Rubella Real-TM Qual

Handbook

Real-Time PCR test for qualitative detection of Rubella Virus

REF TV24-50FRT

Σ 50

NAME

RUBELLA Real-TM Qual

INTENDED USE

RUBELLA Real-TM Qual is a Real-Time test for the qualitative detection of Rubella (Rosolia) Virus RNA in the plasma, serum, umbilical blood, mucosal swabs (nasal, oral), lavages, amniotic liquid, tissue. RUBELLA RNA is extracted from specimens, amplified using RT-amplification and detected using fluorescent reporter dye probes specific for Rubella or Rubella IC.

PRINCIPLE OF ASSAY

Rubella virus detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region by using specific primers. In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that bind specifically to the amplified product. The real-time monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening of the reaction tubes after the PCR run. **RUBELLA Real-TM Qual** PCR kit is a qualitative test that contains the Internal Control (IC), which must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition. **RUBELLA Real-TM Qual** PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase by using a chemically modified polymerase (TaqF), which is activated by heating at 95 °C for 15 min.

MATERIALS PROVIDED

“Ribo-Sorb-50”: isolation of RNA from clinical specimens

- **Lysis Solution**, 22,5 ml;
- **Washing Solution**, 20 ml;
- **Sorbent**, 1,25 ml.
- **RNA-buffer**, 5 x 0,5ml;

Contains reagents for 50 tests.

“RUBELLA Real-TM Qual”: RT Real Time kit

- **RT-G-mix-2**, 0,015ml.
- **RT-PCR-mix-1-FRT Rubella**, 0,6 mL.
- **RT-PCR-mix-2-FEP/FRT**, 0,3 mL.
- **Polymerase (TaqF)**, 0,03 mL
- **TM Revertase (M-MLV)**, 0,015 mL;
- **Positive Control cDNA *Rubella* / STI (C+)**, 0,1 mL
- **RNA-buffer**, 0,6 mL
- **Negative Control (C–)***, 0,5 ml x 2;
- **Positive Control *Rubella* -rec****, 0,1 ml x 2;
- **Internal Control STI-87-rec (IC)*****, 0,5 mL

* *must be used in the isolation procedure as Negative Control of Extraction.*
** *must be used in the isolation procedure as Positive Control of Extraction (add 10 µl of RNA C+ Rec and 90 µl of Negative Control to the tube labeled PCE).*
*** *add 10 µl of Internal Control to each sample during the RNA purification procedure directly to the sample/lysis mixture*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes (RCF max. 16,000 x g); Eppendorf 5415D or equivalent
- 60°C ± 2°C dry heat block
- Vortex mixer
- Pipettors with aerosol barrier
- 1,5 ml polypropylene sterile tubes (Sarstedt, QSP, Eppendorf)
- Disposable gloves, powderless
- Tube racks
- 70% Ethanol (freshly prepared mixture of reagent grade 96% ethanol and distilled water)
- Acetone
- Refrigerator
- Freezer

Zone 2: RT and amplification:

- Real Time Thermal cycler
- Reaction tubes
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes
- Vortex mixer
- Freezer, refrigerator

WARNINGS AND PRECAUTIONS

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

STORAGE INSTRUCTIONS

All components of the **RUBELLA Real-TM Qual** PCR kit (except for RT-G-mix-2, RT-PCR-mix-1-FRT *Rubella virus,* RT-PCR-mix-2-FEP/FRT, polymerase (TaqF), and TM-Revertase (MMlv)) are to be stored at 2–8 ºC. All components of the **RUBELLA Real-TM Qual** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

RT-G-mix-2, RT-PCR-mix-1-FRT *Rubella* virus*,* RT-PCR-mix-2-FEP/FRT, polymerase (TaqF) and TM-Revertase (MMlv) are to be stored from minus 24 to minus 16 °C.

RT-PCR-mix-1-FRT *Rubella virus* is to be kept away from light.

Ribo-Sorb must be stored at 2-25°C.

RUBELLA Real-TM Qual PCR kit and **Ribo-Sorb** should be transported at 2–8 °C for no longer than 5 days but should be stored at suggested temperature immediately on receipt.

STABILITY

RUBELLA Real-TM Qual Test is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

RUBELLA Real-TM Qual can analyze RNA extracted from:

- *Peripheral and umbilical cord blood plasma.* Collect blood to a Vacuett tube (lavender cap, 6% EDTA) after overnight fasting or at least 3 h after the patient had a meal. Invert the tube several times to ensure proper mixing of blood with the anticoagulant. Centrifuge the tube with blood at 800–1600 g at room temperature for 20 min. Take 1.0 ml of plasma and transfer it to a sterile 2.0-ml Eppendorf tube.
- *Saliva.* Collect 0.2–1.0 ml of saliva to a 1.5-ml Eppendorf tube. Have the patient to rinse his mouth with water 3 times before sampling saliva.
- *Oropharyngeal swabs* are obtained with a dry cotton probe from the tonsillar area, palatine arches, and posterior oropharyngeal surface. Have a patient to rinse his mouth with water before swabbing.

  After sampling, the cotton end of the probe should be placed into a sterile tube containing 500 µl of transport medium. Then the probe should be broken off at the score mark and the tube should be tightly closed.
- *Amniotic fluid* should be obtained during amniocentesis by the standard procedure and collected to a sterile Eppendorf tube. Thoroughly resuspend the obtained sample and transfer 1 ml of it to a new sterile tube. Centrifuge the tube at 8,000–9,000 g for 10 min. Remove the supernatant leaving 200 µl of the fluid over the pellet. Use tips with aerosol barrier. Resuspend the pellet.
- *Bronchial lavage, nasal wash:* centrifuge at 2000 g/min for 10-15 min. If the pellet is not visible add 10 ml of liquid and repeat centrifugation. Remove and discard the supernatant. Resuspend the pellet in 100 µl of Saline water.
- *Tissue* homogenized with mechanical homogenizer and dissolved in PBS sterile

Specimens can be stored at +2-8°C for no longer than 12 hours, or frozen at -20°C to -80°C.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

RNA ISOLATION

The following isolation kits are recommended:

- ⇒ **Ribo-Sorb** (Sacace, REF K-2-1)
- ⇒ **Ribo-Virus** (Sacace, REF K-2/C)
- ⇒ **DNA/RNA-Prep** (Sacace, REF K-2-9);
- ⇒ **SaMag Viral Nucleic Acids Extraction kit** (Sacace, REF SM003) for plasma.

Please carry out the RNA extraction according to the manufacturer's instructions.

During extraction, use the following controls:

- Positive Control *Rubella virus*-rec (Positive Control of Extraction, PCE): *add 90 µl of Negative Control (C–) and 10 µl of RUBELLA RNA C+ Rec to the tube labeled PCE;*
- Negative Control (C–): *add 100 µl of Negative Control (C–) to labeled C-*
- Internal Control STI-87-rec (IC): *add 10 µl of Internal Control RNA during the RNA isolation procedure directly to the sample/lysis mixture.*

SPECIMEN AND REAGENT PREPARATION

1. **Lysis Solution** and **Washing Solution** should be warmed up to 60–65°C until disappearance of ice crystals.
2. Prepare required quantity of 1.5 ml polypropylene tubes including one tube for **Negative Control of Extraction** and one tube for **RNA Positive Control of Extraction**.
3. Add to each tube **10 µl** of **Internal Control** and **450 µl Lysis Solution**.
4. Add **100 µl** of **Samples** to the appropriate tube.
5. Prepare Controls as follows:
   - add **100 µl** of **Negative Control** to each of the two control tubes.
   - add **10 µl RUBELLA RNA C+ Rec** to the tube labeled C*pos*.
6. Vortex the tubes and centrifuge for 3-5 sec.
7. Vortex vigorously **Sorbent** and add **25 µl** to each tube.
8. Vortex for 5-7 sec and incubate all tubes for 10 min at room temperature. Vortex periodically.
9. Centrifuge all tubes for 45 sec at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between tubes.
10. Add **400 µl** of **Washing Solution** to each tube. Vortex vigorously and centrifuge for 45 sec at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet.
11. Add **500 µl** of **70% Ethanol** to each tube. Vortex vigorously and centrifuge for 45 sec at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet.
12. Repeat step 11.

13. Add **400 µl** of **Acetone** to each tube. Vortex vigorously and centrifuge for 45 sec at 10000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet.
14. Incubate all tubes with open cap for 10 min at 60°C.
15. Resuspend the pellet in **50 µl** of **RNA-buffer.** Incubate for 5 min at 60°C and vortex periodically.
16. Centrifuge the tubes for 1 min at maximum speed (12000-16000 g). The supernatant contains RNA/DNA ready for use. The amplification can be performed on the same day of extraction. If this is not possible, the RNA preparations can be stored at -80°C for up to one month.

REAGENTS PREPARATION (REACTION VOLUME 25 µL):

1. Prepare required quantity of reaction tubes.
2. Prepare for each sample in the new sterile tube **Reaction Mix**: add **10 µl** of **RT-PCR-mix-1**, **5 µl** of **RT-PCR-mix-2, 0,25 µl** of **RT-G-mix-2**, **0,50 µl** of **TaqF Polymerase** and **0,25 µl** of **M-MLV Revertase**. Vortex thoroughly and centrifuge for 5 sec. This mix must be used immediately. Don't store the prepared mix!

Reagents volume x 1 reaction (µl)		**10,0**	**5,00**	**0,25**	**0,50**	**0,25**
N RNA samples[1]	N reactions[2]	RT-PCR-mix-1	RT-PCR-mix-2	RT-G-mix-2	TaqF Polymerase	M-MLV Revertase
4	6	60	30	1,5	3,0	1,5
6	8	80	40	2,0	4,0	2,0
8	10	100	50	2,5	5,0	2,5
10	12	120	60	3,0	6,0	3,0
12	14	140	70	3,5	7,0	3,5
...58	60	600	300	15,0	30,0	15,0

[1] specimens plus 2 extraction controls (N+2)
[2] specimens plus extraction and amplification controls (N+2+2)

3. Add **15 µl** of **Reaction Mix** into each tube.
4. Add **10 µl** of **extracted RNA** sample to appropriate tubes with Reaction Mix and mix well by pipetting.

(*Re-centrifuge all the tubes with extracted RNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. N.B. don't disturb the pellet, sorbent inhibit reaction!).*

5. Prepare for each panel 2 controls:
   - add **10 µl** of **RNA-buffer** to the tube labeled Negative Control;
   - add **10 µl** of **RUBELLA cDNA Pos & IC Pos** to the tube labeled Positive Control;

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line.

Rubella cDNA is detected on the JOE(Yellow)/HEX/Cy3 channel, IC DNA on the FAM (Green) channel

AMPLIFICATION

Program the real-time instrument according to manufacturer's manual.

Amplification program for rotor-type instruments[1]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
Hold	50	15 min	–	1
Hold 2	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling2	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow	
	72	15 s		

[1] *For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)*

Fluorescence is detected at the 2nd step of Cycling 2 stage (60 °C) in FAM/Green and JOE/Yellow fluorescence channels.

Amplification program for plate-and modular type instruments[2]

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Repeats*
1	50 °C	15 min	–	1
2	95 °C	15 min	–	1
3	95 °C	5 s	–	5
	60 °C	20 s	–	
	72 °C	15 s	–	
4	95 °C	5 s	–	40
	60 °C	30 s	FAM, HEX/Cy3/Joe	
	72 °C	15 s		

[2] *For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid), LineGeneK® (Bioer)*

Fluorescence is detected at stage 4 (60 °C) in FAM and HEX fluorescence channels.

SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct	Dynamic tube
FAM/Green	*from 3 Fl to 8 Fl*	0.03	10 %	On	On
JOE/Yellow	*from 3 Fl to 8 Fl*	0.03	10 %	On	On

Plate-type instruments

Channel	Threshold
FAM	The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.
HEX/Joe/Cy3	

DATA ANALYSIS

Accumulation of ***Rubella virus* cDNA** amplification product is detected in the **JOE/Yellow/HEX** channel, Internal Control amplification product is detected in the **FAM/Green** channel.

The results are interpreted by the software of the PCR instrument used by the crossing (or not crossing) of the fluorescence curve with the threshold line.

The results of analysis are considered reliable only if the results obtained for Positive and Negative Controls of Amplification as well as for the Negative Control of Extraction are correct.

Results for controls

Control	Stage for control	Ct in channel		Interpretation
		FAM/Green	JOE/Yellow/HEX	
NCE	RNA extraction	≤ 35	Neg	OK
PCE	RNA extraction	≤ 35	≤ 35	OK
NCA	RT-PCR	Neg	Neg	OK
C+	RT-PCR	≤ 35	≤ 35	OK

1. The sample is considered **positive** if its Ct value detected in the JOE/Yellow/HEX channel does not exceed the boundary Ct value (< 40 for clinical samples) and the Ct value detected in the FAM/Green channel does not exceed the value specified for the Internal Control (Ct < 35). The fluorescence curve should have a typical sigmoid shape and cross the threshold line once in the region of significant fluorescence increase.
2. The sample is considered **negative** if its Ct in the JOE/Yellow/HEX channel is not detected (the fluorescence curve does not cross the threshold line) and the Ct value detected in the FAM/Green channel does not exceed the boundary Ct value specified for the Internal Control (Ct < 35).

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), positive control of extraction (PCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of **RUBELLA Real-TM Qual** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis. The clinical specificity of **RUBELLA Real-TM Qual** PCR kit was confirmed in laboratory clinical tests.

Analytical sensitivity

The kit **RUBELLA Real-TM Qual** allows to detect *RUBELLA* RNA in 100% of the tests with a sensitivity of not less than 400 copies/ml.

The claimed analytical features of **RUBELLA Real-TM Qual** PCR kit are guaranteed only when an additional reagent kit (DNA/RNA-prep or RIBO-sorb or Ribo-Virus) is used.

Target region: p150 gene

REFERENCES

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TROUBLESHOOTING

The results of analysis are not taken into account in the following cases:

1. If the Ct value of a clinical sample detected in the JOE/Yellow/HEX channel exceeds the boundary Ct value (>37), the result is considered **equivocal**. It is necessary to repeat the analysis twice. If a reproducible positive Ct value is detected, the sample is considered to be **positive**.
2. If any Ct value is detected for the Negative Control of Amplification (NCA) in both channels or the Ct value is detected for Negative Control of Extraction (C–) in the JOE/Yellow/HEX channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples are considered **invalid**. It is necessary to repeat the analysis of all tests and to take measures to detect and eliminate the source of contamination.
3. If the Ct value is absent for the Positive Control of Extraction (PCE), this indicates improper extraction procedure. RNA extraction should be repeated for all samples.
4. If the Ct value is absent for the Positive Control of RT-PCR (C+), this indicates errors in carrying out PCR or an incorrect amplification program. RT-PCR should be repeated for all samples.
5. If the Ct value of a clinical sample is absent or greater than the boundary Ct value (>37) for the JOE/Yellow/HEX channel and the Ct value in the FAM/Green channel is greater than the Ct values specified for the Internal Control (>37), the result is **invalid**. Analysis of such samples should be repeated starting from the RNA extraction stage.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
	Expiration Date	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**C–**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
		IC	Internal Control

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