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For Professional Use Only

GUIDELINES

to **AmpliSens® *Influenza virus* A-type-FRT** PCR kit

for qualitative detection and typing of *Influenza virus* A (identification to subtypes H1N1 and H3N2) RNA in *Influenza virus* cultures and in biological material containing *Influenza virus* A RNA by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

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INTENDED USE

Guidelines describe the procedure of using **AmpliSens® *Influenza virus* A-type-FRT** PCR kit for qualitative detection and typing of *Influenza virus* A (identification to subtypes H1N1 and H3N2) RNA in *Influenza virus* cultures and in biological material containing *Influenza virus* A RNA (nasal and oropharyngeal swabs; sputum or nasopharyngeal or tracheal aspirate; and autopsy material) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000 (three and more channels) (Corbett Research, Australia),
- Rotor-Gene 6000 (five or six channels) (Corbett Research, Australia),
- Rotor-Gene Q (QIAGEN, Germany),
- iCycler iQ (three and more channels), iCycler iQ5 (Bio-Rad, USA),
- SmartCycler II (Cepheid, USA),
- CFX96 (Bio-Rad, USA).

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

Carry out the pretreatment and reaction mixtures preparation according to the instruction manual and Table 1.

Table 1

Correspondence between PCR-mixes-1 and detection channels

PCR-mix-1	Detection in channel		
	FAM/Green	JOE/HEX/ Yellow/Cy3	ROX/Orange/Texas Red
PCR-mix-1-FEP/FRT (F) *Influenza virus* A H1N1	IC cDNA	*Influenza virus* A H1 cDNA	*Influenza virus* A N1 cDNA
PCR-mix-1-FEP/FRT (F) *Influenza virus* A H3N2	IC cDNA	*Influenza virus* A H3 cDNA	*Influenza virus* A N2 cDNA

WORK with the NucliSENS easyMAG AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM

Variant 1

RNA extraction with lysis of sample outside of the instrument

This method allows to reduce the consumption of the NucliSens lysis buffer and is preferred in work with the samples of biological material containing clots (sputum, aspirates).

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*** – 25 µl
   - ***Type*** – Lysed
   - ***Priority*** – Normal.
3. Create a new protocol of RNA/DNA extraction and save it. In protocol select ***On-board Lysis Buffer Dispensing – no***, ***On-board Lysis Incubation – no*****.**
4. Relocate sample table into the created protocol.
5. Take the required number of the specialized disposable tubes for RNA/DNA extraction in the NucliSENS easyMAG instrument including the tube for the negative control of extraction. Add **10 µl** of the **Internal Control STI-rec (IC)** to the internal walls of each tube. Add **550 µl** of **NucliSens lysis buffer** into the tubes.

   NOTE: When working with the material containing clots, It is recommended to carry out the lysis in 1.5-ml tubes. At the end of incubation (see item 8) centrifuge the tubes at 10,000 rpm for 1 min using a microcentrifuge and transfer the supernatant into the specialized tubes intended for the RNA/DNA extraction in the NucliSENS easyMAG instrument.
6. Add **100 µl** of pretreated samples into each tube with lysis buffer and Internal Control STI-rec (IC) using disposable filter tips and carefully mix by pipetting. Avoid transferring the mucous clots and large particles into the tube.
7. Add **100 µl of Negative Control (C–)** to the tube labelled C– (Negative Control of Extraction).
8. Incubate the tubes for 10 min at room temperature.
9. Resuspend the tube with **NucliSens magnetic silica** by vortexing. Add **25 µl** of **NucliSens magnetic silica** into each tube. Use a new filter tip for each tube. Make sure that the silica is evenly dispensed throughout the tube.

10. Place the tubes with samples into the instrument, insert tips and start the RNA extraction program with lysis of samples outside of the instrument **(*off board*)**.
11. After the extraction procedure is completed, remove the reagent cartridge from the instrument and carry out the RT-PCR not later than 30 min after the completion of RNA extraction.

For storing transfer the purified RNA into the sterile tubes within 30 min after extraction. The purified RNA can be stored at 2–8 °C for 8 hour, at ≤–16 °C for 1 month, at ≤–68 °C for a long time.

Variant 2

RNA with lysis of sample in the instrument

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*** – 25 µl
   - ***Type*** – Primary
   - ***Priority*** – Normal.
3. Create a new protocol of RNA/DNA extraction and save it. In protocol select ***On-board Lysis Buffer Dispensing – Yes***, ***On-board Lysis Incubation – Yes*****.**
4. Relocate sample table into the created protocol.
5. Take the required number of the specialized disposable tubes for RNA/DNA extraction in the NucliSENS easyMAG instrument including the tube for the negative control of extraction. Add **10 µl** of the **Internal Control STI-rec (IC)** to the internal walls of each tube.
6. Add **100 µl** of pretreated samples into each tube with Internal Control STI-rec (IC) using disposable filter tips. Avoid transferring the mucous clots and large particles into the tube.
7. Add **100 µl of Negative Control (C–)** to the tube labelled C– (Negative Control of Extraction).
8. Place the tubes with samples into the instrument, insert tips, and run the RNA extraction with sample lysis in the instrument (***on board*** mode).
9. Wait for the NucliSENS easyMAG instrument stop working in ***Instrument State-Idle*** position.
10. Resuspend the tube with **NucliSens magnetic silica** by vortexing. Open the instrument

lid and add **25 µl** of **NucliSens magnetic silica** into each tube. Use a new filter tip for each tube. Thoutoughly mix by pippeting. Make sure that the silica is evenly dispensed throughout the tube.

11. Close the lid and continue the RNA/DNA extraction program.
12. After the extraction procedure is completed, remove the tubes from the instrument and carry out the RT-PCR not later than 30 min after the completion of RNA extraction.

For storing transfer the purified RNA into the sterile tubes within 30 min after extraction. The purified RNA can be stored at 2– 8 °C for 8 hour, at ≤–16 °C for 1 month, at ≤–68 °C for a long time.

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.

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 Rotor-Gene 3000/6000/Q instrument

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".

NOTE: Simultaneous identification of H1N1 and H3N2 subtypes in a single run is not recommended.

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

Creating the template for the run

1. Click the ***New*** button in the main program 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 the ***New*** button.
3. In the opened window select ***36-Well Rotor*** and tick the ***No Domed 0.2 ml Tubes/Locking ring attached*** option. Click the ***Next*** button.
4. In the opened window enter the operator name, select the ***Reaction volume*** as **25 µl**. Tick the ***15 µl oil layer volume*** for Rotor-Gene 6000. 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 (see Table 2).

Table 2

Amplification program

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of 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. After setting up the temperature profile click the ***OK*** button.
7. Click the ***Calibrate/Gain Optimisation*** button in the ***New Run Wizard*** window. In the opened window:
   a) for signal measurement optimisation for the selected channels set calibration:
      - for ***«H1N1»*** test for ***FAM/Green*** and ***ROX/Orange*** channels from 5Fl to 10Fl, for ***JOE/Yellow*** channel from 10Fl to 15Fl (the ***Edit…*** button, ***Auto gain calibration channel settings*** window). Click the ***Close*** button;
      - for ***«H3N2»*** test for ***FAM/Green*** and ***ROX/Orange*** channels from 5Fl to 10Fl, for ***JOE/Yellow*** channel from 10Fl to 15Fl ((the ***Edit…*** button, ***Auto gain calibration channel settings*** window). Click the ***Close*** button.

      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 ***Next.*** 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 of the instrument. 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 presence (absence) of the *Ct* (threshold cycle) value of the DNA sample in the corresponding column of the results grid.

Data analysis in the FAM/Green channel (Internal Control)

1. Activate the ***Analysis*** button in the menu, select the mode of the analysis ***Quantitation***, activate ***Cycling A. FAM*** or the ***Cycling A. Green*** button. Click ***Show***.
2. Cancel the ***Threshold*** automatic choice.
3. Select the ***Linear scale***.
4. Activate the ***Dynamic tube*** button in the main window menu (***Quantitation analysis***). For ***«H3N2»*** test activate the ***Slope Correct*** button.
5. In ***CT Calculation*** menu (in the right part of the window), set ***Threshold = 0.1*****.**
6. Choose the parameter ***More settings/Outlier Removal*** and set **0 %** for the value of negative samples threshold (***NTC/Threshold***).
7. In the results grid (the ***Quant. Results*** window one will be able to see the *Ct* values.

Data analysis in the JOE/Yellow channel

1. Activate the ***Analysis*** button in the menu, select the mode of the analysis ***Quantitation***, activate ***Cycling A. JOE*** or ***Cycling A. Yellow*** button. Click ***Show***.
2. Cancel the ***Threshold*** automatic choice.
3. Select the ***Linear scale***.
4. Activate the ***Dynamic tube*** button in the main window menu (***Quantitation analysis***). For ***«H3N2»*** test activate the ***Slope Correct*** button.
5. In ***CT Calculation*** menu (in the right part of the window) set
   - ***Threshold = 0.1*** for H1N1 test;
   - ***Threshold= 0.03*** for H3N2 test.
6. Choose the ***More settings/Outlier Removal*** parameter and set **5 %** for the value of negative samples threshold (***NTC/Threshold***).

7. In the results grid (***Quant. Results*** window one will be able to see the *Ct* values.

Data analysis in the ROX/Orange channel

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

NOTE: If the fluorescence curves in the FAM/Green, JOE/Yellow or ROX/Orange channels do not correspond to exponential growth (do not have an S-shape), then the ***NTC threshold*** value **can be increased** up to **5-10%**

ANALYSIS OF RESULTS

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 the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

AMPLIFICATION AND DATA ANALYSIS USING SmartCycler II (Cephied, USA)

INSTRUMENTS

Carry out the sample pretreatment and reaction mixture preparation stages according to the *Instruction Manual* to the PCR kit. When carrying out the amplification it is recommended to use 0.025-ml PCR tubes.

Programming the SmartCycler II

1. Insert the tubes into the thermocycler sites. Close the sites' lids.

NOTE: Before placing the tubes into the instrument it is necessary to sediment the reaction mixture to the bottom of the tubes using the microcentrifuge of SmartCycler II instrument.

2. Program the instrument according to the *Instruction Manual* provided by the manufacturer.
3. Select ***Define Protocols*** in the main program menu. Select the ***New Protocol*** button at the left bottom part of the opened window. Name the protocol and set the following

amplification program (see Table 5):

Table 5

Amplification program

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Cycles***
Stage 1 Hold	95	15 min (900 s)	–	1
Stage 2 2-Temperature Cycle	95	15 s	–	42
	54	25 s	FAM, Cy3, TxR (Texas Red)	
	72	25 s	–	

4. Note – Clicking the ***Optics*** cell of the second step and select ***ON*** to enable the signal detection.
5. Select the ***Save Protocol*** button at the bottom of the window**.**
6. Select the ***Create Run*** button in main menu. Entre the file name to save the experiment data in the ***Run Name*** window. Select the ***Dye set*** button in the middle of the left panel of the monitor and select the ***FCTC25*** dye combination from the drop-down menu.
7. Select ***Add/Remove Sites*** button at the center of the monitor. Select the required protocol (program) and select the analyzed wells in the opened window. Click ***OK***.
8. All settings of this experiment are given in the table in the upper part of the window. All samples are indicated (by default) in the ***Sample Type*** column of this table as ***UNKN*** (unknown). Each sample should be indicated in the ***Sample ID*** column.
9. Click ***Start Run*** button at the bottom of the monitor to run the program.

Data analysis

The obtained results are analyzed by the software of the SmartCycler II instrument. 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. Select ***Analysis settings*** in the menu. Check the correctness of threshold line automatic choice for each channel. For **FAM, Cy3, Texas Red** channels at a time set the threshold line (by left mouse button) at the level where the fluorescence have a linear character and do not cross with the curves of the negative samples. As a rule, the threshold line is set at the level of 10-20 % of maximum fluorescence obtained for the Positive control in the last amplification cycle. Make sure that the fluorescence curve of the Positive control has the typical exponential growth of fluorescence.
2. The threshold level can be set also in the ***Manual Thresh Fluor Units*** column. Click the ***Update Analysis*** button at the bottom of the window after the threshold level setting.

3. *Ct* values for each channel will appear in the ***Results Table*** window.

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 the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and 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. Switch on the instrument and the power supply unit of the optical part of the instrument.

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

2. Start the program iCycler iQ/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 in the FAM, JOE/HEX, and ROX channels for all tubes).
   - For **iCycler iQ5** click the ***Create New*** or ***Edit*** button in the ***Selected Plate Setup*** window of the ***Workshop*** module. One can edit the plate setup in the ***Whole Plate loading*** mode. Set the 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.
   - For **iCycler iQ** select the setup of the samples' position in the reaction module by

choosing the ***Samples: Whole Plate Loading*** option in the ***Edit Plate Setup*** window of the ***Workshop*** module. Name each sample in the ***Sample Identifier*** window. Set the fluorescence signal detection in all the tubes in the **FAM-490, JOE-530** and **ROX-575** channels. Save the plate setup by naming the file in the ***Plate Setup Filename*** window (with .pts filename suffix) and clicking the ***Save this plate setup*** button (in the upper part of the screen). One can edit the plate setup which was used before. To do this, choose ***View Plate Setup*** in the ***Library*** window, select the needed setup in ***Plate Setup*** (the file with .pts filename suffix) and click the ***Edit*** button to the right. It is necessary to save the edited file before using. Set the using of the given plate setup by clicking the ***Run with selected protocol*** button.

2. Set **the** amplification program for plate-type instruments (see Table 8).

Table 8

Amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of 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 **iCycler iQ5** to enter an amplification program select the ***Create New*** or ***Edit*** button in the ***Selected Protocol*** window of the ***Workshop*** module. Set the amplification parameters and save the protocol by activating the ***Save&Exit Protocol Editing*** button. Later, for further runs one may select the file containing this program in the ***Protocol*** box (the protocol files are saved in the ***Users*** folder on default).

   – For **iCycler iQ** to enter an amplification program select the ***Edit Protocol*** option of the ***Workshop*** module. Set the amplification parameters (cycles numbers, temperature, and time) in the bottom window and specify ***Cycle 3 - Step 2*** in the right window. To save the protocol, name the file in the ***Protocol Filename*** window and click the ***Save this protocol*** button (at the top of the screen). This file can be used in further runs if selected from the ***View Protocol*** bookmark of the ***Library*** module. Click the ***Run with selected plate setup*** button to assign the program.

3. Start the selected program with the required plate setup.

   – For **iCycler iQ5** ensure the correctness of the ***Selected Protocol*** and ***Selected Plate Setup*** before starting the program. To start the program, click ***Run.*** For detection of

the well factor, select ***Collect Well Factors from Experimental Plate***. Click the ***Begin Run*** button. Name the experiment (the results of the experiment will be automatically saved in this file) and click ***OK***.

- For **iCycler iQ** ensure the correctness of the selected protocol and plate setup in the ***Run Prep*** window. For the detection of the well factor select ***Experimental Plate*** under the ***Select well factor source*** line. Set the reaction mix volume as **25 µ**l. Press ***Begin Run*** to start. Name the experiment (the results of the experiment will be automatically saved to this file) and click ***OK*** button

4. When the temperature in the reaction chamber reaches 95 ºC, click the ***Pause*** button, open the lid, and place the tubes into the wells of the instruments in accordance with plate setup specified.
5. Proceed to the results analysis at the end of the program.

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 results are interpreted by the software of the instrument by the crossing (or not crossing) of the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (absence) of the *Ct* (cycle threshold) value in the results grid.

The level of the threshold line for all channels (FAM, JOE/HEX, and ROX) should be set at 10-20 % of maximum fluorescence of Positive Controls detected in the last amplification cycle (drag the level with the left mouse button). Make sure that the fluorescence curve of the Positive control has the typical exponential growth of fluorescence. Internal Control amplification product is detected in the FAM channel. *Influenza virus* A/H1 (A/H3) cDNA amplification product is detected in the JOE/HEX channel. *Influenza virus* A/N1 (A/N2) cDNA amplification product is detected in the ROX channel.

Analysis of results of the Internal Control amplification

- For **iCycer iQ5:** select the needed file for the analysis in the ***Data File*** window of the ***Workshop*** module, and click the ***Analyze*** button. Select data in the FAM channel. Make sure that ***PCR Base Line Subtracted Curve Fit*** mode is activated (set by default). Set

the level of the threshold line. Click the ***Results*** button to view the results grid.

- For **iCycer iQ:** select the ***FAM-490*** icon in the ***PCR Quantification*** option of the ***Select a Reporter*** menu. Make sure that ***PCR Base Line Subtracted Curve Fit*** (set by default) is activated. In the ***Threshold Cycle Calculation*** menu specify that the threshold level is set manually and the base line is calculated automatically. To do this, select ***Auto Calculated*** in the ***Baseline Cycles*** submenu and select ***User Defined*** in the ***Threshold Position*** submenu. Set the threshold line level. Click the ***Recalculate Threshold Cycles*** button. *Ct* values will appear in the results grid.

Analysis of results of the *Influenza virus* A/H1 (A/H3) cDNA amplification

- For **iCycer iQ5** select data in the **JOE** channel. Make sure that ***PCR Base Line Subtracted Curve Fit*** mode is activated (set by default). Set the level of the threshold line. Click the ***Results*** button to view the results grid.
- For **iCycer iQ** activate the ***View Post-Run Data*** window in the ***Library*** module. Select the required data file in the ***Data Files*** window and click ***Analyze Data***. Select the ***JOE-530*** icon in the ***PCR Quantification*** option of the ***Select a Reporter*** menu. Make sure that ***PCR Base Line Subtracted Curve Fit*** (set by default) is activated. In the ***Threshold Cycle Calculation*** menu specify that the threshold level is set manually and the base line is calculated automatically. To do this, select ***Auto Calculated*** in the ***Baseline Cycles*** submenu and select ***User Defined*** in the ***Threshold Position*** submenu. Set the threshold line level. Click the ***Recalculate Threshold Cycles*** button. *Ct* values will appear in the results grid.

Analysis of results of the *Influenza virus* A/N1 (A/N2) cDNA amplification

- For **iCycer iQ5** select data in the **ROX** channel and disable **FAM** and **JOE** buttons. Make sure that ***PCR Base Line Subtracted Curve Fit*** mode is activated (set by default). Set the level of the threshold line. Click the ***Results*** button to view the results grid.
- For **iCycer iQ** select the ***ROX-575*** icon in the ***PCR Quantification*** option of the ***Select a Reporter*** menu. Make sure that ***PCR Base Line Subtracted Curve Fit*** (set by default) is activated. In the ***Threshold Cycle Calculation*** menu specify that the threshold level is set manually and the base line is calculated automatically. To do this, select ***Auto Calculated*** in the ***Baseline Cycles*** submenu and select ***User Defined*** in the ***Threshold Position*** submenu. Set the threshold line level. Click the ***Recalculate Threshold Cycles*** button. *Ct* values will appear in the results grid.

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 the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

AMPLIFICATION AND DATA ANALYSIS USING CFX96 (Bio-Rad, USA)

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 (detection through the cap of the tube).

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.

Program the instrument according to the *Instruction Manual* provided by the manufacturer.

1. Turn on the instrument and start the ***Bio-Rad CFX Manager*** program.
2. Select ***Create a new Run*** in the ***Startup Wizard*** window. (or select ***New*** and then ***Run…/Experiment***… in the ***File*** menu). Click ***OK***.
3. 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 10). Set ***Sample Volume – 25 µl.***

Table 10

Amplification program

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Number of 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. Click ***OK***.

4. 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.
5. When the required program is entered or edited, click ***OK*** at the bottom of the window.
6. In the ***Plate*** tab click the ***Create new…*** button. Set the tube order in the opened ***Plate***

Editor – New window. In the ***Sample type*** menu select ***Unknown***; click the ***Select Fluorophores…*** button and indicate the required fluorophores with a checkmark; click ***OK***; then indicate with a checkmark the fluorescence signal acquiring for the selected wells in the required channels. Define sample names in the ***Sample name*** window.

7. In the ***Plate Editor New*** window select ***File***, then ***Save As***, name the plate and save.
8. Select the ***Start Run*** tab. Open the lid of the instrument clicking the ***Open Lid*** button***.*** Place the reaction tubes in the wells of the instrument in accordance with the entered plate setup. Close the lid clicking the ***Close Lid*** button.
9. In the ***Start Run*** tab click the ***Start Run*** button then save the file of the experiment.

Using of the template file for test run

For the further runs one should use the test parameters and the plate scheme set earlier. To do this:

Pass to the ***Plate tab*** in the ***Run Setup*** window, select the ***Select Existing… button.*** Select the needed file with the plate scheme, click the ***Open*** button. Select ***Edit selected*** button for editing the scheme.

Data analysis

Obtained data are interpreted by CFX96 PCR instrument 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 and shown as the presence (or absence) of the *Ct* (threshold cycle) value in the results grid.

1. Run the program, open the saved file with analysis data. To do this select the ***File*** button in the menu, then ***Open*** and ***Data file*** and select the needed file.
2. The fluorescence curves, plate setup, and results grid with *Ct* values are displayed in the ***Quantification*** tab of the ***Data Analysis*** window.

   For each channel at a time set the threshold 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 has the typical exponential growth of fluorescence.

   NOTE: Data analysis for each PCR-mix-1 should be carried out individually by marking the area of the tubes relating to the given mix. The simultaneous run with other tests of Influenza line is allowed.
3. Click ***Tools*** on the toolbar, then ***Reports…***, and then save the generated report.

Internal Control amplification product is detected in the FAM channel. *Influenza virus* A/H1 (A/H3) cDNA amplification product is detected in the HEX channel. *Influenza virus* A/N1 (A/N2) cDNA amplification product is detected in the ROX channel.

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 the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

TROUBLESHOOTING

1. The samples (except for NCA) with negative result in all channels should be analyzed once more starting from the nucleic acid extraction stage. If negative result is obtained in the second run, repeat sampling of the biological material. Negative result is acceptable only for the Negative Control of amplification (NCA).
2. If the *Ct* value for the Positive Control of amplification (C+) is absent or exceeds the boundary *Ct* value in the appropriate channel, repeat amplification for all negative test samples.
3. If the *Ct* value is present for the Negative Control of extraction (C–) and/or Negative Control of amplification (NCA) in the channel for detection of any gene target, repeat the analysis for all samples in which the specific gene was detected starting from nucleic acid extraction to rule out possible contamination.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
27.08.24 HM	Through text	The text formatting was changed according to the template
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany)	Information about settings for the Rotor-Gene device for the H3N2 mixture was changed according to changes in the *Important Product Information Bulletin*
08.10.24 HM	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany)	The "Calibrate / Gain Optimisation…" value for JOE channel for Influenza virus A/H1N1 mixture was changed (according to changes in the *Important Product Information Bulletin)*