Annex 7.1

Protocol for amplification of measles sequencing window (N-450)

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

CDC protocol, version 01/26/2015: **RT-PCR Protocol for Measles Genotyping (Version 2)**

Purpose

The following protocol is to be used for the amplification of a fragment of the measles virus (MeV) nucleoprotein (N) gene. This DNA fragment may be used for the genetic characterization of measles virus. The protocol can also be used for detection of MeV RNA; however, this reaction is less sensitive than real-time RT-PCR. For this procedure, RNA is extracted from a clinical sample or from cell culture.

This is a one-tube reaction, so there is a minimum of specimen handling. Primers and control RNA are supplied by CDC as a kit.

Important: This is a general protocol for use with the measles genotyping kit supplied by CDC. Please check the package insert of the kit for updates to the protocol.

What to do with the PCR products

Analyze PCR products on agarose gels (See Agarose Gel Electrophoresis, annex xx). Laboratories with sequencing capacity should purify and sequence the PCR products (See protocols for Clean-up of PCR Products and Measles Sequencing Reactions). Laboratories without sequencing capacity should ship the PCR product to the regional reference laboratory for sequencing. PCR products can be shipped without drying or purification. Transfer the PCR reaction into a 1.5 ml tube, close the lid, seal with parafilm and ship at ambient temperature. PCR products are stable at ambient temperature for at least one month.

Reagents and materials needed

- 70% ethanol
- Lab coat
- Gloves
- RNase inhibitor 2000 units (Life Technologies N8080119)
- RNaseZap (Sigma, # R2020-250ML)
- Sterile 1.5 ml microcentrifuge tubes
- Nuclease-free, deionized water (NF water)
- Qiagen One-step RT-PCR kit (Qiagen CAT# 210210 or 210212)
- Autoclaved PCR tubes (0.2 ml, thin-walled)
- Measles Genotyping RT-PCR Kit, Version 2.0 (primers and control RNA)
- **Equipment needed**

- -70℃ and -20℃ freezers
- Bucket with ice
- Class II biological safety cabinets (BSC) or PCR workstations with UV light
- Micropipettors and sterile pipette tips with aerosol-resistant filters
- Vortex mixer
- Metal cooling rack for 0.2 ml tubes
- Water bath at 50°C
- Thermocycler (e.g. AB GeneAmp PCR System 9700)
- Microcentrifuge, refrigerated to 4°C with rotor for 1.5 ml tubes and 0.2 ml tubes

Recommendations for working with RNA

- Use dedicated equipment, rooms and biosafety cabinets for all pre-PCR procedures. Post amplification analysis and processing should be performed in a separate room using dedicated equipment. Do not share equipment (including lab coats) between pre-PCR and post-PCR procedures.
- Wear gloves throughout experiments to prevent contamination from RNases found on human hands.
- Change gloves after touching skin (e.g. your face), door knobs, and common surfaces.
- Have a dedicated set of pipettors that are used solely for RNA work.
- Use filter tips and tubes that are tested and guaranteed to be RNase-free.
- Use RNase-free chemicals and reagents.
- Reduce RNase contamination by cleaning tube racks, micropipettors, and the work surface of the PCR hood with 70% ethanol and with RNaseZap wipes.
- Reduce DNA contamination with UV light exposure for 15 minutes.

Kit contents

The MeV genotyping RT-PCR kit consists of two boxes.

Box 1 should be opened in the BSC used for preparation of the master mix. It contains:

- 25 µl 10x measles virus forward primer MeV216. Primer needs to be diluted before use (see below).
- 25 µl 10x measles virus reverse primer MeV214. Primer needs to be diluted before use (see below).

Box 2 should be opened in the BSC used for addition of samples to the master mix. It contains:

- Dried measles RNA control. RNA is dried and needs to be rehydrated and diluted before use (see below).
- 1 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.0) for rehydration of controls.

Information about primers

- MeV primer sequences
  - Forward Primer (MeV216): 5’ TGG AGC TAT GCC ATG GGA GT 3’
  - Reverse Primer (MeV214): 5’ TAA CAA TGA TGG AGG GTA GG 3’
- The location of the primer binding sites is described in a separate file (Primers and reference sequences).
- Primer stock solutions are stored at -20°C.
- Final concentration of primers in the reaction mix is 600 nM.

Information about the positive control
The measles RNA control is an RNA transcript of a measles nucleoprotein (N) gene with a 220 nucleotide insert in the 3’ variable region. Amplified products will thus be 220 nucleotides larger than RT-PCR products from patient samples. Using this control simplifies the identification of contamination.

Preparation of working solutions of primers
Primers are supplied as 10X (10 fold) concentrated stocks. The concentration in the stocks is 200 µM. It is necessary to prepare a working solution of each primer prior to setting up a RT-PCR reaction. The concentration of the working dilution is 20 µM. Store diluted primers at -20°C. Do not freeze-thaw more than 5 times.

- MeV214: Mix 10 µl stock solution with 90 µl nuclease-free water. Vortex.
- MeV216: Mix 10 µl stock solution with 90 µl nuclease-free water. Vortex.

Preparation of control RNA stocks
The measles control RNA is supplied as dried RNA. It is necessary to rehydrate it before the first use of the kit. Always work with RNA on ice. Do not work with control RNA in the same PCR hood where master mix preparation is carried out.
1. Add 100 µl nuclease-free TE (supplied in kit) and vortex for 15 seconds.
2. Heat tube at 50°C for 15 minutes in water bath, then vortex for 15 seconds. Spin briefly to collect.
3. Prepare 10 µl aliquots and store at -70°C.

Preparation of working solutions of control RNA
1. Thaw one tube with 10 µl control RNA stock.
2. Add 90 µl nuclease-free TE (supplied in kit). Vortex. Spin briefly to collect.
3. Prepare 10 µl aliquots and store at -70°C for long-term storage and -20°C for short-term storage.
4. Use 1 µl per RT-PCR reaction plus 4 µl nuclease-free water.

Sample Preparation
RNA samples (extracted from a clinical sample or from cell culture, see RNA extraction protocol) are stored at -70°C. Usually, RNA is extracted from one 25 cm2 flask of infected cells or from 100-200 µl of clinical material. Most of the extraction protocols yield 40-50 µl of RNA. For the assay, 5 µl of sample are used per reaction. The volume of RNA can be increased, but this will not significantly improve the sensitivity. Addition of different volumes requires adjustment of added nuclease-free water to result in a final volume of 50 µl.

Assay Controls
The following controls must be run in each assay. They must be included in master mix calculations:

- Negative controls
  - Water control: add 5 µl nuclease-free water instead of RNA.
  - Extraction control: mock-extracted RNA obtained by extraction of water or cell culture medium. In the calculations for the number of reactions (see below) the extraction control is tested as an additional sample.

- Positive control (MeV control RNA)
  - Rehydration and dilutions should be done separately from set up of master mix.
  - Add 1 µl control RNA plus 4 µl of nuclease-free water.

Preparations for Assay Set-up

- Thaw kit reagents: 5X buffer, dNTP mix, and primers and briefly vortex.
- Spin down all reagents (including enzymes) in microcentrifuge and keep on ice until ready to dispense. Enzymes must be kept on ice at all times.
- Thaw RNA samples and keep on ice during assay set up.
- Record the date when reagents were opened on the worksheet for measles genotyping RT-PCR.

Assay Protocol

1. Determine the number of reactions (n) based on the number of RNA samples to be tested. Prepare excess reaction volumes (n + 1) to allow for pipetting errors.

Calculating the number of reactions: the number of reactions is the number of samples (which includes the extraction control) plus 2 for the nuclease-free water control and the positive control plus 1 to allow for pipetting losses.
Example: If there are 4 specimens and 1 extraction control: make a master mix for 8 reactions:

- 5 samples (specimens and extraction control)
- 1 nuclease-free water control
- 1 MeV control RNA
- 1 extra to allow for pipetting losses

2. Enter the number of samples in the Excel worksheet ‘Measles genotyping RT-PCR master mix’ to determine volumes of each reagent to be added.
3. Turn on the thermocycler.
4. In the BSC designated for master mix preparation: Label appropriate number of 0.2 ml thin-walled reaction tubes and place in pre-chilled metal cooling rack. Keep cooling rack on ice for entire protocol.
5. Add appropriate volumes of the first 5 reagents (nuclease-free water through reverse primer) to a pre-chilled 1.5 ml microcentrifuge tube. Vortex and keep tube on ice.

6. Add RNase inhibitor and enzyme mix to master mix tube. Vortex, spin briefly and chill on ice.
7. Dispense 45 µl master mix (see worksheet) to each reaction tube. Keep reaction tubes on ice in the metal cooling rack after the master mix is dispensed.
8. Proceed to the separate BSC designated for template addition. Using a new, clean pipette tip for each transfer, add RNA to each tube and close the cap. The final volume is 50 µl.
9. Add negative controls (nuclease-free water and extraction control), then the positive control (1 µl control RNA and 4 µl nuclease-free water). Mix.
10. Spin the tubes briefly (10,000 rpm for 1 minute) in a chilled microcentrifuge and immediately return the tube to the metal cooling rack. It is important to keep the tubes on ice until they are placed in the thermocycler.
11. Start the appropriate program in the thermocycler (see cycling conditions below). Wait for the block to heat up to about 80ºC, place block on hold. Place the samples in the block and start the run.

Cycling parameters for Qiagen kit-Measles
50°C for 30 minutes
95°C for 15 minutes

40 cycles of:
95°C for 30 seconds
55°C for 30 seconds
72°C for 30 seconds

Followed by 72°C for 10 minutes

Final: hold at 4°C