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.