



PRIMARY HEALTH  
CARE PROJECT



# Iraqi Standard Operating Procedure (S.O.P)

## Serological Investigation and Virus Isolation of Measles

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.....	2
1. Introduction:.....	4
1.1. Outbreaks in IRAQ:.....	4
1.2. National Measles Laboratory (NML):.....	4
2. Purpose:.....	5
3. Responsible positions: .....	5
4. Functions:.....	5
5. Sampling & Documentation: .....	5
5.1. Serum: .....	5
5.2. Urine:.....	5
5.3. Gum Swab (Oral fluid):.....	6
5.4. Nose and throat swabs:.....	6
6. Shipment:.....	6
6.1. Packaging .....	6
6.2. Marking .....	7
6.3. Labeling and Documentation .....	8
7. AGENT & PRECAUTIONS .....	8
7.1. Agent:.....	8
7.2. Employees at risk: .....	8
7.3. Bio-Safety Issues:.....	8
7.4. Vaccinations: .....	9
7.4.1. Vaccination Implementation.....	9
7.4.2. Vaccination doses .....	9
7.5. Assay Precautions: .....	9
8. DIAGNOSTIC PROCEDURES:.....	10
8.1. General Issues: .....	10
8.2. Virus Isolation Steps: .....	10
8.3. VERO/slam cells .....	10
8.4. Contaminated samples:.....	11
8.5. Immunofluorescence: .....	11
8.6. Measles Serology for IgM detection: .....	11
8.6.1. Principle:.....	11
8.6.2. Interpretation of results:.....	12
8.6.3. Procedure: .....	12
8.7. Measles Serology for IgG detection.....	13

8.7.1. Principle:.....	13
8.7.2. Interpretation of results:.....	13
8.8. MEASLES VIRUS PCR.....	13
8.8.1. Measles virus extraction:.....	13
8.8.2. Principle:.....	13
8.8.3. Procedure:.....	14
8.9. Standard Measles RT-PCR Reaction:.....	14
8.9.1. Materials:.....	14
9. APPENDIX.....	18
9.1. APPENDIX A – SAMPLE COLLECTION AND STORAGE.....	18
9.1.1. FOR SEROLOGY.....	18
9.1.2. FOR VIRUS ISOLATION.....	18
9.1.3. FOR RNA EXTRACTION.....	18
9.2. APPENDIX B - ISOLATION OF MONONUCLEAR CELLS FROM CIRCULATING BLOOD.....	19
9.2.1. Procedure:.....	19
9.3. APPENDIX C – MEDIA COMPOSITION.....	20
9.4. APPENDIX D – CELL PROPAGATION.....	21
9.5. APPENDIX E- CELL COUNTING.....	22
9.6. APPENDIX F - PRESERVATION OF CELL CULTURES.....	23
9.7. APPENDIX G - MEASLES IgM SEROLOGY WORKSHEET.....	24
9.8. APPENDIX H - REAGENTS, KITS & SOLUTIONS.....	24
10. 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

## 1. Introduction:

Measles is a highly infectious respiratory virus infection, with typical clinical symptoms including maculopapular rash, fever, cough, coryza, and conjunctivitis. The causative agent of the disease, measles virus (MV), is a negative-strand RNA virus of the genus *Morbillivirus*, family Paramyxoviridae; it has a single serotype with 23 recognized genotypes. Typically, the disease is characterized by a two-phase course. After an average incubation period of 8 to 11 days, initial symptoms such as fever, cough, coryza and conjunctivitis appear. Two thirds of the patients show a white-marked enanthema on the buccal mucosa (Koplik's spots). After disappearance of these symptoms, a second increase of temperature and the typical measles exanthema, a brownish-red maculopapular rash, appear. Infection with measles virus induces transient immunodeficiency that favours the formation of several complications. Some of them, e. g. encephalitic diseases, are severe and associated with a high mortality. Measles are worldwide distributed and belong to the ten most frequent infectious diseases in some less developed countries. The disease is associated with a high mortality in some African and South-East Asian countries, in particular in children aged less than 12 months.

Of particular note, Measles continues to be a major cause of childhood morbidity and mortality worldwide, with an estimated one million fatal cases each year. Although the introduction of live attenuated MV vaccines has largely abrogated the endemic circulation of wild-type MV in the world, on average, each infected person infects nearly 20 others. This is why 95% population immunity is needed to interrupt transmission and hence eliminate measles.

### 1.1. Outbreaks in IRAQ:

Measles is a highly contagious disease; it is endemic in Iraq since decades, the pattern of outbreak every 4 to 5 years.

- **2008:** Iraq faced a huge measles outbreak (38572) reported cases as aggregate data since early 2008, started from the most insecure province (AL-Anbar) in the western part & continue to disseminate to Salahuddin, Kirkuk, Ninawa) during the first half of 2008 it continue to affect the southern governorates in the second half of 2008 then to disseminate to the most eastern & middle governorates during 2009. Out of the (38572) measles cases reported, 18099 have detailed case investigation forms completed, 5000 blood sample were tested.
- **2012:** The second measles outbreak in Iraq was reported at the end of 2012 at Dahuk/Domize camp among Syrian refugee & continue to disseminate to Erbil, Kirkuk, Ninawa till now the virus circulated.

### 1.2. National Measles Laboratory (NML):

NML is part of Virology Department at the Central Public Health Laboratory, it is established since 2004, it is responsible for testing specimens ( Serum , Urine, Gum swab ) related to suspected Measles cases that are referee from all Iraqi governorates ( Hospitals , Primary Health Care ) to confirm the 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 Measles

diagnosis. For the purpose of setting the correct and proper laboratory work in the foundations of the workers in the field of microbiology diagnosis, including diagnosis of Measles virus, it have been prepared in this Standard Operating Procedure (S.O.P.) reference to be Iraqi integrated for workers in this area.

## **2. Purpose:**

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

## **3. Responsible positions:**

- M.Sc./Microbiology/ Director of NML
- B.Sc. of biology (Staff of NML 3)

## **4. Functions:**

- a) Collection and transportation of a serum, urine & 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.

## **5. 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.

### **5.1. Serum:**

A single serum specimen when collected between 72 hours and four weeks after rash onset can be used to diagnose most measles 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.

### **5.2. Urine:**

The isolation of measles 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 10-50ml 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.

### **5.3. Gum Swab (Oral fluid):**

Samples were collected using a foam swab attached to a handle (ORACOL;Malvern Medical Developments, Worcester, United Kingdom). Participants were instructed to rub the swab along the teeth/gum line for approximately 1min, after which the swab was returned to the plastic tube with the foam swab uppermost and transported to the laboratory at 4°C, after which time oral fluid was collected into the bottom of the plastic tube by centrifugation at 3,000 \_ g for 15 min.

### **5.4. Nose and throat swabs:**

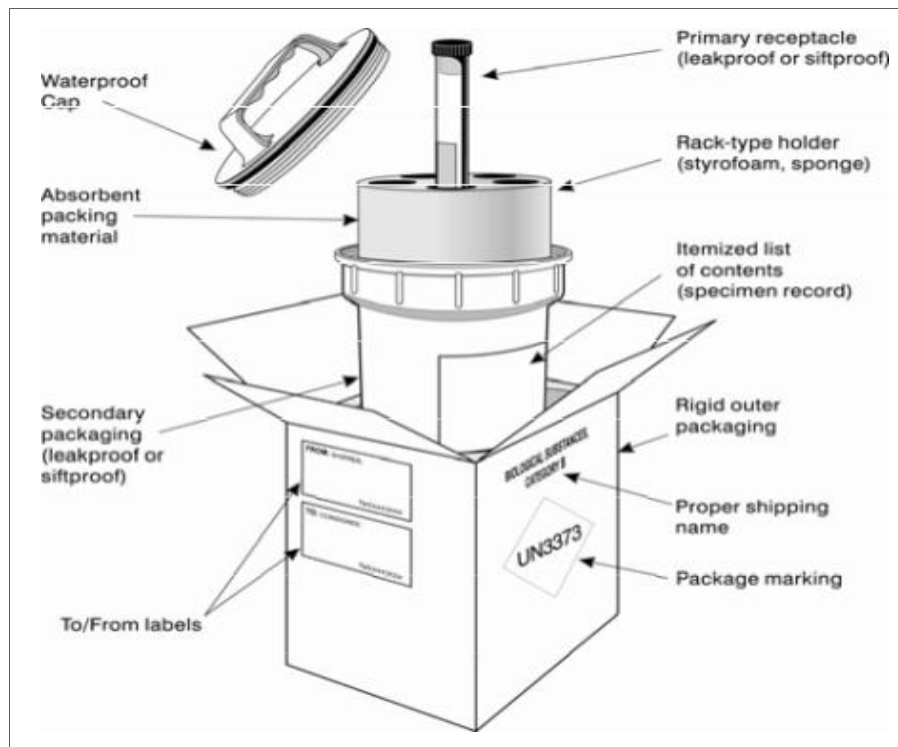
They were pooled in 2.0 ml of viral transport medium at the time of collection, transported to the laboratory at 4°C, tested immediately by PCR, and then stored at \_70°C and arrive at the testing laboratory within 48 hours maximum quantity per package; for air transport.

## **6. Shipment:**

Packaging, Marketing, labeling and documentation.

### **6.1. 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.
- e) 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.

## 6.2. 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) Colour: none specified, provided the mark is displayed on the external surface of the outer packaging on a background of contrasting colour 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 (see section on Refrigerants), the label shown in Figure 4 shall be applied for cryogenic liquids (see section on Refrigerants) the labels shown in Figures 5 and 6 shall also be affixed.

### **6.3. Labeling and 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.

## **7. AGENT & PRECAUTIONS**

### **7.1. Agent:**

Measles or rubeola, genus Morbillivirus, Family Paramyxoviridae.

### **7.2. 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.

### **7.3. Bio-Safety Issues:**

Clinical samples may contain measles 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.

## 7.4. Vaccinations:

Everyone handling clinical specimens must be vaccinated against measles after evaluation of their immune status against measles. 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 measles virus.

### 7.4.1. Vaccination Implementation

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

### 7.4.2. 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 measles will receive two vaccines given 4 weeks apart.
- Even though it is believed that lifelong immunity results from infection, all employees in the measles program will have their titers re-evaluated regularly.

## 7.5. 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 that 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.
- h) Cell cultures are easily contaminated with viruses that have been passed in cell culture.
- i) Therefore, always work with the sample that has the least amount of virus first (usually this is the clinical sample).
- j) 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.
- k) Note that aliquoting first pass is working with a virus in high concentration and the hood.

- l) 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.
- m) Spray your gloves with ethanol after manipulating each first pass isolate.
- n) Measles and most of the viruses may be extremely labile.
- o) Keep samples containing virus on ice.
- p) Dilute virus in polypropylene tubes, **not polystyrene**.

## 8. DIAGNOSTIC PROCEDURES:

### 8.1. General Issues:

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

### 8.2. Virus Isolation Steps:

- a) Obtain cells in Tissue Culture Flasks.
- b) Vero/slam cells should be 70 – 90 % confluent.
- c) Prepare samples: (Refer to 3.3.6, No.2)
- d) Thaw samples. Keep on ice – never bring the samples to room temperature.
- e) Vortex gently (avoid making foam) for 30 seconds. If the swab does not permit the sample to vortex, agitate it.

### 8.3. 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 CO<sub>2</sub> 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, (syncitium 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.

#### 8.4. 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.

#### 8.5. 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.
- l) Positive cells will show a granular green fluorescence in the cytoplasm and the Evans blue counter stain will appear red.

#### 8.6. Measles Serology for IgM detection:

##### 8.6.1. Principle:

Dade Behring Enzygnost Test - Enzyme Immuno Assay for the detection of human IgM antibodies to measles 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 upto 15mg 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 colour is formed which is read at 450nm. 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.

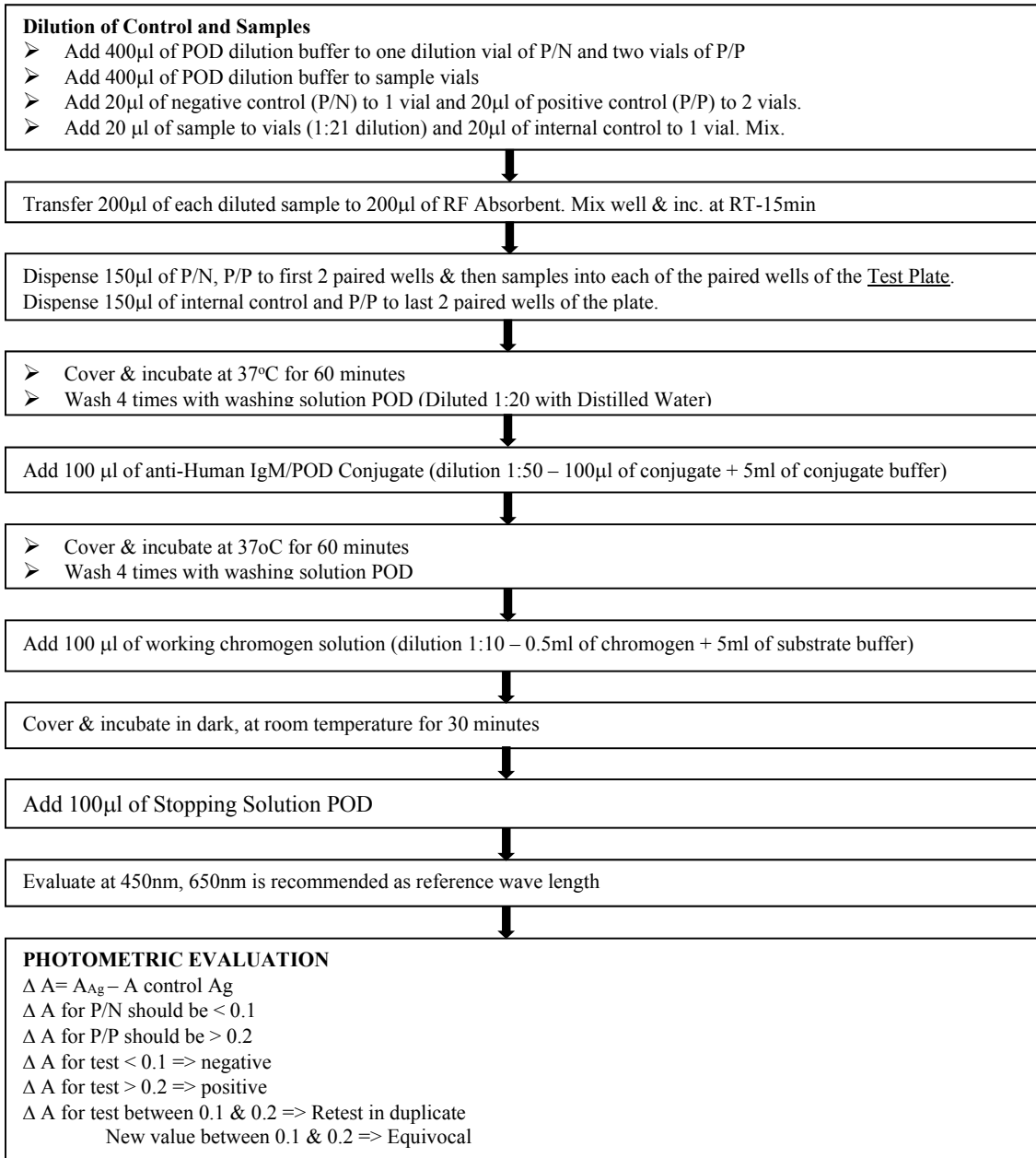
### 8.6.2. Interpretation of results:

A **NEGATIVE** result means that virus specific IgM cannot be detected. The patient either is NOT acutely infected with measles 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 measles patients, anti-measles 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.

### 8.6.3. Procedure:



## 8.7. Measles Serology for IgG detection

### 8.7.1. Principle:

Dade Behring Enzygnost Test - Enzyme Immuno Assay for the detection of IgG antibodies to measles virus in human serum and plasma. This test is useful for decisions concerning vaccine 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 colour. This reaction is terminated by the addition of the stopping solution with colour changing to yellow.

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

### 8.7.2. Interpretation of results:

A **NEGATIVE** qualitative result signifies that measles 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.

## 8.8. MEASLES VIRUS PCR

### 8.8.1. Measles virus extraction:

The QIAamp Viral RNA Mini Spin Kit (Qiagen) is used for virus extraction.

Make sure if samples are frozen, they should be thawed on ice (this is to avoid viral disruption).

### 8.8.2. Principle:

The kit combines the properties of silica-gel-based membrane with the speed of microspin or vacuum technology to purify viral RNA. Samples are first lysed under highly detergent conditions to inactivate RNases and with the application of certain buffering conditions; RNA is able to bind to the QIAamp membrane. Contaminants are then washed away in two steps using two different wash buffers, thus, the template RNA is efficiently free of proteins, nucleases and other contaminants. Then, the QIAamp membrane generates extremely high recovery of intact RNA in twenty minutes without the use of hazardous substances like phenol-chloroform.

### 8.8.3. Procedure:

- Pipette 560ul of prepared Buffer AVL containing Carrier RNA into a 1.5 ml micro-centrifuge tube.
- Add 140ul plasma, serum, urine, cell-culture supernatant or cell-free body fluid to the Buffer AVL/Carrier RNA in the micro centrifuge tube. Mix by pulse-vortexing for 15 sec.
- Incubate at room temperature (15-25 °C) for 10 min.
- Briefly centrifuge tube to remove drops from the inside of the lid.
- Add 560ul of ethanol absolute (96-100%) to the sample, and mix by pulse-vortexing for 15 sec. After mixing, briefly centrifuge tube to remove drops from inside the lid.
- Carefully apply 630ul of the solution from step 5 to the QIAamp spin column (in a 2-ml collection tube) without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp spin column into a clean 2-ml collection tube, and discard the tube containing filtrate.
- Carefully open the QIAamp spin column and repeat the previous step.
- Carefully open the QIAamp spin column and add 500ul of Buffer AW1. Close the cap and centrifuge at full speed 6000 x g (8000 rpm) for 1 min. Place the QIAamp spin column in a clean 2-ml collection tube (provided) and discard the tube containing the filtrate.
- Carefully open the QIAamp spin column, and add 500ul of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g (14,000rpm) for 3 min.
- If concerned about any chance of possible carryover of Buffer AW2, then try to eliminate by placing the QIAamp spin column in a new 2ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
- Place the QIAamp spin column in a clean 1.5ml micro-centrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp spin column and add 60ul of Buffer AVE equilibrated to room temp. Close the cap, and incubate at room temp for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 min.
- Viral RNA is stable for up to one year when stored at -20 or -70°C.

## 8.9. Standard Measles RT-PCR Reaction:

### 8.9.1. Materials:

- 10X PCR Buffer.
- 100mM MgCl<sub>2</sub>.
- dNTP (10mM stock)
- Forward and Reverse Primers.
- RNA (on Ice) → this is your extracted RNA.
- DEPC-H<sub>2</sub>O
- Sterile 1.5ml and 0.2ml tubes (PCR tubes), pipet tips.
- Thermocycler (remember to set the program and note it for your future uses).
- RNase Inhibitor and Reverse Transcriptase (RT) (both should be kept on ice).
- Taq Polymerase (DNA polymerase) (don't take it out until your cDNA is finished, i.e. produced, and ready to make MIX # 2) (always keep on ice).
- Remember never to vortex your enzymes.

**Step 1: DNA reaction:**

**MIX # 1:**

Solution	Amounts	Notes
10X PCR Buffer	2ul	10X PCR:500mM KCL, 100mM Tris-CL [pH 8.3]
100mM MgCl <sub>2</sub>	1ul	15mM MgCl <sub>2</sub> , 0.1%gelatine
dNTP (10mM stock)	2ul	Any company
Forward Primer (20uM)	1ul	MV41
Reverse Primer (20uM)	1ul	MV42
RNA extract up to 5-6ug (up to 13ul)	X ul	1 tube for each nucleotide, then you must add 2ul from each tube (8ml total)
RNase free H <sub>2</sub> O	13-X ul	If so, you can add only up to 7ul of RNA
Total Volume =	20ul	And 7- X ul of H <sub>2</sub> O

**Remember:** RNA is volatile. Prepare mixture of 10X buffer, MgCl, dNTPS, water and primers at room temp. place everything on ice and allow sufficient time to cool. Then add RNA and proceed with the rest of the procedure immediately.

**To prepare 20uM Primers:**

Our stock is 435uM of MV41 →  $IC \times IV = FC \times FV$   
 $435uM \times V1 = 20uM \times 50ul$   
 $= 2.3 \text{ of stock} + 47.7ul \text{ of H}_2\text{O}$

Stock is 549uM of MV42 →  $IC \times IV = FC \times FV$   
 $549uM \times IV = 20uM \times 50ul$   
 $= 1.8ul \text{ of stock} + 48.2ul \text{ of H}_2\text{O}$

**NOTE:** When working with your samples, include appropriate controls such as, known Positive, negative, cell (according to cell line used, if extracted sample is an isolate) and reagent control. This is needed for each set of amplification primers.

**Step 2:** Heat the reaction tubes (with RNA and Mix # 1) to 75°C for 90 seconds.

**Step 3:** Cool slowly for 5-10 minutes on the bench, then place on ice.

**Step 4:** While on ice Add: (RT-REACTION)

0.5-1ul (20units) RNase Inhibitor (Human placental ribonuclease inhibitor)

(Keep on ice)

0.5-1ul (10 units) reverse transcriptase (keep on ice)

**Step 5:** Incubate at 42°C for 45 minutes

**Step 6:** Heat to 75°C for 5 minutes then place on ice.



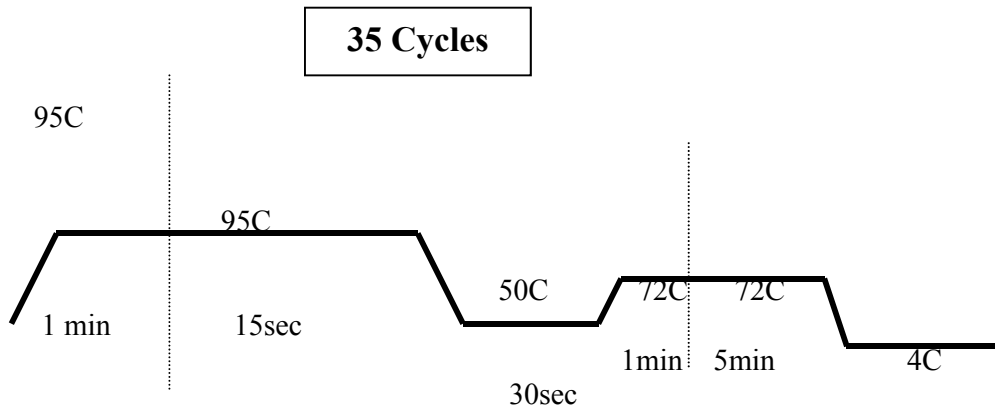
**Step 7: PCR REACTION:**

**MIX # 2:**

	For Whole Reaction (100ul)	For Half Reaction (50ul)
cDNA reaction	20ul	10ul
Forward primer (20uM)	4ul	2ul
Reverse Primer (20uM)	4ul	2ul
10X PCR buffer	8ul	4ul
H2O	64ul	32ul
Taq Polymerase	1ul	1ul

If amplicon product is needed for subsequent sequencing and cloning, then you will need to prepare the 100ul reactions.

Suggested cycling parameters:



Another way of performing measles virus PCR of is to use the “SUPERSCRIT RT-PCR PROTOCOL (RSK/PAR 7/00)”

Using the nested PCR is suggested to be a better way of obtaining a better yield of measles amplicon

**STEP-8: GEL PREPARATION:**

For Agarose Gel Preparation:

**Preparation of 10X TBE (pH 8.4) Gel & Electrophoresis Buffer:**

- Tris Base 108 g
- Boric Acid 55g
- 0.5M EDTA 40 ml (pH8.0)
- Water to 1.0 L

**Buffer preparation:**

Dissolve everything in 2L beaker and add 800ml of d.H<sub>2</sub>O.

Then adjust the volume to 1 liter by adding d.H<sub>2</sub>O.

Adjust pH to 8.4

(There is no need to sterilize the solution. If you notice that white clumps begin to precipitate in the solution, place the bottle in hot water until the clumps dissolve- technically described as the declumping procedure). The solution can be stored at room temperature. Some people report that autoclaving and storing in a refrigerator prevents precipitation.

To use as a buffer, dilute the 10 X stock 10-folds. Commercially available concentrated TBE may be 5X or 20X. Dilute accordingly to make a 1X stock.

**Agarose Gel Preparation:**

In the CPHL laboratory we use 3% of Agarose gel for the detection of nucleotides larger than 200bp:

- 1.5g of Amplizised Agarose + 50 ml of 0.5X TBE Buffer.
- Boil the mixture and cool to 55°C and add 3ul of 10mg/ml of Ethidium Bromide.

**STEP-9: Loading Samples:**

- 10ul of sample + 2ul of loading buffer.
- 5ul of marker (100bp ladder) + 5ul of loading buffer.

**STEP-10: Running the Gel:**

Run the gel with a 100V for 50-60 mins, till the dimmer bands get to almost the end of the gel. Then check your gel.

**STEP-11: Analyzing Results:**

The expected product size should be 333bp.

## 9. APPENDIX

### 9.1. APPENDIX A – SAMPLE COLLECTION AND STORAGE

#### 9.1.1. 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

#### 9.1.2. FOR VIRUS ISOLATION

- Anticoagulated (Heparin) blood for recovery of lymphocytes and macrophages:  
Virus can also be isolated from lymphocytes. Collect 5 ml of heparinized blood and store at 4°C for transport immediately.  
For procedure of lymphocyte separation, refer to appendix B  
After separation of lymphocytes, store the buffy coat at -80°C until inoculation.
- 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. Keep all specimens at 4°C and ship as soon as possible on wet ice.  
Inoculate into cell lines immediately on receipt or store is at -80°C
- Urine: Urine should be collected in the first week after rash onset. First morning voided specimens are ideal. Collect 10-50 ml of urine. It is best to centrifuge at 1500 rpm for 5 minutes at 4°C soon after collection to pellet the sediment. Re-suspend the sediment in 2-3 ml of VTM or culture media with antibiotics. Store at -70°C and ship on dry ice and inoculate immediately. However, if urine has not been centrifuged, store at 4°C and ship immediately on dry ice.

#### 9.1.3. 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.

## 9.2. 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.

### 9.2.1. 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  $4^{\circ}\text{C}$ , 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.

### 9.3. 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 <sub>2</sub> PO <sub>4</sub>	-	0.2 gm
	Na <sub>2</sub> HPO <sub>4</sub>	-	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.

## 9.4. APPENDIX D – CELL PROPAGATION

The Vero/slam cell line is useful in measles virus isolation with syncytial cytopathogenic effect (CPE) sometimes visible as early as 48 hours after inoculation. 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 measles.

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 upto 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 CO<sub>2</sub> incubator at 37°C. Cell stocks can be frozen using standard cryoprotection medium (20% FBS, 10% DMSO).

## 9.5. APPENDIX E- CELL COUNTING

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

- a) Dilute 0.2ml of the cell suspension in 0.2 ml of trypan blue (i.e. 0.1% solution in PBS); non-viable cells are stained blue.
- b) Immediately mix well with a fine Pasteur pipette and aspirate sufficient volume to fill both sides of the haemocytometer chamber:
- c) Count viable cells in each of the four corner squares bordered by triple lines, omitting cells lying on these lines. This is repeated for the second side of the chamber. N.B: cells counts of less than fifty cells are unlikely to be reliable.
- d) If a marked degree of cell “clumping” (aggregation) is observed, discard and re-suspend the original cell suspension.
- e) Calculate the mean count of the total viable cells per four corner squares (NB: Viable cells are not stained by Trypan blue)
- f) 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.
- g) 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  
 $C_1 = 2.4 \times 10^6$  cells/ml

- h) Calculate the dilution factor (d) to obtain the working cell concentration per ml ( $C_2$ ).

Example: Adjust the cell count to  $C_2 = 2 \times 10^5$  cells / ml

$$d = C_2/C_1$$
$$d = \frac{2 \times 10^5}{2.4 \times 10^6}$$
$$d = 1/12$$

(The working concentration can be obtained by mixing 1 volume of the original cell suspension with 11 volumes of the growth medium)

## 9.6. 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  $-70^{\circ}\text{C}$  while frozen and then 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  $-135^{\circ}\text{C}$ .

### 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  $4 \times 10^6$  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  $-70^{\circ}\text{C}$  freezer overnight.
- i) Transfer the vials to the liquid nitrogen ( $-196^{\circ}\text{C}$ ) 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  $36^{\circ}\text{C}$ .
- 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  $25\text{cm}^2$  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  $36^{\circ}\text{C}$ .
- d) Carefully decant medium (to get rid of DMSO present) and add fresh growth medium.



## 9.7. APPENDIX G - MEASLES 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

## 9.8. APPENDIX H: 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.

## 10. References:

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