Annex E9
RT-PCR protocol for rubella genotyping using nested set amplifications
Enzyme kit: Qiagen One-Step RT-PCR

Purpose

This protocol is to be used for the amplification of a portion of the rubella virus (RuV) E1 (envelope) protein coding region in order to generate the recommended 739nt (nt# 9731-9469) sequence for RuV genotype determination. *NOTE: This is a nested set assay, so special precautions must be followed to prevent contamination with amplification products.* Prior to running this assay, sample RNA(s) should be tested using a real-time detection assay. If the Ct value for a sample is over 34/35 and/or the sample cannot be amplified by the two fragment genotyping assay, this nested set assay can be attempted for generating templates for sequencing and genotyping. *Not all samples that are positive by real-time can be amplified by this assay, although the sensitivities between real-time and nested set are very similar. Possible explanations for failure may be that the RNA is partially degraded or nucleotide changes may have occurred in the primer binding sites of the virus from the clinical sample. These changes could lower the efficiency of binding, thus lowering the sensitivity.*

What to do with the PCR products

Analyze PCR products on agarose gels (See Agarose Gel Electrophoresis protocol). Laboratories with sequencing capacity should purify and sequence the PCR products (See Clean-up of PCR Products and Rubella Sequencing Reactions protocol). 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 tube securely, seal with parafilm, and ship at ambient temperature. PCR products are stable at room temperature for at least one month.

Nested Set Precautions

This protocol for nested set amplification should be used only with RNA extracted directly from a clinical sample such as a throat swab, urine, or serum that has tested positive for rubella virus RNA using a detection assay (such as real-time RT-PCR). The overall protocol design is a standard nested set; an aliquot of the first round DNA product is added as template to a second round of amplification using forward and reverse primers that are internal to the primers from the first round. *See the diagram at the end of the protocol*. The first reaction, using an RNA template, will produce an amplicon of 945 nts, which, when run on an agarose gel, may not be visible for the test samples but the positive control product should be visible. The second amplification results in an amplicon of 873 nts, which should be visible on an agarose gel for both test samples and the positive control, and can be used for sequencing. Both rounds should be analyzed on an agarose gel. *See the example at the end of the protocol*.
Avoid template contamination. This is especially important for nested set amplifications due to the high sensitivity of the assay. Use dedicated equipment, rooms and hoods for all pre-PCR procedures. Post amplification analysis and processing must be performed in a separate room using dedicated equipment. Very important: the DNA template for Round 2 must be added in a separate location with dedicated pipettors. Do not share equipment (including lab coats) between pre-PCR and post-PCR procedures. Use filtered pipet tips for all pre-PCR procedures and for setting up RT-PCR reactions. **If your laboratory does not have the facilities to use separate rooms and equipment for pre- and post-amplification, the nested set assay should not be performed.**

Reagents and materials needed

- 70% ethanol
- Lab coats
- Disposable gloves
- RNase inhibitor 2000 units (Applied Biosystems N-808-0119)
- 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)
- Two sets of rubella specific primers described below (can be requested from CDC)
- Rubella virus RNA to serve as a positive control (can be requested from CDC)

Equipment needed

- -70ºC and -20ºC freezers
- Bucket with ice
- Class II biological safety cabinets with UV light (BSC)
- Micropipettors and sterile pipette tips with aerosol-resistant filters
- Vortex mixer
- Metal cooling rack for 0.2 ml tubes
- Thermocycler (AB GeneAmp PCR System 9700 or equivalent)
- 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 separate rooms 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 filtered pipet 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.
- Reduce DNA contamination in the BSC with UV light exposure for 15 minutes between uses.

Information about primers

- RuV primer sequences

Round 1 RT-PCR: 8633F and 9577R

Name	Size	Sequence	NT #s

8633F	20 nts	5’ AGC GAC GCG GCC TGC TGG GG 3’	8633-8652
9577R	21 nts	5’ CGC CCA GGT CTG CCG GGT CTC 3’	9557-9577

Round 2 PCR: 8669F and 9541R

Name	Size	Sequence	NT #s
8669F	20 nts	5’ GTG ATG AGC GTG TTC GCC CT 3’	8669-8688
9541R	21 nts	5’ GTG TGT GCC ATA CAC CAC GCC 3’	9521-9541

- Primer stock solutions are stored at -20ºC.

Sample Preparation

RNAs extracted from clinical samples are stored at -70ºC. Usually, RNA extractions are done using 100-200 µl of clinical material. Most extraction protocols yield 50-60 µl of RNA. For the worksheet for the Qiagen OneStep RT-PCR kit that can be used for this assay, 5 µl of RNA obtained after the elution step are used per 50 µl reactions. The volume of the reaction and RNA added can be reduced by half (2.5 µl RNA/25 µl reaction) to save reagents if desired.
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Assay Controls

- The following controls **must** be run in each assay. They must be included in master mix calculations:
  - Negative controls
    - Water control: add nuclease-free water instead of RNA
    - Extraction control: mock-extracted RNA obtained by extraction of water or media. In the calculations for the number of reactions (see below) the extraction control is tested as an addtional sample.
  - Positive control (RuV control RNA)
    - **Preparation of the RNA and addition of the RNA should be done separately from preparation of master mix.**

Preparations for Assay Set-up

Note: rubella virus has a high GC content and amplification will be enhanced by the addition of a GC melt reagent such as the Qiagen Q solution included in the kit. Always add this reagent for rubella genotyping reactions.

- Thaw kit reagents: 5X buffer, Q solution, dNTP mix, and primers and briefly vortex.
- Spin down all reagents to collect liquid in the bottom of the tubes (including enzymes) in a micro-centrifuge 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 open date of reagents on worksheet for rubella genotyping RT-PCR.

Assay Protocol

1. For RuV nested set genotyping two master mixes need to be set up – one for each primer set (Round 1 and Round 2). *Note: there are differences between the Round 1 master mix and the Round 2 master mix*. For each primer set 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: for each master mix the number of reactions is the sum of the number of samples (which includes the extraction control) plus two for the water control and the positive control plus one to allow for pipetting losses.

Example: If there are four specimens and one extraction control: make master mixes 1 for 8 reactions.

- 5 samples (specimens and extraction control)
- 1 water control
- 1 RuV positive control RNA
- 1 extra to allow for pipetting losses

2. Enter the number of samples in the Excel Genotyping Master Mix worksheet to determine volumes of each reagent to be added.
3. In a 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. Label one set of tubes for Round 1 and one set for Round 2.
4. For each master mix, add appropriate volumes (see Excel Worksheet for Rubella Genotyping RT-PCR) of the first 6 reagents (NF water through reverse primer) to pre-chilled 1.5 ml micro-centrifuge tubes. For master mix Round 1 use primers RV8633/RV9577 and for master mix Round 2 use primers RV8669/RV9541. Vortex and keep tubes on ice. *Note: the water volume added is different between master mixes 1 and 2 and no RNase inhibitor is added to master mix 2.*
5. Add RNase inhibitor to Master Mix 1 and enzyme mix to both master mix tubes. Vortex and chill briefly on ice.
6. Briefly spin down the master mix tubes in a micro-centrifuge. Dispense 45µl master mix 1 to each Round 1 reaction tube. Dispense 49ul of master mix 2 to each Round 2 reaction tube. Keep reaction tubes on ice in the metal cooling rack after the master mix is dispensed.
7. Proceed to a separate BSC designated for template addition. Using a new, clean pipette tip for each transfer, add test sample RNA(s) to the appropriate **Round 1 tubes only** and close the cap. The final volume is 50 µl.
8. Add negative controls (NF water and extraction control). Lastly, add the positive control (5 µl control RNA) to the **Round 1 tubes only**.
9. Close caps tightly; rotate tubes by hand to mix. Spin the tubes briefly in a micro-centrifuge and immediately return the tube to the metal cooling rack.
10. Start the appropriate program in the thermocycler (see cycling conditions below). Place the samples for Round 1 in the block and start the run. Store the tubes for Round 2 on ice or in a 4° C refrigerator during the Round 1 amplification.
11. At the end of the Round 1 amplification, remove the tubes from the thermocycler. Place tubes on ice and take to a designated location for transfer of an aliquot to Round 2. **This should NOT be the same location as the addition of template for Round 1. It should be located in an area for post-PCR processing.**
12. Process the negative controls first, then the test samples using a dedicated pipettor. Open the cap of the Round 1 tube and remove **1µl** of product. Close the cap of Round 1 tube. Open the cap of the equivalent Round 2 tube and add the aliquot. Discard the tip and proceed with the additional samples. Lastly, transfer an aliquot of the Round 1 positive control using a different pipettor (this pipettor should be used only for positive control transfer). Store the Round 1 tubes on ice during Round 2.
13. Place the Round 2 tubes into the thermocycler and start the appropriate Round 2 program in the thermocycler. Note: *The RT portion (50°C for 30 minutes) of the program is removed for Round 2*.

Cycling parameters for Qiagen-Rubella Round 1
50°C for 30 minutes
95°C for 15 minutes
40 cycles of:
95°C for 30 seconds
60°C for 30 seconds
72°C for 1minute
Followed by 72°C for 5 minutes and final hold at 4°C

Cycling parameters for Qiagen-Rubella Round 2
95°C for 15 minutes
40 cycles of:
95°C for 30 seconds
60°C for 30 seconds
72°C for 1minute
Followed by 72°C for 5 minutes and final hold at 4°C

Worksheet for Rubella Genotyping RT-PCR (Qiagen One-step kit)
Below is an example of a worksheet.

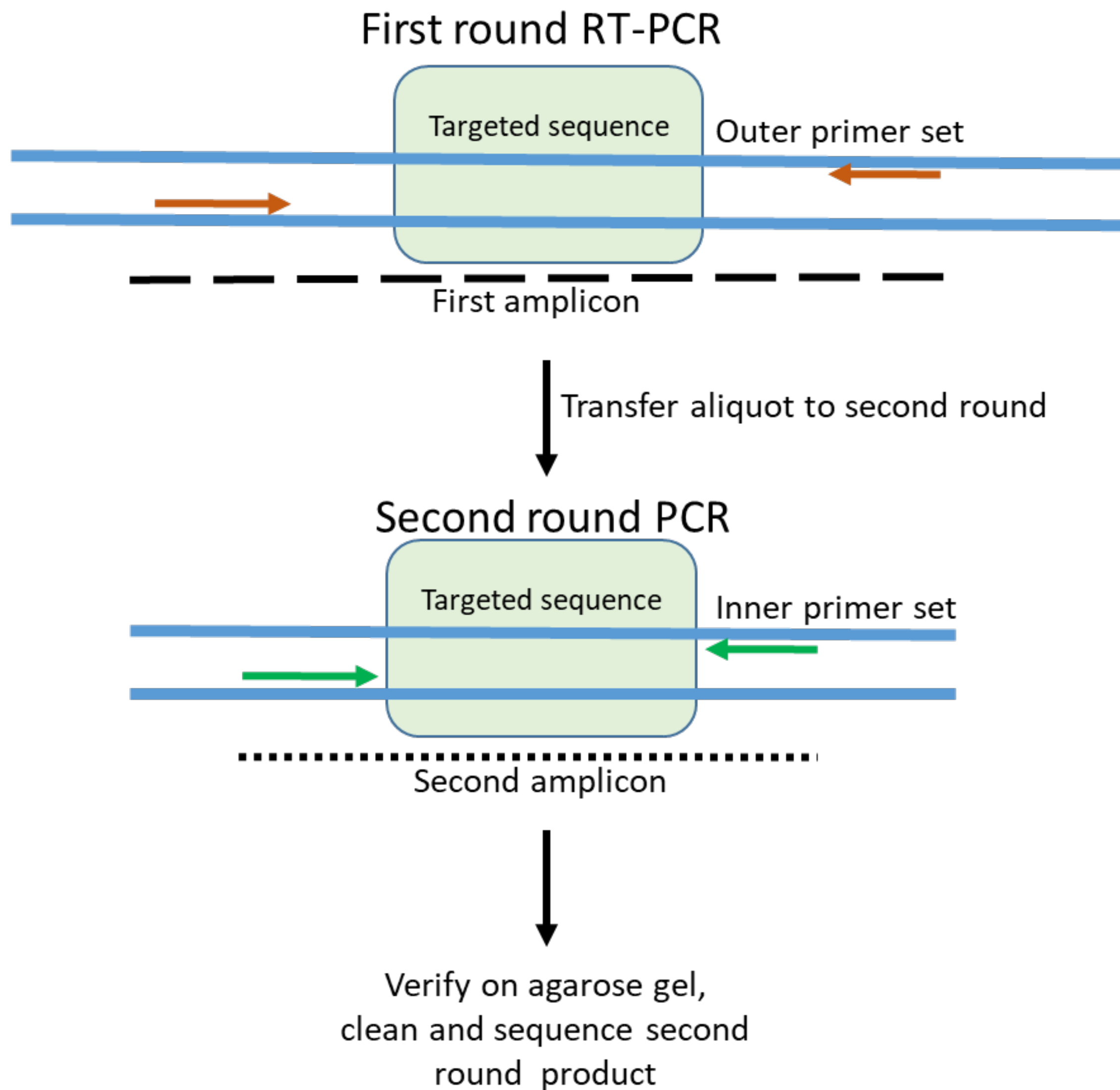
Technician:
Test date:
Kit: Qiagen One-step RT-PCR kit (Qiagen CAT# 210210 or 210212)
Date opened:
Primer lot number:
Number of specimens (including extraction control):
Number of additional reactions: 3

Component: **Master Mix Round 1**	Vol/rxn	Final conc.	# of rxns	Total vol.
1. Nuclease Free water	17.5 µl			
2. 5x Qiagen One-Step RT-PCR buffer	10 µl	1x		
3.Q Solution	10 µl			
4. dNTP mix	2 µl	0.4 mM		
5. Forward primer RV8633 (20 µM)	1.5 µl	0.6 µM		
6. Reverse primer RV9577 (20 µM)	1.5 µl	0.6 µM		
7. RNase inhibitor 40 U/µl	0.5 µl	0.4 U/µl		
8. Qiagen One-Step Enzyme Mix	2 µl			
9. RNA	5 µl		xxxxx	xxxxx

Component: **Master Mix Round 2**	Vol/rxn	Final conc.	# of rxns	Total vol.
1. Nuclease Free water	22 µl			
2. 5x Qiagen One-Step RT-PCR buffer	10 µl	1x		
3.Q Solution	10 µl			
4. dNTP mix	2 µl	0.4 mM		
5. Forward primer RV8669 (20 µM)	1.5 µl	0.6 µM		
6. Reverse primer RV9541 (20 µM)	1.5 µl	0.6 µM		

~~7. RNase inhibitor 40 U/µl~~	~~0.5 µl~~	~~0.4 U/µl~~		
8. Qiagen One-Step Enzyme Mix	2 µl			
9. DNA (from Round 1)	1 µl		xxxxx	xxxxx

Total reaction volume: 50 µl for each Round; dispense 45 ul for Round1, 49 ul for Round 2
Positive control:____________
Negative control:____________



Example of products run on 1.5% gel