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.