Annex B11 Measles real-time RT-PCR, N gene (CDC)

CDC protocols for the molecular epidemiology of measles virus and rubella virus; version of 01/26/2015

Real-time RT-PCR Assays for the Detection of Measles Virus (MeV) N Gene RNA and Human RNase P mRNA (a cellular reference gene) using the ABI 7500 Real-Time Thermocycler

Purpose

The following protocol is to be used to detect RNA of measles virus (MeV) for diagnostic purposes. Primers and control RNA are supplied by CDC as a kit.
For these procedures, RNA is extracted from a clinical sample or from cell culture (See RNA Extraction protocol). If samples give a positive result in the real-time RT-PCR assays, the RNA may be tested in a measles genotyping RT-PCR assay to amplify the target for sequence analysis and genotyping (See Measles Genotyping RT-PCR protocol).

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

Reagents and materials needed

- 70% ethanol
- Aluminum foil
- Gloves
- Lab coat
- MicroAmp optical 96-well reaction plate with barcode (Life Technologies #4306737)
- Nuclease-free water (e. g. Life Technologies #AM9937)
- Optical adhesive cover (Life Technologies #4311971)
- RNase inhibitor 2000 units (e.g. Life Technologies N8080119)
- RNaseZap (Sigma, # R2020-250ML)
- Sterile 1.5 ml microcentrifuge tubes
- SuperScript III Platinum OneStep qRT- PCR Kit (Life Technologies, #11732-020)

Equipment needed

- -70ºC and -20ºC freezers
- ABI 7500 Real-time PCR System
- Bucket with ice
- Centrifuge with holder for 96 well plates
- Class II biological safety cabinets (BSC) or PCR workstations with UV light designated for PCR set up
- Microcentrifuge
- Micropipettors and sterile pipette tips with aerosol-resistant filters
- Vortex
- Water bath

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, pipettors, 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 real-time RT-PCR kit consists of two boxes. Box 1 should be opened in the BSC used for preparation of the master mix. It contains:

- MeV primer/probe mix: One tube with 100 µl of a mix of primers and probe for MeV real-time reactions. Content needs to be diluted before use (see below).
- RNase P primer/probe mix: One tube with 50 µl of a mix of primers and probe for RNase P real-time reactions. Content 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:

- MeV control RNA (high concentration control). Contains synthetic MeV RNA (MeV-N3in) and total human RNA. Content is dried and needs to be rehydrated and diluted before use (see below). This control can be used for both MeV and RNase P reactions.
- MeV control RNA (low concentration control). Contains synthetic MeV RNA (MeV-N3in) and total human RNA. Content is dried and needs to be rehydrated and diluted before use (see below). This tube contains less synthetic MeV RNA than the high control, but the same amount of total human RNA. This control can be used for both MeV and RNase P reactions.
- 2 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.0) for rehydration of controls.

Information about primers and probes

- Primer and probe stock solutions are stored at –20°C. Keep probes protected from light.
- Probes are labeled at the 5' terminus with a fluorescent reporter dye, 6-carboxy-fluorescein (FAM), and at the 3' terminus with a non-fluorescent quencher, black hole quencher-1 (BHQ1).
- Final concentration in the reaction mix:
    - MeV primers and RNase P primers: 300 nM
    - MeV probe: 250 nM
    - RNase P probe: 100 nM
- MeV primer sequences
    - Forward Primer (MVN1139-F): 5' TGG CAT CTG AAC TCG GTA TCA C 3'
    - Reverse Primer (MVN1213-R): 5' TGT CCT CAG TAG TAT GCA TTG CAA 3'
    - Probe (MVNP1163-P): 5' FAM CCG AGG ATG CAA GGC TTG TTT CAG A BHQ 3'
- RNase P primer sequences
    - Forward Primer (HURNASE-P-F): 5' AGA TTT GGA CCT GCG AGC G 3'
    - Reverse Primer (HURNASE-P-R): 5' GAG CGG CTG TCT CCA CAA GT 3'
    - Probe (BHQ1 HURNASE-P): 5' FAM TTC TGA CCT GAA GGC TCT GCG CG BHQ1 3'

Preparation of working solutions of primer/probe mixes

Primer/probe mixes are supplied as 10x (10 fold) concentrated stocks. It is necessary to prepare a working solution of each mix before setting up a real-time RT-PCR reaction. Store diluted primers and probes at -20 ºC. Wrap tubes in aluminum foil to protect from light. Aliquots should be thawed no more than three times.

- MeV primer/probe mix: Mix 10 µl stock solution with 90 µl nuclease-free water. Vortex.
- RNase P primer/probe mix: Mix 10 µl stock solution with 90 µl nuclease-free water. Vortex.

Preparation of control RNA stocks

Control RNAs are supplied as dried RNA. It is necessary to rehydrate these controls before the first use of the kit. Always work with RNA on ice. Do not work with control RNAs in the same PCR hood where master mix preparation is carried out. Refer to package insert of real-time kit for details of preparation of controls.

Preparation of working solutions of control RNAs

1. Thaw one tube with 10 µl high control RNA stock and one tube with 10 µl low control RNA stock.
2. To each tube, add 90 µl nuclease-free TE (supplied in kit). Vortex. Spin briefly to collect.
3. Prepare 10 aliquots of 10 µl each and store at -70ºC.
4. Use one aliquot for each real-time RT-PCR assay. Discard leftover working solution.

Sample Preparation

RNA samples (extracted from a clinical sample or from cell culture, see RNA extraction protocol) are stored at -70ºC. Usually, RNA is extracted from one 25 cm^2 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 25 µl.

Assay Controls

- Every RNA sample should be tested in parallel with the MeV primer/probe and the RNase P primer/probe. The purpose of the RNase P control reaction is to monitor the integrity of the RNA.
- The following standards and controls should be run on each plate as indicated on the Measles Real-time Plate Layout. They must be included in master mix calculations for each primer/probe set:
    - Negative controls
        - NTC: add 2.5 µl/well nuclease-free water instead of RNA to the wells labeled MeV NTC and RNP neg as indicated on Measles Real-time Plate Layout.
        - Extraction control: mock-extracted RNA obtained by extraction of water or medium. In the calculations for the number of reactions (see below) the extraction control is tested as an additional sample.
    - Two control RNAs (high control and low control)
        - Rehydration and dilutions should be done separately from set up of master mix.
        - Either one of the controls can also be used as RNase P controls.

- Add 2.5 µl/well of each control RNA as indicated on Measles Real-time Plate Layout.

Preparations for assay set up

- Thaw kit reagents: 2X reaction mix, ROX, and primer/probe mix and briefly vortex.
- Spin down all reagents (including enzymes) in microcentrifuge and keep on ice until ready to dispense.
- Thaw RNA samples and keep on ice during assay set-up.
- Record the date when reagents were opened on Measles Real-time Master Mix worksheet.

Assay Protocol

1. Determine the number of reactions (n) based on the number of RNA samples to be tested and the format of the Measles Real-time Plate Layout. Each clinical sample and the extraction control should be tested in triplicate with the MeV primer set, and in a single well for the RNase P primer set. Controls for the MeV primer/probe set (MeV high control, MeV low control, MeV-NTC) are tested in duplicate. Controls for the RNase P primer/probe set (RNase P positive control, RNase P-NTC) are tested in a single well. Either the MeV high control or the MeV low control tube can be used as an RNase P control.
   Prepare excess reaction volume (n + 1) for each primer/probe set to allow for pipetting errors.
   Calculating the number of reactions: For the MeV primer set, the number of reactions is 3 times the number of samples (which includes the extraction control) plus 6 for the real-time controls plus 1 to allow for pipetting losses. For the RNase P primer set, the number of reactions is the number of samples (which includes the extraction control) plus 2 for the real-time controls plus 1 to allow for pipetting losses.

   Example: If there are 4 specimens and 1 extraction control: make a master mix for 22 reactions with the MeV primer set:
   - 5 samples (specimens and extraction control) measured in triplicate=15 reactions
   - 2 NTCs
   - 2 reactions high control RNA
   - 2 reactions low control RNA
   - 1 extra to allow for pipetting losses

   Make a master mix for 8 reactions with RNase P primers:
   - 5 samples (specimens and extraction control)
   - 1 NTC
   - 1 reaction with either high or low control RNA
   - 1 extra to allow for pipetting losses

2. Enter the ID# of the sample(s) on the Measles Real-time Plate Layout.
3. Enter the number of samples in the Real-time Master Mix worksheet to determine volumes of each reagent to be added. There are separate calculations for the measles primers and the RNase P primers.

4. In the BSC dedicated for master mix preparation, for the MeV primer/probe set, add the first 4 reagents (nuclease-free water through ROX reference) to a pre-chilled 1.5 ml microcentrifuge tube. Invert, briefly centrifuge, and keep on ice.
5. Add RNase inhibitor and enzyme mix to master mix tube. Vortex and chill briefly on ice.
6. In another 1.5 ml microcentrifuge tube, repeat steps 4 and 5 for the RNase P master mix.
7. Proceed to a separate BSC designated for template addition. Dispense 1 reaction volume of master mix into appropriate wells according to the Measles Real-time Plate Layout, using a new tip for each master mix. Reaction volume is 22.5 µl/well for MeV and RNase P.
8. Add nuclease-free water (NTC), extraction control, sample RNA or positive control standards as indicated on the Measles Real-time Plate Layout, using a new tip for each well. Sample volume is always 2.5 µl/well. The total volume in each well should now be 25 µl.
9. Seal plate with optical adhesive cover.
10. Centrifuge the sealed plate at 1500 rpm for 1 minute at room temperature.

Assay Run

1. Launch software by double-clicking the 7500 software icon on the desktop. The following instructions are for v2.0.4 of the software.
2. Turn on the 7500 thermocycler – connection is usually automatic.
3. Select Advanced Set-Up.
4. The Experiment Properties screen will open. Name the experiment (e.g. date and initials). The settings should be 7500 (96 wells), Quantitation-Standard Curve, TaqMan reagents, Standard Ramp Speed.
5. Go to Plate Setup: Define Targets and Samples. Enter the name of each sample to be tested by clicking on Add New Sample. Include extraction controls. Each sample must be listed twice: once for measles (e.g. sample 1) and once for RNase P (e.g. sample 1-R).
6. Go to Define Targets. There should be one target (FAM) and one quencher (NFQ-MGB, Blackhole Quencher). The target is the fluorescent dye that the instrument will detect. The probes for measles and RNase P are labeled with the same fluorescent dye (FAM), so all wells will be assigned to the same target. It is not necessary to name the target.
7. Go to Assign Targets and Samples. Using the Plate Layout as a guide, highlight all the wells that will contain the test samples, the extraction control, and the RNase P controls. Do not highlight empty wells.
8. Check the box under 'assign'. All the marked wells will display 'U' for 'unknown'.
9. To define standards, highlight the wells with the high control. Under 'Assign Targets', click on 'S' for standard. Under 'quantity', fill in 100000 (10^5). Repeat for the low control with 1000 (10^3) as quantity.
10. To define the NTC, highlight the wells with the measles water control. Under 'Assign Targets', click on 'N' for NTC.
11. To assign samples, highlight the 3 wells that contain the MeV master mix for the first test sample (e.g. sample 1). Under 'Assign samples to the Selected Wells' check the box for sample 1. Repeat for the well that contains sample 1 and RNase P master mix, check the box for sample 1-R. Repeat this process for each sample. Each well in use should now contain a target, a task (NTC, standard or unknown) and a sample name.
12. Make sure ROX is listed as the passive dye.
13. Go to Run Method. Set the Sample Volume to 25 µl. Set the step parameters (time/temp) on the thermal profile to the following:
    - RT Step: 48 °C /30 minutes

- Activation: 95 °C /5 minutes
- PCR (40 cycles): 95 °C /15 seconds, 60 °C /1 minute

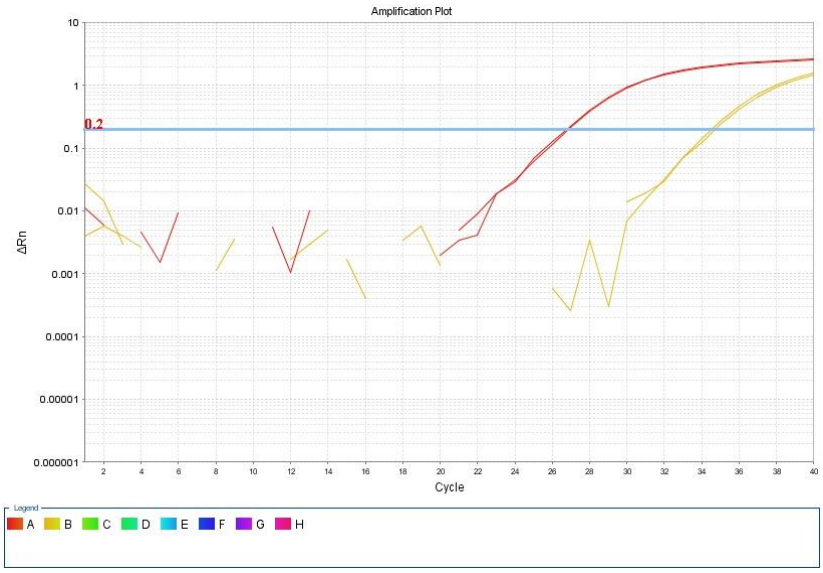
14. Select File > Save As and save file in user folder using a standard format: (e.g. Date_initials.eds)
15. Open the door of the 7500 by pushing in the indentation on the front.
16. Place a plate into the instrument tray. Orient the A1 well on the plate with the A1 position on the instrument tray.
17. Select Start Run. Under Run, select Amplification Plot to monitor the run, which should be complete in approximately 2 hours. The screen will show how much time is left until the end of the run.
18. At the end of the assay, the green Analyze button will appear.
19. Click on Save to save your data.

Assay Quality Control

1. After the run has finished, click on Analyze. The program will calculate an automatic threshold. Do not change the threshold for the analysis of the controls.
2. The results will appear.
3. Checklist for quality control: A real-time RT-PCR assay is valid if:
    - The NTCs for MeV and RNase P and the extraction controls are Undetermined (negative).
    - The automatic threshold is located in the exponential phase of the standard amplification curves.
    - The Ct values for the duplicate samples of the MeV high and low controls fall within the range defined in the measles real-time kit package insert.
    - The Ct value for the single sample of the RNase P control falls within the range defined in the measles real-time kit package insert.

    If any of these criteria are not fulfilled, the assay must be repeated. If the extraction control is positive, the RNA extraction must be repeated.
4. Monitoring real-time RT-PCR assay over time: Ct values for positive controls should be recorded for subsequent runs. After several assays, a typical range of Ct values for each positive control should be determined and used for the analysis of subsequent runs. Changes in these values may indicate problems.
5. Important: the range of Ct values for the positive controls may vary between different lots of the Measles Real-time RT-PCR kit. Please check the package insert for updates.

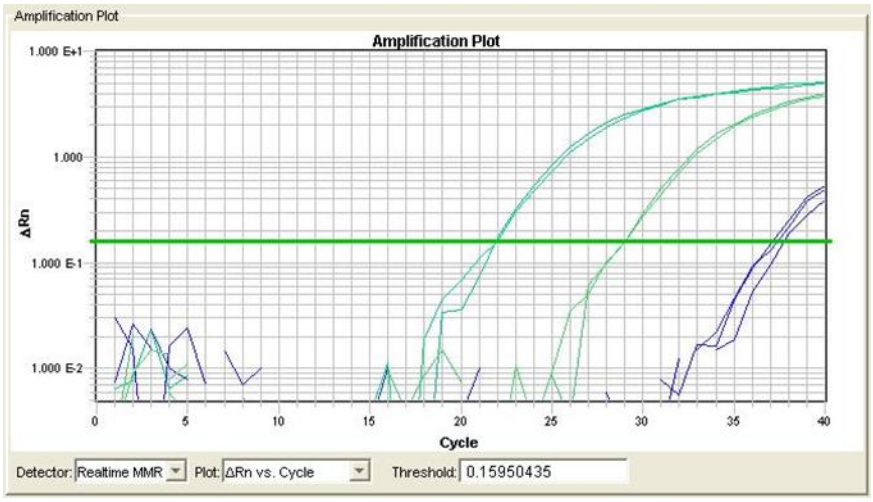
Figure 1: Amplification plot with high and low control curves.



Sample Data Analysis

1. Check the Multicomponent Data Pane icon on all samples with Ct values < 40 to confirm true amplification as indicated by a rise in FAM fluorescence.
2. Some wells may be highlighted by yellow flags. Click on the QC Summary Pane to see the reason why the wells were flagged. For example, if a sample well did not produce a signal, the program will flag this with No amplification. However, if the sample is negative (for example the extraction control), a lack of amplification is the expected result and not a quality control issue.
3. Adjusting the threshold (if necessary): Highlight the wells containing the samples and the NTC and examine the amplification curves. If a sample has an exponential curve that does not cross the threshold, it is possible to lower the threshold for the analysis of patient samples. Only lower the threshold after the Ct values for the standards have been determined as described in **Assay Quality Contro**l.
    - Click on Analysis Settings. Click on Edit Default Settings. Remove the check from Auto Threshold. Click on Save Change and Apply Analysis Settings. Close.
    - In the amplification plot, click on the threshold and drag it to the desired position. Every time the threshold is moved, the Ct values for the samples will be automatically recalculated.
    - Important: The signal for the NTC must remain below the threshold setting. The threshold must be located in the lower third of the exponential phase of the graph. Figure 2 below shows the 2 positive controls and a typical positive test sample.
    - If the threshold has to be moved, write down the value for threshold, because the program does not save this information.

Figure 2: Amplification plot with standards and typical sample



Interpretation of results for MeV, RNase P

Viral gene	RNase P gene	Specimen Result
< 40	< 40	positive
< 40	undetermined	positive
undetermined	< 40	negative
undetermined	undetermined	indeterminate

- If the PCR with the MeV primers/probe produced a Ct below 40, the specimen is positive for measles. It does not matter whether the RNase P reaction is positive or negative.
- If the result of the MeV PCR is undetermined but the RNase P PCR produced a Ct below 40, the specimen is negative for measles. The positive RNase P result indicates that sufficient RNA was extracted from the specimen to allow amplification to occur.
- If the result of the MeV PCR and the RNase P PCR are undetermined, the specimen is judged to be indeterminate. It is not possible determine a result, because there was not enough RNA to allow amplification to occur.
- Clinical samples with 2 or 3 positive reactions (out of the 3 replicates run on the plate) will be reported positive.

Exporting Data

To save the data to an Excel file: Click on Export, then

1. Under: select data to export, choose Results
2. Under: Select one file or separate files, choose One file
3. Choose a file name and browse to the location where the file should be saved.
4. Click Open, then click Start Export.
5. Close the Export Tool.

Worksheet for Measles Real-time Master Mix

Below is an example of a worksheet for Measles Real-time Master-mix. An Excel spreadsheet with the worksheet is provided in a separate file.

Note: Excel will automatically calculate the volume of the reaction components afte			
the number of specimens is inserted into the blue field.			
Technician:			
File name:			
Test date:			
Kit: Superscript III One-Step qRT-PCR (Life Technologies #11732-020)			
Date opened:			
Primer mix lot number:			
Number of specimens (including extraction control):		0	Please insert numbe
Number of reactions for measles reaction mix:		7	specimens in blue fiel
(number of specimens multiplied by 3 plus 7)			
Number of reactions for Rnase P reaction mix:			
(number of specimens plus 3)		3	
Master mix worksheet for measles primer/probe set			

Component	Vol/rxn (µl)	# of rxns	Total vol (µl)
Nuclease-free water	7.2	7	50.4
2x reaction mix	12.5	7	87.5
MeV primer/probe mix	2	7	14
ROX reference	0.05	7	0.35
RNase inhibitor	0.25	7	1.75
SS III/Taq mix	0.5	7	3.5
RNA	2.5	xxxx	xxxx
Total	25		

Master mix worksheet for RNaseP primer/probe set

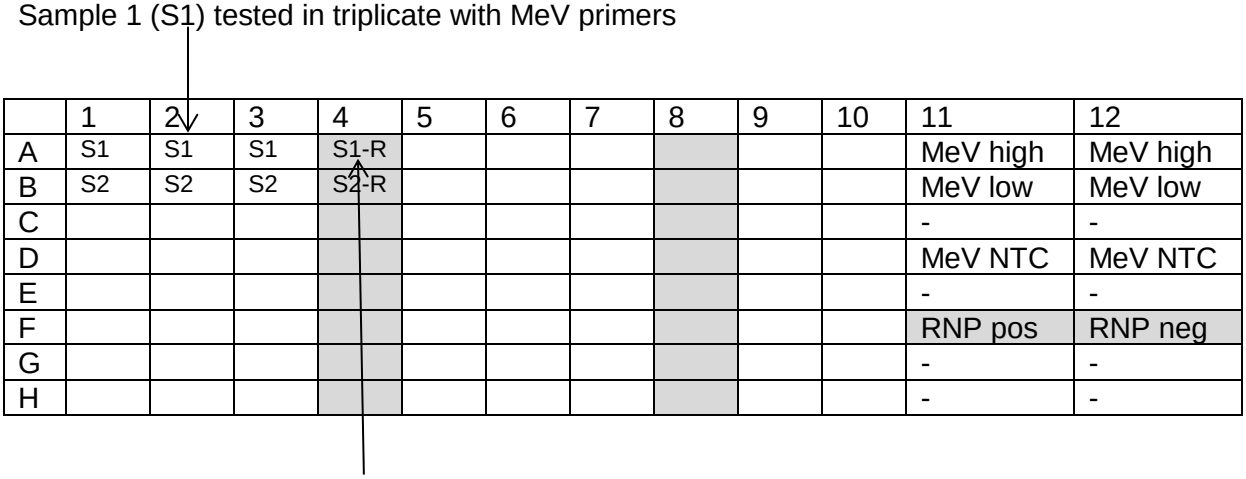
Component	Vol/rxn (µl)	# of rxns	Total vol (µl)
Nuclease-free water	7.2	3	21.6
2x reaction buffer	12.5	3	37.5
RNP primer/probe mix	2	3	6
ROX reference	0.05	3	0.15
RNase inhibitor	0.25	3	0.75
SS III/Taq mix (5U/µl)	0.5	3	1.5
RNA	2.5	xxxx	xxxx
Total	25		

Dispense master mix: 22.5 µl/well for both master mixes. Add 2.5 µl template

Worksheet for Measles Real-time RT-PCR for Measles RNA Detection (page 2 of 2)
Below is an example of a plate layout with two samples. An Excel spreadsheet with the Measles Real-time plate layout is provided in a separate file.

Plate layout for real-time RT-PCR assay

Sample 1 (S1) tested in triplicate with MeV primers



	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	S1-R							MeV high	MeV high
B	S2	S2	S2	S2-R							MeV low	MeV low
C											-	-
D											MeV NTC	MeV NTC
E											-	-
F											RNP pos	RNP neg
G											-	-
H											-	-

Sample 1 (S1) tested with primers for RNaseP

MeV high: MeV control RNA (high)
MeV low: MeV control RNA (low)
NTC: negative control for MeV (water)
RNP pos: positive control for RNase P (MeV control RNA high or low)
RNP neg: negative control for RNase P (water)
White field: MeV master mix
Grey field: RNase P master mix