Concentrations of antibiotics stated are those found in text books⁴

Note: Antibiotics can cause allergic reactions

Phosphate buffered saline (PBS)

Dulbecco A solution

(without calcium and magnesium) pH 7.3.

Versene

Dissolve 10 gms, ethylenediaminetetra-acetic acid disodium salt (Analar grade) in 1000 mL, of distilled water. Distribute into bijou bottles and sterilise by autoclaving at 115°C for 10 mins. Store at 2-8°C. For use, add 0.4 mL to 20 mL of PBS.

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5 SPECIMEN PROCESSING / PROCEDURE

5.1 TEST SELECTION

A suitable reliable identification system, such as colour coding of tubes, should be used to indicate the type of cell line contained within a culture vessel. Vessels should be labelled with the seeding date.

Cells must be obtained from a reputable supplier who can supply a full history and certification of freedom from contaminants; ECACC or similar. Cells may be purchased either in suspension or as a monolayer in culture flasks.

Avoid pouring liquids from or into containers, pipetting should be used wherever practicable.

5.1.1 FLASKS

Follow manufacturer's instructions for the treatment of the culture. Otherwise, the following method may be used.

On arrival, examine flasks to assess the cell growth and condition. On receipt record as per local requirements.

If cells are satisfactory, add sufficient growth medium, if needed, and then incubate at 37°C.

When cell growth reaches confluence, the flasks are ready for use or further passage.

At this stage, the medium may be changed to maintenance medium until the flasks are required. Otherwise they may be subcultured directly into tubes (see below), passaged (if suitable cell line), or disassociated and frozen in liquid nitrogen (see Appendix).

5.1.2 SUSPENSION

1. Dilute the suspension as instructed by the supplier or dilute to a suitable concentration
2. Add the diluted suspension to cell culture flasks or if required directly into tubes. The amount added to each flask is dependent upon the flask size but should be sufficient to cover the entire growth area to a depth of 3-5 mm
3. Incubate at 37°C
4. When cell growth is confluent, the flasks are ready for use

At this stage, the medium may be changed to maintenance medium until the flasks are required. Otherwise they may be subcultured directly into tubes (see below), passaged (if suitable cell line), or disassociated and frozen in liquid nitrogen.

5.2 CULTURE AND INVESTIGATION

These instructions are for a 75 cm² flask, volumes should be adjusted to suit the size of the vessel if different.

1. Select a flask that has achieved confluent growth
2. Remove the medium and rinse with two changes of approx. 35 mL of PBS. Drain the second change completely
3. Add 15 mL of dissociating mixture and rinse all the internal surfaces of the flask carefully, then remove the mixture but do not drain completely
4. Incubate the flask at 37°C until the cells detach, the time will vary according to cell type

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5. Re-suspend the detached cells in 18 mL of growth medium
6. Determine the cell concentration using a counting chamber (modified Fuchs-Rosenthal or similar)
7. Dilute the cells in growth medium to produce the required amount of suspension at a concentration of approx. 1.0×10^5 /mL, (with experience this concentration may be adjusted to achieve the optimum outcome for individual cell lines). With continuous cell lines, any undiluted cells may be returned to the original vessel with sufficient growth medium to continue the culture for future use
8. Mark a line and/or the date on the side of the tubes; this will form a reference so that the tube is always placed in the incubator with the line at the top
9. Seed diluted cells into tissue culture tubes in suitable volumes
10. Incubate at 37°C at a slight angle from horizontal (approx. 5°)
11. When tubes have formed a monolayer which is approximately 80% confluent they are ready to use and should be changed to maintenance medium

5.2.1 DISAGGREGATION¹⁷

Remove cell culture medium from the culture vessel by aspiration and discard, rinse the monolayer with Ca^{++} and Mg^{++} free salt solution (such as 0.1M PBS pH 7.2) to remove all traces of serum. Remove the salt solution by aspiration.

Repeat the step outlined above (these steps are important as they remove traces of serum that might otherwise inhibit the action of Trypsin).

Dispense pre-warmed (to 37°C) 0.25% Trypsin or Trypsin/Versene solution into the cell culture vessel to completely cover the monolayer of cells (5 mL/75 cm² flask) and incubate at 37°C for 1 to 2 minutes.

Remove the Trypsin or Trypsin/Versene solution by aspiration and return the closed culture vessel to the incubator.

The coated cells are allowed to incubate until they round up and detach from the surface. Progress can be checked by examination with an inverted microscope. The time required to remove cells from the surface is dependent on the cell type, population density, potency of Trypsin and time since last subculture. Trypsin causes cellular damage and the time of exposure should be kept to a minimum.

When the trypsinisation process is complete, the cells will be in suspension and appear rounded.

It is advisable to add a medium containing serum to the cell suspension as soon as possible to inhibit further enzyme activity which may damage cells.

Cells can be re-suspended by gently pipetting the cell suspension. Further dilution can be made, if required, for cell (viable) counts or sub-culturing.

If serum free medium is used at this stage a trypsin inhibitor will need to be used.

A suspension of single cells (not clumped) is desirable at sub-culture to ensure an accurate cell count and uniform growth on reseeded.

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5.2.2 AFTERCARE

Tubes should normally be maintained at 37°C until required.

If it is necessary to hold tubes for more than 4-5 days, continuous and semi-continuous cell lines may be moved to 33°C until needed. Primary cells may not tolerate being moved to 33°C; doing so should be based on experience with each cell type.

Change cells onto fresh maintenance medium every 3-4 days. When cells are passaged this should be recorded on the localized record sheet.

5.2.3 PREVENTION OF CONTAMINATION

Laboratory layout

Facilities available may depend on space and economic importance of cell culture system.

Areas to consider:

Designated room for cell culture production

Design of room for ease of cleaning

Use of laminar flow cabinet

Use of UV light for sterilizing cabinet

Prevention of a large number of staff having access to the area

Reduction of risk by discarding continuous cell cultures at regular intervals and bringing in new cell cultures from an approved source

Aseptic technique

All staff must be trained in basic aseptic technique

Use of disinfectants such as hypochlorite and 70% alcohol

Good hand washing practice

Use of disposable gloves where appropriate

Use of Bunsen burner for flaming surfaces

Disinfection of work areas when there is a change of batch number in the cell lines used

Use of antibiotics

It is preferable and in some cases essential that laboratories use antibiotic-free medium for virus culture. However, due to the risks of contamination, most laboratories use some antibiotics. First line antibiotics include Penicillin 100 iu/μL, Streptomycin 100 μg/mL. The additional use of other antibiotics may depend on the isolation of the contaminating organism and a sensitivity test being performed to find the most suitable antibiotic.

5.2.4 DETECTION OF CONTAMINATION

Detection of bacterial or fungal contamination in media

Sterility testing of components of the medium and completed growth and maintenance media should be carried out. A small number of aliquots of the components or the complete medium should be inoculated into media suitable for growing bacteria or fungi, incubated and examined daily for a designated time period. Nutrient broth is suitable for the detection of bacterial contamination. Sabouraud agar is suitable for detection of fungal contamination. All cultures should be incubated at 37°C and room temperature.

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Any component that is found to be contaminated should be discarded. (In exceptional circumstances it may be filtered/re-sterilized and re-tested)¹⁸.

Detection of bacterial and fungal contamination in cell culture

Bacterial (except mycoplasma) and fungal contamination are generally visible in cell culture bottles. Low grade contamination can be detected by inoculating the cell suspension into bacterial or fungal culture media.

Unless the contaminated cells are particularly valuable, they should be discarded. Treatment of an infected cell culture with antibiotics can be attempted but may not be successful.

Detection of mycoplasma in cell culture

Mycoplasma species are difficult to detect visually in cell culture and the first indication of infection is often poor growth of the cells and poor appearance.

Mycoplasma can be detected by growth on specific culture media or with specific staining techniques on the cells. (Mycoplasma testing of media prior to use is difficult and components should be sourced with a Mycoplasma free guarantee).

Detection of viral contamination

The components of the cell culture medium are not routinely tested for viruses except by some commercial sources.

Cell cultures may contain latent viruses, some of which can be detected by light microscopy, electron microscopy or haemadsorption. Some cell lines when obtained from a commercial source will be guaranteed free of certain latent viruses.

Detection of contamination of cells by other cell lines

This contamination may occur prior to receipt in the laboratory or during cell manipulation. Testing of cell lines for purity is not routinely carried out and may only be vital in research work. Detection may be by simple microscopy. More complex methods of detecting extraneous cells involve genetic analysis of the cells, isoenzyme electrophoresis and immunofluorescence¹⁹.

5.3 IDENTIFICATION

NA

6 QUALITY ASSURANCE

6.1 ASSESSMENT OF PREPARATION

A quality system should be in place to ensure that appropriate internal and external quality assessment and quality control procedures are maintained (see QSOP 23 – Quality assurance in the diagnostic virology and serology laboratory)²⁰.

It is essential that laboratories have evidence of adequate validation of methods, equipment and commercial and in-house test procedures demonstrating that they are fit for the purpose²¹.

All cell lines must be checked for virus susceptibility when a new stock is obtained and at the intervals stated below. New stock should be obtained certified free from bacteria, mycoplasma, fungi, and yeasts.

Continuous cell lines may be maintained in medium containing Kanamycin (100 µg/mL) for 4 weeks to remove contamination with mycoplasma³. Cultures suspected of contamination with mycoplasma should be checked using fluorescence microscopy.

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Tubes should be retained from every production batch to check for: -

- Satisfactory morphology, growth, and longevity by microscopic examination
- Freedom from contamination with viruses, bacteria, yeasts, and/or fungi

6.1.1 GROWTH

Cell growth should be assessed by microscopic examination. Cells should achieve semi-confluent growth within 48hrs depending upon cell type.

Morphology should be consistent with the cell type; any variation should be noted. Cells displaying inconsistent or wide variation in morphology should be discarded and a new batch brought into use.

6.1.2 STERILITY

All reagents produced "in-house" must be checked for sterility by culture in a liquid bacterial growth medium (LabLemco broth or similar).

6.1.3 SUSCEPTIBILITY

A suitable panel of viruses should be tested. Such as:

VIRUS PANEL

- Adenovirus
- Herpes Simplex virus
- Respiratory syncytial virus
- Rhinovirus
- Influenza A virus (obtain current strain from Respiratory virus reference Laboratory)

Preparation and storage of virus control panel

Susceptibility testing

CELL LINE	FREQUENCY	TEST VIRUS	COMMENTS
MRC-5	On receipt and following thawing from liquid Nitrogen	HSV RSV	
RhMK	Primary culture and following thawing from liquid Nitrogen	Polio Influenza A	Check haemadsorption
MDCK	On receipt and following thawing from liquid Nitrogen	Influenza A	Check haemadsorption.
HEP-2	On receipt and following thawing from liquid Nitrogen	Adenovirus RSV	
VERO	On receipt and following thawing from liquid Nitrogen	HSV	
PLC-PRF5	On receipt and following thawing from liquid Nitrogen	Adenovirus Polio	

- 1) The virus controls should be inoculated into two tubes of the appropriate cell lines in the same manner as specimens and incubated at the required temperature
- 2) Always include negative controls
- 3) The cell lines should be observed daily for CPE and the results should be recorded
- 4) If a virus control/cell line combination fails to give the expected result and the cause cannot be ascertained or corrected, the cell line should be discarded

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6.2 INTERNAL AND EXTERNAL QUALITY ASSURANCE

N/A

7 LIMITATIONS

Successful isolation of organisms depends on correct specimen collection, transport, storage and processing; the quality and range of cell lines used and the use of correct conditions for culture and the provision of adequate/suitable clinical information.

Susceptibility should be checked on acquisition and at regular intervals while in use.

Cells removed from liquid nitrogen should be checked for sensitivity prior to use.

8 REPORTING PROCEDURE

N/A

9 REPORTING TO THE HPA²² (LOCAL AND REGIONAL SERVICES AND CENTRE FOR INFECTIONS)

N/A

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10 ACKNOWLEDGEMENTS AND CONTACTS

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The National Standard Methods are issued by Standards Unit, Department for Evaluations, Standards and Training, Centre for Infections, Health Protection Agency, London.

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APPENDIX: STORAGE OF CELLS IN LIQUID NITROGEN

Health & Safety

NB Local risk assessments must be carried out on this procedure

These procedures should only be carried out by trained or closely supervised staff.

The temperature of liquid nitrogen is -196°C and the vapour phase in the refrigerators varies between -120°C and -170°C . Therefore cryogenic work must be carried out with extreme caution and using protective equipment, especially gloves and goggles. Upon evaporation, the liquid produces a volume of gas 682 times greater than the original volume; the vapour is heavier than air. Therefore, liquid nitrogen should always be stored and handled in a well-ventilated area where an oxygen monitor and alarm is fitted.

Routine Procedures & Maintenance

The gas phase vessels must be checked every day

The refrigerators must *never* be allowed to become empty or valuable and irreplaceable resources will be lost.

Materials and Equipment

1) Di-methyl sulphoxide (DMSO)

NB DMSO is toxic and should be handled with care. It can be absorbed through the skin and may cause irritation and/or burns. It is teratogenic and an allergen. Wear gloves and if not working in a cabinet, eye protection should be worn

2) MEM growth medium as required for the individual cell line

3) Cryotubes (plastic)

4) Freezing equipment to allow controlled cooling at 1°C per minute

Methods

Actively growing cell cultures should be used for storage in liquid nitrogen. When cells are frozen and stored in liquid nitrogen, complete the appropriate "stock record sheet". Record the storage bin ID, rack number and position on the correct record sheet, and the rack plan for each individual vial.

1) Harvest the cells using the relevant reagent(s). However, when harvesting cells, leave the full volume of reagent(s) in contact with the cells and follow the method below

2) When all the cells are detached, pipette the suspension aseptically into a universal and centrifuge at 1500-2000g for 5 minutes

3) While the cells are centrifuging, prepare a 10% solution of DMSO in the relevant growth medium for the cells

4) Remove the supernatant fluid from the centrifuged cells and resuspend them in the DMSO\GM mixture. Use approximately 1.8mL of medium per ampoule required. The number of ampoules is decided by the number of flasks stripped and density of growth of the cells

5) Dispense the suspension into Cryotubes. Label the tubes with the cell type, passage number. (if required), tube number, and date

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- 6) Place the ampoules in freezing equipment to allow controlled cooling at 1°C per minute
- 7) Remove the ampoules from the cooling equipment when the temperature reaches -60°C or below
- 8) Enter the details of each batch on the correct record chart

Removal from storage

- 1) Locate the required ampoule and carefully remove it from the rack. When vials are removed from liquid nitrogen, cross out the vial removed on the rack plan and record the date of removal on the record sheet
- 2) Rapidly thaw the ampoule in the 37° C water bath, without total immersion
- 3) When thawed centrifuge the ampoule at 1500-2000g for 5 minute
- 4) Remove the supernatant fluid and resuspend the cells in the appropriate growth medium
- 5) Count the cells, dilute to the required concentration and seed into flasks or tubes as required
- 6) Record the removal on the relevant record chart

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