



PRIMARY HEALTH
CARE PROJECT



Iraqi Standard Operating Procedure (SOP)

Laboratory Identification of *Rubella*

December 2014

**SOP: NML
National Measles
Laboratory - IRAQ**

DISCLAIMER

This guideline has made possible through support provided by the U.S. Agency for International Development (USAID) under Primary Health Care Project in Iraq (PHCPI) implemented by University Research Co., LLC. This guideline has been developed in Iraq in close collaboration with the Ministry of Health (MoH) in 2014.

Contents

Acknowledgements	3
Preface	4
1. Introduction	5
1.1. National Measles Laboratory (NML).....	5
1.2. Purpose	5
1.3 Responsible positions	5
1.4. Functions	5
2. Sampling & Documentation	6
2.1 Serum.....	6
2.2 Urine	6
2.3 Gum Swab (Oral fluid).....	6
2.4 Nose and throat swabs	6
3. Shipment	6
3.1 Packaging	6
3.2 Marking	8
3.3 Documentation	9
4. Agents and Precautions	10
4.1 Agent	10
4.2 Employees at risk	10
4.3 Bio-Safety Issues	10
4.4 Vaccinations	10
4.4.1 Vaccination Implementation	10
4.4.2 Vaccination doses	10
4.5 Assay Precautions.....	10
5. Diagnostic Procedures	11
5.1 General Issues.....	11
5.2 Virus Isolation Steps	11
5.3 VERO/slam cells	11
5.4 Contaminated Samples	12
5.5 Immunofluorescence	12
5.6 Rubella Serology for IgM detection	13
5.6.1 Principle.....	13
5.6.2 Interpretation of results	13
5.6.3 Procedure.....	14
5.7.1 Principle.....	15
5.7.2. Interpretation of results	15
6. Appendix	16

6.1 Appendix A – Sample Collection and Storage.....	16
6.1.1 For Serology	16
6.1.2 For Virus Isolation.....	16
6.1.3 For RNA Extraction	16
6.2 Appendix B - Isolation of Mononuclear Cells from Circulating Blood.....	18
6.2.1 Procedure.....	18
6.3 Appendix C – Media Composition.....	19
6.4 Appendix D – Cell Propagation	20
6.5 Appendix E - Cell Counting	21
6.6 Appendix F - Preservation of Cell Cultures	22
6.7 Appendix G - Rubella Virus PCR	23
6.8 Appendix H - Rubella IgM Serology Worksheet	24
6.9 Appendix I - Reagents, kits & Solutions.....	245
7. References	26

Acknowledgements

University Research Co., LLC wishes to thank all the people who have collaborated on the Development of this Document. They have given generously of their time and their experience. Significant contributions to the Technical assistance to develop of this plan were made by USAID/Primary Health Care Project in Iraq (PHCPI) team in the field: Dr. Hala Jassim AlMossawi, Chief of Part, Dr. Ahlam Kadhum and Eng. Saif Ali and HQ team: Dr. Neeraj Kak, and Taylor Price and international Epidemiologist Paul Roddy for their significant assistance in reviewing and revising the document. Special thanks are due to Ministry of Health Public Health Directorate headed by Dr. Ziad Tariq and the technical working group who contributed time and experience to develop this document.

Ministry of Health Technical Working Group:

1. Mrs. Ghada Ghaleb Flaieh / Microbiologist specialist / MSc in Medical Laboratory Technology/National Measles Laboratory Director/CPHL/MoH
2. Mr. Jawad Shahmurad /Quality management section /Directorate of Public Health /MOH

Preface

The United States Agency for International Development (USAID) has funded the Primary Health Care Project in Iraq (PHCPI) to assist the Iraqi Ministry of Health (MOH) achieve its strategic goal of quality primary health care (PHC) services in the country. PHCPI supports the MOH in three key components: 1) strengthening health management systems, 2) improving the quality of clinical services, and 3) encouraging community involvement to increase the demand for and use of PHC services. Since March 2011, PHCPI has assisted the MOH to put in place key building blocks to strengthen the health management systems and to support the delivery of quality PHC services at the community and facility levels. PHCPI has worked to build the capacity of the MOH and primary health care centers (PHCCs) to respond to the needs of the Iraqi population and to assist the MOH in achieving its country-level health priorities, such as meeting its Millennium Development Goals (MDGs) by 2015. Interventions have included updating clinical guidelines, training health providers at the PHC level, implementing supportive supervision and quality improvement models and referral systems to improve the quality of primary care services, particularly for maternal and child health.

In October 2013, a modification to PHCPI's technical scope of work had the project re-focus its efforts to further help the MOH accelerate the achievement of MDGs 4 and 5, reduce child mortality and improving maternal health. Given that large numbers of displaced Iraqis and Syrian refugees have caused a rise in measles cases, especially among vulnerable groups, PHCPI, along with the MOH, has developed a standard operating procedure (SOP) to prepare for potential outbreaks of Rubella. This will assist in the upgrading of actions for microbiology diagnosis, including diagnosis of Rubella, for lab workers. An improved and more streamlined process for transportation and diagnosis will allow the MOH to better and more rapidly respond to detected cases, stemming or preventing potential epidemics or outbreaks.

1. Introduction

Rubella is a mild illness that presents with fever and rash. The public health importance of rubella is because infection in the early months of pregnancy usually affects foetal development [3]. Rubella infection of the fetus can result in miscarriage, foetal death or the birth of an infant with serious congenital birth defects. Congenital rubella syndrome (CRS) is an important cause of blindness, deafness, congenital heart disease, and mental retardation. Worldwide, it is estimated that more than 100 000 infants are born with CRS each year. Most of these cases occur in developing countries that have not yet introduced rubella vaccine. The name rubella is derived from Latin, meaning “little red.” It was initially considered a variant of measles or scarlet fever and was called “third disease.” It was not until 1814 that it was first described as a separate disease in the German medical literature; hence, the common English name of “German measles, the role of rubella in congenital rubella syndrome (CRS), a disease which includes cataracts, heart disease and deafness, was confirmed. To date, no Rubella outbreaks have been reported in Iraq.

1.1. National Measles Laboratory (NML)

The National Measles Laboratory (NML) is part of the Virology Department at the Central Public Health Laboratory (CPHL), it is responsible for testing specimens (serum, urine, and gum swabs) related to suspected Rubella cases that are referred from all Iraqi governorates (hospitals, primary health care centers) to confirm Rubella infection.

The NML laboratory is the only reference laboratory in Iraq that is integrated in terms of availability of staff and specialized laboratory equipment and supplies required for Rubella diagnosis. . In order to instill proper laboratory knowledge and actions, including diagnosis of Rubella virus (*Rubella*) this Standard Operating Procedure (SOP) has been developed for reference and to be integrated into training programs for lab workers.

1.2. Purpose

This SOP provides details and background for isolation and identification of Rubella virus using cell culture, immunofluorescence and molecular techniques. It also describes the determination of Rubella IgM antibody by ELISA.

1.3 Responsible positions

1. M.Sc./Microbiology Director of NML
2. BSc. of biology (Staff of NML 3)

1.4. Functions

A Collection and transportation of a serum, urine and gum swab.

B Detection of Rubella IgM Ab. in serum.

- C Isolation of suspected *Measles virus* on VeroSlam media.
- D Identification of *Measles* by classical and advance methods.
- E Shipment of isolate to a reference lab for confirmation.

2. Sampling & Documentation

All samples collected for analysis must be documented, preferably using a standardized laboratory request form (case investigation form) and to be filled with complete data.

2.1 Serum

A single serum specimen when collected between 72 hours and four weeks after rash onset can be used to diagnose most Rubella cases by ELISA. Serum should be stored at 4–8 °C until shipment takes place, or for a maximum of 7 days. When kept for longer periods, serum samples must be frozen at -20 °C or lower and transported to the testing laboratory on frozen ice packs.

2.2 Urine

The isolation of Rubella virus is most successful if the specimens are collected as soon as possible after the onset of rash, and at least within 5 days after onset. It is preferable to obtain the first urine passed in the morning. About 10-50 ml of urine should be collected in a sterile container and held at 4 to 8 °C before centrifugation. Urine must NOT be frozen before the concentration procedure is carried out. Whole urine samples may be shipped in well-sealed containers at 4 °C.

2.3 Gum Swab (Oral fluid)

Samples are collected using a foam swab attached to a handle (ORACOL; Malvern Medical Developments, Worcester, United Kingdom). Rub the swab along the teeth/gum line for approximately 1 minute, after the swab is replaced in the plastic tube with the foam swab uppermost and transported to the laboratory at 4°C. At this time, oral fluid will be collected into the bottom of the plastic tube by centrifugation at 3,000-g for 15 minutes.

2.4 Nose and throat swabs

Nose and throat swabs should be pooled in 2.0 ml of viral transport medium at the time of collection, transported to the laboratory at 4°C, tested immediately by PCR, and then stored at -70°C and arrive at the testing laboratory within 48 hours maximum quantity per package; for air transport.

3. Shipment

3.1 Packaging

- A. The triple packaging system continues to apply, including for local surface transport.
- B. Testing documents are not required, however. It may be possible to source packaging's locally rather than finding an authorized supplier.
- C. Provided that the packaging manufacturer and the shipper can comply fully with the requirements.

D. As for P620, there is no comprehensive list of suppliers of packaging's that comply with Packing Instruction P650. However, an Internet search using a suitable international or national search engine usually provides appropriate information, as well as access to national regulations. Search phrases such as “UN packaging” and “UN infectious substance packaging” produce extensive results. Carriers and forwarding agents should also be able to supply details of local suppliers or local companies that can provide such information.

To ensure correct preparation for transport, packaging manufacturers and subsequent distributors shall provide clear instructions to the consignor or persons preparing packages (e.g. patients) on how the packaging

- No primary receptacle shall exceed 1L and the outer packaging must not contain more than 4 L (for liquids)
- Except for packages containing body parts, organs or whole bodies, the outer packaging must not contain more than 4 kg (for solids).

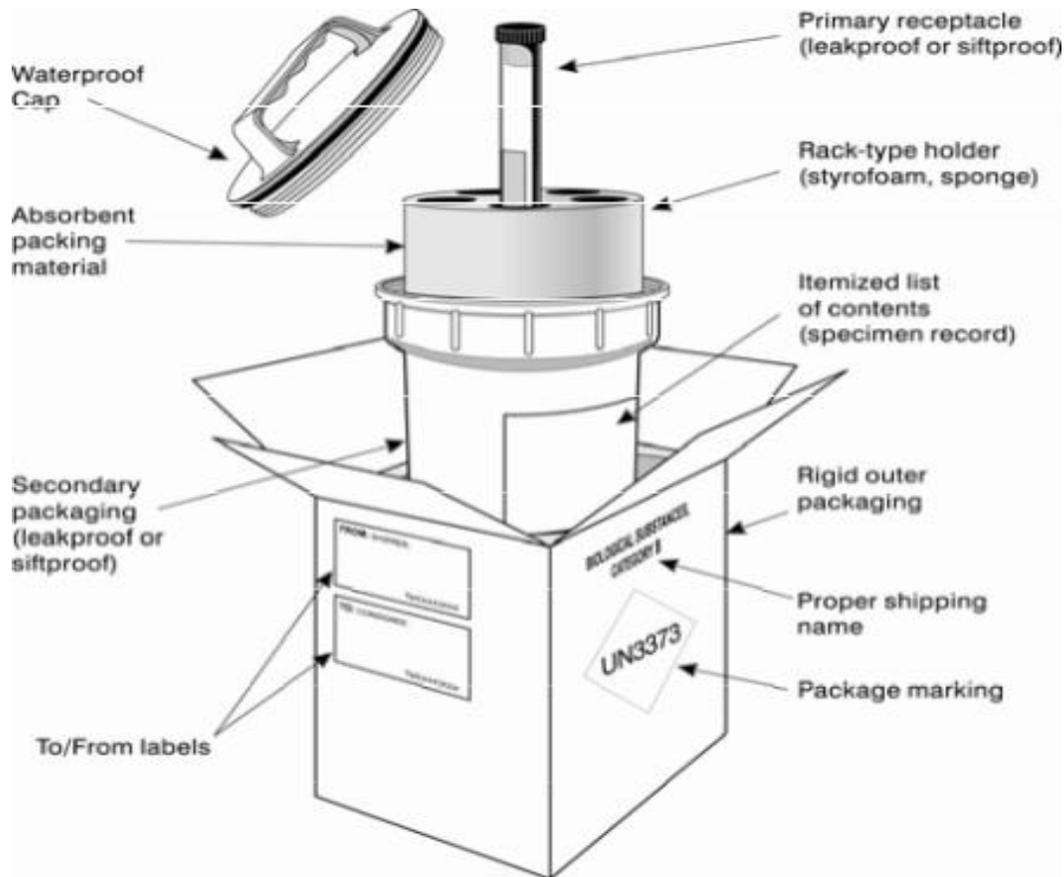


Figure 1. Example of the triple packaging system for the packing and labelling of Category infectious substances (Figure kindly provided by IATA, Montreal, Canada).

Provided all the requirements of P650 are met, there are no other transport requirements. P650 incorporates all that is needed to make a shipment for Category B infectious substances.

3.2 Marking

Each package shall display the following information for air:

- A. The shipper's (sender's, consignor's) name, address and telephone number for air: the telephone number of a responsible person, knowledgeable about the shipment the receiver's (consignee's) name, address and telephone number.
- B. The proper shipping name ("BIOLOGICAL SUBSTANCE, CATEGORY B") adjacent to the diamond-shaped mark shown in Figure 10 temperature storage requirements (optional).
- C. The marking shown in Figure 10 is used for shipments of Category B infectious substances.
- D. Minimum dimension: the width of the line forming the square shall be at least 2 mm, and the letters and numbers shall be at least 6 mm high.
- E. For air transport, each side of the square shall have a length of at least 50 mm.
- F. Color: none specified, provided the mark is displayed on the external surface of the outer packaging on a background of contrasting color and that it is clearly visible and legible The words "BIOLOGICAL SUBSTANCE, CATEGORY B" in letters at least 6 mm high shall be displayed adjacent to the mark.



Figure 2. Marking for infectious substances of Category B and for genetically modified microorganisms or organisms that meet the definition of an infectious substance, Category B.

Note: For air transport: when dry ice (solid carbon dioxide) is used the label shown below shall be applied for cryogenic liquids the labels shown in Figures 4 and 5 shall also be affixed.

Label name: Miscellaneous dangerous substances
Minimum dimensions: 100 × 100 mm
(for small packages: 50 × 50 mm)
No. of labels per package: 1
Color: Black and white

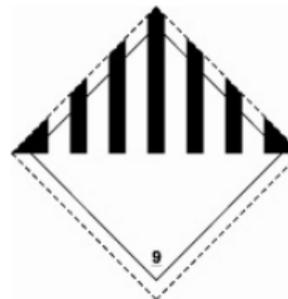


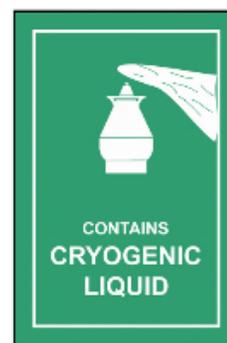
Figure 3: Hazard label for certain noninfectious genetically modified microorganisms and organisms (UN 3245) and for carbon dioxide, solid (dry ice) (UN 1845); substances packed in dry ice shall bear this label in addition to the primary risk label (Figure 2).

Label name: Non flammable, non-toxic gas
Minimum dimensions: 100 × 100 mm
(for small packages: 50 × 50 mm)
of labels per package: 1
Color: Green and white or green and black



Figure 4: Hazard label for liquid nitrogen; substances packed using liquid nitrogen shall bear this label in addition to the primary risk label (Figure 2).

Label name: Cryogenic liquid
Minimum dimensions: Standard A7: 74 × 105 mm
of labels per package: 1
Color: Green and white



3.3 Documentation

Dangerous goods documentation (including a shipper's declaration) is not required for Category B infectious substances. The following shipping documents are required. To be prepared and signed by the shipper (sender, consignor):

- A.** For international shipments: a packing list/proforma invoice that includes the shipper's and the receiver's address, the number of packages, detail of contents, weight, value (Note: the statement "no commercial value" shall appear if the items are supplied free of charge) an import and/or export
- B.** To be prepared by the shipper or the shipper's agent:
 - An air waybill for air transport or equivalent documents for road, rail and sea journeys.

4. Agents and Precautions

4.1 Agent

Rubella (causative agent of German measles) genus Rubivirus, Family Togavirus

4.2 Employees at risk

Clinical specimens for investigations are a potential source of infection to exposed laboratory personnel. It is therefore generally recommended that all clinical specimens should be treated as potentially infectious.

4.3 Bio-Safety Issues

Clinical samples may contain Rubella and/or other dangerous infectious agents. You must be familiar with World Health Organization (WHO) biological safety regulations. Use the precautions for BSL-2 agents. Always wear gloves and a lab coat. Do all work with clinical samples in a biological safety cabinet (BSL – 2). Dispose of all materials that contact specimens in the bio-hazard waste containers. Do not look into the hood when the UV-light is turned on.

4.4 Vaccinations

Everyone handling clinical specimens must be vaccinated against rubella after evaluation of their immune status against rubella. Vaccination cards of all such individuals should be counter checked and/or safely kept by the director or the supervisor of the laboratory. Individuals refusing vaccination or having medical contra-indications may be prohibited from handling clinical specimens suspected of having rubella virus.

4.4.1 Vaccination Implementation

MMR (Measles, Mumps & Rubella) Vaccine will be used to vaccinate individuals identified as having insufficient immunity.

4.4.2 Vaccination doses

Anyone having a 0 titer as demonstrated by an ELISA test will receive two vaccines give 4 weeks apart.

- Individuals with a titer >0 , but $<1:10$ will receive one vaccine booster.
- Anyone who has never been vaccinated and who is not known to have had rubella will receive two vaccines given 4 weeks apart.
- Even though it is believed that lifelong immunity results from infection, all employees in the rubella program will have their titers re-evaluated regularly.

4.5 Assay Precautions

Tissue culture media/cells are easily contaminated with bacteria and fungi.

- a) Use sterile technique at all times.
- b) Wash your hands and arms with soap dry and spray your hands with 70% alcohol before touching tissue culture flasks and reagents.
- c) Turn on the UV light in the hood 10 min prior to working in the hood and for 10 min after working with infectious material.

- d) Immediately before working and after working, wipe the working surface of the hood with 70% ethanol.
- e) Do not place anything into the tissue culture hood that you suspect has more than a minimal amount of bacteria on its surface.
- f) Wipe any container coming from the refrigerator with 70% ethanol before placing it in the hood.
- g) Daily, remove anything that may grow mold from the lab particularly anything that contains even a drop of media.

Cell cultures are easily contaminated with viruses that have been passed in cell culture. Therefore,

- a) Always work with the sample that has the least amount of virus first (usually this is the clinical sample).
- b) Note that when making spot slides a high concentration of virus is present, and because it is being manipulated, it is likely to contaminate any work in the hood.
- c) Note that liquating first pass is working with a virus in high concentration and the hood.
- d) Avoid formation of aerosols. E.g. do not transfer infectious material by dripping into tubes, instead run solution down the side of the tubes; do not allow bubbles or films of liquid to form; et.
- e) Spray your gloves with ethanol after manipulating each first pass isolate.
- f) Rubella and most of the viruses may be extremely labile.
- g) Keep samples containing virus on ice
- h) Dilute virus in polypropylene tubes, **not polystyrene**

5. Diagnostic Procedures

5.1 General Issues

Fill out the worksheet as indicated. Initial the appropriate blank after carrying out each procedure/observation.

5.2 Virus Isolation Steps

- a) Obtain cells in Tissue Culture Flasks.
- b) Vero/slam cells should be 70 – 90 % confluent.
- c) Prepare samples:
 1. Thaw samples. Keep on ice – never bring the samples to room temperature.
 2. Vortex gently (avoid making foam) for 30 seconds. If the swab does not permit the sample to vortex, agitate it.

5.3 VERO/slam cells

- A. Pre-warm maintenance media to RT (room temperature)
- B. Media are different for growth and maintenance of Vero/slam as used in this protocol.
- C. Growth media = DMEM medium (10% FCS)
- D. Maintenance media = DMEM medium (2% FCS)
- E. Pour-off growth media into discard container.

- F. Place negative control flask (inoculated with sterile broth) with each group and process these as the last sample of each group.
- G. Add 5 ml of maintenance medium
- H. Add 0.5ml of specimen to each flask.
- I. Incubate at 36oC and observe the cells under the microscope to ascertain if the sample was toxic to the cells (rounding or floating of cells)
- J. Save the specimens at -20oC. On day 3 or 4, if the samples are not contaminated, refreeze the samples at -80oC.
- K. Observe Cells for Cytopathic Effect (CPE):
 - a) On day one, check that all flasks have equilibrated with the incubator CO₂ and close the caps.
 - b) Observe cells daily for signs of CPE and contamination. If contamination is found, discard the flasks immediately.
 - c) When CPE is observed, (syncytium formation) let it develop to 2 – 3 + (50 – 75%) CPE or until the CPE stops progressing (e.g. day 3 = +1; day 4 = +2; day 5 = +2: Collect the sample on day 5, since CPE has stopped increasing (New virus is not being produced and old virus is dying) Freeze as described below.
 - d) Freeze at -80°C in a freezer box designated “P1 measles isolates”.
 - e) Do not remove floating cells, since these will lyse and may release additional virus.
 - f) Carry out a second pass using 0.5 ml of P1. Also carry out a blind second pass with samples not showing CPE in first pass. If CPE observed in second pass, proceed with immunofluorescence
 - g) Continue observing for 10 days, before designating as negative and discarding.
 - h) Change media every 72 hours.

5.4 Contaminated Samples

- a) To a contaminated sample, add transport broth so there is 0.6ml of liquid.
- b) Filter through a sterile syringe filter into a new sterile tube and transfer the tube’s label, and write filtered on the label.
- c) Inoculate a single flask.
- d) Freeze the remaining sample.

5.5 Immunofluorescence

- a) Scrape the infected cells with a st. cell scraper and add 5ml of PBS. Mix well.
- b) Dispense in screw capped vials and spin for 5 minutes at 1500rpm.
- c) Pour off the PBS and add 200µl of PBS to the pellet.
- d) Mix well to separate cells.
- e) Add 20 µl onto teflon coated spot slide and spread well. Dry at 37oC.
- f) Fix with acetone for 10 min. Remove and dry at room temperature.
- g) Add 20 µl of measles monoclonal antibody and keep in moist chamber for 30 min at 37oC.
- h) Wash 3 times with PBS. Add 25 µl of anti-mouse IgG/FITC.
- i) Incubate in moist chamber for 30 min at 37oC.
- j) Wash 3 times with PBS and keep at room temperature.
- k) Add mounting fluid. Fix with a cover slip and observe in a fluorescent microscope. FITC absorbs at 495 nm with peak emission at 525 nm.

- 1) Positive cells will show a granular green fluorescence in the cytoplasm and the Evans blue counter stain will appear red.

5.6 Rubella Serology for IgM detection

5.6.1 Principle

Dade Behring Enzygnost Test - Enzyme Immuno Assay for the detection of human IgM antibodies to rubella virus in serum and plasma

The RF absorbent binds to the IgG present in the test sample. Any rheumatoid factor in the sample binds to the resulting immune complexes and is thus eliminated. The RF absorbent precipitates up to 15 mg IgG/ml and this enhances the sensitivity of IgM test.

IgM in the test sample which is specific for the virus binds to the virus antigen on the plastic surface of the test plate. The anti-human IgM/POD conjugate binds to this complex. The enzyme component of the conjugate catalyzes the working chromogen solution producing a blue color. This reaction is terminated by the addition of stopping solution POD and the yellow color is formed which is read at 450 nm. IgM directed against the cellular antigen is measured in the same way in the well coated with control antigen the difference between the color intensities in the well coated with antigen and in the well coated with control antigen is the measure of the concentration and immuno chemical reactivity of the virus antibodies detected in the sample.

5.6.2 Interpretation of results

A **NEGATIVE** result means that virus specific IgM cannot be detected. The patient either is NOT acutely infected with rubella virus or, if infected or vaccinated, is still unable to produce IgM specific for the virus. If exposure to the virus is suspected despite a negative finding, a second sample should be collected no less than 7 days later and should be tested together with the first sample.

If a sample is **POSITIVE** it indicates that virus specific IgM has been detected. This is proof of a recent infection. In over half the rubella patients, anti-rubella IgM is detectable on the first day of rash itself and can be detected for about 3 months.

If a result is **EQUIVOCAL** after a re-test this is indicative of virus infection. Here too a second sample must be collected no less than 7 days later and tested together with the first sample.

5.6.3 Procedure

Dilution of Control and Samples

- Add 400µl of POD dilution buffer to one dilution vial each of P/N and P/P
- Add 400µl of POD dilution buffer to sample vials
- Add 20µl of negative control (P/N) to 1 vial and 20µl of positive control (P/P) to 1 vials
- Add 20 µl of sample to vials (1:21 dilution) and 20µl of internal control to 1 vial

Add equal volume of RF Absorbent to all sample vials (200µl). Mix well & inc. at RT-15min

Dispense 150µl of P/N, P/P to first 2 paired wells & then samples into each of the paired wells of the Test Plate. Dispense 150µl of internal control and P/P to last 2 paired wells of the plate

- Cover & incubate at 37 oC for 60 minutes
- Wash 4 times with washing solution POD (Diluted 1:20 with Distilled Water)

Add 100 µl of anti Human IgM/POD Conjugate (dilution 1:50 – 100µl of conjugate + 5ml of conjugate buffer)

- Cover & incubate at 37 oC for 60 minutes
- Wash 4 times with washing solution POD

Add 100 µl of working chromogen solution (dilution 1:10 – 0.5ml of chromogen + 5ml of substrate buffer)

Cover & incubate in dark, at room temperature for 30 minutes

Add 100µl of Stopping Solution POD

Evaluate at 450nm, 650nm is recommended as reference wave length

PHOTOMETRIC EVALUATION

$\Delta A = A_{Ag} - A_{control Ag}$

ΔA for P/N should be < 0.1

ΔA for P/P should be > 0.2

ΔA for test $< 0.1 \Rightarrow$ negative

ΔA for test $> 0.2 \Rightarrow$ positive

ΔA for test between 0.1 & 0.2 \Rightarrow Retest in duplicate

New value between 0.1 & 0.2 \Rightarrow Equivocal

5.7 Rubella Serology for IgG detection

5.7.1 Principle

Dade Behring Enzygnost Test - Enzyme Immuno Assay for the detection of IgG antibodies to rubella virus in human serum and plasma. This test is useful for decisions concerning vaccinee and also provides a basis for monitoring immune status before pregnancy.

IgG antibodies contained in the test sample which are specific for the measles virus bind to the antigen in the wells of the test plate. The anti-human IgG/POD Conjugate binds to these specific antibodies.

The enzyme component of the conjugate catalyzes the working chromogen solution, producing a blue color. This reaction is terminated by the addition of the stopping solution with color changing to yellow.

The intensity of the color formed is proportional to the activity of rubella IgG antibodies contained in the sample. The activity is quantified in international units (IU/ml).

5.7.2. Interpretation of results

A **NEGATIVE** qualitative result signifies that rubella specific IgG cannot be detected. The absence does not exclude the possibility of an acute infection in the incubation phase.

If a sample is **POSITIVE** it indicates that virus specific IgG has been detected. They are indicative of a past or acute rubella infection. Immune status after recent vaccinations can also be detected quantitatively.

If a result is **EQUIVOCAL**, it is recommended that follow-up tests should be run particularly in the case of pregnant women.

6. Appendix

6.1 Appendix A – Sample Collection and Storage

6.1.1 For Serology

Serum / Plasma sample

Single sample obtained at first contact with health care system between day 4- 28 after rash onset

Store serum at 4-8 °C for brief periods, if delay is inevitable, freeze at – 20 °C or below

6.1.2 For Virus Isolation

Anticoagulated blood for recovery of lymphocytes and macrophages:

For procedure, refer to appendix B

After separation of lymphocytes, store the buffy coat at -80 °C until use

Nasal Aspirate / Nasopharyngeal swab:

Nasal aspirate should be collected as soon as possible after onset and not longer than 7 days after appearance of rash, when the virus is present in high concentration

Nasal aspirate is collected by introducing a few ml of st. saline into the nose with a syringe fitted with fine rubber tubing and collecting the fluid into a screw capped centrifuge tube containing viral transport medium.

Nasopharyngeal swabs are obtained by firmly rubbing the nasopharyngeal passage and throat with sterile cotton swabs to dislodge the epithelial cells, the swabs are then placed in sterile VTM and refrigerated

Ideal storage is at -70 °C.

Urine

Urine should be collected in the first week after rash onset though up to 16 days is acceptable.

First morning voided specimens are ideal

Collect 10-50 ml of urine, centrifuge at 1500 rpm for 5 minutes at 40 °C to pellet the sediment

Re-suspend the sediment in 2-3 ml of VTM or culture media with antibiotics. Store at -70 °C

Congenital Rubella cases

All of the above specimens and in addition virus may also be isolated from cataract material, lens fluid, CSF and fetal tissues. From infants with congenital rubella, nasopharyngeal, blood, urine and stool specimens can also be used.

6.1.3 For RNA Extraction

- Nasal Aspirate / Nasopharyngeal swab
- Urine
- Dry blood spots

Four finger pricked blood spots collected on specialized filter paper. The blood should cover the entire area on the filter paper on both sides and transported immediately.

Ideal storage is at room temperature.

- Orocol Swabs

Use like a tooth brush for 1 min. in the interface between gums and teeth to collect the cervicular fluid. Wet swab is placed inside the clear plastic transport tube with patient details. Keep at 4-8°C until shipment.

6.2 Appendix B - Isolation of Mononuclear Cells from Circulating Blood

This procedure employs mixture of polysaccharide and a radiopaque contrast medium. HISTOPAQUE-1077 is a solution of polysucrose and sodium diatrizoate, adjusted to a density of 1.077 ± 0.001 g/ml. This medium facilitates rapid recovery of viable mononuclear cells from small volumes of blood.

Anticoagulated venous blood is layered onto HISTOPAQUE-1077. During centrifugation, erythrocytes and granulocytes are aggregated by polysucrose and rapidly sedimented; whereas, lymphocytes and other mononuclear cells remain at the plasma-HISTOPAQUE-1077 interface. Erythrocyte contamination is negligible. Most extraneous platelets are removed by low speed centrifugation during the washing steps.

6.2.1 Procedure

- To a 15 ml conical centrifuge tube, add 3.0 ml HISTOPAQUE – 1077 and bring to room temperature.
- Carefully layer 3.0 ml whole blood (diluted 1:2) onto the HISTOPAQUE-1077. Centrifuge at $400 \times g$ for exactly 30 minutes at room temperature. Centrifugation at lower temperatures, such as 4°C , may result in cell clumping and poor recovery.
- After centrifugation, carefully aspirate, with a Pasteur pipette, the upper layer to within 0.5cm of the opaque interface containing mononuclear cells. Discard upper layer.
- Carefully transfer the opaque interface, with a Pasteur pipette, into a clean conical centrifuge tube.
- Add to this tube (step 4), 10ml Isotonic Phosphate Buffered Saline Solution and mix by gentle aspiration.
- Centrifuge at $250 \times g$ for 10 minutes.
- Aspirate the supernatant and discard.
- Resuspend cell pellet with 5.0 ml Isotonic Phosphate Buffered Saline Solution and mix by gentle aspiration with a Pasteur pipette.
- Centrifuge at $250 \times g$ for 10 minutes.
- Repeat Steps 7, 8 and 9, discard supernatant and re suspend cell pellet in 0.5ml of freezing medium.

6.3 Appendix C – Media Composition

1) DMEM Growth Medium Fetal calf serum	-	50 ml
Penicillin and Streptomycin (100U/ml) (100 mg/ml)	-	5 ml
Fungizone	-	5 ml
Non-Essential amino acids	-	5 ml
L-Glutamine	-	5 ml
Hepes Buffer	-	10 ml
Vitamins	-	5 ml
Sodium bicarbonate (7.5%)	-	5 ml
Make volume upto 500 ml	-	1X

For DMEM maintenance medium– use 10 ml of FCS. Remaining composition is the same.

2) Viral Transport Medium (VTM)		
Veal Infusion broth	-	1 gm
Bovine Serum Albumin	-	2 gms
Gentamycin sulphate	-	0.8 ml
Fungizone	-	3.2 ml

Make volume upto 500 ml and sterilize through .22µm filter.

3) Phosphate Buffered Saline (PBS)		
NaCl	-	8 gms
KCl	-	0.2 gm
KH ₂ PO ₄	-	0.2 gm
Na ₂ HPO ₄	-	1.15 gm

Make volume upto 1 liter and autoclave

4) Trypsin		
Trypsin 1:250	-	0.05% in PBS
Versene (EDTA)	-	0.02% in PBS

Sterilize through .22µm filter

5) Geneticin, 50 mg/ml, pre-prepared liquid is available or alternative.

6.4 Appendix D – Cell Propagation

The Vero/slam cell line can be used to isolate rubella viruses from clinical specimens with a sensitivity that is similar to that of standard Vero cells or RK13 cells. Unlike measles virus, rubella virus from clinical specimens does not produce cytopathogenic effect (CPE) in the vast majority of cases, even after several passages. These cells are Vero cells, which have been transfected with a plasmid encoding the gene for the human SLAM (signaling lymphocyte-activation molecule) protein. SLAM has been shown to have a receptor for both wild type and laboratory adapted strains of rubella.

Upon receipt from a WHO source, the cells should be passaged in a medium containing 40 ug/ml Geneticin. The cell grows gently attached to the culture surface when grown in DMEM supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 0.25 µg/ml amphotericin (fungizone), and fetal bovine serum. Cell growth is sustained by adding 10% fetal bovine serum (FBS). Laboratories should passage the cells 2-4 times in the presence of Geneticin to prepare cell stock for liquid nitrogen storage. However for routine virus isolation purpose, recovered cells can be passaged up to 15 times in maintenance medium without Geneticin and 2% FBS.

The Vero/slam cells can be passaged by briefly treating the cell monolayers with 0.05% Trypsin – EDTA to release cells from tissue culture surface. Be careful not to over trypsinize. Neutralize Trypsin by adding DMEM containing FBS. Usually the cells from a single monolayer culture can be split 1:3. One will notice that more cells tend to become “floaters”, growing in clumps suspended in the medium as the cell density increases. These cells are viable and can be passaged by gently pipetting to break up the clumps then replating to a lower cell density.

Cells can be transported in a T-75 or T-25 tissue culture flask with additional medium added to help keep cells attached. Upon arrival, look at the cell sheet. If many cells are free-floating, a light spin of the medium will recover cells, which can be added back to the flask (or to another flask for passage). Add 30 – 35 ml of the medium back to the flask for maintenance. Grow cells in a moist CO₂ incubator at 37 oC. Cell stocks can be frozen using standard cryoprotection medium (20% FBS, 10% DMSO).

6.5 Appendix E - Cell Counting

A typical method for enumerating cell concentration using “improved neubauer” haemocytometer is given below:

1. Dilute 0.2 ml of the cell suspension in 0.2 ml of trypan blue (i.e. 0.1% solution in PBS); non-viable cells are stained blue.
2. Immediately mix well with a fine Pasteur pipette and aspirate sufficient volume to fill both sides of the haemocytometer chamber:
3. Count viable cells in each of the four corner squares bordered by triple lines, omitting cells lying on these lines (see Figure 4.2). This is repeated for the second side of the chamber. N.B: cells counts of less than fifty cells are unlikely to be reliable.
4. If a marked degree of cell “clumping” (aggregation) is observed, discard and re-suspend the original cell suspension.
5. Calculate the mean count of the total viable cells per four corner squares (NB: Viable cells are not stained by Trypan blue)
6. Count and calculate the mean count of the other half of the counting chamber. For a valid test, the results of the two counts should be within 20% of the mean value.
7. Calculate the viable cell concentration per ml using the following formula:

$$C_1 = t \times tb \times 1/4 \times 10^4$$

t	=	total viable cell count of four corner squares
tb	=	correction for the trypan blue dilution (counting dilution was $1/tb$)
$1/4$	=	correction to give mean cells per corner square
10^4	=	conversion factor for counting chamber.
C_1	=	initial cell concentration per ml

Example: $t = 480$; $tb = 2$; $C_1 = 480 \times 2 \times 1/4 \times 10^4$ cells per ml.

6.6 Appendix F - Preservation of Cell Cultures

It is possible to maintain stocks of cells in a viable state for long periods at low temperatures by the addition of a cryoprotectant such as dimethyl sulphoxide (DMSO) to the cell growth medium. The essential features of the method are to *freeze the cells slowly* (i.e. at approximately $-1^{\circ}\text{C}/\text{min}$), keep them at a temperature below -70°C while frozen and the *thaw* them rapidly ready for the preparation of fresh cell culture stocks. Long-term storage can only be achieved reliably when cells are stored at or below -135°C .

Freezing cells

- a) Use only cultures of cells that are in a healthy state (i.e. rapidly growing but not completely confluent).
- b) Detach cells with Trypsin (or Trypsin / Versene). Use sufficient flasks to yield a minimum of 4×10^6 cells/ml in the final cryoprotectant solution.
- c) Re-suspend cells in growth medium; centrifuge at $100 \times g$ for 10 minutes.
- d) Discard supernatant and resuspend thoroughly the cell pellet in growth medium containing 20% fetal calf serum and 10% (v/v) dimethyl sulphoxide.
- e) Dilute 0.1 ml cell suspension in trypan blue and count cells in a haemocytometer.
- f) Adjust cell concentration to $4-8 \times 10^6$ cells/ml in growth medium containing DMSO.
- g) Dispense in 1 ml or 2 ml volumes in clearly labelled (cell name, lab. Origin, passage number and date of freezing) screw-capped vials.
- h) Freeze vials slowly. Ideally the temperature should drop at $1^{\circ}\text{C}/\text{minute}$. Place vials in the special container that holds them in the gaseous phase of the liquid nitrogen vessel. Commercial devices are available for which a formula is supplied by the manufacturer for the level vials are held, number to be stored and length of time required to achieve this temperature drop. Alternatively, place vials wrapped in paper towels or cotton wool in a polystyrene container with a wall thickness of $\sim 25\text{mm}$ and place this in the -70°C freezer overnight.
- i) Transfer the vials to the liquid nitrogen (-196°C) storage containers. For long-term storage of cells (i.e. a period of years) liquid nitrogen storage is ideal.

Thawing cells

- a) Remove vial from liquid nitrogen and transfer immediately to a water bath or preferably a beaker of sterile water at 36°C .
- b) When contents are completely thawed, wipe outside of vial with alcohol to reduce bacterial contamination, transfer cell suspension to culture flask. Add drop-wise, sufficient growth medium for the production of a cell monolayer (typically 10- 15ml in a flask of 25cm^2 surface area). The viability of the thawed cells may be significantly reduced if growth medium is added rapidly at this delicate stage.
- c) Incubate bottle until cells are adherent (6 – 8 hours) or overnight at 36°C .
- d) Carefully decant medium (to get rid of DMSO present) and add fresh growth medium.

6.7 Appendix G - Rubella Virus PCR

Rubella virus extraction

Extraction: As mentioned for measles. (Use of **Qiagen RNA Mini-Kit**)

Method obtained from **CDC Rubella Laboratory**.

One Step RT-PCR Procedure by the use of: **Superscript One-Step (Invitrogen)**

Set up Superscript One-Step reactions:

Reagents:	1X
RNase & DNase Free H ₂ O	19.5ul
2X Reaction Buffer	25ul
Forward Primer (RV11- Geneva/ RV71-Proligo) 20uM	0.5ul
Reverse Primer (RV12- Geneva/ RV72- Proligo) 20uM	0.5ul
RNase Inhibitor (40U/ul- Invitrogen / Roche)	0.5ul
SS/Taq Enzyme Mix	1ul
RNA Template	3ul
Total	50ul

Primer used to make a 185bp fragment (8812-8997, E1 coding region)

1. RV11/ RV71 (forward): **5' CAACACGCCGCACGGACAAC 3'** (20mer)
2. RV12/ RV72 (reverse): **5' CCACAAGCCGCGAGCAGTCA 3'** (20mer)

Cycle Parameters: Program # 80

- 1 cycle 55°C for 30 minutes.
- 1 cycle 49°C for 2 minutes.
- 40 Cycles:
 - 95°C for 30 seconds.
 - 60°C for 20 seconds.
 - 72°C for 1 minute.
- Final extension 72°C for 5 minutes and 4°C on Hold.

Finally prepare and run the Gel:

- 1.5% of Agarose gel.
- Load 15ul of 100bp MW.
- Load 10ul sample + 2ul of loading buffer.
- Run at 100V.
- Run for 30 minutes.

6.8 Appendix H - Rubella IgM Serology Worksheet

POD BUFFER

Volume added to control vials – _____

Volume added to sample vials – _____

RF ABSORBENT

Volume added to sample vials – _____

CONTROL / SAMPLES

Volume of P/P – _____

Volume of P/N – _____

Volume of samples – _____

ELISA SET UP

Volume added - _____ Time - _____

INCUBATION

Period - _____ From _____ to _____ Temperature - _____

WASHING

Time - _____ No. of cycles - _____

CONJUGATE

Dilution - _____ Volume added - _____ Time - _____

INCUBATION

Period - _____ From _____ to _____ Temperature - _____

WASHING

Time - _____ No. of cycles - _____

SUBSTRATE

Dilution - _____ Volume added - _____ Time - _____

INCUBATION

Period - _____ From _____ to _____ Temperature - _____

STOPPAGE

Volume added - _____ Time - _____

READING

Wavelength - _____ Time - _____

SIGNATURE

6.9: Appendix I: Reagents, kits & Solutions

No	Item Description	Company/Source	Catalog No. and specification
1	Dulbecco's Modified Eagle Medium (D-MEM), liquid 1X (High glucose), with L-glutamine, 4500 mg/L D-glucose, 25 mM HEPES	GIBCO German	CAT: 42430-025
2	Antibiotic – Antimycotic , prepared with Penicillin/Streptomycin solution, liquid , 10.000units/ml penicillin 10.000µg/ml streptomycin sulfate and 25 µg/ml amphotericin B as fungizone in 0.85% saline	GIBCO German	15240-062
3	Trypsin –EDTA (10X) solution	GIBCO German	CAT: 15400-054
4	Geneticin, selective antibiotic, liquid, 50mg/ml aseptically filtered	Gibco German	10131-027
5	Filter paper dried blood spot collection kits for serological test.	Whatman company German	To get about 50ul of serum
6	Qiagen one step RT-PCR Kit	QIAGEN	210210
7	QIAamp Viral RNA Mini Kit	QIAGEN	52904
8	Anti-measles IgM kits, Indirect IgM (ELISA)	DADE BEHRING USA	OWLI 15
9	Anti-Rubella IgM kits (Indirect ELISA IgM)	DADE BEHRING USA	OWBO 15
10	Anti-Rubella IgG KITS	DADE BEHRING USA	OWBF 15
11	Supplementary Reagents for Enzygnost	DADE BEHRING USA	OUPV17

Manipulation (SOP for preparation):

All reagents for tissue culture should be prepared aseptically in a clean tissue culture cabinet with dedicated equipment

Calibration:

The calibration for the pipettes conducted at CPHL; regarding the devices and biosafety cabinet calibrated by our engineer /maintenance unit.

7. References

1. Centers for Disease Control and Prevention. Isolation and Identification of Measles Virus.<http://www.cdc.gov/ncidod/dvrd/revb/measles/man.htm>
2. Centers for Disease Control and Prevention. Epidemiology and Prevention of Vaccine-
3. Guidance on regulations for the Transport Infectious substances 2011-2012 (WHO/HSE/IHR/2010.8)
4. Guidelines for Biosafety in Teaching Laboratories (American Society for Microbiology 2012)
5. Preventable Diseases. Atkinson W, Hamborsky J, McIntyre L, Wolfe S, eds. 10th ed. Washington DC: Public Health Foundation, 2007.
6. World Health Organization. Western Pacific Regional Plan of Action for Measles Elimination. Manila: WHO, 2003.
7. Manual for the laboratory diagnosis of measles viral infection. Geneva, World Health Organization, 2007. WHO/IVB/07.01.