ANNEX B7
PROTOCOL FOR CULTURE OF VERO/hSLAM CELLS

Purpose
Preparation of frozen stocks of Vero/hSLAM cells, recovery of cells from frozen stocks, passaging of cells for virus isolation and preparation of Vero/hSLAM cells for shipment.

Information about Vero/hSLAM cells
The Vero/hSLAM cell line is recommended for routine isolation of measles or rubella viruses in the WHO Laboratory Network. These cells are Vero cells (African Green Monkey kidney cells) which have been transfected with a plasmid encoding the gene for the human SLAM (signaling lymphocyte-activation molecule) protein (Ono, et al., 2001). hSLAM has been shown to be a receptor for both wild-type and laboratory-adapted strains of measles. Vero/hSLAM cells were developed by Dr. Yusuke Yanagi, Kyushu University, Kukuyoka, Japan. He has kindly agreed to allow the WHO Measles and Rubella Laboratory Network to use these cells in Network Laboratories under the following conditions:

1. The cell line Vero/hSLAM is used only for laboratory diagnosis of measles and rubella viral infection by virus isolation and/or investigation of measles or rubella strains for molecular epidemiological purposes.
2. The cell line is not used for commercial purposes.
3. The cell line is not distributed to laboratories outside the WHO Laboratory Network without Dr. Yanagi's and WHO's permission.
4. Any publication of work using the Vero/hSLAM cell line acknowledges the original publication (Ono et al. J. Virol. 2001, 75:4399-4401).

The sensitivity of Vero/hSLAM cells for isolation of measles virus is equivalent to that of B95a cells and measles infection of Vero/hSLAM results in the characteristic cytopathic effect (CPE), syncytium formation. The advantage of the Vero/hSLAM cells compared to B95a cells is that they are not persistently infected with Epstein-Barr virus, and therefore, are not considered as hazardous material. This provides a significant safety advantage for laboratory workers and greatly facilitates international shipments. The disadvantage of the Vero/hSLAM cells is that they must be cultured in medium containing the antibiotic Geneticin to retain hSLAM expression. This increases the cost of the tissue culture medium.

Vero/hSLAM cells can also be used to isolate rubella viruses from clinical samples with a sensitivity that is similar to that of standard Vero cells. Unlike measles virus, rubella virus from clinical specimens usually does not produce CPE, even after several passages. The presence of rubella virus in the culture must be confirmed by detection methods such as RT-PCR using RNA extracted from the culture or by immunofluorescence or immunocolorimetric assays.

Avoiding contamination
Cells must be passaged in a biological safety cabinet (BSC). Only one cell line should be processed in the BSC at a time. If the same BSC is used for working with infectious material, the surfaces in the BSC and all equipment and containers in the BSC must be thoroughly disinfected and irradiated with UV light before passaging uninfected cells.

Geneticin Requirements for Vero/hSLAM Cells
Passage of Vero/hSLAM cells for routine virus isolation: To prepare cells for virus isolation procedures, Vero/hSLAM cells should be recovered from liquid nitrogen and can be passaged up to 15 times in medium without Geneticin. These cells should be used for virus isolation attempts only and should be discarded after 15 passages. Cells that have been passaged without Geneticin in the medium should never be used to prepare cell stocks for liquid nitrogen storage or shipped to another network laboratory for use in virus isolation.

Preparation of cell stocks for liquid nitrogen storage: Cells to be used to prepare stocks for internal use or distribution to other labs should be passaged in medium containing 400µg/mL Geneticin.

Equipment
- Waterbath set to 37 °C
- Biological safety cabinet (BSC)
- Cell culture incubator, 37 °C, 5% CO_2
- Refrigerated centrifuge with holder for 50 mL tubes
- Device to gradually cool down cells for freezing, e.g. Thermo Scientific Mr. Frosty Freezing Container (catalog number 5100-0001)
- Microscope
- Liquid nitrogen storage
- -70 °C freezer
- -20 °C freezer
- Vortex
- Pipettor and sterile 1, 5, 10, 25 ml plastic pipettes

Reagents and material
- 1 L glass beaker with 10% hypochloride solution
- 15 mL conical tubes (e.g. Falcon, catalog number 352097)
- 162 cm^2 cell culture flasks with vented caps (e.g. Corning Costar catalog number 353112)
- 75 cm^2 cell culture flasks with vented caps (e.g. Corning Costar catalog number 353110)

- 25 cm^2 cell culture flasks with vented caps (e.g. Corning Costar catalog number 353109)
- 25 cm^2 cell culture flasks with non-vented caps (e.g. Falcon catalog number 353014) for shipment
- 50 mL conical tubes (e.g. Falcon, catalog number 35-2098)
- Antibiotics (100X),10,000 units/mL penicillin G and 10,000 µg/mL streptomycin sulfate in 0.85% saline (e.g. Thermo Fisher catalog number 15140122)
- Bucket with dry ice
- Cell scrapers (e.g. Corning Costar catalog number 3010)
- Cryovials (e.g. Sarstedt, catalog number 72.694.006)
- Dulbecco's Modified Eagle Medium (DMEM, e.g. Thermo Fisher catalog number 11965) with 4,500 mg/L D-glucose (high glucose), with L-glutamine, without sodium pyruvate
- Fetal Bovine Serum, heat-inactivated (e.g. Atlanta Biologicals, Optima, heat-inactivated, catalog number S12450H)
- G418 sulfate (Geneticin, 50 mg/mL, e.g. Corning catalog number 30-234-CI)
- Gloves
- Isopropanol (e.g. EMD Millipore, catalog number PX1835-6)
- Lab coat
- Parafilm (e.g. Sigma Aldrich, catalog number P7793-1EA)
- PBS (e.g. Thermo Fisher, catalog number 10010031)
- Trypsin-EDTA: 0.05% Trypsin (porcine pancreas), in 0.53 mM EDTA in HBSS without Ca++ and Mg++ (e.g. ThermoFisher, catalog number 25300)

Medium preparation

- DMEM-PS: Add 5 mL of penicillin/streptomycin solution to 500 mL DMEM.
- DMEM-PSG: To a 500 mL bottle of DMEM-PS, add Geneticin to a final concentration of 400µg/mL (4 ml of 50 mg/mL stock to 500 mL DMEM-PS).
- Prepare fresh daily in a 50 mL tube or cell culture flask:
    - Medium containing 10% FBS: add 5 mL FBS to 45 mL of DMEM-PS or DMEM-PSG
    - Medium containing 5% FBS: add 2.5 mL FBS to 47.5 mL of DMEM-PS or DMEM-PSG
    - Medium containing 2% FBS: add 1 mL FBS to 49 mL of DMEM-PS or DMEM-PSG
    - Medium containing 30% FBS: add 15 mL FBS to 35 mL of DMEM-PSG
    - DMEM-PSG-15%DMSO: add 7.5 mL DMSO to 42.5 mL DMEM-PSG

Mix by shaking. Label medium bottle with your initials, date and the reagents added.
Before use, warm PBS, trypsin and medium to 37 °C in incubator or waterbath.
Discard unused medium two months after opening.

Storage of reagents

- Antibiotics solution is supplied in 100 mL bottles as 100X stock. The solution should be thawed, divided into 5 mL aliquots in 15 mL conical tubes and stored at -20 °C.
- FBS is supplied in 500 mL bottles. The FBS should be thawed and stored as 50 mL aliquots in 50 mL conical tubes at -20 °C.
- Trypsin-EDTA is supplied in 100 mL bottles. It should be thawed and stored as 5 mL aliquots in 15 mL conical tubes at -20 °C.
- Label all tubes with the name of the reagent and the expiration date listed on the original bottle. Do not use expired reagents. Frozen reagents are thawed at room temperature or in a 37 °C incubator.
- After addition of reagents, DMEM is stored at 4 °C.
- All reagents must be kept sterile. Only open reagents in the BSC. Keep all reagents closed as much as possible.

Recovery of Vero/hSLAM cells from liquid nitrogen storage

Note 1: The freezing medium contains DMSO. Once the vial of cells has been thawed, it is important to work very quickly because prolonged exposure to DMSO at room temperature will be toxic to the cells. It is therefore important to have all subsequent steps prepared before the vial of cells is thawed.

Note 2: If cells are thawed to prepare additional frozen stocks or for distribution, use DMEM-**PSG**-10%FBS.

1. Place DMEM-PS-10%FBS in water bath to warm up.
2. Quickly transfer cells from liquid nitrogen to BSC, ideally on dry ice.
3. Add 10 mL warm DMEM-PS-10%FBS to 25 cm^2 flask.
4. Thaw cells quickly in 37 °C water bath. Immediately transfer thawed cells to 25 cm^2 flask containing warm medium.
5. Incubate in cell culture incubator for approximately 4 hours. Check by light microscopy that cells have attached to the flask.
6. Decant medium into hypochlorite solution and replace with 10 mL DMEM-PS-10%FBS.
7. Monitor cells daily by light microscopy.

Maintenance of Vero/hSLAM Cells

Note 1: If cells are passaged to prepare frozen stock, use media containing 400µg/mL Geneticin.

Note 2: For virus isolation, Vero/hSLAM cells can be passaged up to 15 times in medium without Geneticin.

Note 3: Cells are usually maintained in 25 cm^2 or 75 cm^2 flasks, but the volumes given below can be adjusted for larger or smaller vessels.

1. Warm trypsin solution (or PBS) in water bath.

2. Wash cell monolayer with 5 mL warm trypsin solution (or warm PBS) for about 30 sec to 1 minute. Discard wash medium into hypochlorite solution.
3. Add 5 mL warm trypsin solution and allow flask to incubate in the BSC for 4-5 minutes. Decant most of the trypsin leaving just enough fluid in the flask to keep the monolayer wet.
4. Place flask at 37°C for about 3-4 minutes. Observe flask every few minutes to see if cells are detached. When cells begin to detach, hit flask quickly with palm of hand to dislodge cells.
5. Resuspend cells in 5 mL DMEM-PS-10%FBS and pipette up and down to break up clumps.
6. Seed cells into flasks containing DMEM-PS-10%FBS. Split ratios of up to 1:5 are acceptable. A 1:2 or 1:3 split usually will produce monolayers of sufficient density for virus isolation the next day. The total volume of medium required is 30 mL for 75 cm^2 flasks and 10 mL for 25 cm^2 flasks.
7. Cells should be passaged at least one time each week. Cells can be maintained for several days by switching to DMEM-PS-2%FBS when confluent to prevent overgrowth.
8. Label flask with date and passage number. Cell lines should be passaged only 15 times after recovery from liquid nitrogen.

Preparation of frozen stocks of Vero/hSLAM cells

Note 1: This procedure produces 20 vials of frozen cells. It can be scaled up or down if different numbers of vials are needed.

Note 2: Once the DMEM-PSG-15%DMSO has been added to the cells (step 6) is important to work very quickly because prolonged exposure to DMSO at room temperature will be toxic to the cells. It is therefore important to have the cryovials labeled and all subsequent steps prepared before the addition of DMSO to the cells.

Note 3: Continue routine culture of Vero/hSLAM cells with Geneticin until viability of frozen cells has been demonstrated (step 10).

1. Prepare confluent monolayers of Vero/hSLAM cells in two 162 cm^2 or four 75 cm^2 flasks, following the protocol for maintenance of Vero/hSLAM cells above using DMEM-PSG-10%FBS in each passage.
2. Cool centrifuge to 4 ^oC.
3. Label 20 cryovials with name of cell line, passage number and date.
4. Remove cells from flask by trypsinization, following the protocol for maintenance of Vero/hSLAM (take care not to over-trypsinize). After incubation with trypsin, resuspend cells in DMEM-PSG-10%FBS and pipette up and down to break up clumps.
5. Pool resuspended cells from all flasks in a 50 ml conical tube and pellet cells by centrifugation at ca 200 g for 10 min at 4^oC. Discard supernatant.
6. To the cell pellet add 10 mL DMEM-PSG-30%FBS and thoroughly resuspend the cells by vortexing or pipetting up and down. Add 10 mL of DMEM-PSG-

15%DMSO. Pipette gently up and down briefly to mix and dispense 1 mL into each cryovial. It is important to work very quickly because prolonged exposure to DMSO at room temperature will be toxic to the cells.

7. Place vials in freezing container filled with isopropanol to indicated line. Store at -70 °C overnight.
8. Store vials in liquid nitrogen.
9. Maintain an inventory of the number of vials available.
10. Thaw one vial to verify viability (see Recovery of Vero/hSLAM cells from liquid nitrogen storage, above)

Mycoplasma testing

Cell stocks should be tested for the presence of mycoplasma within 2 weeks after being recovered from liquid nitrogen storage. Cells received from other laboratories should be tested before the preparation of frozen stocks. Multiple mycoplasma testing kits are available commercially.

Preparation of Vero/hSLAM cells for shipment to WHO collaborating laboratories.

1. Follow the instructions for recovery of Vero/hSLAM cells from liquid nitrogen storage above to start a new low-passage culture of Vero/hSLAM cells.
2. Verify that the cells are mycoplasma free (see SOP for Mycoplasma Testing).
3. Using a 1:4 split ratio, seed cells into a 25 cm^2 flask with non-vented cap following the protocol for maintenance of Vero/hSLAM cells above. Incubate 24 h and confirm 60-70 % confluency by microscopy.
4. Fill up the flask with DMEM-PSG-5%FBS and seal with parafilm.
5. Arrange the shipment of Vero/hSLAM cells at room temperature.