Annex 5.3

Confirmation of rubella virus in cell culture

NOTE: This document is intended to provide basic test method details and is not an SOP. Laboratories need to develop their own SOPs to suit their needs. The inclusion of reagents and products from specific manufacturers does not constitute an endorsement by the GMRLN or WHO.

A. Indirect Immunofluorescence Assay (IFA) for detection of rubella virus in cell culture

B. Immunocolorimetric Assay (ICA) for detection of rubella virus in cell culture

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A. Indirect Immunofluorescence Assay (IFA) for detection of rubella virus in cell culture

The indirect immunofluorescence assay described here was developed by the Rubella Laboratory, CDC, Atlanta, using a CDC-developed monoclonal antibody specific to the rubella virus E1 glycoprotein. Use of these precise reagents is very important since rubella virus does not produce large amounts of antigen. The protocol for measles IFA using acetone fixation does not work well for rubella virus because background using acetone fixed cells exceeds the signal from rubella virus proteins. The protocol presented here using paraformaldehyde fixation and highly cross-adsorbed fluorescent antibody does work well.

Equipment and reagents required:
• CO₂ Incubator
• 8 chambered slides
• Sterile pipettes
• Vero or Vero/hSLAM cells
• Fluorescent microscope, with appropriate filters
• 2% Paraformaldehyde*
• Foetal bovine serum
• Methanol
• DMEM, PBS
• Antibiotics
• Propidium Iodide** counterstain (stored at 4°C)
• Bovine Serum Albumin (BSA)
• Tween 20
• Fluorescent mounting medium*** (stored at 4°C)
• Cover slips

* Paraformaldehyde can be purchased as a 16% stock solution (e.g. from Electron Microscopy Sciences, #15710). Dilute 1:8 with cold PBS immediately before use.

** e.g. Molecular Probes, #P-3566. Dilute 1:2000 in blocking buffer.

*** e.g. Dako Fluorescent Mounting Medium, #S3023.

Antibodies: Goat anti-mouse IgG conjugate (Alexa Fluor® 488 from Molecular Probes, Eugene, OR, USA, Web: http://www.probes.com (store at 4°C)

Monoclonal antibody (anti-rubella E1 glycoprotein) from CDC rubella lab (stored at -20°C).

Growth of Vero or Vero/hSLAM cells and virus infection:

Growth of rubella virus in the following protocols is at 35°C because some rubella virus strains grow best at this temperature. If a 35°C incubator is not available, 37°C is an acceptable alternative temperature.

1) Grow Vero or Vero/hSLAM cells to about 50% confluence in an 8-chambered slide, e.g. Lab-Tek Chamber Slide (catalogue number 177445). Do not allow cells to overgrow. Typically, this is done using DMEM with 5% FBS and Pen/Strep in 35°C, CO₂ incubator.

Note: If a CO₂ incubator is not available, the slide will have to be sealed to allow cell growth and virus growth after inoculation (see #2 below). The seal needs to be gas impermeable, for example coating the edges of the chamber slide lid with petroleum jelly.

2) Remove medium and add 100 µl of specimen (media from passage 2 of the culture of a clinical specimen inoculated on Vero or Vero/SLAM cells) to one chamber.
3) A known positive specimen (such as a well characterized wild-type strain) should be added to one of the corner chambers. Three negative control chambers (add media only) should surround the positive control to reduce the chance of the positive control contaminating a specimen chamber. If a high titre virus is used as a control, it should be diluted before adding to chamber. Incubate 1 hour in 35°C, CO₂ incubator, in order to allow virus attachment.

4) Add 200 µl DMEM containing 5 % FCS. Incubate for 3 days in 35°C, CO₂ incubator (or 37°C). At the end of this 3 day incubation, IFA results will be optimal if cell monolayer is about 80% confluent, but a completely confluent monolayer is acceptable.

5) If the tissue culture medium in the virus-containing wells becomes acidic (yellow), this may indicate that virus is present.

Fixing cells:
The cells are fixed using 2% paraformaldehyde in PBS that has been chilled at 4°C. Cells are then permeabilized with -20°C methanol.

1) Remove chambered slide from incubator and place on ice for 10 minutes; support the slide above the ice by laying the slide on a metal or foil strip in contact with ice.

2) In a Biological Safety Cabinet, remove the tissue culture medium and wash 1X with cold PBS. For media removal, insert pipette tip into one corner of chamber and continue to use this one corner throughout the procedure to minimize cell loss. Add reagents down the side of the chamber for the same reason.

3) Add 200 µl of 2% paraformaldehyde for 30 minutes on ice.

4) Remove paraformaldehyde and wash 1X with cold PBS. The rest of procedure can be done on the bench top as virus has been inactivated by the paraformaldehyde.

5) Add 200 µl of -20°C methanol and incubate for 10 minutes at -20°C. This is easily done by placing the slide in a -20°C freezer. If a freezer is not available, placing the slide on a frozen (-20°C) freezer pack (e.g. Frigid Brick manufactured by Touchpad Solutions) on the lab bench is acceptable.

6) Remove the methanol and wash 1X with room temperature PBS.

7) Fixed cells may be stored at this point, covered with blocking buffer (1% BSA, 0.5% FBS, 0.1% Tween 20 in PBS). Fill the chambers/wells with blocking buffer; fixed cells covered with blocking buffer can be stored in a humidified chamber at 4°C for at least 1 month. For long term storage, add 1X Pen/Strep to blocking buffer.
Note: A humidified chamber can be as simple as placing the slide on damp paper towels and storing in a plastic box or wrapped with plastic wrap.

IFA Procedure:

Blocking, dilutions of antibodies and propidium iodide, and washes are all performed in blocking buffer at room temperature. Final washes after propidium iodide are with PBS only.

1) Block non-specific antibody reactions by adding 200 µl of blocking buffer per chamber for 1 hour at room temperature (if the slide has been stored in blocking buffer, this step can be omitted.)

2) Remove blocking buffer and add diluted monoclonal antibody (100 µl per well) in blocking buffer. Incubate 1 hour at room temperature. Dilute monoclonal stock 1:1000 to use in assay (for lot # 03-031). Note that the dilution of the monoclonal may change depending on the lot used.

3) Wash 2 times with blocking buffer, and then add second fluorescent antibody in blocking buffer (100 µl per well). Typically, this is Alexa Fluor 488 goat anti-mouse IgG (H+L) “highly cross-adsorbed” at a 1:500 dilution. Incubate 30 minutes at room temperature; cover slide with foil to keep it in the dark.

4) Wash 2 times with blocking buffer, and then add propidium iodide at 0.5 µg/ml in blocking buffer. Incubate 5-15 minutes at room temperature covered with foil.

5) Wash 2 times with PBS. Remove chambers and gasket from slide.

Wick off excess PBS with absorbent tissue or filter paper. Allow slide to dry completely, and then add 2-3 drops of mounting media (e.g. Dako). Carefully add the cover slip and press slightly to remove air bubbles. Wipe off excess mounting media from edges with absorbent paper.

6) Observe results with fluorescent microscope using blue light (e.g. Zeiss Axiovert BlueH 485 filter).

Note: Rubella virus infected cells will be green; propidium iodide-stained nuclei will be red. There should be no green background antibody staining in uninfected cells (negative control chambers), although there is sometimes some staining near edges of chambers, presumably due to the gasket. If there is background antibody staining, most likely it is too much fluorescent second antibody. The utility of IFA test is dependent on low background antibody staining in uninfected cells. Depending on how much virus was contained in the inoculum, all cells may not exhibit green fluorescence (in fact, with clinical samples only a very small number of cells may exhibit fluorescence, e.g. 2 foci of 10 cells each on the entire lawn of cells).
B. Immunocolorimetric Assay (ICA) for detection of rubella virus in cell culture

This is an indirect colorimetric assay using monoclonal antibody developed by the Rubella Laboratory, CDC, Atlanta which is specific to the rubella E1 glycoprotein. The procedure is the nearly the same as the rubella IFA up to the step where the secondary antibody is added, which in this case is HRP conjugated rather than fluorescent. Twice the amount of monoclonal antibody is used in the immunocolorimetric assay. The immunocolorimetric assay is described for both chamber slides and 48-well plates. If using 48-well plates instead of chamber slides, there is a difference in the way the positive controls are processed.

Equipment and reagents required:

- CO₂ Incubator
- 8 chambered slides or 48-well tissue culture plates
- Sterile pipettes
- Vero or Vero/hSLAM cells
- 2% Paraformaldehyde*
- Foetal bovine serum
- Methanol
- DMEM, PBS
- Antibiotics
- Bovine Serum Albumin (BSA)
- Tween 20
- BM Blue POD substrate, precipitating: Roche, cat# 1442066.

* Paraformaldehyde can be purchased as a 16% stock solution (e.g. from Electron Microscopy Sciences, #15710). Dilute 1:8 with cold PBS immediately before use.

Antibodies: Goat anti-mouse, HRP conjugated antibody: Molecular Probes: cat# G21040.

Monoclonal antibody (anti-rubella E1 glycoprotein) from CDC rubella lab (stored at 20°C).

Growth of Vero or Vero/hSLAM cells and virus infection:

Growth of rubella virus in the following protocols is at 35°C because some rubella virus strains grow best at this temperature. If a 35°C incubator is not available, 37°C is an acceptable alternative temperature.

1) Grow Vero or Vero/SLAM cells to about 75% confluence in an 8-chambered slide, e.g. Lab-Tek Chamber Slide (catalogue number 177445) or wells of a 48 well tissue culture plate. Do not allow cells to overgrow. Typically, this is done using DMEM with 5% foetal bovine serum (FBS) and Pen/Strep in 35°C, CO₂ incubator.
Note: If a CO₂ incubator is not available, the slide or plate will have to be sealed to allow cell growth and virus growth after inoculation (see #2 below). The seal needs to be gas impermeable, for example coating the edges of the chamber slide lid or edges of 48 well plate lid with petroleum jelly.

2) Remove medium and add 100 µl of specimen (media from passage 2 of the culture of a clinical specimen inoculated on Vero or Vero/hSLAM cells) to one chamber/well.

3) A known positive specimen (such as a well characterized wild type strain) should be added to one of the corner chambers. Three negative control chambers (add media only) should surround the positive control to reduce the chance of the positive control contaminating a specimen chamber. If a high titre virus is used as a control, it should be diluted before adding to chamber. Incubate 1 hour in 35°C, CO₂ incubator, in order to allow virus attachment.

4) Add 200 µl DMEM containing 5% FCS. Incubate for 5 days in 35°C, CO₂ incubator (or 37°C). At the end of this 5 day incubation, immunocolorimetric results will be optimal if cell monolayer is about 80% confluent, but a completely confluent monolayer is acceptable.

5) If the tissue culture medium in the virus-containing wells becomes acidic (yellow), this may indicate that virus is present.

Note: Positive controls for 48 well plates should be prepared in advance and stored for use in specimen testing as described below. The separate preparation of positive controls greatly reduces possible contamination of specimens by positive controls. A known positive specimen (such as vaccine virus or lab strain) should be prepared on a separate 48 well plate. If a high titer lab strain of virus is used, dilute 1:10 to 1:1000 with PBS, 1% FBS before adding to cells. Several wells of the 48 well plate can be infected with the positive control virus. After the cell fixation step (see below) plates can be stored at 4°C for at least 1 month, provided wells are covered with blocking solution. To use as a positive control, remove the blocking buffer from 1 fixed well and complete the colorimetric assay, starting with the monoclonal antibody addition step. The plate can then be refrigerated until the next assay.

Fixing cells:
The cells are fixed using 2% paraformaldehyde in PBS that has been chilled at 4°C. Cells are then permeabilized with -20°C methanol.

1) Remove chambered slide or tissue culture plate from incubator and place on ice for 10 minutes; support above the ice by laying the slide on a metal or foil strip in contact with ice.

2) In a Biological Safety Cabinet, remove the tissue culture medium and wash 1X with cold PBS. For media removal, insert pipette tip into one corner of chamber (or a position on the well) and continue to use this one corner/position throughout the procedure to minimize cell loss. Add reagents down the side of the chamber/well for the same reason.

3) Add 200 µl of 2% paraformaldehyde for 30 minutes on ice.
4) Remove paraformaldehyde and wash 1X with cold PBS. The rest of procedure can be done on the bench top as virus has been inactivated by the paraformaldehyde.

5) Add 200 µl of -20°C methanol and incubate for 10 minutes at -20°C. This is easily done by placing the slide/plate in a -20°C freezer. If a freezer is not available, placing the slide/plate on a frozen (-20°C) freezer pack on the lab bench is acceptable.

6) Remove the methanol and wash 1X with room temperature PBS.

7) Fixed cells may be stored at this point, covered with blocking buffer (1% BSA, 0.5% FBS, 0.1% Tween 20 in PBS). Fill the chambers/wells with blocking buffer; fixed cells covered with blocking buffer can be stored in a humidified chamber at 4°C for at least 1 month. For long term storage, add 1X Pen/Strep to blocking buffer.

*Note:* A humidified chamber can be as simple as placing the slide/plate on damp paper towels and storing in a plastic box or wrapped with plastic wrap.