

Annex B12.2

Real-time RT-PCR Assays for the Detection of Rubella Virus RNA and Human RNase P mRNA using the ABI 7500 Real-time Thermocycler

This is an updated version of the rubella real-time RT-PCR protocol (Annex B12).

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Purpose

The following protocol is to be used to detect rubella virus (RuV) RNA from clinical samples for diagnostic purposes. The primers target a region in the non-structural protein coding region of RuV. Samples that give a positive result in the real-time RT-PCR assay should be tested in a rubella genotyping RT-PCR assay to amplify the target for sequence analysis and genotyping.

Important: This is a general protocol for use with the rubella real-time RT-PCR kit supplied by CDC. Please check the WHO website for updates to the protocol. Instructions in this protocol are given for use with the ABI 7500 Thermocycler; however, other real-time platforms have been used successfully with the CDC kit.

Reagents and materials needed (listing of manufacturer does not imply endorsement by CDC or the US government)

- SuperScript Platinum OneStep qRT- PCR Kit (Invitrogen, #11732-020)
- RNase inhibitor 2000 units (for example: Applied Biosystems N-808-0119)
- MicroAmp optical 96-well reaction plate with barcode (Applied Biosystems #4306737)
- Optical adhesive cover (Applied Biosystems #4311971)
- 70% ethanol
- Aluminum foil
- Gloves
- Lab coat
- Nuclease-free water (for example: Ambion #9937)
- RNaseZap (Sigma, # R2020-250ML)
- Sterile 1.5 ml microcentrifuge tubes

Equipment needed

- -70°C and -20°C freezers
- ABI 7500 real-time PCR System
- Bucket with ice
- Centrifuge with holder for 96 well plates or plate spinner
- Class II BSC with UV light designated for PCR set up
- Microcentrifuge
- Micropipettors and sterile pipette tips with aerosol-resistant filters
- Vortex
- Waterbath

Recommendations for working with RNA

- Wear gloves throughout experiments to prevent contamination from RNases found on human hands.
- Change gloves after touching skin (e.g. your face), doorknobs, and common surfaces.
- Have a dedicated set of pipettors that are used solely for RNA work.
- Use filter tips and tubes that are 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.
- Reduce DNA contamination in the BSC with UV light exposure for 15 minutes.

Kit contents

The RuV real-time RT-PCR kit contains

- RuV primer/probe mix: One tube with 100 µl of a mix of primers and probe for RuV 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).
- RuV high control RNA. Contains synthetic RuV RNA 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 RuV and RNase P reactions.
- RuV low control RNA. Contains synthetic RuV RNA and total human RNA. Content is dried and needs to be rehydrated and diluted before use (see below). This tube contains less synthetic RuV RNA than the high control, but the same amount of total human RNA. This control can be used for both RuV and RNase P reactions.
- One tube with 2ml 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:
 - RuV primers: 400 nM
 - RNase P primers: 300 nM
 - RuV probe: 200 nM
 - RNase P probe: 100 nM
- RuV primer sequences
 - Forward primer (RV98F): 5' GGC AGT TGG GTA AGA GAC CA 3'
 - Reverse primer (RV251R): 5' CGT GGA GTG CTG GGT GAT 3'
 - Probe (RuV): 5' FAM ACA TCG CGC ACT TCC CAC GBHQ 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 prior to setting up a real-time RT-PCR reaction. Store diluted primers and probes at -20°C. Wrap tubes in aluminum foil to protect from light.

- RuV primer/probe mix: Mix 10 µl stock solution with 90 µl nuclease-free water. Vortex.
- RNase P primer/probe mix: Mix 5 µl stock solution with 45 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.

1. To each tube (high and low control RNA) add 100 µl nuclease-free TE (supplied in kit) and vortex for 15 seconds.
2. Heat tubes to 50°C for 10 minutes in water bath then vortex for 15 seconds. Spin briefly to collect.
3. Prepare 10 aliquots of 10 µl each and store at -70°C. Each aliquot should be thawed only once to make a working solution (see below).

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 -70°C.
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) are stored at -70°C. For the assay, 2.5 µl of sample are used per reaction. Addition of different volumes requires adjustment of added water to result in final volume of 25 µl.

Assay Controls

- Every RNA sample should be tested in parallel with the RuV 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 real-time 7500 plate layout. They must be included in master mix calculations for each primer/probe set:
 - Negative controls
 - Non-template control (NTC): nuclease-free water
 - Negative extraction control obtained by extraction of water along with the test samples
 - Add 2.5 µl/well of each control
 - Positive controls
 - Positive extraction control (to yield a positive RNase P signal): to be tested with RNase P primer set only
 - Option 1: lysate from uninfected cells, such as A549 cells
 - Option 2: re-extract RNA from a previous sample or pool of samples that tested negative for rubella, but positive with RNaseP primers
 - *Note: an internal control, such as Phocine Distemper virus which is added to every sample prior to extraction, can be used instead, but will not be covered in this protocol.*
 - Two RuV standard RNAs (high and low)
 - Rehydration and dilutions should be done separately from set up of master mix
 - Either one of the controls can also be used as an RNase P positive control
 - Add 2.5 µl/well of each control RNA

Preparations for assay set up

- Thaw kit reagents: 2X SS buffer, 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 open date of reagents on real-time 7500 control sheet.
- For more accurate pipetting of the ROX reagent, dilute 1:10 in nuclease-free water and stored wrapped in foil (see master mix with diluted ROX 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 real-time 7500 control sheet.
 - a. All reactions with the rubella primer set should be tested in duplicate wells: clinical samples, NTC, high and low controls, negative extraction control.

- b. All reactions with the RNase P primer set should be tested in single wells: clinical samples, NTC, RNase P positive control (either high or low control), negative and positive extraction controls.

Prepare excess reaction volume ($n + 1$) for each primer/probe set to allow for pipetting errors.

Calculating the number of reactions:

- a. For the rubella primer set, the number of reactions is 2 times the number of samples plus 8 for the real-time run controls (NTC, high control, low control, negative extraction control), plus 1 to allow for pipetting losses.
- b. For the RNase P primer set, the number of reactions is the number of samples plus 4 for the controls (positive extraction control, negative extraction control, NTC and either high or low control) plus 1 to allow for pipetting losses.

Example: If there are 2 specimens: make a master mix for 13 reactions with the RuV primer set (duplicate wells for each):

- 2 samples (in duplicate) =4 reactions
- 2 duplicate negative extraction control
- 2 duplicate NTC
- 2 duplicate high control RNA
- 2 duplicate low control RNA
- 1 extra to allow for pipetting losses

Make a master mix for 7 reactions with RNase P primers (single well for each):

- 2 samples (single well)
- 1 negative extraction control
- 1 NTC
- 1 positive extraction control
- 1 with either high or low control RNA
- 1 extra to allow for pipetting losses

2. Enter the ID# of the sample(s) on the control sheet in the Plate Layout section.
3. Enter the number of samples and controls in the Master Mix worksheet to determine volumes of each reagent to be added. There are separate calculations for the rubella primers and the RNase P primers.
4. In the BSC dedicated for master mix preparation, for the RuV primer/probe set, add reagents in a 1.5 ml microcentrifuge tube in the order indicated on the worksheet except for the RNA. Vortex, briefly centrifuge, and keep on ice.
5. In another 1.5 ml microcentrifuge tube, add reagents for the RNase P master mix.
6. Dispense 1 reaction volume of master mix into appropriate wells according to the real-time 7500 Plate Layout, using a new tip for each master mix. Reaction volume is 22.5 μ l/well for RuV and RNase P.
7. Add 2.5 μ L/well nuclease-free water (NTC) to the designated wells on the Plate Layout.
8. Proceed to a separate BSC designated for template addition.

9. Add the extraction controls, sample RNA and high and low standards as indicated on the 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.
10. Seal plate with optical adhesive cover.
11. Centrifuge the sealed plate at 1500 rpm for 1 minute at room temperature or spin in a plate spinner.

Assay Run

1. Launch software by double-clicking the 7500 software icon on the desktop. The following instructions are for v2.3 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 (usually 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 name of each test sample and the extraction control to be tested by clicking on Add New Sample. Each test sample and the negative extraction control (NEC) must be listed twice: once for rubella (e.g. sample 1) and once for RNase P (e.g. sample 1-R). Also, add new samples named RuV NTC, RuV High, RuV Low, RNP PC, RNP PEC, and RNP NTC.
6. Go to Define Targets. Under Target Name, change the name of 'Target 1' to 'RuV'. The target for rubella is FAM and the quencher NFQ-MGB (Blackhole Quencher). The target is the fluorescent dye that the instrument will detect. Click on Add Target. Name the new target 'RNase P'. The target for RNase P is FAM and the quencher NFQ-MGB (Blackhole Quencher). Choose different colors for the RuV and RNase P targets.
7. Go to Assign Targets and Samples. Using the Plate Layout as a guide, highlight all the wells that will contain RuV reaction mix. Under Assign Target(s) to the Selected Wells check the box for the RuV target.
8. Highlight all the wells that will contain RNase P reaction mix. Under Assign Target(s) to the Selected Wells check the box for the RNase P target.
9. The wells will be labeled with the colors representing the two different targets. All the marked wells will display 'U' for 'unknown'. **Do not add anything to empty wells.**
10. To define standards, highlight the wells with the RuV target that are for 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. To define the RNase P control, highlight the well, click on 'S' for standard. It is not necessary to change the quantity.
11. To define the NTC, highlight the wells with the RuV and RNase P water controls. Under 'Assign Targets', click on 'N' for NTC.
12. To assign samples, highlight the two wells that contain the RuV 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 RNaseP 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

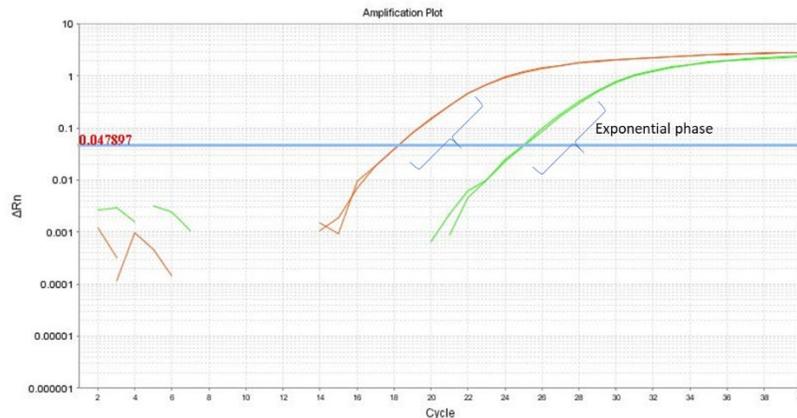
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1 U RuV	S1 U RuV	S1-R U RNP								RuV High S RuV 1	RuV High S RuV 1
B	S2 U RuV	S2 U RuV	S2-R U RNP								RuV Low S RuV 1	RuV Low S RuV 1
C	NEC U RuV	NEC U RuV	NEC-r U RNP									
D											RuV NTC N RuV	RuV NTC N RuV
E												
F											RNP PC U RNP	RNP PEC U RNP
G												RNP NTC N RNP
H												

13. Make sure ROX is listed as the passive dye (on lower left)
14. Go to Run Method. Set the Sample Volume to 25 µl. Set the step parameters (time/temp) on the thermal profile to the following:
 - a. RT Step: 48 °C /30 minutes
 - b. Activation: 95 °C /5 minutes
 - c. PCR (40 cycles): 95 °C /15 seconds, 60 °C /1 minute
15. Select File > Save As and save file in user folder using a standard format: (e.g. Date_initials.eds).
16. *Note: the plate layout with assigned standards, controls, and run method can be saved as a Template file (.edt). Use of the Template file can save set-up time. To use, add new test samples to the plate layout; save the altered file under a new name (.eds).*
17. Open the door of the 7500 by pushing in the indentation on the front.
18. Place a plate into the instrument tray. Orient the A1 well on the plate with the A1 position on the instrument tray and push the door closed.
19. Select Start Run. Under Run, select Amplification Plot to monitor the run, which should be complete in approximately 2 hours. Time left in run will be given.
20. At the end of the assay, the green Analyze button will appear.
21. Click on Save to save your data.

Checking the threshold

1. After the run has finished, click on Analyze. The program will calculate an automatic threshold. The results will appear.
2. Under Plot Settings, verify that the graph type is 'log'.
3. Below the amplification curve, under Options, choose the RuV target. You will only be able to see the amplification curves from the wells containing RuV primer mix.
4. Highlight only the wells with the RuV high and low controls and the RuV NTC. Do not include the sample wells.

- a. Under **Options**, uncheck **Threshold Auto**. Move the cursor to the threshold line. The cursor will change to a hand symbol. Click and drag the threshold to the appropriate place in the lower third of the exponential phase of the positive controls and above the signal for the NTC. Every time the threshold is moved, the Ct values for the samples will be automatically recalculated.
- b. Save the file under a new name because the program will not retain the information for the new threshold.



6. Repeat steps 3-5 for the RNP threshold.

Assay Quality Control

Visual inspection of the amplification plots is important. Positive samples and controls should show exponential amplification curves. Negative samples and controls should show no evidence of amplification.

Checklist for quality control: a real-time RT-PCR assay is valid if:

- The threshold is located in the exponential phase of the standard amplification curves.
- The NTCs for RuV and RNase P are Undetermined (negative).
- The negative extraction control is Undetermined (negative).
- The positive extraction control is positive.
- The Ct values for the duplicate samples of the RuV high and low controls fall within the range defined in the rubella real-time kit package insert.
- The Ct value for the RNase P high or low positive control falls within the range defined in the rubella real-time kit package insert.

If any of these criteria are not fulfilled, the assay is invalid.

If the negative extraction control is positive, this indicates contamination during the RNA extraction. The RNA extraction should be repeated.

If the positive extraction control is negative, this indicates problems with the RNA extraction. The RNA extraction should be repeated.

Monitoring real-time RT-PCR assay over time: Ct values for at least one of the RuV positive controls should be recorded for subsequent runs. It is also useful to track the Ct value of the positive extraction control. After several assays, a typical range of Ct values for the control should be determined and used for the analysis of subsequent runs. Changes in these values may indicate problems. It is recommended to use Levy-Jennings charts to track the performance of the control.

Important: the range of Ct values for the high and low positive controls may vary between different lots of the rubella Real-time RT-PCR kit. Please check the package insert for updates.

Sample Data Quality Control

After the assay has been determined to be valid, quality control must be performed for each sample.

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 negative extraction control), a lack of amplification is the expected result and not a quality control issue.
3. Samples must be retested if:
 - a. replicates are discordant (>2 Ct apart)
 - b. one replicate is < 40 and the other is 'undetermined'
 - c. the interpretation of the test is 'inconclusive' (see below)

Interpretation of results

Viral gene	RNase P gene	Specimen Result
< 40	< 40	Positive
< 40	Undetermined	Positive
Undetermined	< 40	Negative
Undetermined	Undetermined	Inconclusive

- If the PCR with the RuV primers/probe produced a Ct below 40 for both replicates, the specimen is positive for rubella. It does not matter whether the RNase P reaction is positive or negative. If one replicate is positive and one replicate is negative, that sample must be retested. If the repeated test gives the same result, the sample should be reported as inconclusive. If both replicates are positive, report the average Ct value for the repeated run as the result.

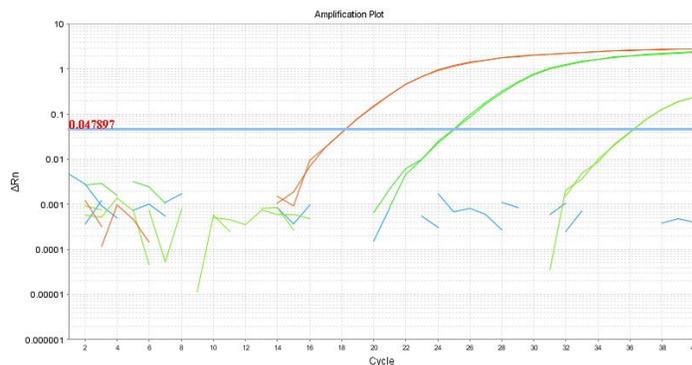
- If the result of the RuV PCR is undetermined for both replicates, but the RNase P PCR produced a Ct below 40, the specimen is negative for rubella. The positive RNase P result indicates that insufficient RNA was extracted from the specimen to allow amplification to occur.
- If the result of both the RuV PCR and the RNase P PCR are undetermined, the specimen result is judged to be inconclusive. It is not possible determine a result because there was not enough RNA to allow amplification to occur. It is recommended to repeat the RNA extraction.

Exporting Data

To save the data to an Excel file:

1. Select all wells containing data; do not include empty wells
2. Click on Export in menu bar
3. Under: select data to export, choose Results
4. Under: Select one file or separate files, choose One file
5. Choose a file name and browse to the location where the file should be saved.
6. Click Open, then click Start Export.
7. Close the Export Tool.

Example of rubella standards, NTC, and positive rubella sample (far right):



Troubleshooting

Problem	Possible causes
All reactions negative, including positive control	Component missing in master mix set up Wrong thermocycler settings Expired or inappropriately stored reagent
No amplification (i.e. negative) of a positive PCR control	High or low control RNA degraded or not added to wells
Amplification in a NTC well	Contamination of the reagents Contamination of the equipment (PCR block, pipettes) Contamination due to technician error during the PCR set up
Amplification in the negative extraction control	Contamination due to technician error during the extraction procedure
No amplification (i.e. negative) of the positive extraction control	Error during the RNA extraction Extraction control sample degraded or pipetting error

