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For Professional Use Only

GUIDELINES

to **AmpliSens®** ***Rubella virus*-FRT** PCR kit

for qualitative detection of *Rubella virus* RNA in the biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *Rubella virus*-FRT** PCR kit for qualitative detection of *Rubella virus* RNA in the biological material (peripheral and umbilical cord blood plasma, saliva, oropharyngeal swabs, and amniotic fluid) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- RotorGene Q (QIAGEN, Germany);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA);
- Mx3000P, Mx3005P (Stratagene, USA)

and also in combination with the automatic station for the nucleic acids extraction NucliSENS easyMAG (bioMérieux, France).

Correspondence of targets and detection channels

Fluorophore	**FAM**	**JOE**
Detection channel name for different instrument models	**FAM/Green**	**JOE/HEX/R6G/Yellow/Cy3**
cDNA-target	Internal Control STI-87-rec cDNA	*Rubella virus** cDNA
Target gene	Artificially synthesized sequence	*Rubella virus* nonstructural protein P150

* In March 2021, International Committee on Taxonomy of Viruses (ICTV) changed the species name of *Rubella virus* to *Rubivirus rubella.*

WORK with the NucliSENS easyMAG AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM

Variant 1

RNA extraction with lysis of sample outside of the instrument (off-board mode)

This method of extraction allows reducing the consumption of NucliSens lysis buffer. It is preferred for working with test samples which contain clots (saliva).

1. Switch on the NucliSENS easyMAG instrument and prepare it to the RNA extraction according to the instruction manual.
2. In the window for input of test samples, enter the following parameters:
   - Sample name
   - ***Matrix*** for RNA extraction (select ***Other***)
   - ***Volume*** – ***0.1 ml***
   - ***Eluate*** – ***55 µl***
   - ***Type*** – ***Lysed***
   - ***Priority*** – ***Normal***.
3. Create a new protocol of RNA extraction and save it. In the protocol select ***On-board Lysis Buffer Dispensing*** - ***No***, ***On-board Lysis Incubation*** - ***No***.
4. Relocate the sample table into the created protocol.
5. Take the required quantity of special disposable tubes intended for RNA extraction in the NucliSENS easyMAG instrument (including Negative Control of extraction). Add **10 µl** of **Internal Control STI-87** to inner walls of each tube and then add **550 µl** of **NucliSens lysis buffer**.

NOTE: When working with material which contains clots, lysis should be carried out in 1.5-ml tubes. After finishing the incubation (see item 8), tubes should be centrifuged at 10,000 rpm for 1 min. Then transfer the supernatant into special tubes intended for RNA/DNA extraction in the NucliSENS easyMAG instrument

6. Add **100 µl** of prepared samples into each tube with **Lysis buffer** and **Internal Control STI-87-rec** by using disposable tips with aerosol barriers and carefully mix by pipetting (avoid getting mucus clots and big particles into the tube).
7. Add **100 µl** of **Negative Control** into the tube with the Negative Control of Extraction (C–). Add **90 µl** of **Negative Control** and **10 µl** of **Positive Control *Rubella virus*-rec (PCE)** into the tube with the Positive Control of Extraction (PCE).
8. Incubate the tubes for 10 min at room temperature.
9. Resuspend the tube with **magnetic silica NucliSens** (bioMérieux) by intensive vortexing. Add **25 µl** of **magnetic silica** by using disposable tips with aerosol barriers

and carefully mix by pipetting. Magnetic silica should be distributed evenly over the tube volume.

10. Place the tubes with samples into the instrument and start the RNA extraction program with lysis of samples by selecting the ***Off-board*** mode.
11. After the extraction is finished, take the tubes out of the instrument and carry out the RT-PCR not later than 30 min after RNA extraction.

If necessary to store, transfer purified RNA into sterile tubes within 30 min after RNA extraction.

The purified RNA should be stored at 2–8° C for 8 hours, at the temperature ≤ –16 °C for 1 month, and at the temperature ≤ –70° C for a long time.

Variant 2

RNA extraction with automated lysis of sample in the instrument (on-board mode)

1. Switch on the NucliSENS easyMAG instrument and prepare it for the RNA extraction according to the instruction manual.
2. In the window for input of test samples, enter the following parameters:
   - Sample name
   - ***Matrix*** for RNA extraction (select ***Other***)
   - ***Volume*** – 0.1 or 1 ml
   - ***Eluate*** – 55 µl
   - ***Type*** – ***Primary***
   - ***Priority*** – ***Normal***.
3. Create a new protocol of RNA extraction and save it. In the protocol, select ***On-board Lysis Buffer Dispensing*** - ***Yes***, ***On-board Lysis Incubation*** - ***Yes***.
4. Relocate the programmed sample into the created protocol.
5. Take the required quantity of special disposable tubes intended for RNA extraction in NucliSENS easyMAG instrument (including negative control of extraction). Add **10 µl** of **Internal Control STI-87-rec** into each tube along the internal walls.
6. Add **100 µl** of prepared samples into each tube with **lysis buffer** and **Internal Control** by using disposable tips with aerosol barriers and carefully mix by pipetting (avoid getting mucus clots and big particles into the tube).
7. Add **100 µl** of **Negative Control** into the tube with Negative Control of Extraction (C-). Add **90 µl** of **Negative Control** and **10 µl** of **Positive Control *Rubella virus*-rec (PCE)** into the tube with the Positive Control of Extraction (PCE).
8. Place the tubes with samples into the instrument, place the tips and run the RNA

extraction with lysis of samples in the instrument (the ***On-board*** mode).

9. Wait until the NucliSENS easyMAG instrument proceeds to the ***Instrument State–Idle*** option and pauses.
10. Thoroughly vortex the tube with **magnetic silica NucliSens** (bioMérieux). Open the lid of instrument and add **25 µl** of **magnetic silica** into each tube by using disposable tips with aerosol barriers (or by using multichannel pipettes with disposable tips with aerosol barriers for **200 µl**) and carefully mix by pipetting. Magnetic silica should be distributed evenly over the tube volume.
11. Close the lid of the instrument and continue the RNA isolation program.
12. After the extraction is finished, take the tubes out of the instrument and carry out the RT-PCR not later than 30 min after RNA extraction.

AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) INSTRUMENTS

When working with Rotor-Gene 3000 one should use the Rotor-Gene version 6.1 and higher software and the Rotor-Gene 6000 versions 1.7 (build 67) software or higher for Rotor-Gene 6000 and Rotor-Gene Q instruments.

Hereinafter, all the terms corresponding to different instruments and software are indicated in the following order: for Rotor-Gene 3000 / for Rotor-Gene 6000/Q.

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with flat caps (e.g. Axygen, USA), or PCR tubes (0.1 ml) with caps from the four-pieces-strips (e.g. Corbett Research, Australia; QIAGEN, Germany) (detection through the bottom of the tube).

Programming the thermocycler

1. Turn on the instrument, run the Rotor-Gene software.
2. Insert the tubes or strips into the rotor of the Rotor-Gene 3000/6000/Q instrument beginning from the first well (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler). Insert the rotor into the instrument, close the lid.

NOTE: Well 1 must be filled with any test tube except for an empty one. If the tubes with reagents from different PCR kits or with different PCR-mixes are inserted into the rotor then the tubes' numbers for calibration in each detection channel should be indicated in the Rotor-Gene software. Recommendations about the calibration are described in the information list "Calibration priorities for the AmpliSens kits for Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) thermocyclers".

3. Program the instrument according to the *Instruction Manual* given by the manufacturer of the instrument.

Creating the template for the run

1. Click the ***New*** button in the software main menu. To create the template select the ***Advanced*** tab in the opened window ***New run***.
2. Select the ***TwoStep/Hidrolysis Probes*** template in the tab for edition and click The ***New*** button.
3. In the opened window select the ***36-Well Rotor (or 72-Well Rotor)*** and tick the ***No Domed 0,2ml Tubes / Locking Ring Attached*** option. Click the ***Next*** button.
4. In the opened window enter the operator name, select the reaction volume – 25 µl. Tick the ***15 µl oil layer volume*** option. Click the ***Next*** button.

5. In the ***New Run Wizard*** window set the temperature profile of the experiment. To do this click the ***Edit profile*** button and set the amplification program:

AmpliSens-2 amplification program

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
Hold	50	15 min	–	1
Hold 2	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s	–	

NOTE: It is possible to carry out any combination of tests that use the AmpliSens-2 universal amplification program within the same run.

Note – The ROX/Orange and Cy5/Red channels are enabled when required if the "multiprime" format tests are performed.

6. After setting up the temperature profile click the ***OK*** button.
7. In the ***New Run Wizard*** window, click the ***Calibrate*** or ***Gain Optimisation*** button. In the opened window:
   a) for signal measurement optimisation for the selected channels set calibration from **5Fl** to **10Fl** for FAM/Green, JOE/Yellow channels.
   To do this, click the ***Calibrate Acquiring/Optimise Acquiring*** button. In the opened window for first channel (***Auto Gain Optimisation Channel Settings/Auto Gain Calibration Channel Settings***) indicate the values of minimum and maximum signal in the ***Target Sample Range*** line. Click the ***OK*** button. The window for the next channel will open automatically. The selected values for all the channels can be checked in the ***Min Reading, Max Reading*** boxes.

NOTE: The additional requirements for setting the channels' calibration ranges are specified in the information list "Calibration priorities for the AmpliSens kits for Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) thermocyclers"

   b) perform the calibration in the selected channels before the first detection (tick the ***Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition*** option). Click the ***Close*** button.
8. Click the ***Next*** button. For saving the programmed template it is necessary to click the ***Save Template*** button and enter the template file name, corresponding to the amplification program – **AmpliSens**. Save the file into a proposed folder: ***Templates\Quick Start Templates***; close the ***New Run Wizard window.*** After that the

programmed template will appear in the template list in the ***New Run*** window.

Using the ready template for the run

1. Click the ***New*** button in the software main menu. In the opened ***New Run*** window select the ***Advanced*** tab. Then select the **AmpliSens** template (which is programmed as described in the “Creating the template for the run” section) in the template list.
2. In the opened window select the ***36-Well Rotor*** (***or 72-Well Rotor***) and tick the ***No Domed 0,2ml Tubes / Locking Ring Attached*** option. Click the ***Next*** button.
3. In the opened window check that the reaction volume is 25 µl and the ***15 µl oil layer volume*** option is activated. Click the ***Next*** button.
4. In the next window the correctness of the amplification program and signal level auto-optimisation parameters can be checked. Go to the next window clicking the ***Next*** button. Start the amplification by the ***Start run*** button. Herewith, the rotor with the samples should be already fixed and the lid should be closed. Name the experiment and save it to the disc (the results of the experiment will be automatically saved in this file).
5. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Enter the names/numbers of the test samples in the ***Name*** column. Define the Negative control of amplification as NCA, the Positive control of amplification as C+. Set the type ***Unknown*** opposite all the test samples, the type ***Positive control*** – for the Positive control of amplification, the type ***Negative control*** – for the Negative control of amplification. Set the type ***Standard*** for DNA-calibrators C1 and C2 and enter the values from the *Important product information bulletin* enclosed to the given lot of the PCR kit into the ***Given conc.*** column. Set the type ***None*** for the cells matching with the corresponding empty tubes. Click the ***Finish/OK*** button.

NOTE: Samples indicated as ***None*** won’t be analysed.

Note – To edit the table of samples before the start it is needed previously to select the ***Edit Samples Before Run Started*** option in the ***User preferences*** submenu of the ***File*** menu.

Data analysis:

The obtained results are analyzed by the Rotor-Gene software. The results are interpreted according to the crossing (or not-crossing) of the S-shaped (sigmoid) fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value of the DNA sample in the corresponding column of the results table.

Amplification data analysis in the FAM/Green channel:

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the buttons ***Cycling A. FAM/Cycling A. Green***, ***Show***.
2. Cancel the automatic choice of the threshold line level ***Threshold***.
3. Select the ***Linear scale***.
4. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the menu of main window (***Quantitation analysis***).
5. In the ***Calculation*** menu (in the right part of the window) indicate the threshold line level **0.03** in the ***Threshold*** box.
6. Choose the parameter ***More settings/Outlier Removal*** and set **10 %** for the value of negative samples threshold (***NTC/Threshold***).
7. Set 1 in the ***Eliminate cycles before:*** menu (in the right part of the window).
8. In the results grid (the ***Quantitation Results*** window) one will be able to see the *Ct* values.

Results analysis in the JOE/Yellow channel is carried out similarly to results analysis in the FAM/Green channel in accordance with the settings in the table below.

Канал	***Threshold***	***Dynamic tube***	***Slope Correct***	***More Settings/Outlier Removal***	***Eliminate cycles before:***
FAM/Green	0.03	on	on	10%	1
JOE/Yellow	0.03	on	on	10%	1

NOTE: If the fluorescence curves by the FAM/Green and JOE/Yellow channels do not correspond to exponential growth (they do not have an S-shape), it is allowed to increase the value of the threshold of negative samples (***NTC/Threshold***) to 20%.

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (see the *Instruction manual*) and boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The interpretation of the test samples is to be carried out in accordance with table for results' interpretation of test samples (Table 2) the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

Table 1

Results for controls

Control	**Stage for control**	***Ct* value in the channel**	
		FAM/Green	**JOE/Yellow**
C-	RNA extraction	< boundary value	Absent
PCE	RNA extraction	< boundary value	< boundary value
NCA	PCR	Absent	Absent
C+	PCR	< boundary value	< boundary value

Table 2

Results analysis interpretation for test samples

***Ct* value in the channel**		**Result**
FAM/Green	**JOE/Yellow**	
<boundary value	<boundary value	*Rubella virus* cDNA is **detected**
<boundary value	absent	*Rubella virus* cDNA is **NOT detected**
> boundary value or absent	> boundary value or absent	Invalid
<boundary value	> boundary value	Equivocal

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ5 (Bio-Rad, USA) INSTRUMENTS

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with domed or flat optically transparent caps, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

Programming the thermocycler

1. Turn on the instrument and the power supply unit of the optical block of the instrument.

NOTE: The lamp is to be warmed up during 15 min before starting the experiment.

2. Start the program iCycler iQ5.
3. Insert the tubes or strips into the reaction module of the thermocycler and program the instrument according to the *Instruction Manual* given by the manufacturer of the instrument.

NOTE: Monitor the tubes. There must not be drops left on the walls of the tubes as falling drops during the amplification process may lead to the signal failure and complicate the results analysis. Don't turn the tubes (strips) upside down while inserting them into the instrument.

Creating the template for the run

1. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal).
   - click the ***Create New*** button in the ***Selected Plate Setup*** window of the ***Workshop*** module;
   - in the opened window click the ***Whole Plate loading*** button and set the plate setup using the buttons of the upper toolbar. Enter the samples' names in the ***Identifier/Condition*** column in the bar appeared in the screen bottom. Select the fluorescent signal detection in the FAM, JOE/HEX channels. Click the ***Select/Add Fluorophores*** button, select the fluorophore and tick it in the ***Selected*** column. Click ***OK***. The fluorophore name will appear in the ***Fluorophore*** window. For addition of fluorescence signal measuring for each sample it is necessary to click the fluorophore (activate it) and select the samples on the plate using the ***Fluorophore loading in whole Plate mode***  button under the scheme;
   - set the reaction volume (***Sample Volume***) as **25 µl**, the caps type (***Seal Type***) as ***Domed Cap***, and the tubes type (***Vessel Type***) as ***Tubes***;
   - Save the set plate setup by clicking the ***Save&Exit Plate Editing*** button. Enter the file name and click ***Save***.

2. Set all the biological samples as ***Unknown***, positive controls as «**+**», and negative controls as «**–**».
3. Set the amplification program. To do this, in the ***Selected Protocol*** window of the ***Workshop*** module click the ***Create New*** button. Set the amplification parameters and save the protocol by activating the ***Save&Exit Protocol Editing*** button. Enter the name of the file and then click ***Save***.

AmpliSens-2 amplification program

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
1	50	15 min	–	1
2	95	15 min	–	1
3	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
4	95	5 s	–	40
	60	20 s	FAM, JOE/HEX, ROX, Cy5	
	72	15 s	–	

NOTE: It is possible to carry out any combination of tests that use the AmpliSens-2 universal amplification program within the same run. ROX and Cy5 channels are activated if necessary (for “multiprime” format tests).

Note – The ROX and Cy5 channels are enabled when required if the “multiprime” format tests are performed.

4. Before a run it is obligatory to check if the selected protocol (***Selected Protocol***) and the plate scheme (***Selected Plate Setup***) are correct. To begin a run click the ***Run*** button. For the well factors measurement the ***Use Persistent Well Factors*** type is selected by default. Click the ***Begin Run*** button, save the experiment (the results of this experiment will be automatically saved in this file) and click ***OK***.
5. At the end of the run it is necessary to close the software and turn off the instrument (the thermocycler and the optical block).

Using the ready template for the run

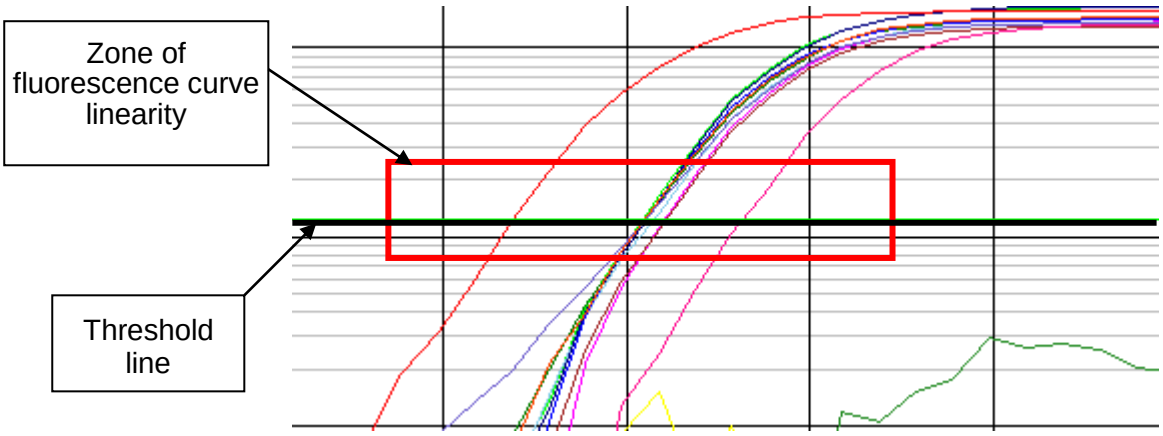
The test parameters and the plate setup set earlier can be used for the further runs. To do this:

- select the needed file with the run in the upper left window of the ***Workshop*** module;
- click the ***Edit*** button in the ***Selected Plate Setup*** area of the ***Workshop*** module and edit the plate setup (the files of protocols are saved in the ***SampleFiles*** folder by default);
- click the ***Edit*** button in the ***Selected Protocol*** area of the ***Workshop*** module and check the correctness of the selected protocol (the files of protocols are saved in the ***Users*** folder by default).

Data analysis

The obtained results are analyzed by the iCycler iQ5 software. The results are interpreted according to the crossing (or not-crossing) of the S-shaped (sigmoid) fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value in the corresponding column of the results table.

1. Start the software and open the needed file with data of the analysis in the ***Data File*** window of the ***Workshop*** module. Click the ***Analyze*** button.
2. Select the ***Analysis Mode: PCR Base Line Subtracted Curve Fit*** (is set by default).
3. Check the correctness of threshold line automatic choice for each channel. The threshold line is to cross only with S-shaped (sigmoid) curves describing the accumulation of the signal detecting positive samples and controls. The threshold line is not to cross the base line. If it happens, it is necessary to set the threshold line level for each channel manually. To do this, click the ***Log View*** (logarithmic scale selection) and set (with the left mouse button) the threshold line at the level where the fluorescence curves have a linear character and do not cross with the curves of the negative samples (see the figure below).



4. In order to analyze the results click the ***Results*** button which is situated under the buttons with the fluorophores' names (for **iCycler iQ5**).

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (Table 3).

The interpretation of the test samples is to be carried out in accordance with table for results' interpretation of test samples (Table 4) the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

Table 3

Results for controls

Control	Stage for control	*Ct* value in the channel

		FAM	JOE/HEX
C-	RNA extraction	< boundary value	Absent
PCE	RNA extraction	< boundary value	< boundary value
NCA	PCR	Absent	Absent
C+	PCR	< boundary value	< boundary value

Table 4

Results analysis interpretation for test samples

***Ct* value in the channel**		**Result**
FAM	**JOE/HEX**	
<boundary value	<boundary value	*Rubella virus* cDNA is **detected**
<boundary value	absent	*Rubella virus* cDNA is **NOT detected**
> boundary value or absent	> boundary value or absent	Invalid
<boundary value	> boundary value	Equivocal

AMPLIFICATION AND DATA ANALYSIS USING Mx3000P, Mx3005P (Stratagene, USA) INSTRUMENT

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit Instruction Manual. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with domed or flat optically transparent caps, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

1. Switch on the instrument. Run the software Stratagene Mx3000P.
2. Select ***Quantitative PCR (Multiple Standards)*** and ***Turn lamp on for warm-up*** in ***New Experiment Options*** window.

NOTE: The lamp is to be warmed up during 15 min before starting the experiment.

3. Insert the tubes into the instrument and close the lid.
4. Select ***Optics Configuration*** in the ***Options*** menu and in ***Dye Assignment*** tab set **JOE** parameters opposite to the ***HEX/JOE filter set*** item, **FAM** parameters in front of the ***FAM filter set*** item.

NOTE: Don't turn the strips/plate upside down while inserting them into the instrument.

5. Lock the fixing arm and the door of the instrument
6. Select ***Quantitative PCR (Multiple Standards)*** in ***New Experiment Options*** window and set ***Turn lamp on for warm-up***.
7. Set fluorescence detection parameters in the ***Plate Setup*** menu. For this:
   - Select all wells with the test tubes or strips (hold ***Ctrl*** button down and select the necessary region with the mouse).
   - Mark all selected cells as ***Unknown*** in the ***Well type*** window. Set **FAM** and **JOE** in ***Collect fluorescence data*** option. Name each sample by double clicking on each cell (***Well Information*** window).
8. Set fluorescence detection parameters for tubes in the ***Plate Setup*** tab. For this:
   - Select all cells with analysis tubes or strips (holding ***Ctrl*** down and selecting the required range)
   - Mark all selected cells as ***Unknown*** in the ***Well type*** window. Set **FAM** and **JOE** in ***Collect fluorescence data*** option. Name each sample by double clicking on each cell (***Well Information*** window), positive control indicate as **+**, negative - as **-**.
9. In the ***Thermal Profile Setup*** tab, set the amplification program according to the one of this modes:

<u>Using of the template file</u> for setting the amplification program (is recommended).

Press the ***Import…*** button right to thermocycling profile picture. Proceed to the folder containing previous experimental file and open it. In the ***Thermal Profile*** window required thermocycling profile will appear.

Individual programming.

1. After setting all necessary values and parameters, select all wells with tested microtubes again. Proceed to ***Thermal Profile Setup*** menu, set the amplification program:

AmpliSens-2 amplification program

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Number of cycles*
1	50	15 min	–	1
2	95	15 min	–	1
3	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
4	95	5 s	–	40
	60	20 s	FAM, JOE/HEX, ROX, Cy5	
	72	15 s	–	

NOTE: It is possible to carry out any combination of tests that use the AmpliSens-2 universal amplification program within the same run. ROX and Cy5 channels are activated if necessary (for “multiprime” format tests).

Note – The ROX and Cy5 channels are enabled when required if the “multiprime” format tests are performed.

2. To set detection parameter of fluorescent signal at desired temperature, select the ***All points*** option for ***Data collection by marker dragging*** parameter and move it by mouse from right side to the shelf with desired temperature.
3. Select ***Run*** and ***Start*** and name the experiment file.

Data analysis

The obtained results (fluorescence accumulation curves for two channels) are analyzed by the instrument software. The results are interpreted according to the crossing (or not-crossing) of the fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value of the RNA sample in the corresponding column of the results table.

Data processing

1. In **Mx3000P** software, select ***Analysis*** by clicking the corresponding button of the tool bar.
2. The ***Analysis Selection/Setup*** tab will open. Make sure that all the test samples are active (the cells corresponding to the samples should be of a different colour).

Otherwise select all the test samples by holding down the ***Ctrl*** button and selecting the needed range with the mouse.

3. Select the ***Results*** tab.
4. Make sure that two fluorescence channels are active (the **FAM, JOE/HEX** buttons are activated in the ***Dyes Shown*** field in the lower part of the program's window).
5. Select the ***Threshold fluorescense*** field and make sure that tick marks are put against two fluorescence channels: FAM and JOE/HEX. Check the correctness of the automatically chosen threshold line. Normally, the threshold line is to intersect only with S-shaped curves describing the accumulation of the signal detecting positive samples and controls The threshold line is not to cross the base line. If it happens, it is necessary to raise the threshold level.
6. Select ***Text Report*** point in the ***Area to analyze*** window. Visually ensure that all data is sorted by the name of dyes (the ***Dye*** column). To do this, press once the name of the column (***Dye***).
7. Select ***Export Text Report*** in the ***File*** menu, then select ***Export Text Report to Excel*.** A Microsoft Excel window will open.

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (Table 3).

The interpretation of the test samples is to be carried out in accordance with table for results' interpretation of test samples (Table 6) the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

Table 5

Results for controls

Control	Stage for control	*Ct* value in the channel	
		FAM	JOE/HEX
C-	RNA extraction	< boundary value	Absent
PCE	RNA extraction	< boundary value	< boundary value
NCA	PCR	Absent	Absent
C+	PCR	< boundary value	< boundary value

Table 6

Results analysis interpretation for test samples

Ct value in the channel		Result
FAM	JOE/HEX	
<boundary value	<boundary value	*Rubella virus* cDNA is **detected**
<boundary value	absent	*Rubella virus* cDNA is **NOT detected**

Ct value in the channel		Result
FAM	**JOE/HEX**	
> boundary value or absent	> boundary value or absent	Invalid
<boundary value	> boundary value	Equivocal

TROUBLESHOOTING

1. The *Ct* value determined for the Positive Control of RT-PCR (C+) in the channels for the FAM and/or JOE fluorophores is greater than the boundary *Ct* value specified in the *Important Product Information Bulletin* or absent. The amplification and detection should be repeated for all the samples.
2. The *Ct* value determined for the Positive Control of Extraction (PCE) in the channels for the FAM and/or JOE fluorophores is greater than the boundary *Ct* value specified in the *Important Product Information Bulletin* or absent. The PCR analysis (beginning with the RNA extraction stage) should be repeated for all samples.
3. If the *Ct* value is determined for the Negative Control of Extraction (C–) in the channel for the JOE fluorophore. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The PCR analysis (beginning with the RNA extraction stage) should be repeated for all samples.
4. If the *Ct* value determined for the Negative Control of Extraction (C–) in the channel for the FAM fluorophore is greater than the boundary *Ct* value specified in the *Important Product Information Bulletin* or absent. The PCR analysis (beginning with the RNA extraction stage) should be repeated for all samples in which specific RNA was not detected.
5. The *Ct* value is determined for the Negative Control of RT-PCR (NCA) in the channels for the FAM and/or JOE fluorophores. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The PCR analysis should be repeated for all samples.
6. If the *Ct* value is determined for the test sample, whereas the area of typical exponential growth of fluorescence is absent (the graphic looks like approximate straight line). It is necessary to check the correctness of selected threshold line level or parameters of base line calculation. If the result has been obtained with the correct level of threshold line (base line), the amplification and detection should be repeated for this sample.
7. The *Ct* value determined for the test sample in the channels for the FAM and/or JOE fluorophores is greater than the boundary *Ct* value specified in the *Important Product Information Bulletin*. The PCR analysis (beginning with the RNA extraction stage) should be repeated for the appropriate test sample.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
11.05.11 RT	Amplification and data analysis using Rotor-Gene 3000/6000 instrument (Corbett Research, Australia). Programming the Rotor-Gene 3000/6000 instrument	In item 5 table 1 was deleted (Amplification program)
	Amplification and data analysis using iCycler iQ/iQ5 instruments (Bio-Rad, USA)	In item 2 table 3 was deleted (Amplification program)
	Amplification and data analysis using Mx3000P, Mx3005P (Stratagene, USA). Manual programming	In item 1 table 5 was deleted (Amplification program)
23.06.11 LA	Cover page	The name of Institute was changed to Federal Budget Institute of Science "Central Research Institute for Epidemiology"
01.02.12 VV	Amplification and data analysis using Rotor-Gene 3000/6000 instrument (Corbett Research, Australia) Data analysis of the *Rubella virus* cDNA (JOE/Yellow channel).	The phrase "The *NTC threshold* value can be raised to 20% if a weak signal (Ct) from the Green/FAM channel is detected for the positive control of RT-PCR in the Yellow/JOE channel. The nonspecific signal associated with cross-detection has a lower rise value than the specific signal" was deleted.
31.07.13 ME	Cover page	**RUO** symbol was added
	Footer	**REF** R-V24-S(RG,iQ,Mx)-CE was deleted
02.07.15 PM	Through the text	Corrections according to the template
31.05.21 VA	Cover page	The phrase "For research use only. Not for diagnostic procedures" was added
22.06.23 EM	Footer	**REF** R-V24-S(RG,iQ,Mx)-CE was added
18.08.25 PM	Through the text	Corrections according to the template
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) instruments	The optimization of signal measurement using the FAM/Green, JOE/Yellow channels has been changed from «3 Fl to 8 Fl» to «5Fl to 10Fl». The the value for ***Eliminate cycles before*** parameter have been added
	Troubleshooting	The section has been added