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For Professional Use Only

GUIDELINES

to AmpliSens® ARVI-screen-FRT PCR kit

for detection of ARVI pathogens: *human Respiratory Syncytial virus – hRSv* RNA, *human Metapneumovirus – hMpv* RNA*, human Parainfluenza virus-1-4 – hPiv* RNA*,* HKUI *human Coronavirus – hCov* RNA, *human Rhinovirus – hRv* RNA, *human B, C, E Adenovirus – hAdv* DNA and *human Bocavirus – hBov* DNA 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 ARVI-screen-FRT** PCR kit for detection of ARVI pathogens: *human Respiratory Syncytial virus – hRSv* RNA, *human Metapneumovirus – hMpv* RNA, *human Parainfluenza virus-1-4 – hPiv* RNA, OC43, E229, NL63, HKUI *human Coronavirus – hCov* RNA, *human Rhinovirus – hRv* RNA, *human B, C, E Adenovirus – hAdv* DNA and *human Bocavirus – hBov* DNA in the biological material by means of polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000/6000 (Corbett Research, Australia);
- Rotor-Gene Q (Qiagen, Germany);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA);
- CFX96 (Bio-Rad, USA);

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

Table 1

Correspondence of PCR-mixes-1 and channels for ARVI pathogen detection

PCR-mix-1-FL	**Channel**		
	FAM/Green	**JOE/HEX/Yellow**	**ROX/Orange**
	Detection of IC	**Detection of pathogen**	**Detection of pathogen**
hRSv - hMpv	IC	*hRSv*	*hMpv*
hAdv - hBov	IC	*hBov*	*hAdv*
hRv	IC	–	*hRv*
hPiv 1/3	IC	*hPiv3*	*hPiv1*
hPiv 2/4	IC	*hPiv2*	*hPiv4*
hCov	IC	*NL-63, 229E*	*HKU-1, OC 43*

WORK WITH NucliSENS easyMAG AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM

Variant 1.

RNA extraction with off-board sample lysis (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 (sputum, aspirates).

1. Switch on the NucliSENS easyMAG instrument and prepare it for the RNA/DNA extraction according to the instruction manual.
2. In the window for input of test samples, enter the following parameters for each sample:
   - Sample name;
   - ***Matrix*** for RNA/DNA extraction (select ***Other***);
   - ***Volume*** – 0.1 ml;
   - ***Eluate*** – 25 µl;
   - ***Type*** – Lysed;
   - ***Priority*** – Normal.
3. Create a new protocol of RNA/DNA extraction and save it. Select ***On-board Lysis Buffer Dispensing – No***, ***On-board Lysis Incubation – No*** in the protocol.
4. Relocate sample table into the created protocol.
5. Take the required number of specialized disposable tubes intended for RNA/DNA extraction in the NucliSENS easyMAG instrument (include negative control of extraction). Add **10 µl of Internal Control STI-rec** 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 the prepared samples into each tube with **lysis buffer** and **Internal Control STI-rec (IC)** using tips with aerosol filters and carefully mix by pipetting (avoid adding mucus clots and large particles to the tube).
7. Add **100 µl** of **Negative Control (C–)** into the tube with Negative Control of Extraction (C-).
8. Incubate tubes for 10 min at room temperature.
9. Resuspend the tube with **magnetic silica NucliSens** by intensive vortexing. Add **25 µl** of **magnetic silica** using a new one filter tip for each sample, carefully mix by pipetting. Magnetic silica should be distributed evenly throughout the tube volume.
10. Place the tubes with samples into the instrument, insert the tips, and start the RNA

extraction program with lysis of samples by selecting the ***off board*** mode.

11. After finishing RNA/DNA extraction take the tubes out of the instrument. The supernatant contains purified RNA and DNA. **Carry out reverse transcription immediately after RNA extraction.** If necessary to store, purified RNA should be transferred into sterile tubes no later than 30 min after extraction. The purified RNA can be stored:
    - at 2-8 °C for 4 hours;
    - at not more than minus 16 °C for 1 month;
    - at not more than minus 68 °C for a long time.

Variant 2.

RNA extraction with on-board sample lysis (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/DNA extraction (select ***Other***);
    - ***Volume*** – 0.1 ml;
    - ***Eluate*** – 25 µl;
    - ***Type*** – Primary;
    - ***Priority*** – Normal.
3. Create a new protocol of RNA/DNA extraction and save it. Select ***On-board Lysis Buffer Dispensing – Yes***, ***On-board Lysis Incubation – Yes*** in the protocol.
4. Relocate the sample table into the created protocol.
5. Take the required number of special disposable tubes intended for RNA/DNA extraction in the NucliSENS easyMAG instrument (including negative control of extraction). Add **10 µl of Internal Control STI-rec (IC)** to inner walls of each tube.
6. Add **100 µl** of the **prepared samples** into each tube with **Internal Control** using tips with aerosol filters (avoid adding mucus clots and large particles to the tube).
7. Add **100 µl** of **Negative Control (C–)** into the tube intended for the **Negative Control of Extraction (C–).**
8. Place tubes with samples into the instrument, insert the tips, and start the RNA extraction program with lysis of samples by selecting the ***on board*** mode.
9. Wait until NucliSENS easyMAG instrument proceeds to the ***Instrument State - Idle*** option and pauses.
10. Thoroughly vortex the tube with **magnetic silica NucliSens**. Open the lid of the instrument and transfer **25 µl of magnetic silica** into each tube using a new one filter tip

for each sample and mix thoroughly by pipetting. Make sure that magnetic silica is evenly distributed throughout the tube.

11. Close the lid and continue the RNA extraction program.
12. After finishing RNA/DNA extraction take the tubes out of the instrument. The supernatant contains purified RNA and DNA. **Carry out reverse transcription immediately after RNA extraction.** If necessary to store, purified RNA should be transferred into sterile tubes no later than 30 min after extraction. The purified RNA can be stored:
    - at 2-8 °C for 4 hours;
    - at not more than minus 16 °C for 1 month;
    - at not more than minus 68 °C for a long time.

AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) 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 0.2-ml clear tubes with flat caps (detection trough the bottom of the tube), or 0.1 ml tubes.

Insert the tubes 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.

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

Programming the thermocycler

1. Click the ***New*** button in the main menu.
2. In the opened window, select the template of the experiment start-up ***Advanced*** and mark ***Dual Labeled Probe/Hydrolysis probes/***. Press the ***New*** button.
3. In the opened window, select **36-Well Rotor** (or **72-Well Rotor**) and ***No Domed 0.2 ml Tubes/Locking ring attached***. Click ***Next***.
4. In the opened window, set the operator and select the reaction mixture volume: ***Reaction volume*** – **25 µl**. For Rotor-Gene 6000 set check in front of ***15 µl oil layer volume***. Click ***Next***.
5. In the opened window, set the temperature profile of experiment: press the ***Edit profile*** button and set the following parameters (see Table 2):

Table 2

Amplification program

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycles*

Hold	95	15 min	–	1
Cycling	95	10 s	–	10
	54	20 s	–	
	72	10 s	–	
Cycling 2	95	10 s	–	35
	54	20 s	FAM/Green, JOE/Yellow, ROX/Orange	
	72	10 s	–	

6. Click the ***OK*** button
7. In the window ***New Run Wizard***, click the ***Calibrate/Gain Optimisation…*** button.
   - Perform calibration in FAM/Green, JOE/Yellow and ROX/Orange channels (click the ***Calibrate Acquiring/Optimise Acquiring*** button);
   - Perform calibration before the first measurement (***Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition***);
   - Set channel calibrations for all dyes from 5Fl to 10Fl (***Edit…*** button, ***Auto gain calibration channel settings*** window). Click the ***Close*** button.

NOTE: In case of simultaneous amplification of different types of PCR-mix-1-FL, do not perform calibration for PCR-mix-1-FL *hRv*.

8. Click the ***Next*** button, click ***Start run*** for amplification run.
9. Name the experiment and save it on the hard drive (results of the experiment will be automatically saved in this file).
10. Enter data into the table of samples (opens automatically after the amplification start). Set names/numbers of test and control samples in ***Name*** column. For empty wells indicate ***None***.

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

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 fluorescence curve with the threshold line, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value in the corresponding column of the results table.

Amplification data analysis in the FAM/Green channel:

1. Activate the ***Analysis*** button 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. Activate the ***Dynamic tube*** button in the menu of main window (***Quantitation analysis***).
4. In ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level

***Threshold* = 0.1**.

5. Choose the parameter ***More settings/Outlier Removal*** and set **0 %** for the value of negative samples threshold (***NTC/Threshold***).
6. In the results grid (***Quant. Results*** window), *Ct* values will appear.

Amplification data analysis in the JOE/Yellow channel:

1. Activate the ***Analysis*** button in the menu, select the mode of the analysis ***Quantitation***, activate the buttons ***Cycling A. JOE/Cycling A. Yellow, Show***.
2. Cancel the automatic choice of the threshold line level ***Threshold***.
3. Activate the ***Dynamic tube*** button in the menu of main window (***Quantitation analysis***).
4. In ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level ***Threshold* = 0.1**.
5. Choose the parameter ***More settings/Outlier Removal*** and set **5 %** for the value of negative samples threshold (***NTC/Threshold***).
6. In the results grid (***Quant. Results*** window), *Ct* values will appear.

Amplification data analysis in the ROX/Orange channel:

1. Activate the ***Analysis*** button in the menu, select the mode of the analysis ***Quantitation***, activate the buttons ***Cycling A. ROX/Cycling A. Orange***, ***Show***.
2. Cancel the automatic choice of the threshold line level ***Threshold***.
3. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the main window menu (***Quantitation analysis***).
4. In ***CT Calculation*** (in the right part of the window) indicate the threshold line level ***Threshold* = 0.1**.
5. Choose the parameter ***More settings/Outlier Removal*** and set **5 %** for the value of negative samples threshold (***NTC/Threshold***).
6. In the results grid (***Quant. Results*** window) *Ct* values will appear.

NOTE: For analysis of results of *Parainfluenza virus* type 1 and *human Coronavirus* cDNA amplification reaction (the ROX/Orange channel), the ***Slope Correct*** button should be inactive. For *Parainfluenza virus* type 1 cDNA the ***More settings/Outlier Removal*** value should be **10 %**.

NOTE: For analysis of results of *Parainfluenza virus* type 3 cDNA amplification reaction (the JOE/Yellow channel), the ***Slope Correct*** button should be active, and the ***More settings/Outlier Removal*** value should be **10 %**.

NOTE: For analysis of results of *human Adenovirus – hAdv* DNA amplification reaction (the ROX/Orange channel), the ***More settings/Outlier Removal*** value should be **3 %**, the threshold line level should be **0.05**.

Interpretation of results for control samples

Results of analysis are accepted as relevant if the results obtained for positive and negative controls of amplification as well as negative and positive controls of extraction are correct (see Table 3). The results for positive and negative control samples should not exceed the *Ct* values specified in Table 3 (for Rotor-Gene 3000, Rotor-Gene 6000, and Rotor-Gene Q).

Table 3

Results for controls for Rotor-Gene 3000/6000/Q

Control	**Stage for control**	***Ct* value in channel**		
		FAM/Green	**HEX/Yellow**	**ROX/Orange**
		IC STI-rec detection	**Pathogen detection**	**Pathogen detection**
For all PCR-mixes-1-FL				
NCA	amplification	neg	neg	neg
C-	RNA/DNA extraction	< 30	neg	neg
CS+	amplification	< 29	neg	neg
For corresponding PCR-mix-1-FL				
C+$_{hRSv\text{-}hMpv}$	amplification	neg	< 24	< 24
C+$_{hAdv\text{-}hBov}$	amplification	neg	< 22	< 24
C+$_{hRv}$	amplification	neg	–	< 21
C+$_{hPiv\ 1/3}$	amplification	neg	< 24	< 24
C+$_{hPiv\ 2/4}$	amplification	neg	< 24	< 24
C+$_{hCov}$	amplification	neg	< 22	< 22

Interpretation of results for test samples

1. **Sample is considered positive** if the *Ct* value determined in the results grid in the **JOE/Yellow** and/or **ROX/Orange** channels does not exceed the specified boundary value (see Table 4).
2. **Sample is considered negative** if the *Ct* value is not determined in the **JOE/Yellow** and/or **ROX/Orange** channels (the fluorescence curve does not cross the threshold line) and the *Ct* value determined in the FAM/Green channel does not exceed the specified boundary value (see table 4).
3. **Sample is considered invalid** if the *Ct* value is not determined (absent) in all channels for detection of ARVI pathogens and the *Ct* value in the FAM/Green channel is also absent or exceeds the specified boundary value. In this case, repeat the test for this sample starting from the DNA/RNA extraction stage.
4. **Sample is considered equivocal** if the *Ct* value determined in the **JOE/Yellow** and/or **ROX/Orange** channels in the results grid exceeds the specified boundary value (see table 4). Repeat the test starting from the DNA/RNA extraction stage. If the obtained result is the same, the sample is considered positive. If the obtained result is negative,

the sample is considered equivocal.

Table 4

Results for test samples for Rotor-Gene 3000/6000/Q

PCR-mix-1-FL	***Ct* value in channel**		
	FAM/Green	**JOE/Yellow**	**ROX/Orange**
	IC STI-rec detection	**Pathogen detection**	**Pathogen detection**
hRSv - hMpv	< 30	***hRSv*** < 28	***hMpv*** < 31
hAdv - hBov	< 30	***hBov*** < 28	***hAdv*** < 31
hRv	< 30	–	***hRv*** < 27
hPiv 1/3	< 30	***hPiv3*** < 31	***hPiv1*** < 30
hPiv 2/4	< 30	***hPiv2*** < 30	***hPiv4*** < 30
hCov	< 30	***NL-63, 229E*** < 30	***HKU-1, OC43*** < 30

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

1. Analysis for samples (except NCA) with negative results in all detection channels should be repeated starting from the DNA amplification step. If the same result was obtained again, repeat analysis starting from the RNA/DNA extraction step. For the NCA sample, negative result in all fluorescence detection channels is normal.
2. If the *Ct* value obtained for C+ in the corresponding channel is absent or exceeds the boundary value of the threshold cycle, repeat amplification for all negative samples.
3. If the *Ct* value is determined for the Negative Control of extraction (C-) and/or Negative Control of Amplification (NCA) in the channel for ARVI pathogen detection, repeat the test for all samples where DNA/RNA of the corresponding pathogen was detected from RNA/DNA extraction step to avoid possible contamination.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and 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 optically transparent domed or flat 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).

NOTE:	**It is not allowed** to perform «*Rhinovirus»* test together with other tests from AmpliSens® ARVI-screen-FRT PCR kit when working with **iCycler iQ** and **iQ5** instruments.

1. Turn on the instrument and optical module 20–30 min before measurement.
2. Start the program iCycler/iQ5.
3. Set plate setup (arrangement of the tubes in the reaction chamber and fluorescence measurement in all tubes and in **FAM, HEX** and **ROX** channels).

NOTE:	For the «*Rhinovirus»* (*hRv*) test analysis, it is necessary to use **ONLY FAM** and **ROX** channels. **It is not allowed** to perform «*Rhinovirus»* test together with other tests from AmpliSens® ARVI-screen-FRT PCR kit when working with **iCycler iQ** and **iQ5** instruments.

- In case of the **iCycler iQ5** instrument, for plate setup creation press the ***Create New*** or ***Edit*** button in the ***Selected Plate Setup*** window of the ***Workshop*** module*.* It is possible to edit plate setup in the ***Whole Plate loading*** mode*.* Set the reaction volume *(**Sample Volume**)* **25 µl**, ***Seal Type**:* **Domed Cap***,* and the type of tubes *(**Vessel Type**):* **Tubes***.* Save the plate setup: press the ***Save&Exit Plate Editing*** button.
- for the **iCycler iQ** instrument**,** edit the plate setup in ***Edit Plate Setup*** window of the ***Workshop*** module*.* In option ***Samples: Whole Plate Loading*** set arrangement of the samples in reaction chamber and name every sample in the ***Sample Identifier*** window. In the ***Select and load Fluorophores*** option, set fluorescence measurement for all tubes in **FAM, JOE/HEX** and **ROX** channels**.** Save the plate setup: set the file name with .pts extension in the ***Plate Setup Filename*** window, and press the ***Save this plate setup*** button. The previously used ***Plate Setup*** can be edited: in ***Library*** window, open ***View Plate Setup***, select the required ***Plate Setup*** (file with .pts extension), and press the ***Edit*** button. The edited file should be saved before use. Press the ***Run with selected protocol*** button in order to start working with the selected plate setup.

4. Set the amplification program (see Table 5).

Table 5

Amplification program

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Cycles***
1	95	15 min	–	1
2	95	10 s	–	10
	54	25 s	–	
	72	25 s	–	
3	95	10 s	–	35
	54	25 s	FAM, JOE/HEX, ROX	
	72	25 s	–	

- For **the iCycler iQ5** instrument, in order to create a protocol, press the ***Create New*** or ***Edit*** button in the ***Selected Protocol*** window of the ***Workshop*** module. Set the amplification parameters and save the protocol by pressing the ***Save&Exit Protocol Editing*** button. For further experiments, the file with this program can be selected in the ***Protocol*** block (protocol files are saved in ***Users*** folder by default).
- For the **iCycler iQ** instrument, create the amplification program: select ***Edit Protocol*** option of ***Workshop*** module. Set the amplification parameters in the bottom window (cycle repeats, time, and temperature), in the right window specify the scanning step for the fluorescent signal: Cycle 3 – Step 2. Save the protocol, name the file in ***Protocol Filename*** window (file with .tmo extension), and press the ***Save this protocol*** button (in the upper part of the screen). For further experiments you can select the file with this program in the ***View Protocol*** tab in the ***Library*** module. Press the ***Run with selected plate setup*** button after selecting or editing the selected program to start it.

5. Put the prepared tubes into the reaction module according to the selected plate setup.

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.

- For the **iCycler iQ5** instrument, before running the program, ensure that the protocol (***Selected Protocol***) and the plate setup (***Selected Plate Setup)*** were selected. Press the ***Run*** button to start the program. Select the ***Collect Well Factors from Experimental Plate*** variant. Press the ***Begin Run*** button, name the experiment (results of the experiment will be saved in this file automatically), and press the ***OK*** button.
- For the **iCycler iQ** instrument**,** before running the program, ensure that the name of the protocol and the plate setup were selected correctly in the ***Run Prep*** window. For well factor measurement, select the ***Experimental Plate*** variant in the ***Select well factor source*** menu. Set the reaction mix volume in the window ***Sample Volume* – 25 µl**. Press the ***Begin Run*** to start the program, name the experiment (results of the experiment will

be saved in this file automatically), and press the ***OK*** button*.*

Proceed to the results analysis at the end of the program

Data processing and analysis

The obtained results are analyzed by the iCycler iQ5 / iQ software. The results are interpreted according to the crossing (or not-crossing) of the S-shaped 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.

NOTE: Data analysis for each PCR-mix-1 should be carried out individually; use only the group of PCR tubes referring to the given PCR-mix-1.

Data processing

- For the **iCycler iQ5** instrument, select the required file with data for analysis (in the ***Data File*** window of the ***Workshop*** module*)* and press the ***Analyze*** button. Select data for the corresponding channel in the module window. Data analysis mode ***PCR Base Line Subtracted Curve Fit*** should be selected (by default). To set the threshold line level, click on it and then drag it by holding the left mouse button down. Set the threshold line sequentially for FAM, JOE/HEX, and ROX channels (by pressing the left mouse button down and dragging) at a level corresponding to 10–20 % of the maximum fluorescence level recorded for the positive control samples, C+, during the last amplification cycle. The fluorescent curve of C+ should cross the threshold line on the interval of exponential growth of fluorescence coming into the linear growth phase. Press the ***Results*** button to display the results grid.
- For the **iCycler iQ** instrument, activate the ***View Post-Run Data*** window in the ***Library*** module. In the ***Data Files*** window, select the required file with data analysis and press the ***Analyse Data*** button. In the ***PCR Quantification*** option (***Select a Reporter*** menu) select the icon of the corresponding channel. The ***PCR Base Line Subtracted Curve Fit*** data analysis mode should be selected (by default). In the ***Threshold Cycle Calculation*** menu, select manual setting of the threshold line and automatic baseline calculation. Select the ***Auto Calculated*** in the ***Baseline Cycles*** submenu, select ***User Defined*** in the ***Threshold Position*** submenu. click on it and then drag it by holding the left mouse button down. Set the threshold line sequentially for FAM, JOE/HEX, and ROX channels (by pressing the left mouse button down and dragging) at a level corresponding to 10–20 % of the maximum fluorescence level obtained for the positive control samples, C+, during the last amplification cycle. Make sure that fluorescence curve of the Positive Control crosses the threshold line at the zone of exponential growth of fluorescence passing onto linear growth. Press the ***Recalculate Threshold Cycles*** button ***Ct*** values

will appear in the results grid.

Interpretation of results for control samples

Results of analysis are accepted as relevant only if the results obtained for positive and negative controls of amplification as well as negative and positive controls of nucleic acid extraction are correct (see Table 6). Results for positive and negative control samples should not exceed the Ct values specified in Table 6 for **iCycler iQ and iQ5** instruments **(Bio-Rad, USA)**.

Set the threshold level alternately for FAM, JOE/HEX and ROX channels at 10–20 % of the maximal fluorescence level for the positive control sample in the last amplification cycle. The fluorescence curve for the Positive control sample should cross the threshold level during the exponential fluorescence growth, coming into the linear growth phase.

Table 6

Results for controls for iCycler iQ and iQ5 (Bio-Rad, USA)

Control	**Stage for control**	**Signal in channel**		
		FAM	**JOE/HEX**	**ROX**
		IC detection	**Pathogen detection**	**Pathogen detection**
For all PCR-mixes-1-FL				
NCA	Amplification	neg	neg	neg
C-	RNA/DNA extraction	< 31	neg	neg
CS+	Amplification	< 25	neg	neg
For given PCR-mix-1-FL				
C+ $_{hRSv-\ hMpv}$	Amplification	neg	< 25	< 25
C+ $_{hAdv-hBov}$	Amplification	neg	< 24	< 24
C+ $_{hRv}$	Amplification	neg	—	< 24
C+ $_{hPiv\ 1/3}$	Amplification	neg	< 26	< 26
C+ $_{hPiv\ 2/4}$	Amplification	neg	< 26	< 26
C+ $_{hCov}$	Amplification	neg	< 22	< 22

Interpretation of results for test samples

1. **Sample is considered positive** if the *Ct* value determined in the results grid in the **JOE/HEX** and/or **ROX** channels does not exceed the specified boundary value (see Table 7).
2. **Sample is considered negative** if the *Ct* value is not determined in the **JOE/HEX** and/or **ROX** channels (the fluorescence curve does not cross the threshold line) and the *Ct* value determined in the FAM channel does not exceed the specified boundary value (see table 7).
3. **Sample is considered invalid** if the *Ct* value is not determined (absent) in all channels

for detection of ARVI pathogens and the *Ct* value in the FAM channel is also absent or exceeds the specified boundary value. In this case, repeat the test for this sample starting from the DNA/RNA extraction stage.

4. **Sample is considered equivocal** if the *Ct* value determined in the **JOE/HEX** and/or **ROX** channels in the results grid exceeds the specified boundary value (see table 7). Repeat the test starting from the DNA/RNA extraction stage. If the obtained result is the same, the sample is considered positive. If the obtained result is negative, the sample is considered equivocal.

Table 7

Results for test samples for iCycler iQ and iQ5 (Bio-Rad, USA)

PCR-mix-1-FL	***Ct* value in channel**		
	FAM/Green	**HEX/Yellow**	**ROX/Orange**
	IC detection	**Pathogen detection**	**Pathogen detection**
hRSv - hMpv	IC < 31	***hRSv*** < 31	***hMpv*** < 31
hAdv - hBov	IC < 31	***hBov*** < 29	***hAdv*** < 30
hRv	IC < 31	–	***hRv*** < 30
hPiv 1/3	IC < 31	***hPiv3*** < 32	***hPiv1*** < 30
hPiv 2/4	IC < 31	***hPiv2*** < 30	***hPiv4*** < 30
hCov	IC < 31	***NL-63, 229E*** < 32	***HKU-1, OC43*** < 30

Troubleshooting

1. Analysis for samples (except NCA) with negative results in all detection channels should be repeated starting from the DNA amplification step. If the same result is obtained again, repeat analysis starting from the RNA/DNA extraction step. Negative result for the NCA sample in all detection channels is normal.
2. If the *Ct* value obtained for C+ in the corresponding channel is absent or exceeds the boundary value of the threshold cycle, repeat amplification for all negative samples.
3. If *Ct* value is determined for Negative Control of extraction (C-) and/or Negative Control of Amplification (NCA) in the channel for ARVI pathogen detection, repeat the test for all samples where DNA/RNA of the corresponding pathogen was detected from RNA/DNA extraction step to avoid possible contamination.

AMPLIFICATION AND DATA ANALYSIS USING CFX96 (Bio-Rad, 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 optically transparent flat 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 start the Bio-Rad CFX Manager.
2. Program the instrument according to the manufacturer instrument.

Creating a template for the test

1. In the ***Startup Wizard*** window select the position ***Create a new Run/Experiment*** (or select ***New*** and then ***Run…/Experiment…*** in the ***File*** menu)/ Click ***OK.***
2. In the ***Run Setup*** window, select ***Protocol*** and click the ***Create new…*** button. Set amplification parameters (time, temperature, cycles, and fluorescence acquiring cycle) in the opened ***Protocol Editor – New*** window (see Table 8). Set ***Sample Volume* – 25 µl.**

Table 8

Amplification program

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycles*
1	95	15 min	–	1
2	95	10 s	–	10
	54	25 s	–	
	72	25 s	–	
3	95	10 s	–	35
	54	25 s	FAM, HEX, ROX	
	72	25 s	–	

NOTE! Set ***Ramp Rate* 2,5 °C/s** by clicking the ***Step Options*** button for each step of cycling.

3. In the ***Protocol Editor New*** window select ***File***, then ***Save As***, and name the protocol. This protocol can be used for further runs by clicking the ***Select Existing…*** button in the ***Protocol*** tab.

   When the required program is entered or edited, click ***OK*** at the bottom of the window.
4. Select scheme of a tablet. In the ***Plate*** tab click the ***Create new…*** button. Set the tube order in the opened ***Plate Editor – New*** window. Click the ***Select Fluorophore*** button**,** and then indicate with a checkmark ***Selected*** fluorophores: ***FAM, HEX, ROX*** and click ***OK.*** In the ***Sample type*** menu select ***Unknown*** for all samples. Then indicate with a checkmark ***Load*** (in the right part of window) measuring the fluorescence signal of all

samples in the required channels. Define sample names in the ***Sample name*** window, with a ***Load*** must be indicate with a checkmark.

5. Save scheme of a tablet: in the ***Plate Editor New*** window select ***File***, then ***Save As***, and name the file, click ***Save.***
6. Select ***Start Run*** tab. Click ***Open Lid*** button, open the lid instrument. Click ***Close Lid*** button, close the lid instrument. According to a preprogrammed scheme tablet put the reaction tubes in the thermocycler cells.

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 strips upside down while inserting them into the instrument.

7. Click ***Start Run*** button start the selected program with the specified scheme tablet, select the directory to save the fail staging, names file, click ***Save.***

Use templates for the test

In subsequent productions to run the instrument may be used previously set parameters for the test and the programmed scheme plate. To do this:

– In the ***Run Setup*** window, select ***Protocol*** and click the ***Select Existing…,*** button, in the ***Select Protocol*** window select necessary file with amplification program, click ***Open*** button;

– In the ***Run Setup*** window, select ***Plate***, click the ***Select Existing…,*** button, in the ***Select Plate*** window select necessary file with scheme plate click ***Open*** button. Click ***Edit selected*** button to edit the scheme.

Data analysis

Obtained data are analyzed by the software the instrument CFX96. The results are interpreted according to the crossing (or not-crossing) of the S-shaped (sigma form) fluorescence curve with the installed at the relevant level threshold line and shown as the presence (or absence) of the *Ct* (threshold cycle) value in the results grid.

1. Start the program and open the saved file with the data analysis. To do this, select the menu ***File***, then ***Open*** and ***Data file*** and select necessary file.
2. In the ***Data Analysis*** window tab ***Quantification*** shows the curves of fluorescence, location of the test tubes in the plate and the table with the value of the threshold cycles.

For each channel FAM, HEX and ROX set the threshold level line (drag it with a cursor while pressing the left mouse button) at the level of 10-20% of maximum fluorescence obtained for the Positive Controls in the last amplification cycle. Make sure that fluorescence curve of the Positive Control crosses the threshold line at the zone of exponential growth of fluorescence passing onto linear growth.

Results of analysis are accepted as relevant only if the results obtained for negative and positive controls of amplification as well as negative controls of DNA extraction are correct according with the table of assessing results test samples (see Table 9).

To forming report on the setting you need to select from the toolbar ***Tools***, then ***Reports***, and save the created document.

Table 9

Results for controls for CFX96 (Bio-Rad, USA)

Control	**Stage for control**	**Signal in channel**		
		FAM	**HEX**	**ROX**
		IC detection	**Pathogen detection**	**Pathogen detection**
For all PCR-mixes-1-FL				
NCA	Amplification	neg	neg	neg
C-	RNA/DNA extraction	< 31	neg	neg
CS+	Amplification	< 25	neg	neg
For given PCR-mix-1-FL				
C+ ***hRSv- hMpv***	Amplification	neg	< 25	< 25
C+ ***hAdv-hBov***	Amplification	neg	< 24	< 24
C+ ***hRv***	Amplification	neg	—	< 24
C+ ***hPiv* 1/3**	Amplification	neg	< 26	< 26
C+ ***hPiv* 2/4**	Amplification	neg	< 26	< 26
C+ ***hCov***	Amplification	neg	< 22	< 22

Interpretation of results for test samples

1. **Sample is considered positive** if the *Ct* value determined in the results grid in the **HEX** and/or **ROX** channels does not exceed the specified boundary value (see Table 10).
2. **Sample is considered negative** if the *Ct* value is not determined in the **HEX** and/or **ROX** channels (the fluorescence curve does not cross the threshold line) and the *Ct* value determined in the FAM channel does not exceed the specified boundary value (see table 7).
3. **Sample is considered invalid** if the *Ct* value is not determined (absent) in all channels for detection of ARVI pathogens and the *Ct* value in the FAM channel is also absent or exceeds the specified boundary value. In this case, repeat the test for this sample starting from the DNA/RNA extraction stage.
4. **Sample is considered equivocal** if the *Ct* value determined in the **HEX** and/or **ROX** channels in the results grid exceeds the specified boundary value (see table 7). Repeat the test starting from the DNA/RNA extraction stage. If the obtained result is the same,

the sample is considered positive. If the obtained result is negative, the sample is considered equivocal.

Table 10

Results for test samples for CFX96 (Bio-Rad, USA)

PCR-mix-1-FL	***Ct* value in channel**		
	FAM	**HEX**	**ROX**
	IC detection	**Pathogen detection**	**Pathogen detection**
hRSv - hMpv	IC < 31	***hRSv*** < 31	***hMpv*** < 31
hAdv - hBov	IC < 31	***hBov*** < 29	***hAdv*** < 30
hRv	IC < 31	–	***hRv*** < 30
hPiv 1/3	IC < 31	***hPiv3*** < 32	***hPiv1*** < 30
hPiv 2/4	IC < 31	***hPiv2*** < 30	***hPiv4*** < 30
hCov	IC < 31	***NL-63, 229E*** < 32	***HKU-1, OC43*** < 30

Troubleshooting

1. Analysis for samples (except NCA) with negative results in all detection channels should be repeated PCR and detection. If the same result is obtained again, repeat analysis starting from the RNA/DNA extraction step. Negative result for the NCA sample in all detection channels is normal.
2. If the *Ct* value obtained for C+ in the corresponding channel is absent or exceeds the boundary value of the threshold cycle, repeat amplification for all negative samples.
3. If *Ct* value is determined for Negative Control of extraction (C-) and/or Negative Control of amplification (NCA) in the channel for ARVI pathogen detection, repeat the test for all samples where DNA/RNA of the corresponding pathogen was detected from RNA/DNA extraction step to avoid possible contamination.