VIROLOGY MANUAL

TABLE OF CONTENTS

APPENDICES:

INTRODUCTION

Diagnostic Virology is performed for a variety of reasons, ranging from the diagnosis of an acute illness to the determination of asymptomatic carrier state.

The methods used to diagnose viral infections are based on the fact that many viruses produce characteristic changes in cells of the host and that most of them induce the production of infectious viruses or viral antigens in body tissues, secretions and excretions. This in turn is usually followed by the production of antibodies, which are specific for the virus and its associated antigens. The many diagnostic procedures used in Diagnostic Virology can be grouped into 4 categories, each with its particular role, limitations and advantages. These include:

I. Microscopic examination of infected tissues and exudates from the patient for evidence of viral inclusions or other pathologic alterations which may be characteristic of certain viruses.

II. Isolation (Propagation) and identification of virus from infected tissues or other specimens obtained from the patient.

III. Serologic studies for detection of virus-specific antibodies or antigens in patient's serum. (See Serology Manual).

IV. Direct detection of virus, viral antigens or viral nucleic acids (DNA or RNA) in tissues or other specimens from patients, independent of the propagation of these viruses in the laboratory. (eg. Direct Antigen Detection [Direct Smear], Centrifugation enhancement, Polymerase Chain Reaction [PCR], etc).

TML/MSH Virology Lab is a Containment Level 2* facility that performs or reports the following procedures:

1. Virology Direct Smear:
   Antigen Detection done directly on specimens using Cytospin centrifugation and Immunofluorescence staining performed only on cell-containing materials such as Bronco-Alveolar Lavage (BAL), vesicular aspirates and buffy coat.

2. Virology PCR:
   Nucleic Acid Amplification using PCR performed usually on specimens lacking in cellular materials, viable viruses or viral antigens such as CSF and plasma.

3. Virology Shell Vial Assay:
   Centrifugation-enhanced culture that detects antigens during early stages of viral propagation. Identification is done by immunofluoresent staining.

4. Virology Tube Culture Assay:
   Many clinically significant viruses can be propagated and detected in appropriately selected cell lines. Identification is done by immunofluoresent staining..

5. Virology Referred Out Tests:
   Assays that are not performed in this lab, such as Electron Microscopy (EM), are usually sent to the Public Health Laboratory (PHL). EM is done on viruses that cannot be propagated such as Norwalk, rota and other viruses causing gastro-enteritis. Other viruses requiring EM include BK, JC and Papovavirus.

Selection of these assays is driven by the nature of the specimen (eg. type and quantity); seasonality (eg. influenza in winter, enterovirus in summer); information supplied (eg. suspected viruses, symptoms and the degree of urgency); availability of resources and constrains placed on the laboratory. Samples that are improperly identified; improperly transported or unsafe will not be processed. Specimens containing agents requiring higher than Level 2 Containment* or assays not available in this lab may be referred to other laboratories for testing.

* Agents that are transmitted through the air, and can cause serious or life threatening disease (Level 3); viral agents causing haemorrhagic fevers such as the Ebola virus, Marburg virus and Lassa Fever (Level 4) will not be processed in this laboratory. Refer to Safety Manual or Health Canada web site for a more complete list and Laboratory Biosafety Guidelines www.hc-sc.gc.ca/pphb-dgspsp/publicat/lbg-ldmbl-96/index.html

CEREBRAL SPINAL FLUID

I. Introduction

Cerebral Spinal Fluid (CSF) will be routinely cultured for cytomegalovirus (CMV), herpes simplex virus (HSV), varicella-zoster virus (VZV) and enteroviruses (coxsackie, echo and polio virus). PCR for these viruses will be performed if specifically requested. Other viruses that may be isolated from CSF include mumps virus and adenovirus. Requests for Rubella virus, JC virus, BK virus and arbovirus should be referred to the Public Health Laboratory (PHL).

II. Collection and Transport

Specimens should be collected in a clean, sterile container and sent to the laboratory as soon as possible. If a delay in transport or processing is anticipated, the specimen should be kept at 4^{o}C until processed. If a delay of more than 72 hours is anticipated, the specimen should be frozen at –70^{o}C. Avoid repeated freeze-thaw cycles.

III. Procedure

A. Processing of Specimens:

Specimens should be set up as soon as possible after arriving in virology laboratory. After processing, an aliquot of up to 2 mL of the left-over specimen should be stored at -70°C in a cryovial.

a. If the specimen is requested for PCR and viral culture and
   i. The amount of specimen is <0.5 mL, perform PCR only.
   ii. The amount of specimen is between 0.5-1.0 mL, perform PCR and tube cultures.
   iii. The amount of specimen is >1.0 mL, perform PCR, tube culture and shell vial assay.

b. If specimen is requested for viral culture only and
   iv. The amount of specimen is <0.5mL, perform the culture only.
   v. If the amount of specimen is >0.5mL, perform tube culture and shell vial assay.

c. If PCR is requested, aliquot 0.2-1 mL first (freeze aliquot unless PCR can be performed immediately) before proceeding.

d. CSF specimens will be inoculated directly into shell vials and tube cultures without further processing.

B. Direct Examination:

Method	Virus(es)	Location
PCR	HSV / CMV / EBV/VZV	Research Lab
PCR	HHV6,7,8	Hospital for Sick Children
PCR	Adenovirus	Research Lab
RT-PCR*	Enteroviruses	Research Lab
RT-PCR*	West Nile virus	In-house

*RT-PCR = Reverse Transcription PCR using Qiagen Isolation Kit, RealArt reagents and Roche LightCycler. CMV= cytomegalovirus; EBV= Epstein-Barr virus; HSV= Herpes simplex virus; VZV= Varicella-zoster virus; HHV6,7,8= Human herpes virus types 6,7,8

Note: PCR and RT-PCR are performed only upon request and only for those viruses requested.

C. Isolation and Identification:

Method	**Cell Line[a]**	**Incubation at 36°C**	**Stain used/Read**
Shell Vial	MRC-5 MRC-5 (if requested) MRC-5 (if requested)	2 days 1 day 2 days	CMV-IE HSV-bivalent VZV
Tube	CMK HFF RD[b]	14 days 14 days 7 days	3 x Reads/week 3 x Reads/week 3 x Reads/week

[a]MRC-5 = Human Fibroblast cells; HEp 2 = Human Laryngeal Epidermoid Carcinoma Cells; CMK = Cynomolgus Monkey Kidney;
[b] RD = Rhabdomyosarcoma cells are inoculated from May to November (and from December to April if enterovirus is specifically requested).

D. Interpretation and Processing of Cultures:

a) Shell vial procedure:

i) For CMV, fix and stain 1 shell vial after 2 days (or next working day).
ii) If HSV is requested, fix and stain 1 shell vial after 1 day (or next working day).
iii) If VZV is requested, fix and stain 1 shell vial after 2 days (or next working day).

See Appendix II for detailed shell vial procedure.

b) Tube cultures should be examined a minimum of 3x per week for Cytopathic effect (CPE). Any culture demonstrating 2+ or more CPE should be confirmed using appropriate monoclonal antibodies immunofluorescent staining (Refer to Appendices IV and V). If positive, record in freezer program and freeze the cells and supernate (Refer to Appendix X and XII).

c) Any culture demonstrating CPE for which a virus cannot be detected using monoclonal antibodies or other in-house methods and toxicity has been ruled out (see below) should be referred to the Public Health Laboratory (PHL) for electron microscopy and further work-up. Consult the charge/senior technologist or medical microbiologist.

d) **Culture Toxicity:**If toxicity is suspected in a tube culture (rounding of cells, sloughing of cells, granular cytoplasm of cells or unusual CPE), the cells should be scraped and appropriate monoclonal antibody staining performed. Negative stain results indicate the need for a passage. Scrape cells and add 0.2 ml of these scraped cells to a fresh tube containing 2 ml of media (1:10 dilution) and proceed again with tube culture method. (Appendix III). If toxicity or CPE persists, refer to the charge/senior technologist for review.

e) **Contaminated Culture:** If the tube culture is visibly contaminated and uninterpretable, replant the specimen.

IV. Reporting Results

PCR: Negative Report: "Negative for _____virus. This is a research test"

Positive Report*: "POSITIVE for _____ virus. This is a research test."

Indeterminate Report: "Indeterminate by PCR. This is a research test"

Shell vial: Negative Report: "Negative for _____ virus."

Positive Report*: "POSITIVE for _____ virus."

Tube Culture: Negative Report: "No virus isolated," OR "See Shell Vial Assay."

Positive Report*: "_____ virus isolated"

Toxicity Report: "Specimen toxic to cell culture."

Contaminated Report: "Specimen is heavily contaminated with bacteria and/or fungus. Unable to perform Virology Tube Culture."

*** Telephone all positive results to ward/ordering physician**.
* When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window (under F7). See LIS Manual for entering results.

V. References

1. Gleaves, Curt A. et al. Cumitech 15A "Lab Diagnosis of Viral Infections". American Society for Microbiology, August 1994.

2. Collier L, Balows A, Sussman M. Topley's and Wilson's Microbiology and Microbial Infections. Volume 1, Ninth Ed. 1998.

GENITAL/PERI-ANAL/MOUTH/NOSE/SKIN LESIONS

I. Introduction

Specimens from genital, perianal and oro-labial (mouth/nose) lesions will only be examined for herpes simplex virus (HSV) unless otherwise requested. Specimens from skin lesions will be examined for both herpes simplex virus (HSV) and varicella-zoster virus (VZV). For other viruses requested, refer to Appendix XV (Virus Isolation and Identification) to ensure that the appropriate media is inoculated.

II. Collection and Transport

The roof of the vesicle(s) is disrupted. The fluid and cells released from the base of the lesion are collected using a sterile syringe and needle or a sterile swab. If the specimen is collected with a syringe and needle, aspirate viral transport medium into and out of the syringe several times, then express the contents into the viral transport container. Do not leave the needle and syringe in the transport container. (These should be discarded in an appropriate sharps container). If a swab is used, place the swab immediately into viral transport medium. Transport to the lab as soon as possible. If a delay in transport or processing is anticipated, the specimen should be kept at 4^{o}C until processed. If a delay of more than 72 hours is anticipated, the specimen should be frozen at -70^{o}C. Avoid repeated freeze-thaw cycles.

III. Procedure

A. Processing of Specimens:

Specimens for HSV and other viruses can be kept refrigerated for up to 72 hours. However, if VZV is specifically requested, the specimen should be set up immediately or stored at -70°C. Vortex patient sample in transport medium for 30 seconds. Remove excess fluid from the swab and discard the swab. Specimen in the transport medium can be transferred to a 2 mL cryovial. After preparation of slide (if needed) and inoculation of cultures, store cryovial at -70°C for 6 months. The original specimen container should be kept at 4°C for 1 week.

Refer to Appendix II for Shell Vial inoculation.

B. Direct Examination:

i) For genital, perianal and oro-labial (mouth/nose) lesions, prepare one double-well cytospin **only if requested**. Stain one well with HSV 1 monoclonal antibody and the other with HSV 2 monoclonal antibody.

ii) For all other skin lesions, **always** prepare one double-well cytospin. Stain one well with herpes 1/2 bivalent antibody and the other well with VZV monoclonal antibody.

If a slide comes with the original specimen, it will be fixed in acetone for 10 minutes and stained in addition to the in-house prepared slide. The slide will be stained for VZV primarily and HSV if possible.

Refer to Appendix V for immunofluorescent staining techniques.

C. Isolation and Identification:

Specimen	**Method**	**Cell Lines[a]**	**Incubation at 36°C**	**Stain[b] used**
Oro-facial/genital	Shell Vial	MRC-5	1 day 1 day	HSV1 HSV2
Skin	Shell Vial	MRC-5 MRC-5 MRC-5	1 day 1 day 2 days	HSV1 HSV2 VZV

[a] MRC-5 = Human fibroblast cells

[b] HSV1= Monoclonal antibody DFA stain for Herpes simplex 1

HSV2= Monoclonal antibody DFA stain for Herpes simplex 2

VZV= Monoclonal antibody DFA stain for Varicella zoster

See Appendix II for detailed shell vial procedure

IV. Reporting Results

A. For genital, perianal, oro-labial specimens

a. Direct Examination:
Negative Report: "Negative for Herpes Simplex virus".

Positive Report*: "Positive for _____ virus".
Insufficient Cells Report: "Insufficient cellular material to interpret test. Culture to follow".

b. Shell Vial:
Negative Report: "Negative for Herpes Simplex virus"
Positive Report*: "POSITIVE for _____virus."

B. For skin lesion specimens

a. Direct Examination:
Negative Report: "Negative for Varicella-Zoster virus".
"Negative for Herpes Simplex virus".
Positive Report* "Positive for _____ virus".
Insufficient Cells Report: "Insufficient cellular material to interpret test. Culture to follow".

c. Shell Vial:
Negative Report: "Negative for Varicella-Zoster virus"
"Negative for Herpes Simplex virus"
Positive Report*: "POSITIVE for _____virus."

***Telephone all positive VZV results to ward/ordering physician and Infection Control.**
Telephone all positive HSV results from neonates and post-partum women to appropriate ward/ordering physician.
When entering results in LIS use "control G" to send to appropriate infection control queue.
* When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window (under F7). See LIS Manual for entering results.

V. Reference

1. Gleaves, Curt A. et al. Cumitech 15A "Lab Diagnosis of Viral Infections". American Society for Microbiology, August 1994.

OCULAR SPECIMENS

I. Introduction

Viral infections of the eye (conjunctivitis, corneal ulcers, etc) are usually due to herpes simplex virus (HSV), varicella-zoster virus (VZV) and adenoviruses. Ulcerative lesions are usually due to HSV and VZV. For vitreous fluid, the two most common viruses isolated are cytomegalovirus (CMV) and varicella-zoster (VZV). Other viruses which may cause conjunctivitis, such as enteroviruses, will be looked for only if specifically requested.

II. Collection and Transport

Specimens should be collected using a clean, sterile swab and gently swabbing the conjunctiva or ulcerative lesion. Place the swab in viral transport medium and send to the laboratory as soon as possible. Vitreous fluid should be collected in a clean, sterile container. If a delay in transport or processing is anticipated, the specimen should be kept at 4^{o}C until processed. If a delay of more than 72 hours is anticipated, the specimen should be frozen at -70^{o}C. Avoid repeated freeze-thaw cycles.

III. Procedure

A. Processing of Specimens:

Specimens should be set up immediately or stored at -70°C. Vortex patient sample in transport medium for 30 seconds. Remove excess fluid from the swab and discard the swab. Refer to Appendices II and III for Shell Vial and Tube culture inoculation, respectively.

B. Direct Examination:

If requested, prepare one double-well cytospin. Stain one well with HSV bivalent 1/2 antibody and the other with VZV monoclonal antibody.

Refer to Appendix V for immunofluorescent staining technique.

C. Isolation and Identification:

Specimen	**Method**	**Cell Line[a]**	**Incubation at 36°C**	**Stain[b] used/Read**
Conjunctiva, cornea	Shell Vial	MRC-5 MRC-5	1 day 2 days	HSV-bivalent VZV
	Tube	HEp2 CMK (if enterovirus is requested) RD (if enterovirus is requested)	10 days 14 days 7 days	3 x Reads/week 3 x Reads/week 3 x Reads/week
Vitreous fluid	Shell Vial	MRC-5 MRC-5 MRC-5	1 day 2 days 2 days	HSV-bivalent VZV CMV-IE
	Tube	HEp2 CMK (if enterovirus is requested) RD (if enterovirus is requested)	10 days 14 days 7 days	3 x Reads/week 3 x Reads/week 3 x Reads/week

[a]MRC-5 = Human Fibroblast cells; HEp 2 = Human Laryngeal Epidermoid Carcinoma Cells; CMK = Cynomolgus Monkey Kidney cells; RD = Rhabdomyosarcoma cells
[b]HSVbivalent= Monoclonal DFA stain for HSV1 and HSV2
[b]VZV= Monoclonal DFA stain for Varicella zoster virus
[b]CMV-IE= Monoclonal IFA stain for Cytomagalovirus Immediate Early antigen

D. Interpretation and Processing of Cultures:

a. For shell vial procedure:

If CMV is requested, fix and stain after 2 days (or next working day)

See Appendix II for detailed shell vial procedure.

b. Tube cultures should be examined a minimum of 3x per week for Cytopathic effect (CPE). Any culture demonstrating 2+ or more CPE should be confirmed using appropriate monoclonal antibodies and immunofluorescent staining (Refer to Appendices IV and V). If positive, record in freezer program and freeze the cells and supernate (Refer to Appendix X and XII).

c. Any culture demonstrating CPE for which a virus cannot be detected using monoclonal antibodies or other in-house methods and toxicity has been ruled out (see below) should be referred to the Public Health Laboratory (PHL) for electron microscopy and further work-up. Consult the charge/senior technologist or medical microbiologist.

d. **Culture Toxicity:** If toxicity is suspected in a tube culture (rounding of cells, sloughing of cells, granular cytoplasm of cells or unusual CPE), the cells should be scraped and appropriate monoclonal antibody staining performed. Negative stain results indicate the need for a passage. Scrape cells and add 0.2 ml of these scraped cells to a fresh tube containing 2 ml of media (1:10 dilution) and proceed again with tube culture method. (Appendix III). If toxicity or CPE persists, refer to the charge/senior technologist for review.

e **Contaminated Culture:** If the tube culture is visibly contaminated and uninterpretable, issue a report indicating contamination.

IV. Reporting Results

Direct:	Negative Report:	"Negative for _____ virus."
	Positive Report*:	"POSITIVE for _____virus."
Shell Vial:	Negative Report:	"Negative for _____ virus."
	Positive Report*:	"POSITIVE for _____ virus."
Tube Culture:	Negative Report:	"No virus isolated"
	Positive Report*:	"_____ virus isolated."
	Toxicity Report:	"Virology Tube Culture: Specimen toxic to cell culture."

Contaminated Report: "Virology Tube Culture: Specimen is heavily contaminated with bacteria and/or fungus. Unable to perform Virology Tube Culture."

*** Telephone all positive results to ward/ordering physician**.
* When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window (under F7). See LIS Manual for entering results.

V. Reference

1. Gleaves, Curt A. et al. Cumitech 15A "Lab Diagnosis of Viral Infections". American Society for Microbiology, August 1994.

BRONCHO-ALVEOLAR LAVAGE (BAL) - CMV SURVEILLANCE

I. Introduction

Broncho-alveolar lavage specimens (BAL) for CMV surveillance collected on day 35 post-bone marrow transplant patients or routine screening of solid organ transplant patients are examined for CMV only.

II. Collection and Transport

BAL fluid should be collected into a clean, sterile container. If a delay in transport or processing is anticipated, keep the specimen at 4 ^{o}C.

III. Procedure

A. Processing of Specimen:
   a) Approximately 2-3 mL of centrifuged sediment should be received from the specimen receiving planting area.
   b) Transfer 2 mL of sediment to a sterile freezer vial containing 4 drops (0.2 mL) gentamicin (1 mg/mL) and 2 drops (0.1 mL) of fungizone (250 µg/mL) to a final concentration of 100 µg/mL and 10µg/mL respectively.
   c) Allow to stand at room temperature for 10 minutes
   d) Refer to Appendix II for Shell Vial inoculation.

B. Direct Examination:
   Not done.

C. Isolation and Identification:

Method	**Cell Lines[a]**	**Incubation at 36^{o}C**	**Stain[b] used**
Shell Vial	MRC-5	2 days	CMV-IE

[a]MRC-5 = Human fibroblast cells
[b]CMV-IE= Monoclonal antibody IFA stain for CMV immediate early antigen

Refer to Appendix II for Shell Vial staining and interpretation.

D. Interpretation and Processing of Cultures:

a) At 48 hours observe shell vial coverslips using the inverted microscope:

i) If <50% or no CPE - Perform DFA for CMV immediate early antigen (See Appendix V).
ii) If >50% CPE - Prepare a cytospin slide of scraped cells and then perform DFA for CMV immediate early antigen.
iii) If positive, freeze shell vial supernate.

IV. Reporting Results

Shell Vial:	Negative report:	"Negative for Cytomegalovirus."
	Positive report*:	"POSITIVE for Cytomegalovirus."

*** Telephone all positive results to ward/ordering physician**.
* When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window (under F7). See LIS Manual for entering results.

V. Reference

1. Gleaves, Curt A. et al. Cumitech 15A "Lab Diagnosis of Viral Infections". American Society for Microbiology, August 1994.

THROAT/NASOPHARYNGEAL/NASAL SWABS (SYMPTOMATIC)

I. Introduction

These specimens are set up for routine isolation of respiratory viruses (Influenza A and B, Parainfluenza 1, 2, and 3, Respiratory Syncytial Virus, and Adenovirus). Direct antigen detection will be performed during the winter months (November to April, inclusive) and upon request only from May to October. Additional cell lines or shell vials may be required if specific viruses are requested. For other viruses requested, refer to Appendix XV (Isolation and Identification) to ensure that the appropriate media is inoculated.

II. Collection And Transport

Specimens should be obtained as early in the patient's illness as possible. Throat, nasopharyngeal or nasal swabs should be collected using a clean, sterile swab and placed into viral transport medium and transported to the laboratory as soon as possible. If a delay in transport or processing for up to 72 hours is anticipated, keep the specimen at $4^{o}C$.

III. Procedure

A. Processing of Specimen:
Specimens can be kept refrigerated for up to 72 hours. Vortex patient sample in transport medium for 30 seconds. Remove excess fluid from the swab and discard the swab. Specimen in the transport medium can be transferred to a 2 mL cryovial. After preparation of slide (if needed) and inoculation of cultures, store cryovial at -70°C for 6 months. The original specimen container should be kept at 4°C for 1 week.

B. Direct Examination:
Prepare one double-well cytospin slide for immunofluorescent staining using the SimulFluor influenza A/B monoclonal antibody for one well and SimulFluor respiratory virus screen monoclonal antibody for the second well. Refer to Appendix VI for SimulFluor Screen Protocol.

NB: If a specific respiratory virus(es) is/are requested, prepare appropriate number of additional slides and stain using specific individual monoclonal antibodies.

Complete direct smear results the same day for specimens received in the virology section by 14:00 hours. For specimens received between 14:00 – 15:30 hours, processing and smear preparation should be completed, however, staining, reading and reporting results may be completed the next day (except on Fridays, consult charge/senior).

C. Isolation and Identification:

Specimens	Method	Cell Line[a]	Incubation at 36°C	Stain[b] used/Read
Specimens from infants in NICU	Shell Vial	MRC-5	2 days	CMV-IE
All patients	Tube	CMK CMK HEp2	5 days 10 days 10 days	RS/HSVbivalent RS/HSVbivalent 3 x Reads/week

[a]MRC-5 = Human Fibroblast cells; CMK = Cynomolgus Monkey Kidney; HEp2= Human Laryngeal Epidermoid Carcinoma cells
[b] CMV-IE = Monoclonal IFA stain for Cytomegalovirus Immediate Early antigen
[b] RS= SimulFluor Respiratory virus Screen DFA staining
[b] HSVbivalent= Monoclonal DFA stain for HSV1 and HSV2

D. Interpretation and Processing of Cultures:

a) For shell vial procedure:

i) If HSV requested, fix and stain at 24 hours (or next working day).

ii) If CMV requested, fix and stain at 48 hours (if >48 hours, or next working day) .

See Appendix II for detailed shell vial procedure.

b) Tube cultures should be examined a minimum of 3x per week for Cytopathic effect (CPE). Any culture demonstrating 2+ or more CPE should be confirmed using appropriate monoclonal antibodies and immunofluorescent staining (Refer to Appendices IV and V). If positive, record in freezer program and freeze the cells and supernate (Refer to Appendix X and XII).

c) Any culture demonstrating CPE for which a virus cannot be detected using monoclonal antibodies or other in-house methods and toxicity has been ruled out (see below) should be referred to the Public Health Laboratory (PHL) for electron microscopy and further work-up. Consult the charge/senior technologist or medical microbiologist.

d) **Culture Toxicity:** If toxicity is suspected in a tube culture (rounding of cells, sloughing of cells, granular cytoplasm of cells or unusual CPE), the cells should be scraped and appropriate monoclonal antibody staining performed. Negative stain results indicate the need for a passage. Scrape cells and add 0.2 ml of these scraped cells to a fresh tube containing 2 ml of media (1:10 dilution) and proceed again with tube culture method. (Appendix III). If toxicity or CPE persists, refer to the charge/senior technologist for review.

e) **Contaminated Culture:** If the tube culture is visibly contaminated and uninterpretable, replant the specimen.

IV. Reporting Results

Direct:	Negative Report:	"Negative for respiratory viruses."
	Positive* Report:	"POSITIVE for _____virus."
Tube Culture:	Negative Report:	"No virus isolated"
	Positive* Report:	"_____ virus isolated."
	Toxicity Report:	"Specimen toxic to cell culture."
	Contaminated Report:	"Specimen is heavily contaminated with bacteria and/or fungus. Unable to perform Virology Tube Culture."

***Telephone all positive results to ward/ordering physician.**
***Notify Infection Control of all positive respiratory virus results**.
*When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window (under F7). See LIS Manual for entering results.

V. References

1. Gleaves, Curt A. et al. Cumitech 15A "Lab Diagnosis of Viral Infections". American Society for Microbiology, August 1994.

2. Greenberg, S. et al. Cumitech 21 "Lab Diagnosis of Viral Respiratory Disease". American Society for Microbiology, March 1986.

ETT/AUGER SUCTION (INFANTS)

I. Introduction

Viral bronchitis, bronchiolitis and pneumonia are commonly caused by RSV or Parainfluenza in infants and young children. CMV pneumomitis may be seen in newborn/premature infants.

II. Collection and Transport

Endotracheal tube (ETT) aspirates or auger suctions are generally collected into a sterile container. If a delay in transport or processing is anticipated, keep the specimen at 4^0C.

III. Procedure

A. Processing of Specimens:

a) If sample is for virology only, flush approximately 2 mL of viral transport media through tubing and use for inoculation. If sample is to be split for other tests (i.e. C&S) use sterile saline.
b) For samples in saline add 0.2 ml (4 drops) of gentamicin (1 mg/mL) and 0.1 ml (2 drops) of fungizone (250 μg/mL) to a final concentration of 100 μg/ml and 10 μg/mL respectively.
c) Let stand at room temperature for 10 minutes before inoculating.
d) Refer to Appendix II and III for Shell Vial and Tube Culture inoculation, respectively.

B. Direct Examination:

Prepare one double-well cytospin slide for immunofluorescent staining. Stain one well with RSV/Para3 SimulFluor monoclonal antibody and one with SimulFluor Respiratory virus Screen monoclonal antibody. If a specific virus is requested, prepare appropriate number of additional slides and stain using specific individual monoclonal antibodies (Refer to Appendix VI Direct antigen Detection from specimens – SimulFluor Respiratory Screen Protocol Scheme 2)

Complete direct smear results the same day for specimens received in the virology section by 14:00 hours. For specimens received between 14:00 and 15:30

processing and smear preparation should be completed, however staining, reading and reporting results may be completed the next day (except Fridays).

C. Isolation and Identification:

Method	**Cell Line[a]**	**Incubation at 36°C**	**Stain[b] used/Read**
Shell Vial	MRC-5	2 days	CMV-IE
Tube	CMK CMK HEp2	5 days 10 days 10 days	RS/HSVbivalent RS/HSVbivalent 3 x Reads/week

[a]MRC-5 = Human Fibroblast cells; CMK = Cynomolgus Monkey Kidney; HEp2= Human Laryngeal Epidermoid Carcinoma cells
[b] CMV-IE = Monoclonal IFA stain for Cytomegalovirus Immediate Early antigen
[b] RS= SimulFluor Respiratory virus Screen DFA staining
[b] HSVbivalent= Monoclonal DFA stain for HSV1 and HSV2

D. Interpretation and Processing of Cultures:

a) For shell vial procedure:

If HSV requested, fix and stain at 24 hours (or next working day).

See Appendix II for detailed shell vial procedure.

b) Tube cultures should be examined a minimum of 3x per week for Cytopathic effect (CPE). Any culture demonstrating 2+ or more CPE should be confirmed using appropriate monoclonal antibodies and immunofluorescent staining (Refer to Appendices IV and V). If positive, record in freezer program and freeze the cells and supernate (Refer to Appendix X and XII).

c) Any culture demonstrating CPE for which a virus cannot be detected using monoclonal antibodies or other in-house methods and toxicity has been ruled out (see below) should be referred to the Public Health Laboratory (PHL) for electron

microscopy and further work-up. Consult the charge/senior technologist or medical microbiologist.

d) **Culture Toxicity:** If toxicity is suspected in a tube culture (rounding of cells, sloughing of cells, granular cytoplasm of cells or unusual CPE), the cells should be scraped and appropriate monoclonal antibody staining performed. Negative stain results indicate the need for a passage. Scrape cells and add 0.2 ml of these scraped cells to a fresh tube containing 2 ml of media (1:10 dilution) and proceed again with tube culture method. (Appendix III). If toxicity or CPE persists, refer to the charge/senior technologist for review.

e) **Contaminated Culture:** If the tube culture is visibly contaminated and uninterpretable, issue a report indicating contamination.

1. **Reporting Results**

Direct:	Negative Report:	"Negative for respiratory virus"
	PositiveReport*:	"POSITIVE for _____virus."
Shell Vial:	Negative Report:	"Negative for Cytomegalovirus."
	Positive Report*:	"POSITIVE for Cytomegalovirus."
Tube Culture:	Negative Report:	"No virus isolated"
	Positive Report:	"_____ virus isolated."
	Toxicity Report:	"Virology Tube Culture: Specimen toxic to cell culture.
	Contaminated Report:	"Virology Tube Culture: Specimen is heavily contaminated with bacteria and/or fungus. Unable to perform Virology Tube Culture.

***Telephone all positive results to ward/ordering physician.**
***Notifiy Infection Control of all positive respiratory virus results**.
* When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window (under F7). See LIS Manual for entering results.

V. Reference

1. Greenberg, S. et al. Cumitech 21 "Lab Diagnosis of Viral Respiratory Disease". American Society for Microbiology, March 1986.

BRONCHOSCOPY / BAL / SPUTUM / WASHINGS (SYMPTOMATIC)

I. Introduction

Viral pneumonia in immunocompromised patients is commonly due to cytomegalovirus (CMV) or respiratory syncytial virus (RSV). Other viruses that may be detected include influenza, parainfluenza, adenovirus, enterovirus and measles viruses. Requests for viruses other than the above may require the use of additional media. Refer to Appendix XV (Virus Isolation and Identification).

II. Collection and Transport

Sputum, bronchial washes and aspirates are to be submitted in a clean, sterile container. If a delay in transport or processing is anticipated, keep the specimen at 4^0C. Multiple bronchial washes and/or BAL samples received from the same patient at the same time should be pooled and processed as a single BAL sample.

III. Procedure

A. Processing of Specimens:

i) Bronchial washes and aspirates:

a) Approximately 2-3 mL of centrifuged sediment is to be received from the specimen receiving area (planting).
b) Prepare direct smears (if required) from the sediment. Very thick/mucoid specimens may require dilution of a portion of the sediment with sputolysin prior to direct smear preparation to avoid excessively thick smears. (See Appendix XXII)
c) Transfer 2 mL of sediment to a sterile freezer vial. Add 4 drops gentamicin and 2 drops of fungizone to a final concentration of 100μg/mL and 10 μg/mL respectively.
d) Allow to stand at room temperature for 10 minutes.

ii) Sputum:

a) Combine equal amounts of sputum and a 1:10 dilution of sputolysin and vortex gently.
b) Prepare direct smears, if required, from the above mixture.
c) Process as for Bronchoscopy specimens above steps c) and d)

B. Direct Examination:

Complete direct smear results the same day for specimens received in the virology section by 14:00 hours. For specimens received between 14:00 and 15:30 hrs, processing and smear preparation should be completed, however staining , reading and reporting results may be completed the next day (except on Fridays, consult Charge technologist).

C. Isolation and Identification:

Method	**Cell Line[a]**	**Incubation at 36°C**	**Stain[b] used/Read**
Shell Vial	MRC-5	2 days	CMV-IE
Tube	CMK CMK HEp2	5 days 10 days 10 days	RS/HSVbivalent RS/HSVbivalent 3 x Reads/week

[a]MRC-5 = Human Fibroblast cells; CMK = Cynomolgus Monkey Kidney; HEp2= Hep2 = Human Laryngeal Epidermoid Carcinoma cells
[b] CMV-IE = Monoclonal IFA stain for Cytomegalovirus Immediate Early antigen
[b] RS= SimulFluor Respiratory virus Screen DFA staining
[b] HSVbivalent= Monoclonal DFA stain for HSV1 and HSV2

D. Interpretation and Processing of Cultures:

a) For shell vial procedure:

If HSV requested, fix and stain after 1 day (or next working day).

See Appendix II for detailed shell vial procedure.

b) Tube cultures should be examined a minimum of 3x per week for Cytopathic effect (CPE). Any culture demonstrating 2+ or more CPE should be confirmed using appropriate monoclonal antibodies and immunofluorescent staining (Refer to Appendices IV and V). If positive, record in freezer program and freeze the cells and supernate (Refer to Appendix X and XII).

c) Any culture demonstrating CPE for which a virus cannot be detected using monoclonal antibodies or other in-house methods and toxicity has been ruled out (see below) should be referred to the Public Health Laboratory (PHL) for electron microscopy and further work-up. Consult the charge/senior technologist or medical microbiologist.

d) **Culture Toxicity:** If toxicity is suspected in a tube culture (rounding of cells, sloughing of cells, granular cytoplasm of cells or unusual CPE), the cells should be scraped and appropriate monoclonal antibody staining performed. Negative stain results indicate the need for a passage. Scrape cells and add 0.2 ml of these scraped cells to a fresh tube containing 2 ml of media (1:10 dilution) and proceed again with tube culture method. (Appendix III). If toxicity or CPE persists, refer to the charge/senior technologist for review.

e) **Contaminated Culture:** If the tube culture is visibly contaminated and uninterpretable, send out report indicating contamination.

IV. Reporting Results

Direct:	Negative Report:	"Negative for respiratory viruses."
	Positive Report*:	"POSITIVE for _____ virus."
Shell Vial:	Negative Report:	"Negative for Cytomegalovirus."
	Positive Report*:	"POSITIVE for Cytomegalovirus."
Tube Culture:	Negative Report:	"No virus isolated"

Positive Report*:	"_____ virus isolated."
Toxicity Report:	"Virology Tube Culture: Specimen toxic to cell culture.
Contaminated Report:	"Virology Tube Culture: Specimen is heavily contaminated with bacteria and/or fungus. Unable to perform Virology Tube Culture.

*** Telephone all positive results to ward/ordering physician**.
***Notify Infection Control of all positive respiratory virus results**.
***** When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window (under F7). See LIS Manual for entering results.

V. References

1. Gleaves, Curt A. et al. Cumitech 15A "Lab Diagnosis of Viral Infections". American Society for Microbiology, August 1994.

2. Greenberg, S. et al. Cumitech 21 "Lab Diagnosis of Viral Respiratory Disease". American Society for Microbiology, March 1986.

THROAT/MOUTH/WASHINGS (PMH)

I. Introduction

These specimens are routinely set up for herpes simplex virus only. Other viruses such as enteroviruses will be looked for only if specifically requested. Refer to Appendix XV (Virus Isolation and Identification) to ensure that the appropriate media is inoculated.

II. Collection and Transport

Mouth or throat washings are collected using sterile saline and placed in a clean, sterile container and transported to the laboratory as soon as possible. If a delay in transport or processing is anticipated, keep the specimen at 4^{o}C.

III. Procedure

A. Processing of Specimen:

a) Transfer 2mL of specimen to a sterile freezer vial.
b) Add 0.2 mL (4 drops) of gentamicin and 0.1 mL (2 drops) of fungizone to a final concentration of 100 µg/mL and 10 µg/mL respectively.
c) Let stand at room temperature for 10 minutes.

B. Direct Examination:
Not done.

C. Isolation and Identification:

Method	Cell Lines[a]	Incubation at 36^{o}C	Stain[b] used
Shell Vial	MRC-5	1 day	HSV1
	MRC-5	1 day	HSV2

[a]MRC-5 = Human diploid fibroblast cells
[b] HSV1= Monoclonal antibody DFA stain for Herpes simplex 1
[b] HSV2= Monoclonal antibody DFA stain for Herpes simplex 2

Refer to Appendix II for Shell Vial staining and interpretation.

IV. Reporting Results

Shell Vial:	Negative Report:	"Negative for Herpes Simplex virus"
	Positive Report*:	"POSITIVE for Herpes Simplex virus type____."

*** Telephone all positive results to ward/ordering physician**.
***** When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window (under F7). See LIS Manual for entering results.

V. Reference

1. Isenberg, HO. 1992. Clinical Microbiology Procedure Handbook, Vol. 2. ASM.

STERILE FLUIDS

I. Introduction

Viruses which may be isolated from pleural, peritoneal and pericardial fluid are enteroviruses (Coxsackie Group A and B, poliovirus, and echoviruses). Viral isolation from joint fluid is exceptionally rare. Amniotic fluid will be set up for cytomegalovirus shell vial and tube culture. Specimens with requests for HSV or VZV also necessitate inoculation of shell vials. Refer to Appendix XV (Virus isolation and isolation and identification) to ensure the appropriate media are inoculated.

II. Collection and Transport

Specimens should be collected in a clean, sterile container and sent to the laboratory as soon as possible. If a delay in transport or processing is anticipated, keep the specimen at $4^{o}C$.

III. Procedure

A. Processing of Specimens:

Specimens should be set up as soon as possible or stored at 4ºC for up to 72 hours. All processed specimens should be stored at -70ºC after inoculation. Bloody fluids should be spun at 2000 rpm (700x*g*) for 10 minutes and the supernate used for inoculation.

Refer to Appendix II for Shell Vial inoculation and staining.
Refer to Appendix III for Tube Culture inoculation.

B. Direct Examination:

Not done.

C. Isolation and Identification:

Specimens	Method	Cell Line[a]	Incubation at 36°C	Stain used/Read
Amniotic fluid	Shell Vial	MRC-5 MRC-5 (if requested) MRC-5 (if requested) MRC-5 (if requested)	2 days 1 day 1 day 2 days	CMV-IE HSV1 HSV2 VZV
	Tube	CMK HFF RD[b]	14 days 14 days 7 days	3 x Reads/week 3 x Reads/week 3 x Reads/week
Pleural , Peritoneal , Pericardial, other fluids	Shell Vial	MRC-5 (if requested) MRC-5 (if requested) MRC-5 (if requested) MRC-5 (if requested)	2 days 1 day 1 day 2 days	CMV-IE HSV1 HSV2 VZV
	Tube	CMK HFF RD[b]	14 days 14 days 7 days	3 x Reads/week 3 x Reads/week 3 x Reads/week

[a]MRC-5 = Human Fibroblast cells; CMK = Cynomolgus Monkey Kidney; HFF= Human Foreskin Fibroblast cells
[b] RD = Rhabdomyosarcoma cells are inoculated from May to November (and from December to April if enterovirus is specifically requested).

D. Interpretation and Processing of Cultures:

a) For shell vial procedure:

i) For amniotic fluid (or other fetal specimens), always set up CMV shell vial. Fix and stain after 2 days (or next working day).

ii) For other fluids, set up CMV, HSV1, HSV2 and VZV if requested. Fix and stain as indicated.

See Appendix II for detailed shell vial procedure.

b) Tube Cultures:
Tube cultures should be examined a minimum of 3x per week for Cytopathic effect (CPE). Any culture demonstrating 2+ or more CPE should be confirmed using appropriate monoclonal antibodies immunofluorescent staining (Refer to Appendices IV and V). If positive, record in freezer program and freeze the cells and supernate (Refer to Appendix X and XII).

c) Any culture demonstrating CPE for which a virus cannot be detected using monoclonal antibodies or other in-house methods and toxicity has been ruled out (see below) should be referred to the Public Health Laboratory (PHL) for electron microscopy and further work-up. Consult the charge/senior technologist or medical microbiologist.

d) **Culture Toxicity:** If toxicity is suspected in a tube culture (rounding of cells, sloughing of cells, granular cytoplasm of cells or unusual CPE), the cells should be scraped and appropriate monoclonal antibody staining performed. Negative stain results indicate the need for a passage. Scrape cells and add 0.2 ml of these scraped cells to a fresh tube containing 2 ml of media (1:10 dilution) and proceed again with tube culture method. (Appendix III). If toxicity or CPE persists, refer to the charge/senior technologist for review.

e) **Contaminated Culture:** If the tube culture is visibly contaminated and uninterpretable, replant the specimen.
Refer to Appendix II for Shell Vial staining interpretation.
Refer to Appendix III for Tube culture reading and interpretation.

IV. Reporting Results

Shell Vial:	Negative Report:	"Negative for _____virus."
	Positive Report*:	"POSITIVE for _____virus."
Tube Culture:	Negative Report:	"No virus isolated"
	Positive Report*:	"_____ virus isolated."

Toxicity Report: "Virology Tube Culture: Specimen toxic to cell culture.

Contaminated Report: "Virology Tube Culture: Specimen is heavily contaminated with bacteria and/or fungus. Unable to perform Virology Tube Culture.

*** Telephone all positive results to ward/ordering physician**.

***** When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window (under F7). See LIS Manual for entering results.

V. Reference

1. Gleaves, Curt A. et al. Cumitech 15A "Lab Diagnosis of Viral Infections". American Society for Microbiology, August 1994.

FAECES/RECTAL

I. Introduction

Faecal specimens may be submitted for the detection of viruses from patients with gastroenteritis, aseptic meningitis, viral encephalitis, or exanthem type rashes. Most cases of viral gastroenteritis are caused by viruses which do not propagate in cell culture. Faecal specimens from such cases can be examined directly by antigen detection methods for rotavirus, Norwalk-like agents and adenovirus type 40 and 41 or by electron microscopy (EM). Aseptic meningitis may be due to enteroviruses and many can propagate in routine cell cultures. Herpes simplex virus is commonly implicated in cases of viral encephalitis and is not isolated from faecal specimens. Enteroviruses may cause exanthem type rashes and stool may be cultured for these viruses.

II. Collection and Transport

Stool is collected in a dry sterile container. Although rectal swab is less satisfactory than stool specimen, it will be processed if a stool sample cannot be obtained. Rectal swab should be placed in viral transport media. Specimens should be transported to the laboratory as soon as possible. If a delay in transport or processing is anticipated, keep the specimen at 4^{o}C.

III. Procedure

A. Processing of Specimens:

a. **Stool**

i. Stool specimens will be processed according to the clinical information provided. Specimens from patients with gastroenteritis or diarrhea will be referred to the Public Health Laboratory (PHL) for rotavirus, Norwalk-like agents or enteric adenoviruses.

ii. Specimens from patients with rashes, aseptic meningitis or other symptoms involving the central nervous system will be processed in-house for enteroviruses.

iii. Specimens received without any clinical information will be referred to PHL for Electron Microscopy.

Stool specimens being processed in-house should be processed as follows:

a) Transfer 1.5 to 2.0 g of stool to a clean, sterile 50 ml tube containing 4 to 6 sterile glass beads.

b) Add 15 ml of maintenance medium.

c) Vortex for 30 to 60 seconds.

d) Centrifuge at 3000 rpm (700 x g) for 15 minutes.

e) Transfer 2 ml supernatant to a sterile freezer vial. Add 4 drops gentamicin and 2 drops fungizone to a final concentration of 100 μg/ml and 10 μg/ml respectively.

f) Allow to stand at room temperature for 10 minutes.

ii) **Rectal swab**

a) Vortex the swab in transport medium for 30 seconds. Remove excess fluid from the swab and discard the swab.

b) Transfer maintenance medium to a conical bottom centrifuge tube and centrifuge at 3000 rpm (700 x g) for 15 minutes.

c) Transfer 2 ml supernate to a sterile freezer vial. Add 4 drop gentamicin and 2 drops fungizone to a final concentration of 100 mg and 10 mg respectively.

d) Allow to stand at room temperature for 10 minutes.

B. Direct Examination:

If electron microscopy is requested or symptoms of gastroenteritis reported, forward specimen to PHL. Otherwise, direct examination is not indicated.

C. Isolation and Identification:

Method	**Cell Lines[a]**	**Incubation at 36°C**	**Stain used/Read**
Shell Vial	MRC-5 (only if requested)	2 days	CMV-IE
Tube	CMK RD	14 days 7 days	3x Reads/week 3x Reads/week

[a]MRC-5 = Human diploid fibroblast cells; CMK = Cynomolgus Monkey Kidney cells; RD = Rhabdomyosarcoma cells

D. Interpretation and Processing of Cultures:

a) For shell vial procedure:

i) If CMV is requested, fix and stain after 2 days (or next working day) .

See Appendix II for detailed shell vial procedure.

b) Tube cultures should be examined a minimum of 3x per week for Cytopathic effect (CPE). Any culture demonstrating 2+ to 3+ CPE should be confirmed using appropriate monoclonal antibodies and immunofluorescent staining (Refer to Appendices IV and V). If positive, record in freezer program and freeze the cells and supernate (Refer to Appendix X and XII).

c) Any culture demonstrating CPE for which a virus cannot be detected using monoclonal antibodies or other in-house methods and toxicity has been ruled out (see below) should be referred to the Public Health Laboratory (PHL) for electron microscopy and further work-up. Consult the charge/senior technologist or medical microbiologist.

d) **Culture Toxicity:** If toxicity is suspected in a tube culture (rounding of cells, sloughing of cells, granular cytoplasm of cells or unusual CPE), the cells should be scraped and appropriate monoclonal antibody staining performed. Negative stain results indicate the need for a passage. Scrape cells and add 0.2 ml of these scraped cells to a fresh tube containing 2 ml of media (1:10 dilution) and proceed again with tube culture method. (Appendix III). If toxicity or CPE persists, refer to the charge/senior technologist for review.

i) **Contaminated Culture:** If the tube culture is visibly contaminated and uninterpretable, issue a report indicating contamination.

IV. Reporting Results

Tube Culture:

Negative Report:	"No virus isolated"
Positive* Report:	"_____ virus isolated."
Toxicity Report:	" Specimen toxic to cell culture."
Contaminated Report:	"Specimen is heavily contaminated with bacteria and/or fungus. Unable to perform Virology Tube Culture."

*** Telephone all positive results to ward/ordering physician.**
***** When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window (under F7). See LIS Manual for entering results.

V. Reference

1. Isenberg, HO. 1992. Clinical Microbiology Procedure Handbook, Vol. 2. American Society of Microbiology.

TISSUE/BIOPSY SPECIMENS

I. Introduction

The following viruses may be detected from various tissue or biopsy sources:
Lung: Adenovirus/CMV/enterovirus/respiratory viruses
Liver: CMV/non-culturable viral hepatitis
Brain: HSV/VZV/enterovirus/measles
Kidney: CMV
Heart: Enterovirus
Postpartum: CMV
Bone Marrow: CMV

For other viruses requested, refer to Appendix XV (Virus isolation and identification) to ensure the appropriate media is inoculated.

II. Collection and Transport

The sample should be collected directly adjacent to the affected tissue and placed in viral transport medium. If a delay in transport or processing is anticipated, keep the specimen at $4^{o}C$.

III. Procedure

A. Processing of Specimens:

a) If the tissue is received in a dry container, add 1-3 ml of maintenance medium.
b) Macerate the tissue using the small tissue grinder.
c) If a large amount of tissue/cellular material is present, transfer suspension to a sterile 15 ml centrifuge tube and spin at 700 x g for 10 minutes
c) Use the supernate as inoculum.

If Fetal / Post-partum tissue is received and Parvovirus PCR is requested, forward specimen to the Hospital for Sick Children for processing.

B. Direct Examination:

Not usually performed.

C. Isolation and Identification:

Specimens	Methods	Cell Lines[a]	Incubation at 36°C	Stain used/Read
Lung[b]Liver/Bone Marrow/Kidney/ FetalTissue	Shell Vial	MRC-5 MRC-5 (if requested) MRC-5 (if requested) MRC-5 (if requested)	2 days 1 day 1 day 2 days	CMV-IE HSV1 HSV2 VZV
	Tube	CMK[b] HFF RD	14 days 14 days 7 days	3 x Reads/week [b]**RS/HSV-bvalent** 3 x Reads/week 3 x Reads/week
Brain	Shell Vial	MRC-5 MRC-5 MRC-5	2 days 1 day 2 days	CMV-IE HSV-bivalent VZV
	Tube	CMK HFF RD	14 days 14 days 7 days	3 x Reads/week 3 x Reads/week 3 x Reads/week

[a]MRC-5 = Human Fibroblast cells; CMK = Cynomolgus Monkey Kidney; HFF= Human Foreskin Fibroblast cells; RD = Rhabdomyosarcoma cells

[b] CMK tube inoculated with **Lung tissue** should be **read** for 2 weeks **and** if no CPE is observed, **stain** a double Cytoprep with SimulFluor Respiratory virus Screen and HSV bivalent monoclonal antibody stains.

See Appendix II for detailed shell vial procedure.
See Appendix III for detailed tube culture procedure.

IV. Reporting Results

Shell Vial:	Negative Report:	"Negative for _____virus."
	Positive Report*:	"POSITIVE for _____virus."
Tube Culture:	Negative Report:	"No virus isolated"
	Positive Report*:	"_____ virus isolated."

Toxicity Report: "Virology Tube Culture: Specimen toxic to cell culture.

Contaminated Report: "Virology Tube Culture: Specimen is heavily contaminated with bacteria and/or fungus. Unable to perform Virology Tube Culture.

*** Telephone all positive results to ward/ordering physician**.
*When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window (under F7). See LIS Manual for entering results.

V. References

1. Leland D. 1996. Clinical Virology. W.B. Saunders Company.

2. Gleaves, Curt A. et al. Cumitech 15A "Lab Diagnosis of Viral Infections". American Society for Microbiology, August 1994.

URINE

I. Introduction

Urine is the specimen of choice for neonates suspected of congenital cytomegalovirus (CMV) infection. Other viruses recovered from urine that may be representative of a systemic infection include enteroviruses, rubella, measles, and mumps. In addition, adenovirus may be cultured from urine specimens. If no virus is requested, urine specimens will be processed for CMV only. Additional cell lines or shell vials may be required if other specific viruses are requested. Refer to Appendix XV (Isolation and Identification) to ensure that the appropriate media is inoculated. Urine for which polyomavirus (BK or JC) is requested should be sent to the Public Health Laboratory (PHL) for electron microscopy.

II. Collection and Transport

The first morning voided urine sample is best for recovery of CMV. Urine should be collected in a clean, sterile container. If a delay in transport or processing is anticipated, keep the sample at 4^{o}C.

III. Procedure

A. Processing of Specimens:

Do not freeze the urine before processing. Urine can be left at 4º C for no more than 48 hours before inoculation. A fresh specimen should be requested from patients for which a urine is received that cannot be set up within this time frame (i.e. Friday evening, long weekends).

a) Gently mix and transfer 2 ml of urine to a sterile freezer vial. Add 4 drops gentamicin and 2 drops of fungizone to a final concentration of 100 µg/ml and 10 µg/ml respectively.

b) Allow to stand at room temperature for 10 minutes before inoculating

B. Direct Examination:

Not done.

D. Isolation and Identification:

Method	**Cell Lines[a]**	**Incubation at 36°C**	**Stain[b] used**
Shell Vial	MRC-5	2 days	CMV-IE

[a]MRC-5 = Human diploid fibroblast cells
[b] CMV-IE= Monoclonal antibody IFA stain for CMV immediate early antigen

Refer to Appendix II for Shell Vial staining and interpretation.

IV. Reporting Results

Shell Vial: Negative Report: "Negative for Cytomegalovirus."

Positive Report*: "POSITIVE for Cytomegalovirus."

*** Telephone all positive results to ward/ordering physician**.
***** When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window (under F7). See Appendix for entering LIS results.

V. Reference

1. Gleaves, Curt A. et al. Cumitech 15A "Lab Diagnosis of Viral Infections". American Society for Microbiology, August 1994.

BLOOD / BONE MARROW FOR CMV ANTIGENEMIA

I. Introduction

Human cytomegalovirus (CMV) can cause severe, life-threatening disease in immunocompromised patients such as transplant patients and patients with AIDS. Systemic infections are characterized by carriage of CMV in the polymorphonuclear leukocytes (PMNL) of peripheral blood (viremia). Infected PMNL can be detected by direct detection of CMV pp 65 antigen (CMV antigenemia) using an indirect immunofluorescence (IFA) technique. CMV antigenemia can also be used to monitor the course of CMV infection during and after treatment. Antigenemia will be performed on EDTA or heparinized blood requesting CMV. Antigenemia and culture will be performed on bone marrow samples requesting CMV.

II. Collection and Transport

A minimum of 5 mL of blood is collected in an EDTA Vacutainer® tube (purple top). Samples should be transported to the laboratory as soon as possible at room temperature. Smaller volumes of blood from infants will be accepted and the procedure will be completed despite a small number of PMNL's. Blood samples received 14:00–13:00 hrs will be processed as far as preparation and fixing of slides. Staining and reading will be done the next day. Samples received after 15:00 hrs will be refrigerated and processed the next working day or a fresh sample requested (except on Fridays, consult Charge technologist).

Note:
Toronto Hospital Division of UHN: Reject (Transplant patients, In or Out patients)

1. Specimens received after 2 p.m., before 12 a.m. (previous night)
2. Specimens received over the weekend (Friday after 2 p.m. to Sunday midnight)

Phone Ward if specimen is rejected!!

Refer complaints to Dr Atul Humar (pager no. 416-664-8211) or Dr. Mazzulli @17-4695.

Other sites (PMH, MSH): Only reject specimens received over the weekend.

III. Materials:

i) Phosphate Buffered Saline:

For 1000 mL - 10 mM PBS powder, pH 7.4 (Sigma p-3813) in a glass vial
- 1000 mL dH_20
Autoclave and store at room temperature.

OR

Obtain sterile PBS from Rm. 977, ext. 8257.

ii) Erythrocyte Lysing Solution:

For 2000 mL -16.6 gm NH_4CL power, M.W.53.49 (Sigma A-0171)
-2.0 gm Potassium bicarbonate
-2000 mL dH_20

Filter sterilize and store at 4^oC. Stable for 2 months.

iii) Fixative Solution:

For 500 mL -25 mL Formaldehyde (Sigma Cat. F-1268)
-10 gm Sucrose, M.W. 342.3 (Sigma S-1888)
-500 mL PBS buffer

Store at room temperature. Stable for 1 month.
Use fresh aliquot of fixative each day.

iv) Wash Solution

For 500 mL -5.0 mL FCS
-500 mL PBS

Store at 4^oC. Stable for 1 week.

v) Permeabilization Solution

For 500 mL -2.5 mL NP40 (ICN cat# 198596 formerly Nonidet, sigma)
-50 gm Sucrose (Sigma S-1888)
-5.0 mL Fetal Calf Serum (FCS)
-500 mL PBS

Store at 4°C. Stable for 1 month.

vi) Antibody 1

Monoclonal Anti-HCMV pp65, (Biotest Clonab CMV, Cat No: P/N 912600). Mix 1 mL of pp65 with 4 mL of PBS. Aliquot and store at 4°C. Test with a CMV positive control slide. Enter lot no. and QC slide results in Reagent History Form.

vii) Antibody 2

Monoclonal Anti-Mouse – FITC Conjugate with Evans Blue (Baxter B1029-86B). Store at 4°C.

viii) Control slides

Double-well slide with CMV positive and negative wells are stored at 4°C and at -70°C.

IV. CMV Antigenemia Procedures:

A. Cell Separation Procedure

1. Invert blood tube several times to mix.
2. Transfer ~ 2.0 mL of blood to a 15 mL graduated centrifuge tube which contains 10 mL of ELS.
3. Mix and put on a rotator for 5 minutes (or until RBC's are lysed).
4. Spin at 1300 rpm for 7 minutes.
5. Gently pour supernate into a discard container.
6. Add ELS to 10 mL level.
7. Mix and spin at 1300 rpm for 7 minutes.
8. Gently pour supernate into a discard container.
9. Using transfer pipette, carefully remove excess red blood cells from suspension above the WBC deposit.

10. Add PBS solution to 10 mL level.
11. Mix and spin at 1300 rpm for 7 minutes.
12. Gently pour supernate into a discard container.
13. Gradually add PBS to cell pellet until it reaches a turbidity of ~ 0.5 to 1.0 MacFarland Standard.
14. Proceed to cell counting procedure

B. Cell Counting Procedure

1. Before using Coulter Counter:
   - Replace the blue Cleaner Solution with Isotonic Diluent in an ACCUVETTE II vial.
   - Run the Isotonic Diluent 3 times using the Start/Stop key.
2. Dispense 10 mL of Isotonic Solution into an ACCUVETTE II vial for each sample.
3. Invert cell suspension, dispense 50 ul of cell suspension into each ACCUVETTE II vial. Pipette up and down several times.
4. Add 3 drops of ZAP-OGLOBIN (which will lyse any remaining RBC's).
5. Stir with transfer pipette to mix. Avoid introducing air bubbles.
6. Read immediately two times and calculate the average WBC count (e.g. Reading of 2,200 E6 means the cell suspension has a WBC count of 2.2×10^6 /ml). Write down the average WBC count on the centrifuge tube.

   Acceptable limit is between 1.0×10^6 to 2.0×10^6/ml
7. Increase the volume added to the cytospin funnel if a specimen has a very low WBC count (maximum 300 ul). Each slide should have 200, 000 WBC's.
8. At the end of the day:
   - Immerse the Aperature Tube of the Coulter Counter into an ACCUVETTE II vial full of blue Cleaner Solution.
   - Run the Cleaner Solution 3 times using the Start/Stop key.
   - Leave the Aperature Tube immersed in the Cleaner Solution overnight.

C. Preparation of Slides

1. Dispense 200 ul of cell suspension from ACCUVETTE II vial (see above) into a single cytospin funnel.
2. Spin at 900 rpm for 3 minutes in a SHANDON Cytocentrifuge.
3. Air dry slide for 5 minutes inside the Laminar Flow Hood.

D. Fixation and Permeabilization of Slides

1. Immerse prepared slides in Fixative Solution for 10 minutes.
2. Rinse in Wash Solution for 5 minutes.
3. Immerse in Permeabilization Solution for 5 minutes.
4. Rinse in Wash Solution for 5 minutes.
5. Let slides dry in Laminar Flow Hood for 5 minutes and proceed to staining, or store dried slides in fridge at 4^{o}C for up to 72 hr. Store in -70^{o}C freezer if slides cannot be stained within 72 hr.

E. Staining of Slides

1. Place one double-well control slide with CMV positive and negative cell spots at a random position within each batch of slides to be stained.
2. Add 20 µl of working solution monoclonal antibody # 1 (anti-pp65) onto the sample and control slide. Incubate in a humidified chamber at 37°C for 30 minutes. FROM THIS POINT, DO NOT ALLOW THE CYTOPREP TO DRY AT ANY TIME DURING THE STAINING PROCESS.
3. Wash by immersion in fresh PBS 3 times for 1 minute each.
4. Wipe excess PBS off slide and add 20 µl of fluorescein conjugated antibody # 2 to the cytoprep. DO THIS ONE SLIDE AT A TIME, DO NOT ALLOW CELL SPOT TO DRY IN BETWEEN.
5. Incubate for 30 minutes at 37°C in a humidified chamber.
6. Wash by immersion in fresh PBS 3 times for 2 minutes each. Wash in fresh dH_2O briefly.
7. Allow slides to dry under hood about 5 minutes.
8. Coverslip slides with FA mounting media.

F. Reading of Slides

a. Reading is performed with the fluorescence microscope using the #4 filter (λ 490/575). Scan the entire surface of the cytoprep with the 40x objective, counting the number of infected cells.

The fluorescent, green polylobate nuclei are the infected and stained PMNL's. A single fluorescent PMNL is sufficient to indicate a positive antigenemia result.

The following appearances **DO NOT** constitute a positive:
- cytoplasmic fluorescence in large granules (eosinphils)
- slightly greenish periphery of PMNL
- all of the PMNLs appear greenish
- peri-nuclear staining of PMNLs

b. Calculation of positive CMV antigenemia cell count performed in LIS:

Under DPP 65, at the media screen:
WBC # : WBC count from Coulter Counter, written on each centrifuge tube after counting.
PREP : # of positive cells seen in cytospin slide
DIV2 : # of positive cells divided by 2
POS # : DIV2/ WBC#.
F7, put 'V' in isolate # field, Enter, Enter, F2 under 'Org id', F12, choose 1 for CMV, F8, V,and '8' , F12, F12.

G. Daily Quality Control:

a. Check reagent expiratory date and verify that Reagent QC is satisfactory for the reagent lot/kit being used

b. Appropriate control slide with positive and negative CMV wells (commercial; home-made slide with ATCC strain or buffer coat of known CMV positive are acceptable) must be stained with each batch. Place QC slide at a random position within each batch

c. Examine the negative control well first to establish the dull red colour (Evans blue counterstain) and to determine if there is any nonspecific staining.
The positive control must be clearly distinguishable from the negative control or the test is invalid.

d. Record daily QC results in LIS (under procedure VPP65D, CMV pp65 - daily QC STN Staining Reaction).

Failed Daily QC:

a. Do not release patient results pending resolution of QC error.
b. Inform charge/senior technologist.
c. Record in Reagent Log, Instrument Log if microscope/incubator is involved in the failure and file incident report as appropriate.
d. Re-run failed controls in parallel to fresh controls (and/or external QC) to evaluate the QC material itself.
e. If the re-run shows the old QC material still fails, fresh QC passes and nothing else is wrong with the batch (only the old QC material failed, patient results valid) patient results may be released.

Marked decrease/absence in fluorescence can be due to:

a. Reagent deterioration/skipping (did not apply primary/secondary stain)
b. Microscope (filter, bulb, alignment)
c. Other equipment, reagents or technique

IV. Reporting Results

CMV Antigenemia: Negative Report: "Negative for Cytomegalovirus."

Positive Report*: "POSITIVE, - # positive cells/100,000

***Telephone all antigenemia results to Bone Marrow Transplant Clinic. Telephone all positive results to UHN (TGH & TWH).**
When entering positive results in the Lab Information System (LIS), enter the virus name in the isolate window.

V. References

1. Clonatec Co.: Detection of HCMV 65 Kda protein kinase in peripheral blood polymorphonuclear leukocytes by indirect immunofluorescence. Clonatec, Biosoft Department Siege social: 60 rue de Wattignies, 75580, Paris Cedex 12 Tel. (1) 43 42 38 30, Fax (1) 43 40 48 86.

2. Gerna, G. et al. Comparison of different immunostaining techniques and monoclonal antibodies to the lower matrix phosphoprotein (pp65) for optimal quantitative of human cytomegalovirus antigenemia. J. Clin. Micr. 1992; 30: 1232 – 1237.

3. Niubo J. et al. Association of quantitative cytomegalovirus antigenemia with symptomatic infection in solid organ transplant patients. Diagn. Microbiol. Infect. Dis. 1996; 24: 19-24.

4. Ho S. et al. Rapid cytomegalovirus pp65 antigenemia assay by direct lysis and immunofluoresence staining. J. Clin. Micro. 1998; 36;638-640.

Appendix I

REAGENTS/KITS

Chemical Reagents

1. Formaldehyde, Sigma
2. Sucrose, Sigma
3. Nonidet, ICN (changed to IGEPAL CA-630, Sigma cat. # 198596)
4. Ammonium Chloride powder, Sigma
5. Potassium bicarbonate, Sigma
6. DMSO freezing Medium, Sigma
7. Phosphate Buffered Saline (PBS):
   - 8.06 g/L Sodium Chloride
   - 0.20 g/L Potassium Chloride
   - 1.36 g/L KH_2PO_4
   - 1.42 g/L Na_2HPO_4
   - Adjust pH to 7.4 and autoclave.

Reagent Kits

1. FITC – Anti Mouse-(Intermedico, Bartels, Dade-Behring)
2. Herpes simplex I and II DFA-(Dade-Behring)*
3. Varicella zoster DFA-(Oxoid)*
4. CMV, DFA-Bartels (Intermedico)
5. CMV Early Antigen (CMVEA), IFA-Bartels (Intermedico)*
6. Herpes simplex Bivalent DFA-Intermedico*
7. Pan-Enterovirus Blend-(Chemicon)
8. Anti-Echovirus-(Chemicon)
9. Anti-Coxsackie A9-(Chemicon)
10. Anti-Coxsackie B Blend-(Chemicon)
11. Anti-Enterovirus Blend 70/71-(Chemicon)
12. Anti-Poliovirus Blend-(Chemicon)
13. Clonab®, CMV pp 65, Biotest-(RWR Scientific)*
14. Respiratory Virus Antibody Pool – Bartels (Intermedico)
15. Adenovirus Antibody – Bartels (Intermedico)
16. Anti-Influenza A Monoclonal Antibody – Bartels (Intermedico)
17. Anti-Influenza B Monoclonal Antibody – Bartels (Intermedico)

18. Anti-Parainfluenza 1 Monoclonal Antibody – Bartels (Intermedico)
19. Anti-Parainfluenza 2 Monoclonal Antibody – Bartels (Intermedico
20. Anti-Parainfluenza 3 Monoclonal Antibody – Bartels (Intermedico)
21. Anti-RSV Monoclonal Antibody-Bartels (Intermedico)
22. Anti-Adenovirus Antibody-Bartels (Intermedico)
23. Anti-measles Antibody-(Chemicon)
24. SimulFluor DFA Respiratory Viral Screen/RSV panel-(Chemicon)*
25. SimulFluor DFA FluA/B panel-(Chemicon)
26. SimulFluor DFA RSV/para3 panel-(Chemicon)
27. SimulFluor DFA Parainfluenza 1,2,3/Adeno panel-(Chemicon)
28. Specific Parainfluenza 1 DFA-(Chemicon)
29. Specific Parainfluenza 2 DFA-(Chemicon)

Reagent Quality Control:

Performed prior to patient testing and must fall within range of expected results before reagents are released for use. External QC materials are used unless not available. QC materials supplied with reagent kits (usually used as daily QC) must also be done (may be done with the first batch of testing if external QC passes).

Reagent Quality Control Procedure:

a. Check and make sure received date, expiratory date and lot number are recorded in the Reagent Log as well as in LIS micqc.
b. Perform the assay to verify that the results are satisfactory for the reagent lot/kit and record in Reagent Log, and/or LIS and/or on the kit.
c. Use external QC materials (different supplier and to test all components e.g. Bion 14-well Respiratory Panel slides for SimulFluor Resp Screen).
d. Record QC results in Reagent Log and/or LIS as appropriate.
e. Record “in use” date in Reagent Log, and/or LIS (by entering lot active:“yes”) and/or on the kit when placed in service.

Failed Reagent QC:

Test is invalid without satisfactory Reagent QC results.

a. Do not release reagent lot for use pending resolution of QC error.
b. Inform charge/senior technologist.
c. Record in Reagent Log Chart, Instrument Maintenance Log if microscope/incubator is involved in the failure (and Incident Report if necessary).

d. Re-run failed control materials in parallel to fresh controls to evaluate the QC material itself.
e. If the re-run shows the old QC material still fails and fresh QC is satisfactory, the error may be attributed to the old QC material itself and the reagent is satisfactory.
f. If the re-run shows both the old and fresh QC material fail (or other QC not satisfactory), the error may be attributed to the reagent then the reagent cannot be released for use.

Appendix II

SHELL VIAL PROCEDURE

I. Introduction

The shell vial method employs centrifugation of the patient specimen onto a cell monolayer contained in a vial. In general, the centrifugation step shortens the time to a positive culture result. Virus may be detected by direct fluorescent antibody (DFA) or indirect fluorescent antibody (IFA) staining within hours or days of inoculation. The shell vial method is used primarily for detection of CMV, HSV, and VZV. The current cell line used for shell vials is MRC-5 (Human Fibroblast cells).

II. Reagents and Materials

Fluorescence microscope with filter for FITC/Evans blue
Inverted microscope
FITC-conjugated virus-specific antibody stains (HSV1,2 VZV, CMV-IE)
Phosphate buffered saline (PBS)
Distilled water
Cold acetone ($4^{o}C$)
Mounting fluid
Sterile pipettes
Cytospin and accessories
Humidified chamber
Sterile freezer vial
Sterile shell vials with round coverslips and caps
Needle with hooked end attached to syringe
Maintenance media
Glass slides
Coverslips
Paper towels for blotting
Humidified chamber

III. Procedure

1. Registration

   i) Upon receipt of a shipment of cells, initial and date the record sheet accompanying the shipment. The record should contain vendor, lot number, passage number and QC data. File in the Cell Culture QC binder.

   ii) Register the cell suspension lot in the lab information system (LIS), this is necessary to generate the Shell Vial MRC-5 QC procedure. Print labels. Refer to virology LIS manual for procedure.

2. Seeding of shell vials

   i) Before seeding the shell vials, aspirate about 15 mL of MRC-5 cell suspension into a 125 cm^2 tissue culture flask, place label **on the side** of flask and/or write "MRC-5, date and '**Pre**'".

   ii) After seeding shell vials, aspirate about 15 mL of MRC-5 cell suspension into another 125 cm^2 tissue culture flask, label on the side of flask with "MRC-5, date and '**Post**'".

   iii) Aliquot the MRC-5 cells in 1 to 2 mL volumes into a sterile shell vials containing round cover-slips.

   iv) Each shell vial is capped tightly (CO_2 produced by growing cells is needed to maintain proper pH for optimal cell growth) and incubated at 36ºC for 2-3 days before use.

3. Inoculation of shell vials

   i) Refer to specimen protocol or Appendix XXI Specimen Cell Line Stain Table for the number of shell vials to be inoculated.

   ii) Prior to inoculation, check for confluent monolayer formation, sterility and for presence of a coverslip. Record results daily under Shell Vial MRC-5 QC procedure in LIS.

iii) Apply a specimen label (LIS barcode) to the shell vial(s) and a corresponding plane glass slide. Label slides and shell vials for HSV1; HSV2; VZ; CMV accordingly.

iv) Aspirate medium from shell vial using a sterile pipette and discard. Inoculate 0.2 ml of processed specimen into shell vial. Inoculate one specimen at a time, recapping immediately afterward.

v) Centrifuge at room temperature for 15 minutes at 4300 rpm (3500 x *g*).

vi) Afterward, add 1.5 ml of the aliquotted maintenance medium to each shell vial.

vii) Use a new, sterile pipette for each vial. Process one specimen at a time, recapping immediately afterward. After set up is complete, discard any remaining maintenance medium. For specimens that have excess blood or mucous, check with charge/senior technologist before incubating shell vials.

viii) Incubate the shell vials at 36ºC lined up in rows of HSV1, HSV2, VZ. CMV should be lined up in a second cluster plate (CMV-IE requires an extra step in IFA staining and an extra day of incubation):

Virus	**# of Vials**	**Incubation Time**
HSV 1, 2	2	1 day
HSV bivalent	1	1 day
VZV	1	2 days
CMV	1	2 days

4. Staining of shell vials

Prior to staining, examine the shell vial monolayer using the inverted microscope:

a) If there is <75% CPE, perform IFA or DFA staining on the shell vial monolayer using the required antibody conjugate. For CMV, see shell vial staining under Appendix IV (IFA) and for HSV 1/2 and VZV, see shell vial staining under Appendix V (DFA).

b) If >75% of the monoloayer has lifted from the coverslip, check the colour of the maintenance media and proceed as follows:

i) If the maintenance media is bright pink (suggesting alkaline pH), yellow or cloudy, check with charge/senior technologist before proceeding further.

ii) If the maintenance media is appropriately coloured (salmon pink), perform IFA or DFA staining using cytospin preparations of scraped shell vial cells. Follow the staining procedure for prepared cytospin slides as outlined in the tube culture section in Appendix IV (IFA) and Appendix V (DFA).

c) Discard cap. Remove maintenance medium from the shell vials, using a different sterile pipette for shell vials of the same specimen number.

d) Add 1 mL of cold acetone to each shell vial. Cover and fix for 10 minutes.

e) Decant acetone and blot on paper towel.

f) Gently rinse with PBS from squirt bottle, filling vial 3/4 full. Make sure the stream is gentle enough not to flip the cover-slip. Decant PBS.

g) Add 75 µl (2 drops from bottle) of HSV1, HSV2 and VZ to the appropriate row of shell vials in the DFA cluster plate (including QC shell vials, if done on that day). Cover.

h) Add 75 µl (2 drops from bottle) of CMV-IE to the appropriate row of shell vials in the IFA (CMV, 2 day) cluster plate (including QC shell vials, if done on that day). Cover.

i) Incubate both DFA and IFA shell vials at 36^{o}C for 30 minutes.

j) Gently rinse with PBS from squirt bottle, filling vial 3/4 full. Make sure the stream is gentle enough not to flip the cover-slip. Decant PBS. Repeat.

k) For the DFA shell vials (HSV1, HSV2, VZV) remove the coverslip and place cell side down onto a drop of mounting fluid on the pre-labelled glass slide.

l) For the IFA (CMV 2 day) shell vials, add 75µl (2 drops from bottle) of appropriate FITC-conjugated anti-mouse antibodies, cover and repeat the incubation and wash steps (i and j).

m) Remove the coverslip and place cell side down onto a drop of mounting fluid on a glass slide.

n) Read using fluorescence microscope with the FITC/Evans Blue filter and the 40x objective.

III. Reading of Stained Shell Vials

i) CMV – Immediate Early Antigen (CMV-IE)

Using the fluorescence microscope with the FITC/Evans Blue filter, scan the entire field using the 25x objective. Use the 40x objective to investigate any fluorescing cells.

POSITIVE: An even matte apple-green fluorescence covering the entire kidney bean shaped/oval nucleus. May include specks of brighter fluorescence.

NEGATIVE: No typical cells with apple-green fluorescence

INVALID: If no counterstain is not visible or only the edge of cover slip is stained, inform senior/charge technologist. The cover-slip may have flipped before being stained

ii) HSV/VZV

Using the fluorescence microscope with the FITC/Evans Blue filter, scan the entire field using the 10x or 25x objective. Use the 40x objective to investigate any fluorescing cells.

POSITIVE: Distinct apple-green fluorescence of the cytoplasma and /or nucleus of the infected cells. Dull red Evans blue counter stain should be visible for stained nonfluorescent cells.

NEGATIVE: No typical cells with apple-green fluorescence.
Dull red Evans blue counter stain should be visible for negative cells.

INVALID: If no counterstain is not visible or only the edge of cover slip is stained, inform senior/charge technologist. The cover-slip may have flipped before being stained.

IV. Quality Control

A. Shell Vial MRC-5 Quality Control: (unopened shell vial)

This is done weekly when cell shipments are received to monitor cell growth. Record daily in LIS.

Examine daily (for 7 days) for:	Expected results:	Shell Vial MRC5 QC- expected results (LIS entry):
Absence of contamination	Visual inspection: (1) medium colour not yellow (2) medium not cloudy	OK*
Healthy cell growth	Under inverted microscopy: (1) confluent monolayer (2) medium colour pink	OK*
Cover slip	Under inverted microscopy: cover slip present	OK*

At the end of 7 days, one unopened shell vial in good condition is used as "Previous lot MRC-5" for the following week.

B. Shell Vial Inoculation QC procedure (6 shell vials + 1 previous lot):

This QC procedure is performed once a week utilizing HSV-1 (ATCC 539) to:

1. Show that each MRC-5 lot supports the propagation of the intended viruses.
2. Monitor entire shell vial procedures from inoculation to reading including incubation, staining and reading (HSV1 & 2 are DFA, CMV-IE is IFA).
3. The inoculation part is done by Tube Culture bench, the Shell Vial bench completes the procedure including reporting in the LIS.

Shell Vials:		Each week, inoculated with 4 drops of:	After 1 day at 36°C, stain with:	Shell Vial **MRC5** QC-expected results (LIS entry):	HSV1 daily SLIDE SV QC-expected results on **Staining Reaction** (LIS entry):
1	Previous lot MRC-5	HSV-1 ATCC 539	HSV-1	HSV1 old ATCC539: **Gr***	
2	New lot MRC-5 (pos con)	HSV-1 ATCC 539	HSV-1	HSV1 ATCC539: **Gr***	**Pos***
3	New lot MRC-5 (neg con)	None	HSV-1		**Neg***
4	New lot MRC-5 (pos con)	HSV-2 ATCC 539	HSV-2		**Pos***
5	New lot MRC-5 (neg con)	None	HSV-2		**Neg***
6	New lot MRC-5 (pos con)	CMV ATCC Davis	CMV-IE		**Pos***
7	New lot MRC-5 (neg con)	None	CMV-IE		**Neg***

Gr* = stained positive for the intended virus
Pos*= stained positive with the specified stain
Neg*= stained negative with the specified stain

C. Daily Slide Shell Vial QC procedure:

Done and recorded each work day to monitor the staining of each batch (except the day when Inoculated Shell Vial QC procedure is done).

4-well HSV daily QC slide	2-well CMV daily QC slide	Well containing:	Stain with:	HSV1 daily SLIDE SV QC- expected results on **Staining Reaction** (LIS entry):
1		HSV-1 (ATCC 539)	HSV-1	Pos*
2		Uninoculated MRC-5 cells	HSV-1	Neg*
3		HSV-2	HSV-2	Pos*
4		Uninoculated MRC-5 cells	HSV-2	Neg*
	1	CMV (ATCC 807)	CMV-IE	Pos*
	2	Uninoculated MRC-5 cells	CMV-IE	Neg*

Gr* = stained positive for the intended virus
Pos*= stained positive with the specified stain
Neg*= stained negative with the specified stain

D. Reagent QC (HSV1, HSV2, HSV bivalent, CMV-IE and VZ stains):

a. Performed prior to patient testing and must pass before reagents are released for use.
b. Done on external QC slides.
c. Record QC results in Reagent Log and LIS.

Failed QCs:

a. Do not release patient results pending resolution of QC failure.
b. Inform charge/senior technologist.
c. Record in Reagent Log Chart, Instrument Maintenance Log (if eg. microscope/incubator is involved in the failure) and file incident report if necessary.
d. Re-run failed controls in parallel to fresh controls (and/or external QC) to evaluate the QC material itself (already done routinely for MRC5 cells).
e. If the re-run shows the old QC material still fails, fresh QC passes and nothing else is wrong with the batch (only the old QC material failed, patient results valid) patient results may be released.

Marked decrease/absence in fluorescence can be due to:
a. Reagent deterioration/skipping (did not apply primary/secondary stain)
b. Microscope (filter, bulb, alignment)
c. Other equipment, reagents or technique

V. Reference

1. Isenberg, H.D. 1992. Clinical Microbiology Procedure Handbook Vol. 2. ASM Press.

2. Gleaves, Curt A. et al, J Clin Micro., Feb. 1985. Comparison of Standard Tube and Shell Vial Cell Culture Techniques for the Detection of Cytomegalovirus in Clinical Specimens.

3. Engler, Howard D., Selepak, Sally T., J Clin Micro., June 1994. Effect of Centrifuging Shell Vials at 3,500 x *g* on Detection of Viruses in Clinical Specimens.

Appendix III

TUBE CULTURE PROCEDURE

I. Introduction

Tube culture is the conventional method used by diagnostic virology laboratories for virus isolation. Since there is no universal cell line for recovery of all clinically significant viruses, a combination of cell types is used routinely depending on the symptoms, clinical specimen type and specific viruses being sought.

II. Reagents and Materials

Fluorescence microscope Leica DBRB with #2 filter for Rodamine/FITC Evans blue and #4 filter for FITC Evans blue or
Fluorescence microscope Leica DC300F with #3 filter for Rodamine/FITC Evans blue and #1 filter for FITC Evans blue
Inverted microscope
Control slides
Virus-specific antibody
FITC-conjugated antimouse antibody
Phosphate buffered saline (PBS)
Distilled water
Cold acetone ($4^{o}C$)
Mounting fluid
Sterile pipettes
Cytospin and accessories
Vortex
Sterile freezer vial
Glass slides
Coverslips
Paper towels for blotting
Humidified chamber

III Procedure

1. Registration

   i) Upon receipt in the lab, register cell culture tubes received from the supplier in the lab information system (LIS). Refer to virology LIS manual for procedure.
   ii) File the vendor QC sheet (received with the shipment) in the QC binder.
   iii) Randomly, select two tubes from each lot and check the monolayer microscopically for confluent growth and quality of cells. Use these two tubes as the "unopened controls" outlined under the Quality Control section below.
   iv) Human Foreskin Fibroblasts (HFF) and Cynomolgus Monkey Kidney (CMK) cell lines are stored at 36^{o} C in O_2 until expiry. Rhabdomyosarcoma (RD) and Human Laryngeal Epidermoid Carcinoma (Hep-2) cell lines are stored for 18-24 hours at 36^{o} C in O_2 (or until cell lines reach >50% confluency) and then are kept at room temperature until expiry.

2. Inoculation of cell culture tubes

   i) Aliquot 50 mL maintenance medium and allow to come to room temperature before using.
   ii) Refer to the protocol for each specimen type to determine the number of tubes and types of cell lines to be inoculated. Also refer to Appendix XV if needed.
   iii) Prior to inoculation, check the cell culture tubes for acceptable confluent monolayer formation and sterility.
   iv) Decant the medium from the tube.

   v) Using a clean, sterile pipette for each tube, add 1.5 mL of the aliquotted maintenance medium to each tube and re-cap. After set up is complete, discard any remaining maintenance medium.
   vi) Inoculate 0.2 mL (4 drops) of processed specimen into each tube, recapping immediately afterward.
   vii) Incubate the tubes in the roller drum at 36^{o}C. Refer to the appropriate specimen protocol for the incubation time for each tube.
   viii) Refeed CMK and HFF tubes minimally once per week. Refeed HEp-2 and RD tubes minimally twice per week. QC tubes (N1, N2 and N3 of each cell line) should be refed with patients' samples. Tubes showing signs of chemical toxicity (red media / sloughing cells), bacterial / fungal contamination (yellow / turbid media) or aging should be refed within the day.

III. Reading of Tube Cultures

i) **Cytopathic effect (CPE)**: Tube cultures should be examined minimally three times (3x) per week for CPE. Any culture demonstrating ≥ 2+ CPE should be confirmed by staining. The cells should be scraped, a cytospin slide prepared and appropriate monoclonal antibody staining performed. If no CPE is present, change maintenance medium (refeed) weekly for CMK, HFF and bi-weekly for HEp-2, RD. Reincubate according to appropriate specimen protocol for each cell line.

ii) **Respiratory virus screens (RS)**: For respiratory specimens, perform respiratory virus screens with SimulFluor RS when ≥2+ CPE is observed or on days 5, 10 (which ever comes first) on the 2 CMK tubes:

a. Prepare cytospin preparation from cell culture tube as outlined below:
b. Remove all except 1 ml maintenance media from the culture tube using a sterile pipette.
c. Scrape cells from side of tube using a sterile pipette. Break up cell clumps by pipetting the cells up and down several times.
d. Pipette 200 ul (4 drops) of scraped cells into funnel for each well.
e. Cytospin at 2000 rpm (700 x g) for 5 minutes.
f. Remove slide and air dry.
g. Fix in cold acetone for 10 minutes in a coplin jar. Remove slide and air dry.
h. Tube Culture-RSV Slide QC (rsv ATCC 1302) stain reaction (LIScode) should be stained and recorded with each batch of respiratory virus screen.
i. Stain by adding 20 µl of HSV bivalent and SimulFluor RS onto the fixed cytospin cell spots.
j. Incubate in a humidified chamber for 30 minutes at 36°C.
k. Wash each slide 3 times with fresh PBS for 2 minutes each in a coplin jar.
l. Wipe excess PBS from the slide without touching the cell spot.
m. Add 20 µl of appropriate FITC-conjugated antibodies.
n. Incubate in a humidified chamber at 36°C for 30 minutes.
o. Wash each slide 3 times with fresh PBS for 2 minutes each in a coplin jar.
p. Wash with distilled water for 1 minute in a coplin jar.
q. Wipe excess water from the slide without touching the cell spot.
r. Mount using coverslip and mounting fluid.
s. Read with fluorescence microscope Leica DC300F with #3 filter for Rodamine/FITC Evans blue and the 40x objective (**warning:#1 filter is for FITC Evans blue only**).

Interpretation of Results
(Also see Appendix VI for interpretation of SimulFluor RS stain results and further staining if only the resp screen is positive)

Positive for RSV:	Gold fluorescence
Positive for respiratory virus other than RSV:	Green fluorescence, proceed to Appendix VI for further ID
Negative:	Dull-red counterstained cells with no apple-green fluorescence.
Invalid:	If no counterstain is visible, repeat staining QC slide failed, report to senior/charge

If positive, record in freezer program and freeze cells and supernate. Refer to Appendix X and XII for procedure.

iii) **Confirmation by PHL**: Any culture demonstrating CPE for which a virus cannot be detected using monoclonal antibodies or other in-house methods and for which toxicity has been ruled out (see below) should be referred to the Public Health Laboratory (PHL) for further work-up. Pass cells to a new tube before sending. Scrape and add 0.2 ml (4 drops) of scraped cells to a fresh tube containing 2 mL of fresh maintenance media (1:10 dilution). Consult the charge/senior technologist or medical microbiologist before referring the specimen to PHL.

iv) **Culture Toxicity:** If chemical toxicity is suspected in a tube culture (rounding of cells, sloughing of cells, granular cytoplasm of cells or unusual CPE, consult senior/charge technologist if unsure), proceed as follows:

v) Pass cells by scraping and adding 0.2 ml (4 drops) of these scraped cells to a fresh tube containing 2 mL of fresh maintenance media (1:10 dilution). Proceed with tube culture method as outlined above.

vi) The effects of chemical toxicity would be reduced by dilution whereas the effects of CPE (caused by viral replication) would be the same, if not accelerated on passage. If CPE is suspected, identify virus by antibody stains. If chemical toxicity is suspected, continue to incubate (may need further refeeding to reduce toxicity). If unsure of cell toxicity or CPE , refer to the charge/senior technologist for review.

vii) **Contaminated Culture:** If the tube culture appears visibly contaminated (eg. cloudy and/or yellow medium) and thus uninterpretable, proceed as follows:

a. On 1st or 2nd reading - change the maintenance medium, and reincubate.
b. On 3rd or later reading or recurrence - issue a final report stating: "Virology tube culture: Specimen is heavily contaminated with bacteria and/or fungus. Unable to interpret virology tube culture."
c. Replant if specimen is from a sterile site or contamination is attributed to the lab. If multiple specimens are contaminated, report to senior/charge.

IV. Quality Control

Record all results of QC in LIS under VTCCMK; VTCHEp; VTCHFF and VTCRD. Refer to virology LIS manual for procedure. Report any abnormal results to charge/senior technologist.

	Day of Receipt: Mon/Tue	Start on Wed	Start on Fri	Start on Mon
	Unopened	Refeed	Refeed	Refeed
CMK	C	N1	N2	N3
	V			
HEp2	C	N1	N2	N3
	V			
HFF	C	N1	N2	N3
	V			
RD	C	N1	N2	N3
	V			

LIS entry codes:
C = unopenC
V = unopenV
N1=negcon1
N2=negcon2
N3=negcon3

Five tubes are reserved from each lot of cell culture tubes received, and used as controls as follows:

i) Negative controls: (tubes labelled N1, N2, N3)

On Wednesday, Friday and Monday, an uninoculated tube from each cell line used that day is placed in the roller drum with the inoculated specimens. These tubes are incubated, read and refed with the patient inoculated cultures to monitor the monolayer quality, medium toxicity/contamination. They can also be used to provide a baseline for comparison for inoculated cultures when reading for CPE. HFF, CMK, HEp-2 and RD tubes are kept for 5, 2, 2 and 1 weeks respectively.

ii) Unopened Controls: (2 tubes labelled C and V respectively)

These tubes are not opened. One tube is kept at 36^{o} C in O_2 in the clean room (C) and one is placed on the roller drum (V) at 36^{o} C in O_2. These tubes are observed for 1 week to identify toxicity and contamination originating with the vendor.

iii) Positive Controls:

Each week HSV-1 ATCC strain # VR- 539 is scraped from the previous week's positive control tube and used to inoculate a fresh HFF tube. If the control fails to propagate, a new vial can be retrieved from liquid N_2 tank MINS shelf 6.

Additional positive controls may be set up for the following reasons:

a) Low isolation rates
b) Comparison of cell lines
c) Vendor changes
d) Proficiency test failures
e) Training
f) Continuing problems with negative controls
g) Preparation of QC material (i.e. positive control slides)

Consult a charge/senior technologist to determine the cell lines and viruses to be set up.

V. Reference

1) Isenberg, H.D. 1992. Clinical Microbiology Procedures Handbook. Vol. 2. ASM Press.

Appendix IV

INDIRECT IMMUNOFLUORESCENT ANTIBODY (IFA) STAINING FOR VIRAL CULTURE CONFIRMATION

I. Introduction

The IFA technique is used to identify viral isolates in the cells obtained from shell vials and tube cultures. The indirect method consists of two steps. In the first step, primary antibodies are allowed to react with viral antigens in the cells. These specific complexes are detected in a second step using a species-specific antibody conjugated with a fluorochrome. Viruses which we currently identify by IFA staining include cytomegalovirus immediate early antigen (CMV-IE) and enteroviruses.

II. Reagents and Materials

Virus-specific antibody
FITC-conjugated antimouse antibody
Phosphate buffered saline (PBS)
Distilled water
Cold acetone ($4^{o}C$)
Mounting fluid
Sterile pipettes
Cytospin and accessories (for tube cultures)
Humidified chamber
Sterile freezer vial
Glass slides
Coverslips
Paper towels for blotting
Humidified chamber (for tube culture)

II. Procedure

1. Shell Vial

Follow outline in Appendix II to determine if staining should be done in the shell vial itself or if a cytospin needs to be prepared. If the staining is to be done in the shell vial itself, proceed to step i) below.

i. Discard cap. Remove maintenance medium from the shell vial using a clean sterile pipette.

c) Add 1 mL of cold acetone. Cover and fix for 10 minutes.

d) Decant acetone and blot on paper towel.

e) Gently rinse with PBS from squirt bottle, filling vial 3/4 full. Decant PBS.

f) Add 75 µl (2 drops from bottle) of appropriate antibody. Cover.

vi. Incubate at 36^{o}C for 30 minutes.

vii. Gently rinse with PBS from squirt bottle, filling vial 3/4 full. Decant PBS. Repeat.

viii. Add 75µl (2 drops from bottle) of appropriate FITC -conjugated antibodies, cover and repeat steps vi and vii.

x. Remove the coverslip and place cell side down onto a drop of mounting fluid on a glass slide.

xi. Read using fluorescence microscope with the FITC/Evans Blue filter and the 40x objective.

2. Tube Culture

i. Prepare cytospin preparation from cell culture tube as outlined in Appendix XX.

ii. Add 20 µl of appropriate antibodies onto the fixed cytospin slide.

iii. Incubate in a humidified chamber for 30 minutes at 36^{o}C.

iv. Wash each slide 3 times with fresh PBS for 2 minutes each in a coplin jar.

v. Wipe excess PBS from the slide without touching the cell spot.

vi. Add 20 µl of appropriate FITC-conjugated antibodies.

vii. Incubate in a humidified chamber at 36^{o}C for 30 minutes.

viii. Wash each slide 3 times with fresh PBS for 2 minutes each in a coplin jar.

ix. Wash with distilled water for 1 minute in a coplin jar.

x. Wipe excess water from the slide without touching the cell spot.

xi. Mount using coverslip and mounting fluid.

xii. Read with fluorescence microscope with the FITC/Evans Blue filter and the 40x objective.

Interpretation of Results

Positive: CMV-IE:
An even matte green fluorescence covering the entire nucleus which may include specks of brighter fluorescence.

Enteroviruses:
Distinct apple green fluorescence of the cytoplasm and/or nucleus of the infected cells.

Negative: Red cells with no apple-green fluorescence.

IV. Quality Control

Appropriate positive and negative control slides should be stained with each batch.

V. Reporting

See individual specimen protocols.

VI. Reference

Isenberg, H.D., 1992. Clinical Microbiology Procedures Handbook Vol. 2. ASM Press.

Appendix V

DIRECT IMMUNOFLUORESCENT ANTIBODY (DFA) STAINING

I. Introduction

The DFA staining technique is used to detect viruses either directly in patient specimens or which have been isolated in shell vial or tube cultures. The method consists of a single staining step using a virus-specific antibody which is conjugated with a fluorochrome. Viruses which we currently identify by DFA staining include HSV-1, HSV-2, VZV, CMV (late antigen) and respiratory viruses (SimulFluor stains for respiratory syncytial virus, parainfluenza, influenza, adenovirus).

II. Reagents and Materials

FITC-conjugated virus-specific antibody
FITC/Rodamine-conjugated virus-specific antibody (SimulFluor)
Phosphate Buffered Saline (PBS)
dH_2O
cold acetone (4^oC)
mounting fluid
sterile pipettes
cytospin and accessories (for tube culture)
humidified chamber
glass slides
coverslips
paper towels for blotting

III. Procedure

1. Shell Vial

This procedure is for staining of cells directly in shell vial. If staining a cytospin slide or slide made directly from a patient specimen, follow the tube culture procedure below.

i. Discard cap. Remove maintenance medium from the shell vial using sterile pipette.

ii. Add 1 mL of cold acetone. Cover with tray lid and let sit for 10 minutes.

iii. Decant acetone and blot shell vial on paper towel.

iv. Gently rinse with PBS from squirt bottle, filling vial 3/4 full. Decant PBS.

v. Add 75µl (2 drops from bottle) of appropriate FITC-conjugated virus-specific antibody. Cover with tray lid.

vi. Incubate at 36^{o}C for 30 minutes.

vii. Gently rinse with PBS from squirt bottle, filling vial 3/4 full. Decant PBS. Repeat.

viii. Remove the coverslip from each shell vial and place cell side down onto a drop of mounting fluid on a glass slide.

ix. For HSV 1, HSV 2, VZ and CMV, read using fluorescence microscope with the FITC/Evans Blue filter and the 40x objective.

x. For respiratory viruses, read using fluorescence microscope with the FITC/Evans Blue filter and the 40x objective.

2. Tube Culture

i. Prepare cytospin slide from cell culture tube as outlined in Appendix XX.

ii. Fix slide in cold acetone for 10 minutes in a coplin jar. Remove slide and air dry.

iii. Add 20µl of appropriate FITC-conjugated antibody onto the fixed cytospin slide.

iii. Incubate in a humidified chamber at 36^{o}C for 30 minutes.

iv. Wash each slide 3 times with fresh PBS for 2 minutes each in a coplin jar.

v. Wash with distilled water for 1 minute in a coplin jar.

vi. Wipe excess water from the slide without touching the cytospin preparation.

vii. Mount using coverslip and mounting fluid.

xi. For HSV 1, HSV 2, VZ and CMV, read using fluorescence microscope with the FITC/Evans Blue filter and the 40x objective.

viii. For respiratory viruses, read using fluorescence microscope with the FITC/Evans Blue filter.

Interpretation of Results

Positive: Bartel CMV monoclonal antibody: Bright apple green fluorescence of cytoplasmic inclusion (late antigen) and homogenous early nuclear antigen in CMV-CPE cells.

Chemicon SimulFluor Respiratory Screen:
All respiratory viruses except RSV show bright apple green fluorescence of the cytoplasm and/or nucleus of the infected cell.
RSV shows bright gold fluorescence of the cytoplasm and/or nucleus of the infected cell.

Chemicon SimulFluor Flu A/Flu B:
Influenzae A virus shows bright apple green fluorescence.
Influenzae B virus shows bright gold fluorescence.

Chemicon SimulFluor RSV/Para 3:
RSV virus shows bright apple green fluorescence.
Parainfluenzae 3 shows bright gold fluorescence.

Chemicon SimulFluor Para 123/Adeno:
Parainfluenza 1,2,3 viruses show bright apple green fluorescence.
Adenovirus shows bright gold fluorescence.

Chemicon individual monoclonal antibodies:
Parainfluenzae 1 and 2, and adenovirus show bright apple green fluorescence.

Negative: Red Cells with no apple-green fluorescence.

IV. Quality Control

Appropriate positive and negative control slides should be stained with each batch.

VI. Reference

Isenberg, H.D., 1992, ASM. Clinical Microbiology Procedures Handbook Vol. 2.

Appendix VI

DIRECT ANTIGEN DETECTION FROM SPECIMENS

I. Introduction

Immunofluorescent staining may be used to rapidly detect viral antigen directly in a clinical specimen. Smears prepared at the patient's bedside may be used, or a smear may be prepared in the laboratory from cellular material in a specimen. Specimens for which direct antigen detection may be requested include bronchoscopy or nasopharyngeal specimens for respiratory virus detection or vesicular lesion scraping for HSV/VZV antigen detection. Cells are stained using pooled or specific monoclonal antibody stains and examined under the fluorescence microscope looking for specific fluorescence of cell cytoplasm or nucleus.

II. Reagents and Materials

Virus-specific or pooled FITC and Rodamine conjugated antibody stains (with Evans blue counter stain):

- Respiratory Viral Screen/RSV panel
- FluA/B panel
- RSV/para3 panel
- Para1,2,3/Adeno panel
- Specific Parainfluenza 1
- Specific Parainfluenza 2
- Herpes simplex 1
- Herpes simplex 2
- Herpes simplex bivalent
- Varicella zoster virus

Phosphate buffered saline (PBS)
Cold acetone (4^{o}C)
Distilled water
FA mounting fluid
Vortex
Sterile pipettes
Cytospin and accessories
Humidified chamber
Coplin jars
Fuorescence microscope with FITC/Rodamine/Evans blue filters

III. Procedure

A. Preparation of slide

a) For swabs:

i. Vortex patient sample in transport medium for 30 seconds. Remove excess fluid from the swab and discard the swab.

ii. Transfer 0.5 - l.0 ml of this specimen to a microcentrifuge tube. Centrifuge 1 minute at 14,000 rpm.

iii. Remove supernatant (can be placed back with original specimen to be processed further) down to 400 ul (8 drops). Vortex 5-10 seconds.

iv. Pipette 200 ul (4 drops) of this sediment into funnel for each well. Prepare the appropriate number of cytospin wells according to table below.

v. Cytospin at 2000 rpm (700g) for 5 minutes.

vi. Remove slide and air dry.

vii. Fix in cold acetone for 10 minutes in a coplin jar.

viii. Remove slide and air dry.

ix. Proceed to staining. Refer to Appendix V (DFA) for staining procedures.

b) For Bronchoscopy Specimens (BAL,Washings):

i. Mix specimen gently and examine for cellular turbidity. Specimens which are more turbid than a 0.5 McFarland standard are diluted to that approximate turbidity using Hank's Balanced Salt Solution (Gibco BRL).

ii. Pipette 200 ul (4 drops) of specimen into funnel for each well. Prepare the appropriate number of cytospin wells according to table below.

iii. Cytospin 200 uL at 2000 rpm (700 x g) for 5 minutes. Prepare the appropriate number of cytospin preparations according to the table below.

iv. Remove slide and air dry.

v. Fix in cold acetone 10 minutes in coplin jar.

vi. Remove and air dry.

vii. Proceed to staining. Refer to Appendix V (DFA) for staining procedures.

c) For Smears Prepared Outside of the Laboratory

For smears which have been prepared outside of the laboratory, examine macroscopically for evidence of material on slide. Specimens for which only one slide is received may be stained with ONE monoclonal antibody only according to the physician's request. It is not possible to stain a negative control for such specimens. A swab for viral culture should be requested if not received along with the smear.
Draw a circle around material on slide with a diamond pencil prior to staining.

i. Fix in cold acetone 10 minutes in coplin jar.
ii. Remove and air dry
iii. Proceed to staining. Refer to Appendix V (DFA) for staining procedures.

2. Staining Protocol

Prepare appropriate number of cytospin wells and stain as per chart. Refer to Appendix V (DFA) for staining procedures.

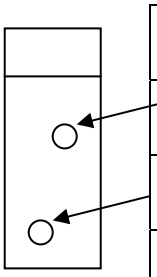
Specimen	Monoclonal Antibody	
Nasopharyngeal/ Bronchoscopy/ Throat (prepare 2 cytospin wells)	Respiratory screen [a] (November to April)	SimulFluor, Light Diagnostics
	Flu A/ Flu B	SimulFluor, Light Diagnostics
Vesicular Lesion Swab (prepare 2 cytospin wells)	HSV-bivalent	Bartels
	VZV	Meridan Bioscience Inc.

[a] If respiratory screen or Flu A/B is positive, prepare more cytospin preparations and stain according to interpretation chart that follows.

SimulFluor Respiratory Screen Staining Protocol and Interpretations – Scheme 1

Using filter for FITC/Evans Blue (Filter no. 3 in fluorescence microscope no.2, Leica I)

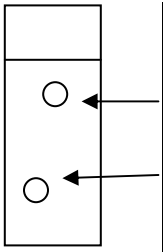
First double-well cytospin slide



Cell spot	**SimulFluor Mab Panels**	**Scenario I**	**Scenario II**	**Scenario III**	**Scenario IV**	**Scenario V**
1	Flu A/FluB	Not green Not gold	Not green Not gold	Green	Gold	Not green Not gold
2	Resp Screen	Not green Not gold	Gold	Green	Green	Green
	Interpretations:	Report: Negative	Report: RSV	Report: Flu A	Report: Flu B	*Adeno;, Para 1,2 or 3 →Proceed to second slide*

Scenario V: Spot 1 (A/B) is negative and spot 2 (RS) is green, proceed

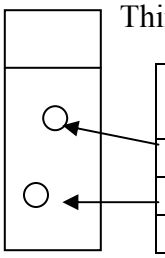
Second double-well cytospin slide



Cell spot	**SimulFluor Mab Panels**	**Scenario V-A**	**Scenario V-B**	**Scenario V-C**
3	Para1,2,3/Ad	Green	Gold	Green
4	RSV/Para3	Gold	Not green Not gold	Not green or gold
	Interpretations:	Report: Para 3	Report: Adeno	*Para1,2 → Proceed to third slide*

Scenario V-C: Spot 1 (Para123/Ad) is green and spot 2 (RSV/P3) is negative, proceed

Third double cytospin slide

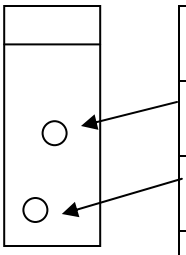


Cell spot	**Individual Mab**	**Scenario V**	**Scenario VI**
5	Para 1	Apple-Green	Not green
6	Para 2	Not green	Apple-Green
	Interpretations:	Report: Para 1	Report: Para 2

SimulFluor Respiratory Screen Staining Protocol and Interpretations- Scheme 2

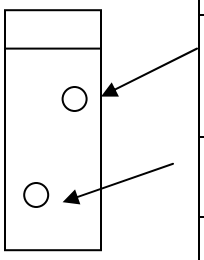
Using filter for FITC/Evans Blue (Filter no. 3 in fluorescence microscope no.2, Leica I)

First double cytospin slide



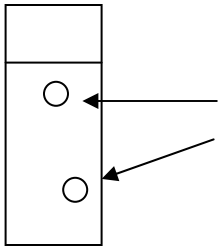
Cell spot	SimulFluor Mab Panels	Scenario I	Scenario II	Scenario III	Scenario IV
1	RSV/Para3	Not green Not gold	Green	Gold	Not green Not gold
2	Resp Screen	Not green Not gold	Gold	Green	Green
	Interpretations:	Report: Negative	Report: RSV	Report: Para 3	*Adeno; P1,2; FluA,B Proceed to second slide*

Second double cytospin slide



Cell spot	SimulFluor Mab Panels	Scenario IV-A	Scenario IV-B	Scenario IV-C	Scenario IV-D
3	Para 1,2.3/Ad	Not green Not gold	Not green Not gold	Gold	Green
4	FluA/FluB	Gold	Green	Not green Not gold	Not green Not gold
	Interpretations:	Report:Flu B	Report: Flu A	Report: Adeno	*Para 1,2 Proceed to third slide*

Third double cytospin slide:



Cell spot	Individual Mab IFA	Scenario V	Scenario VI
5	Para 1	Green	Not green
6	Para 2	Not green	Green
	Interpretations:	Report: Para 1	Report: Para 2

III. Interpretation of Results

Examine smear for adequate numbers and types of cells. Respiratory specimens should consist of columnar (ciliated or goblet) epithelial cells. Scrapings from lesions should contain basal epithelial cells. (See reference 2, page 69). **Adequate interpretation of results requires a minimum of approximately 20-50 cells per smear.** Samples with inadequate numbers of cells should be shown to a charge/senior technologist and reported as having insufficient cellular material.

Positive:	Specific, apple-green fluorescence in the cytoplasm and/or nucleus of the exfoliated cells. This specific fluorescence must be absent in the negative control and/or smears stained with other antibodies.
Negative:	Dull-red stained cells with no viral specific apple-green fluorescence. OR Dull-red stained cells with pinpoint non-specific nuclear staining.
Insufficient cells:	Fewer than 20 epithelial cells per smear

IV. Quality Control

Reagent QC:

a. Check expiratory date.
b. Verify that Reagent QC is satisfactory for the reagent lot/kit being used (recorded in Reagent Log and/or on the kit).
c. If necessary, perform the Reagent QC procedure (external QC slide with all components eg. Bion 14-well Respiratory Panel).

Failed Reagent QC:

Test is invalid without satisfactory Reagent QC results.

a. Do not release reagent lot for use pending resolution of QC error.
b. Inform charge/senior technologist.

c. Record in Reagent Log Chart, Instrument Maintenance Log if microscope/incubator is involved in the failure (and Incident Report if necessary).
d. Re-run failed control materials in parallel to fresh controls to evaluate the QC material itself.
e. If the re-run shows the old QC material still fails and fresh QC is satisfactory, the error may be attributed to the old QC material itself and the reagent is satisfactory.
f. If the re-run shows both the old and fresh QC material fail (or other QC not satisfactory), the error may be attributed to the reagent then the reagent cannot be released for use.

Daily QC:

a. Appropriate positive and negative control slides (eg. ATCC 4-well slide with RSV/Para3 for SimulF RS stain) should be stained with each batch.

b. Examine the negative control well first to establish the dull red colour (Evans blue counterstained) and to determine if there is any nonspecific staining.
The positive control must be clearly distinguishable from the negative control or the test is invalid.

Failed Daily QC:

a. Do not release patient results pending resolution of QC error.
b. Inform charge/senior technologist.
c. Record in Reagent Log Chart (and Instrument Maintenance Log if microscope/incubator is causing the failure).
d. Re-run failed controls in parallel to fresh controls (and/or external QC) to evaluate the QC material itself.
e. If the re-run shows the old QC material still fails and fresh QC passes and nothing else is wrong with the batch (only the old QC material failed, patient results valid) patient results may be released.

Marked decrease/absence in fluorescence can be due to:

a. Reagent deterioration/skipping (did not apply the correct stain)
b. Microscope (filter, bulb, alignment)
c. Other equipment, reagents or technique

V. Reporting

See individual specimen protocols.

VI. Reference

1. Wiedbrauk, D.L. 1993, Raven Press. Manual of Clinical Virology.

2. Rossier, E., Miller, H., Phipps, P. 1989, University of Ottawa Press. Rapid Viral Diagnosis by Immunofluorescence: An Atlas & Practical Guide.

3. SimulFluor Product Insert for cat. no. 3296, June 2002, Revision C: 40729 Light Diagnostics Chemicon International Temecula, CA 92590

Appendix VII

HEMADSORPTION OF TUBE CULTURE MONOLAYERS (NOT ROUTINELY USED)

The hemadsorption (HAD) technique is used primarily to detect viruses that produce little or no cytopathic effect (CPE) in tube culture monolayers. Using guinea pig RBC, it is used to screen inoculated cell cultures for the presence of influenza, parainfluenza, mumps and Newcastle disease viruses.

I. Procedure

Reagents

guinea pig RBC in Alsever's solution
sterile phosphate buffered saline(PBS)

Materials

sterile pipettes
precision pipettes
inverted microscope
centrifuge
15 mL sterile centrifuge tube

Preparation of 10% stock guinea pig RBC suspension

The stock suspension should be prepared every Monday and stored at 4^{o}C and used within 7 days of preparation.

1. Transfer 5 mL of blood to 15 mL tube and add equal volume of PBS.

2. Centrifuge at 3000 rpm (700 x *g*) for 5 minutes at room temperature.

3. Discard the supernate and add 10 mL of PBS.

4. Centrifuge and wash the cells until the supernatant is clear. (approx. 2-3 washings).

5. Determine the packed-cell volume and add PBS equal to 9 times the packed-cell volume to yield a 10% suspension.

Preparation of 0.4% working guinea pig RBC suspension

The working suspension should be prepared from 10% suspension on the day of testing.

1. Add 0.4 mL of the 10% suspension to 9.6 mL of PBS.

HAD Test

The HAD test is performed on day 5 and day 10 for respiratory specimens with no evidence of CPE.

1. Select one RMK tube to be used for HAD. Transfer the medium in the tube to another sterile, labelled capped tube. Place medium at $4^{o}C$ pending HAD results.

2. Add 0.2 mL of the 0.4% RBC suspension to each culture tube, to be tested.

3. Incubate the tubes horizontally at $4^{o}C$ for 30 minutes. Make sure the RBC suspension is distributed over the monolayer.

4. Gently rotate or tap the tubes to resuspend nonadsorbed cells. Immediately examine the tubes with inverted microscope with the 40X objective. Do not handle the tubes in such a way that the monolayers will become warm.

Interpretation of Results

1. Positive HAD test should show RBCs firmly attached to the monolayer. Hemagglutinated cells (clumped RBCs) are also seen in the fluid overlaying the monolayer.

2. Negative HAD test should show no or minimal RBCs attached to the monolayers, with almost all cells floating above the monolayers.

II. Quality Control

Positive and negative controls should be set up for HAD test prior to the expected "flu" season.

Positive HAD Test

1. Place the tube in a 36^{o}C water bath for 15 minutes.

2. Wash the eluted monolayers twice with PBS.

3. Perform indirect immunofluorescence test for influenzae and parainfluenzae viruses. See Appendix III.

4. The culture medium harvested prior to the HAD test may be used for subpassage, storage or identification by hemagglutination inhibition.

Negative HAD Test

1. Discard tube that is HAD negative at 10 days after inoculation.

2. Tube inoculated after 5 days should be washed 3 times with 5 mL Hank's Balanced Salt Solution to remove all RBCs.

3. Refeed tubes with 2 mL of maintenance medium and reincubate the cultures for another 5 days.

4. Discard the culture fluid harvested from negative cultures unless subpassage is to be performed.

V. Reference

Isenberg, H.D. 1992. Clinical Microbiology Procedures Handbook. Vol. 2. ASM.

Appendix VIII

MEDIA

GROWTH MEDIUM (Not in routine use)

500 mL Eagles' MEM with Hanks' salt/without glutamine
10 mL Vitamins
5 mL L-glutamine (200 mM)
50 mL Inactivated fetal calf serum

Store at 4°C. Stable for 2 weeks.

MAINTENANCE MEDIUM

500 mL Eagles' MEM with Hanks' salt/without glutamine (stored at 4°C)
5 mL L-glutamine (200 mM, stored frozen at -20 °C in clean room freezer)
10 mL Inactivated fetal calf serum (stored frozen at -20 °C in clean room freezer)
5 mL Fungizone (250 µg/mL, stored frozen at -20 °C in clean room freezer)
*5 mL Gentamicin (1 mg/mL, stored frozen at -20 °C in clean room freezer)
**5 mL Vancomycin (10 mg/mL, stored frozen at -20 °C in clean room freezer)

Store at 4°C. Stable for 2 weeks.

* **Gentamicin Solution (1 mg/mL):**
Dilute entire contents (10 mL) of 10 mg/mL Gentamicin Sulphate vial into 90 mL of d. H_2O to achieve 1 mg/mL. Filter sterilize, then dispense into 5 mL aliquots (stored frozen at -20 °C in clean room freezer).

** **Vancomycin Solution (10 mg/mL):**
Add contents of 1 gram vial of Vancomycin to 100 mL of distilled H_2O. Filter sterilize, then dispense into 5 mL aliquots (stored frozen at -20 °C in clean room freezer.

Media Quality Control:

a. A 2 mL aliquot of maintenance media is to be placed into each of CMK, HFF, HEp-2 and RD tubes for a check on sterility/toxicity before the media is used.
b. Maintenance Media is to be registered into the LIS (micqc) using the date made as the lot number. The individual components and their lot numbers are recorded at this time.
c. QC results are all entered into the LIS when reading tube culture QC on Mondays, Wednesdays and Fridays.

Failed Media QC:

a. Any cell lines showing microbial contamination or toxicity is considered abnormal.
b. Do not release media lot for use pending resolution of the QC failure.
c. Inform charge/senior technologist to discuss further actions including the following.
d. Record in Incident Report (if necessary).
e. Repeat QC/re-make media to evaluate if it is the cell media, the supplement(s) or other materials causing the problem.

CELL WASHING MEDIUM (Purchased)

Hanks' Balanced Salt solution without $CaCL_2$, $MgCL_2$ and $MgSO_4.7H_2O$

CELL FREEZING MEDIUM (SIGMA C6164) (Purchased)

MEM supplemented with a mixture of fetal bovine serum and calf serum containing DMSO.

VIRAL FREEZING MEDIUM (SIGMA C6039) (Purchased)

MEM supplemented with a mixture of fetal bovine serum and calf serum containing 10% glycerol.

TRANSPORT MEDIA:

BARTEL'S VIRAL TRANSPORT MEDIUM (B1029-36A) (Purchased)

Eagles' MEM with nonessential amino-acid and L-glutamine in HBSS.
2% FBS
10 µg/mL Gentamicin
50 µg/mL Streptomycin
50 µg/mL Penicillin
4 µg/mL Amphotericin B
15 mM HEPES
9.5 mM Sodium bicarbonate

CHLAMYDIA TRANSPORT MEDIUM (Not in routine use)

500 mL Eagles' MEM with Hank's salt/without glutamine.
50 mL Fetal Calf Serum (normal)
5 mL 3M Glucose
5 mL Gentamicin (1000 µg/mL)
5 mL Vancomycin (1000 µg/mL)
5 mL Fungizone (250 µg/mL)

Combine all components aseptically. Dispense 1.5 mL transport medium into sterile screw-cap vials containing 3 glass beads. Store at -20^{o}C.

Appendix IX

TRYPSINIZATION AND MAINTENANCE OF MONOLAYER CELL CULTURES

Monolayer cell cultures may be kept active and available for seeding of tubes, vials, dishes and plates. A constant supply of cell cultures can be maintained by routine subpassage of cell lines to new flasks. (Generally research use only.)

1. Discard medium from the cell culture flask (125 cm^2). Rinse monolayer with 15 mL of Hank's Balanced Salt Solution and discard.

2. Add 5 mL of trypsin EDTA mixture (pre-warmed to 36^oC) to flask.

3. Incubate the culture flask for 3 minutes (no more than 5 minutes) at 36^oC. Observe after 3 minutes to see whether the cell sheet is breaking loose from the flask surface. Tap flask sharply against palm of hand to aid in loosening tissue.

4. When tissue has loosened completely, add 15 mL of pre-warmed growth medium into the flask.

5. Mix cells by drawing cells and fluid up and down in a pipette.

6. Adjust the volume to 90 mL with growth medium.

7. Aliquot 30 mL of suspended cell mixture into at least one new 125 cm^2 culture flask. Aliquot the remaining to shell vials at 1.5 mL each or tube culture (16 x 125 mm) at 2 mL each.

8. Incubate the culture flasks, shell vials or tube cultures at 36^oC.

9. Observe daily for growth of cells (3 - 5 days) and for change in pH of medium. If the medium becomes acidic or basic, replace it with fresh growth medium.

10. Replace growth medium with maintenance medium when a confluent monolayer is obtained (usually after 2 - 3 days).

11. Replace with fresh maintenance medium once a week.

Note: Freeze first passage of cell culture whenever a new shipment is received. (See Appendix VIII).

Appendix X

CRYOPRESERVATION OF CELL CULTURES

Generally research use only:

1. When a new bottle of cells to be frozen arrives, transfer 30 mL aliquots of cells into 125 cm^2 flasks (about 3 flasks).

2. Incubate at 36^oC until a complete monolayer is obtained (approximately 4 - 5 days).

3. Follow steps 1 - 5 in Appendix IX (Trypsinization and maintenance of monolayer cell cultures).

4. Transfer the trypsinized monolayer to a sterile 15 mL centrifuge tube.

5. Centrifuge at 2000 rpm (700 x *g*) for 10 minutes.

6. Discard the supernate and resuspend the cells in 10 mL cell freezing medium.

7. Distribute 1.0 mL volume into freezing vials labelled with cell line information.

8. Place the vials into a "Mr. Frosty" freezing container and hold in -70^oC freezer for 2 - 3 hours.

9. Record in the computer Freezer Program details of the cell lines stored. Use study "CEL".

10. Transfer the vials to liquid nitrogen freezer.

Reference

Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infection. A.P.H.A. 1989. Sixth Edition. Pg. 72.

Appendix XI

RECOVERY OF CRYOPRESERVED CELLS

Generally research use only:

1. Obtain the frozen cells from liquid nitrogen storage and immediately place the vial in a 37^{o}C water bath **WITH A PROTECTIVE COVER. **** Allow to thaw for 2 minutes (no more than 3 minutes).

2. Wipe the outside of the vial with 95% alcohol.

3. Transfer the contents of the vial to a new 125 cm^2 flask using a sterile transfer pipette.

4. Gradually add 30 mL growth medium to the cells (slowly over 2 minutes) to dilute the cells.

5. Incubate at 37^{o}C and observe at 24 hours for cell adherence and growth.

6. Discard old medium and refeed cells with 30 mL of growth medium at 24 hours to remove all traces of DMSO and reincubate the cells.

7. Replace growth medium with maintenance medium when the monolayer is confluent (usually after 2-4 days).

8. Replace maintenance medium with fresh maintenance medium once a week.

Reference

Isenberg, HD: Clinical Microbiology Procedures Handbook. American Society of Microbiology, 1992. Pg. 8.20.7.

Appendix XII

CRYOPRESERVATION OF VIRUS ISOLATES

1. Record in the computer Freezer Program details of the isolate stored.

    a. Select "A. Clinical Records".
    b. "Add new entries?": enter "Yes".
    c. Enter lab number, ensure patient data is correct, enter date frozen, enter study (ie "VIR"), ensure specimen type is correct.
    d. Enter name of isolate to be frozen once.
    e. Note the freezer locations in which the frozen isolates are to be placed.
    - Freezer "G" & "J" refer to Liquid Nitrogen freezers.
    - Shelf no. refers to the canister number.
    - Box no. refers to the cane number. Each cane holds 5 freezer vials.

2. Discard maintenance medium on monolayer to be frozen.

3. Add 1.0 mL viral freezing medium (see Appendix VII) to positive cells. Scrape monolayer and mix well with freezing medium.

4. Distribute into freezing vial labelled with LIS label.

5. Store the vial immediately in liquid nitrogen freezer in the location specified by the freezer program.

<u>FREEZING CAP SPECIMENS AND ISOLATES</u>:

1. After planting CAP specimens, add 1 mL of viral freezing medium to the sample and transfer to a freezing vial. Enter into the freezer program as described above using "VQC" for study. Enter "CAPSAM" as isolate #1.

2. Freeze one vial of CAP viral isolates after identification using steps 2 - 5 described above. The frozen isolate will be isolate #2 and is entered using the code for the name of the virus. (The original sample and frozen isolate may be on different canes, this is acceptable).

Reference

Laboratory Diagnosis of Infectious Diseases. 1988 Volume II. Pg. 268.

Appendix XIII

PRESERVATION OF CELL CULTURE MONOLAYERS

1. Aspirate the medium from the cell culture tubes to be preserved.

2. Add 8 mL buffered formaldehyde preservative medium to each tube.

3. Record the following information on the tube:
   - virus
   - number of days incubation
   - lab number

4. Store preserved culture at room temperature.

Formaldehyde Preservative

100 mL	Formaldehyde Solution (37 - 40%)
900 mL	Distilled Water
20 mL	Phenol Red (0.5%)
4.0 g	$NaH_2PO_4 - H_2O$
6.5 g	Na_2HPO_4

Materials

Vortex
Sterile pipettes
10 - 100 uL Eppendorf pipette
Humidified chamber
Coplin jars
Fluorescent microscope

Appendix XIV

QUALITY CONTROL OF CELL CULTURES USED FOR ROUTINE VIRUS ISOLATES

Tube Culture

Upon receipt of cell culture tubes, the record of the date received, vendor, lot number, and passage number is kept in the QC binder for cell lines. The monolayer is checked microscopically for sterility and appearance of an acceptable confluent monolayer.

A. Uninoculated Negative Controls:

Reserve 4 tubes of each lot cells for use as controls and label as follows:

N1, date;
N2, date;
C, date;
V, date

a. Negative Controls with refeed: (2 tubes, N1; N2)

Select one tube on Thursday and one tube on Monday to set up along with inoculated specimens. Incubate, observe and refeed these tubes in parallel with patient inoculated cultures to monitor monolayer quality. They can also be used to provide a baseline for comparison of inoculated cultures when reading for CPE and immunostaining. CMK and HFF tubes are kept for 3 weeks, HEp-2 and RD for 2 weeks

b. Unopened Negative Controls without refeed: (2 tubes, C; V)

These tubes are left unopened and observed to identify toxicity and contamination originating with the vendor. All tubes, CMK, HEp-2 , HFF and RD tubes are kept for only one week. One tube (C) is kept in the clean room and one (V) is placed on the Virology drum.

Record sterility and cell appearance for these tubes into the LIS Manual (REGISTRATION OF TUBE CULTURE MEDIA)

B. <u>Positive Controls:</u>

Each week scrape from an HFF tubes containing the following QC strains to propagate the QC strains in the new lot of HFF tubes. Examine microscopically for CPE and record the results into the LIS as growth control. At the same time also inoculate MRC-5 shell vials to perform quality control for MRC-5 shell vials (see II. **<u>Shell Vial Cell Lines (MRC-5 cell suspension below)</u>**

HSV-1 (ATCC VR-5539)
HSV-2 (ATCC VR-540)
CMV (ATCC VR-807)

The shell vials are then stained with HSV-1, HSV-2 and CMV IEA monoclonal antibodies at 24 hours. Record the results into the LIS as shell vial growth control as well as stain controls.

Each month (and whenever necessary), remove from the liquid nitrogen storage, cryovials of above strains to propagate the QC strains in HFF tubes.

Additional positive controls may be set up with the following strains and for the following reasons:

Coxsackie B1 (ATCC VR-28)
RSV (ATCC VR-284)
Influenza A (ATCC VR-544)
Varicella-zoster (ATCC VR-1367)

i) Low isolation rates
ii) Comparison of cell lines
iii) Vendor changes
iv) Proficiency test failures
v) Training purposes
vi) Consistent problems with negative controls
vii) Preparation of QC material (i.e. Positive control slides)

Consult a senior technologist to determine the cell lines and viruses to be set up.

II. Shell Vial Cell Lines (MRC-5 cell suspension)

Upon receipt of a shipment of cells, initial and date the record sheet accompanying the shipment. The record should contain vendor, lot number, passage number and QC data. File in the QC binder for cell lines.

Before seeding the shell vials, aspirate about 30 mL of MRC-5 cell suspension into a 125 cm^2 tissue culture flask, label on the side of flask with "MRC-5, date and '**Pre**'".

After seeding shell vials, aspirate about 30 mL of MRC-5 cell suspension into another 125 cm^2 tissue culture flask, label on the side of flask with "MRC-5, date and '**Post**'".

Reserve 6 shell vials for use as negative and positive controls as follows:

A. Negative Controls for this week, also become Positive Old Lot for following week (3 Vials)

These are incubated at 36^oC and observed daily for one week or more to identify toxicity and contamination originating with the vendor. Results are recorded on the QC chart.
Three shell vials are reserved to QC the next shipment in parallel after the cells are added.

B. Positive Controls (3 Vials)

Each week, usually 2-3 days after seeding the shell vials, HFF tubes containing HSV-1 (ATCC VR-5539), HSV-2 (ATCC VR-540) and CMV (ATCC VR-807) are scraped from and used to inoculate 6 MRC-5 shell vials, 3 from the current lot and 3 from the previous lot (the same 3 vials were used as Negative Controls for a week). The shell vials are then stained with HSV-1, HSV-2 and CMV IEA monoclonal antibodies after 1 day incubation. Record the results into the LIS daily QC under the following codes:

a. VHSV1D HSV1 daily SLIDE SV QC
b. VHSV1D HSV1 daily SLIDE SV QC
c. VCMV-D CMV-IE daily SLIDE SV QC
d. VQCSV Shell Vial MRC-5 Quality Control

Appendix XV

VIRUS ISOLATION AND IDENTIFICATION CHARACTERISTICS

Virus	Minimum Cell Lines(s) Required	Method of Detection	Approx. Time to Detection	Typical CPE Appearance	Typical IF Appearance
Adenovirus	Hep-2 x 1 2 weeks	- CPE - Adenovirus DFA Mab	1 - 2 weeks	- rounding, enlarging, clustering, granular - acid media	-nuclear and/or cytoplasmic -may see extracellular fluorescence
	CMK x 1, 5 days CMK x 1, 10 days	-respiratory screen IFA plus specific Mab	5 - 10 days	-poor development of CPE	"
Enterovirus					
Polio	CMK x 1, 2 weeks	-CPE -Poliovirus IFA Mab	1 - 3 days	-small, rounded cells, floating	IFA: Nuclear and/or cytoplasmic
	HHF x 1, 2 weeks	"	"	"	"
	Hep-2 x 1, 2 weeks	"	"	"	"
Coxsackie A	CMK x 1, 2 weeks	+/- CPE difficult to grow	1 - 7 days	-some strains produce CPE, some do not	"
	HHF x 1 2 weeks	+/- CPE	1 - 7 days	"	"
	Hep-2 x 1, 2 weeks	No CPE seen	--	--	--
	Suckling mouse	Signs of illness	3 - 5 days	-required for some types of coxsackie A	--

IF = Immunofluorescence; SV = Shell Vial; CPE = Cytopathic Effect; IFA = Indirect Fluorecent Antibody; DFA = Direct Fluorescent Antibody

Appendix XV (Cont'd)

VIRUS ISOLATION AND IDENTIFICATION CHARACTERISTICS

Virus	Minimum Cell Lines(s) Required	Method of Detection	Approx. Time to Detection	Typical CPE Appearance	Typical IF Appearance
Coxsackie B	RMK x 1 2 weeks	-CPE -Coxsackie B IFA Mab	4 - 7 days	-teardrop shape	IFA: Nuclear and/or cytoplasmic
	HHF x 1 2 weeks	(+/- CPE)	"	-teardrop shape	"
	Hep 2 x 1, 2 weeks	"	"	-teardrop shape	"
Echovirus	RMK x 1 2 weeks	-CPE -Echovirus IFA Mab	1 - 7 days	-teardrop, rounding	IFA: Nuclear and/or cytoplasmic
	HHF x 1, 2 weeks	"	"	"	"
	Hep-2 x 1, 2 weeks	No CPE	--	--	--
Herpesvirus Family					
Herpes Simplex	HHF x 1 7 days	-CPE -HSV - 1 and 2 DFA Mab	-usually 24-72 hrs. -sometimes longer	-swelling, rounding, refractile	-nuclear & cytoplasmic staining
	MRC-5 (SV) x 2	-HSV-1 and 2 DFA Mab on coverslip	24 hours	-may or may not see CPE	-pale yellow-green staining of cells and periphery
Cytomegalo-virus	HHF x1 3 weeks	-CPE -CMV DFA Mab	1-3 weeks	-small round or elongated foci -spreads slowly -rarely involves entire monolayer	-Early: nuclear -Late: nuclear and cytoplamic
	MRC-5 (SV)x2 24-48 hours	-CMV IEA IFA Mab on coverslip	24-48 hours	None	-pale matte green nuclear fluorescence inclusion

IF = Immunofluorescence; SV = Shell Vial; CPE = Cytopathic Effect; IFA = Indirect Fluorecent Antibody; DFA = Direct Fluorescent Antibody

Appendix XV (Cont'd)

VIRUS ISOLATION AND IDENTIFICATION CHARACTERISTICS

Virus	Minimum Cell Lines(s) Required	Method of Detection	Approx. Time to Detection	Typical CPE Appearance	Typical IF Appearance
Varicella Zoster	HHF x 2 weeks	-CPE -VZV DFA Mab	5 days - 3 weeks	-Small foci -Slow -Rounded	-nuclear and cytoplasmic
	MRC-5 (SV) x1 48 hours	-VZV DFA Mab on coverslip	48 hours	-CPE rarely produced by 48 hours	-nuclear and cytplasmic -Direct Smear: cydtplasmic "halo" appearance
Human Herpes-6	Isolation Not Done				
Influenza A/B	RMK x 1, 5 days RMK x1, 10 days	IFA: Resp. screen and specific Mab	5 - 10 days	-CPE may not be present -cells may appear toxic or -granular, vaculoated	-nuclear and cytoplasmic
Measles	Send to PHL RMK x 1, 2 weeks	CPE	5 - 10 days	-syncytia -giant cells with ring of nuclei -intranuclear inclusions	-cytoplasmic
Mumps	Send to PHL RMK x 1, 2 weeks	Hemadsorp-tion	6 - 8 days	-CPE production not predictable -may see synctyia, cell degeneration	-cytoplamsmic
Norwalk	Send Stool to PHL	EM	---	---	---
Para-influenza 1,2,3	RMK x 1, 5 days RMK x 1, 10 days	IFA: Resp. screen & specific Mab	5 - 10 days	- little or none	-cytoplasmic
Parvovirus B19	-serum for to PHL -fluid/tissue for PCR, Virology Lab, St. Joseph's Hospital, Hamilton	---	---	---	---

IF = Immunofluorescence; SV = Shell Vial; CPE = Cytopathic Effect; IFA = Indirect Fluorecent Antibody; DFA = Direct Fluorescent Antibody

VIRUS ISOLATION AND IDENTIFICATION CHARACTERISTICS					
Virus	**Minimum Cell Lines(s) Required**	**Method of Detection**	**Approx. Time to Detection**	**Typical CPE Appearance**	**Typical IF Appearance**
Rhinovirus	Not Done Send to PHL	---	---	---	---
Rotavirus	Send Stool to PHL	EM	---	---	---
RSV	HEP-2 x 1 2 weeks	CPE RSV DFA Mab	2 - 7 days	-syncytia (Multi-nucleated fused cells)	-cytoplasmic -dense cytoplasmic inclusions
Rubella	Send to PHL Isolation rarely done	(Interference)	---	---	---

IF = Immunofluorescence; SV = Shell Vial; CPE = Cytopathic Effect; IFA = Indirect Fluorecent Antibody; DFA = Direct Fluorescent Antibody

Appendix XVI

WEEKLY WORK SCHEDULE

1. Specimen Management Bench - Monday to Friday :
    a) Accessioning and processing all Virology specimens including PCR, HIV NASBA and send-out.
    b) Preparing Direct Smears, PCP slides and necessary materials (eg. Fungizone, gentamicin mixtures).

2. Shell Vial Bench – Monday to Friday :
    a) Staining and reading of all shell vials.
    b) Staining and reading of all Direct Smears and PCP slides.
    c) Enter all shell vial related (eg. new reagent lots, daily staining, weekly viral propagation and MRC-5 growth) QC results either in LIS and/or Data Sheet.
    d) Tabulate and fax weekly viral isolates data (on/after Friday).

3. Tube Culture Bench :
    a) Read tube cultures on Monday, Wednesday, and Friday.
    b) Freeze isolates and enter into LIS store program.
    c) Enter new cell lots into LIS when received (Monday/Tuesday).
    d) Seed shell vials with MRC-5 (Tuesday).
    e) Set up QCs: inoculate HSV1, HSV2, CMV in S/Vs and HFFs, read and enter results into LIS of all new cell cultures on Wednesday.
    f) Perform EBV on Thursday.
    g) Maintain virology inventories especially liquid N_2 (Tuesday / Thursday).
    h) Prepare Maintenance Media on Friday, and set up appropriate QC.

4. CMV Antigenemia Bench :
    a) Monday to Friday: Record temperature on all freezers and fridge every morning.
    b) Monday to Friday : Process, stain and read all CMV antigenemia specimens.
    c) Perform RPR on Wednesday.
    d) Perform HTLV on Thursday.
    e) Perform VZ Ab testing using VIDAS

5. Molecular Bench:
   a) Perform HCV RNA PCR using COBAS when there are enough of specimens for a full run .
   b) Perform HBV DNA Assay when there are enough specimens for a full run.
   c) Perform Chlamydia PCR when there are enough specimens for a full run.
   d) Perform *C.difficile* EIA Assay (using BEP 2000) if not done by floater.
   e) Maintain non-AxSYM serology inventories.
6. AXSYM Bench- Monday to Friday :
   a) Perform all Hepatitis markers, HIV1&2, CMV IgG and Rubella IgG assays using AxSYM.
   b) Perform weekly maintenance on Monday (if no stat) / Tuesday.
   c) Perform monthly maintenance at beginning of each month.
   b) Maintain AxSYM inventories.

7. Eye Bank:

   Perform Eye Bank (BioRad EIA) tests and stat HTLV (Transplants), stat RPR and report these stat results (fax and/or phone).

8. West Nile:

   Perform WNV serology and/or WNV PCR; freeze samples and enter into LIS store program.

9. Technologist Floater:

   Perform C.difficile EIA Assay (BEP2000).

10. Technician 1- Monday to Friday :
    a) Spin and accession all Serology specimens.
    b) Send out all PHL specimens.
    c) Process Viral Load specimens.

11. Technician 2- Monday to Friday :
    a) Help to unload Translogic and centrifuge Serology bloods.
    b) Spin,accession,and freeze sera for HBV DNA and HCV RNA.
    c) Disinfect, check centrifuges and record in LIS QC.

Appendix XVII

VIROLOGY TRAINING GUIDE (optional)

WEEK	ACTIVITY	MATERIALS
1	SHELL VIAL UNKNOWNS	CMV / HHF HSV / HHF
1 - 2	TUBE CULTURE UNKNOWNS	CMV / HHF HSV / HHF ECHOVIRUS / HHF, RMK, HEP-2 COXSACKIE B / HHF, RMK, HEP-2 ADENOVIRUS / HFF, RMK, HEP-2 *INFLUENZA A / HHF, RMK, HEP-2 *PARAINFLUENZA 3 / HHF, RMK, HEP-2 *RSV / HHF, RMK, HEP-2
START 1ST WEEK	SPECIMEN PLANTING	PATIENT SAMPLES
START 2ND WEEK	SHELL VIAL STAINING	PATIENT SAMPLES
START 3RD WEEK	TUBE CULTURE READING	PATIENT SAMPLES
1 - 8	READ LAP - 1, 2, 3	CACMILE SELF-STUDY VIROLOGY COURSE
8 (END OF ROTATION)	WRITTEN EXCERCISES / SELF-EXAM	CACMILE SELF-STUDY VIROLOGY COURSE

*November to April training periods only.

Appendix XVIII

QUALITY CONTROL OF MONOCLONAL ANTIBODIES

Monoclonal Antibodies	Method	Expected Use
Respiratory Viral Screen/RSV panel FluA/B panel RSV/para3 panel Para123/Adeno panel Specific Parainfluenza 1 Specific Parainfluenza 2	SimulFluor DFA SimulFluor DFA SimulFluor DFA SimulFluor DFA DFA DFA	Tube culture / direct specimen
Coxsackie A9 Coxsackie B Echovirus Poliovirus Enterovirus 70 / 71 Mumps (not in routine use)	IFA	Tube culture
CMV pp65 CMV Immediate Early	IFA IFA	Direct polymorph, leukocytes shell vial
Specific Herpes simplex 1 Specific Herpes simplex 2 Specific Varicella zoster virus CMV early & late Herpes simplex bivalent	DFA	Shell vial / tube / direct specimen Shell vial / tube / direct specimen Shell vial / tube / direct specimen Tube Shell vial / tube / direct specimen

Appendix XVIII (Cont'd)

QUALITY CONTROL OF MONOCLONAL ANTIBODIES

Reagent quality controls:

These must be perform prior to patient testing to ensure each component of the reagent performs as expected.

a. Check expiratory date then perform DFA, SimulFlour DFA or IFA accordingly.
b. External QC slides (different manufacturer, unless not available) of the same batch are used to test both current and the new reagents in parallel
c. Results must fall within range of expected results before reagents are released for use (eg. all 7 viruses must be positive and negative wells are negative for the Bion 14-well Respiratory Panel).
d. Record reagent expiry date and QC results in Reagent Log and/or LIS.
e. Report abnormal QC results to Charge/Senior technologist.

Expected reagent QC results:

External (commercial) QC slide	Current Reagent	New Reagent
Positive well (for each virus)	+	+ (no weaker than current reagent)
Negative well	-	-

Failed reagent QC results:

i. Inform charge/senior technologist to investigate cause of failed QC.
ii. Record in Reagent Log Chart. (Instrument Maintenance Log if microscope/incubator is involved in the failure and Incident Report if necessary).
iii. May need to re-run failed control materials in parallel to fresh controls to evaluate the QC material itself.

iv. If the re-run shows the old QC material still fails and fresh QC is satisfactory, the error may be attributed to the old QC material itself and the reagent is satisfactory.

v. If the re-run shows both the old and fresh QC material fail (or other QC not satisfactory), the error may be attributed to the reagent then the reagent cannot be released for use. Supplier of the reagent should be contacted and the appropriate incident report should be filled.

Daily QCs:

These are performed within each batch of patient samples to monitor assay performance and techniques within the batch.

a. Check reagent expiratory date and verify that Reagent QC is satisfactory for the reagent lot/kit being used.

b. Appropriate positive and negative control slides (eg. ATCC 4-well slide with RSV/Para3 for SimulF RS stain) should be stained with each batch. These slides should be placed in various random positions within the batch.

c. Examine the negative control well first to establish the dull red colour (Evans blue counterstained) and to determine if there is any nonspecific staining.
The positive control must be clearly distinguishable from the negative control or the test is invalid.

d. Record QC results in LIS and/or wosksheet.

Failed Daily QC:

i. Do not release patient results pending resolution of QC error.
ii. Inform charge/senior technologist.
iii. Record in Reagent Log Chart (and Instrument Maintenance Log if microscope/incubator is involved in the failure).
iv. Re-run failed controls in parallel to fresh controls (and/or external QC) to evaluate the QC material itself.
v. If the re-run shows the old QC material still fails, fresh QC passes and nothing else is wrong with the batch (only the old QC material failed, patient results valid) patient results may be released.

Marked decrease/absence in fluorescence can be due to:

a. Reagent deterioration/skipping (did not apply primary/secondary stain)
b. Microscope (filter, bulb, alignment)
c. Other equipment, reagents or technique

Appendix XIX

PNEUMOCYSTIS CARINII DFA TEST

I. Introduction

The Merifluor-Pneumocystis DFA test is an in vitro test for the direct detection of Pneumocystis carini cysts and trophozoites in bronchoalveolar lavage (BAL), bronchial wash (BW); sputum or biopsy specimen.

II. Collection and Transport

BAL, wash and sputum should be collected using standard procedures. Biopsy specimens e.g. transbronchial, open lung or others must not be fixed and are transported to the lab on a saline moistened piece of gauze in a sterile container. Tissue should not be allowed to dry or become immersed in saline. All specimens should be transported as soon as possible to the laboratory. PCP testing can be done on the day after receipt except specimens received Friday or the day before a holiday must be stained and read that day.

III. Procedure

Reagents

FITC- *P. carinii* conjugate
Control slides
Distilled water
FA mounting fluid
Sputolysin: diluted 1:10 (i.e. 300 Ul sputolysin 3.0 mL distilled water)

Materials

Vortex
Sterile pipettes
10 - 100 uL Eppendorf pipette
Humidified chamber
Coplin jars
Fluorescent microscope

Preparation of Slides

BAL and BW:

1. Centrifuge the BAL or BW for 10 minutes at 1800 x g.

2. Remove and discard all but 0.5 mL of the supernatant. Thoroughly resuspend the pellet in the remaining 0.5 mL of fluid.

3. Make a thin smear twice the size of a cytospin spot and allow to air dry.

4. Fix in acetone for 5 minutes in a coplin jar, then air dry.

5. Slide must be stained within 8 hours or freeze at -20^{0}C.

Sputum .. See Sputolysin Procedures AppXXII

1. Combine equal volumes (3 mL each) of sputum and diluted sputolysin. Vortex mixture.

2. Incubate for 3 minutes at 35^{0}C.

3. Vortex the mixture briefly and add an equal volume of PBS and entrifuge at 1300 x g for 5 minutes.

4. Remove the supernatant, leaving 0.5 mL to resuspend the pellet.

5. Make a smear twice the size of a cytospin spot. Allow to air dry.

6. Fix in acetone for 5 minutes in a coplin jar, then air dry.

7. Slide must be stained within 8 hours or freeze at -20^{0}C.

Biopsy Specimen

1. Prepare a freshly cut surface on a fragment of tissue.

2. Touch the cut surface to a FA slide. Make several non-overlapping imprints within the well, avoiding smearing using several cuts.

3. While imprints are still moist on the slide, fix by adding 1 - 2 drops of acetone and allow to air dry.

4. Slide must be stained within 8 hours or freeze at -20^{0}C.

Staining - DFA

1. Cover the smear with 30 uL of *P. carinii* FITC-conjugate antibody.

2. Incubate in a humidified chamber for 30 minutes at 36^{0}C.

3. Wash slide twice with distilled water for 2 minutes in a coplin jar.

4. Allow the slide to dry.

5. Mount using coverslip and mounting fluid.

6. Read with fluorescence microscope with the FITC / Evans Blue filter and 40x objective.

Interpretation of Results

POSITIVE: Any specimen which contains two typical cysts exhibiting apple-green fluorescence of characteristic morphology. Generally cysts, 5 - 8 um diameter, are found together with trophozoites in clusters. Clusters can be variable in size and may appear with or without "honeycomb" like structure. Some cysts fluoresce evenly throughout their structure whereas other cysts may fluoresce mainly on their periphery and produce a "honeycomb" appearance within the clusters.

NEGATIVE: Red fluorescence without any characteristic apple-green fluorescence as described above.

IV. Reporting

POSITIVE: "*Pneumocystis carini* positive by immunofluorescence".

NEGATIVE: "*Pneumocystis carinii* negative by immunofluorescence".

Telephone all results.

V. Quality Control

Positive and negative control slides should be stained each time the staining procedure is performed. Refer to a senior technologist if controls do not work or for any other problems with staining, reading or reporting results.

VI. Reference

1. Merifluor Pneumocystis, Meridian Diagnostics, Inc. 3471 River Hills Drive, Cincinnati, Ohio, 45244. Tel. 513-271-3700.

Appendix XX

CYTOSPIN PREPARATION

I. Introduction

A cytospin preparation is a concentration of cells taken directly from specimens or from scraped cell cultures.

II. Reagents and Materials

Virus-specific or pooled antibody
Phosphate buffered saline (PBS)
Cold acetone (4^{o}C)
Distilled water
Mounting fluid
Non-immune antibody
vortex
sterile pipettes
cytospin and accessories
humidified chamber
coplin jars
fluorescence microscope

III. Procedure

1. Shell Vial

i. Remove all except 1 ml maintenance media from shell vial using a sterile pipette.
ii. Scrape cells from top of coverslip using a sterile pipette. Break up cell clumps by pipetting the cells up and down several times.
iii. Pipette 200 ul (4 drops) of scraped cells into funnel for each well.
iv. Cytospin at 2000 rpm (700g) for 5 minutes.
v. Remove slide and air dry.
vi. Fix in cold acetone for 10 minuets in a coplin jar. Remove slide and air dry.

vii. Proceed to staining. Refer to Appendix IV for Indirect fluorescent antibody staining techniques or Appendix V for Direct fluorescent antibody staining techniques.

or

Refer to Appendices IV and V for immunofluorescent staining techniques for shell vials.

2. Tube culture

i. Remove all except 1 ml maintenance media from the culture tube using a sterile pipette.
ii. Scrape cells from side of tube using a sterile pipette. Break up cell clumps by pipetting the cells up and down several times.
iii. Pipette 200 ul (4 drops) of scraped cells into funnel for each well.
iv. Cytospin at 2000 rpm (700 x g) for 5 minutes.
v. Remove slide and air dry.
vi. Fix in cold acetone for 10 minutes in a coplin jar. Remove slide and air dry.
vii. Proceed to staining. Refer to Appendix IV for Indirect fluorescent antibody stains or Appendix V for Direct fluorescent antibody stains.

or

Refer to Appendices IV and V for immunofluorescent staining techniques for shell vials.

3. Direct from specimen

IV. Reference

Thermo Shandon, cytospin. Manufacturer's manual. Refer to Appendix VI for procedure.

Appendix XXI – Specimens, Cell Lines and Stain Table

Source	Direct	S/V	S/V Stain	Tube
Amniotic fluid		MRC-5 48 hr	CMV-IEA	HFF CMK RD
Auger suction	DFA*	MRC-5 48 hr	CMV-IEA	Hep-2 CMK x2
BAL(CMV) only-Tx		MRC-5 48 hr	CMV-IEA	
Throat/mouth/oral washing from PMH		MRC-5 24 hr MRC-5 24 hr	HSV-1 HSV-2	
BAL/ Throat swab/Sputum/lung aspirate	DFA during Flu season only, otherwise only if requested	MRC-5 48 hr	CMV-IEA	Hep-2 CMK x2
Nasopharyngeal/ Nasal swab (Respiratory viruses)	DFA during Flu season only, otherwise only if requested			Hep-2 CMK x2
Blood(CMV Antigenemia Assay)	CMV pp-65			
CSF	HSV,CMV/VZ PCR (MSH) Enterovirus PCR(MSH) WNV PCR (MSH)	MRC-5 48 hr	CMV-IEA	HFF CMK RD **
Genital swab for HSV	DFA	MRC-5 24 hr MRC-5 24 hr	HSV-1 HSV-2	
Lesion swab/fluid Vesicle swab/fluid		MRC-5 24 hr MRC-5 24 hr MRC-5 48h	HSV-1 HSV-2 VZV	
Ocular	DFA if requested	MRC-5 24 hr MRC-5 24 hr MRC-5 48h	HSV-1 HSV-2 VZV	Hep-2 RD-if Enteroviruses req'd
Stool/Rectal swab	send to PHL for EM (Rotavirus,Norwalk Virus,Calicivirus)	MRC-5 48 hr if CMV req'd	CMV-IEA	if enteroviruses req'd set up RD & CMK
TissueBiopsy, Lung, Liver, Kidney, fetal & others		MRC-5 48 hr	CMV-IEA	HFF CMK RD
Brain		MRC-5 48 hr MRC-5 24 hr MRC-5 48h	CMV-IEA HSV- Bivalent VZV	HFF CMK RD
Sterile fluids		MRC-5 48 hr	CMV-IEA	HFF CMK RD
Urine		MRC-5 48 hr	CMV-IEA	

* DFA= SimulFluor DFA Flu season is from the 1st of November to the end of April.
** RD for CSF May to Nov Treat sputum with sputolysin if is purulent.

Appendix XXII

SPUTOLYSIN PROCEDURE

Sputolysin Reagent is a concentrate of dithiothreitol (DTT) in phosphate buffer used in the isolation of pathogens from mucoid sputum , BAL and other fluids.

Materials

a. Sputolysin Reagent (Cat. No. 560000, CALBIOCHEM) - dilute a 10 mL vial to 100 mL with sterile dH_2O. Stable for up to 48 hr. at 2 – 8^oC.
b. Microcentrifuge tubes
c. Microcentrifuge
d. Vortex mixer

Procedures

Sputum / mucoid BAL for PCP

a. Adjust specimen s to approximately 0.5 McFarland standard with Hank's Buffered Saline Solution (HBSS) or maintenance medium.
b. Suspend specimen (approximately 1 mL) with an equal volume of Sputolysin in a microcentrifuge tube by vortexing for 30 seconds.
b. Incubate at room temperature for 15 minutes.
c. Centrifuge at 5000 rpm for 5 minutes and remove supenate.
d. Make a thick and a thin smear on the 2 wells of a PCP slide.

Reference

CALBIOCHEM Product Insert, revised 21 Jan 2000 (http://www.calbiochem.com)