Annex B8
Measles or rubella virus isolation and preparation of virus stocks

Purpose
Inoculation of Vero/hSLAM cells with patient samples to isolate measles or rubella virus.
Production of virus stocks for long-term storage.

Reagents and material needed
- 1 L glass beaker with 10% hypochlorite solution
- 15mL conical tubes (e.g. Falcon, catalog number 35-2097)
- 25 cm^2 cell culture flasks with vented caps (e.g. Corning Costar, catalog number 3056)
- 50 mL conical tubes (e.g. Falcon, catalog number 35-2098)
- Antibiotics (100X),10,000 units/mL penicillin G and10,000 µg/mL streptomycin sulfate in 0.85% saline (e.g. ThermoFisher, catalog number 15140122)
- 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. ThermoFisher, catalog number 11965) with 4,500 mg/L D-glucose (high glucose), with L-glutamine, without sodium pyruvate
- Fetal Bovine Serum (FBS) (e.g. Atlanta Biologicals, Optima, Heat-inactivated catalog number S12450H)
- Gloves
- Lab coat
- Trypsin-EDTA : 0.05% Trypsin (porcine pancreas), in 0.53 mM EDTA in HBSS without Ca++ and Mg++ (e.g. ThermoFisher, catalog number 25300)

Equipment needed
- -70 °C freezer
- Biological safety cabinet (BSC)
- Cell culture incubator set to 37 °C and 5% CO_2
- Microscope
- Pipettor and sterile pipettes (5, 10, 25 ml)
- Refrigerated centrifuge cooled to 4 °C
- Water bath set to 37 °C

Medium preparation
- DMEM-PS: Add 5 mL of penicillin/streptomycin solution to 500 mL DMEM
- DMEM-PS-2%FBS: To 500 mL of DMEM-PS, add 10 mL of FBS

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

Storage of reagents
- Antibiotics solution is supplied as 100X stock. The solution should be 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 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.

Avoiding contamination

Preparation of samples for virus isolation and the process of virus inoculation and subsequent passaging of virus in Vero/hSLAM cells should be carried out only in a BSC specifically used for handling infectious material. Potentially infectious material should be kept completely separate from locations where "clean" cell stocks are being handled. Measles or rubella isolation must not be carried out simultaneously with isolation of other viruses.

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.

Controls

Always include a negative control of uninfected Vero/hSLAM cells during virus isolation attempts.

A positive control may be included, but the operator should be aware of the possibility for cross contamination. Positive controls, if used, should be wild-type viruses that have been characterized genetically.

Procedure

Note 1: The CPE (cytopathic effect) caused by measles viruses is syncytium formation. If successful measles virus isolation has been achieved, an immunological assay such as immunofluorescence or molecular techniques can be used to confirm the presence of measles virus

Note 2: Rubella virus most likely will <u>not</u> cause CPE in Vero/hSLAM cells. Virus must be passaged “blind”. For each passage, supernatant can be saved and tested for the presence of rubella virus. Successful virus isolation must be confirmed with immunological (Immunocolorimetric assay) or molecular methods (real-time RT-qPCR assay).

Note 3: For measles, the terms “virus stock” and “virus lysate” are equivalent. For rubella virus, stocks can be prepared from cell lysate or from supernatant, but cell lysates should be used for RNA extraction.

Note 4: Record all relevant information (sample ID, date of inoculation, passage number, CPE, harvest etc.)

1. For each sample seed cells into a 25 cm^2 tissue culture flask (See SOP for Culture of Vero/hSLAM Cells). Add one flask for the negative control. Cells should be at approximately 85-90% of confluency and at least one day after seeding. If cells are overgrown, virus isolation will be unsuccessful.
2. Decant growth medium into hypochlorite solution. To the cells, add 5 mL of DMEM-PS-2%FBS and 0.5 - 1 mL of specimen. This step equals virus passage number 1.
3. Incubate at 37˚ C in BSC for 1 hour, then observe the cells by light microscopy to see if the sample was toxic to the cells (rounding of cells, cells floating).
    a. Optional: Replace medium with 5 mL of DMEM-PS-2%FBS after 1-2 hours to reduce toxicity.
4. Continue to incubate cells for 4-5 days. Cells should be observed by light microscopy for CPE on a daily basis. If measles CPE becomes visible, proceed to step 8.

5. If no CPE is visible after 4-5 days of incubation, passage the infected Vero/hSLAM cells (See SOP for Culture of Vero/hSLAM Cells) at a 1:3 split ratio. This passage should be carried out in a BSC used specifically for handling potentially infectious material. This passage equals virus passage 2.
6. Incubate cells for 4-5 days and monitor infected cells by light microscopy for CPE on a daily basis. If CPE is observed, proceed to step 8.
7. If no CPE is observed, repeat steps 5 and 6. This passage equals virus passage 3. If CPE is observed, proceed to step 8. For measles, if no CPE is observed, discard the culture and record result as negative. For rubella, prepare a virus stock (step 9) and confirm by immunological or molecular methods.
8. When CPE is visible, continue to incubate until the CPE becomes extensive. If the medium turns yellow after several days of incubation, replace the medium with fresh DMEM-PS-2% FBS. It may be necessary to passage the cells one more time to allow the infection to spread before cells become overgrown. When CPE is visible over at least 50-75% of the cell layer, cells can be harvested for preparation of a viral stock.
9. To prepare a viral stock, scrape the cells into the medium with a cell scraper or 1 mL pipette. Transfer medium and cells to a sterile, 15 mL conical tube and centrifuge the cells at approximately 1000 x g for 10 minutes at 4 °C.
10. Discard the supernatant into hypochlorite solution and resuspend the cell pellet in 1 mL of DMEM-PS-2%FBS.
11. Label two cryovials per sample with date, passage number and source material.
12. Transfer 0.5 mL of the cell suspension to each of 2 cryovials and store at –70° C.

Alternative method for preparation of viral stock from cell lysate
Decant all but about 1 mL of the supernatant medium into hypochlorite solution. Scrape the cells into the remaining medium and pipette 0.5 mL each into 2 cryovials and store at -70° C.

Alternative method for preparation of rubella virus stocks
Transfer 0.5 mL cell culture supernatant to each of 2 cryovials and store at –70° C.