IVD

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 clinical material containing *Influenza virus* A RNA by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

EC REP

Ecoli Dx, s.r.o., Purkyňova 74/2
110 00 Praha 1, Czech Republic
Tel.: +420 325 209 912
Cell: +420 739 802 523

Federal Budget Institute of Science “Central Research Institute for Epidemiology”
3A Novogireevskaya Street
Moscow 111123 Russia

Not for use in the Russian Federation

TABLE OF CONTENTS

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 clinical 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 clinical 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

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) (detection through the bottom of the tube).

Insert the tubes into the carousel of the Rotor-Gene 3000/6000/Q instrument so that the first tube is in No. 1 well (the carousel cells are numbered, the numbers are used for the further programming of the samples' position in the thermocycler). Program the instrument.

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

Programming the Rotor-Gene 3000/6000/Q instrument

1. Click the ***New*** button in the main program menu.
2. In the opened window, select ***Advanced*** menu and ***Dual Labeled Probe/Hydrolysis probes***. Click the ***New*** button.
3. Select ***36-Well Rotor*** and ***No Domed 0.2 ml Tubes/Locking ring attached***. Click ***Next***.
4. Select the operator and set the ***Reaction volume*** as **25 µl**. Tick the ***15 µl oil layer volume*** for Rotor-Gene 6000. Click **Next**.
5. Select the ***Edit profile*** button and set the temperature profile of the experiment (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. Click ***OK***.
7. Select the ***Calibrate/Gain Optimisation*** button in the ***New Run Wizard*** window:
   - perform the fluorescence detection in FAM/Green, JOE/Yellow and ROX/Orange channels (activate the ***Calibrate Acquiring/Optimise Acquiring*** button);
   - perform the calibration in FAM/Green, JOE/Yellow and ROX/Orange channels before the first detection (activate the ***Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition*** button);
   - set channels calibration from 5Fl to 10Fl for all dyes (the ***Edit*** button of the ***Auto gain calibration channel settings***). Click ***Close***.
8. Click ***Next.*** Start the amplification program by activating the ***Start Run*** button.
9. Name the experiment and save it to the disk (results of the run will be automatically saved in this file).
10. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Indicate the names/numbers of the test and control samples in the box ***Name***. Set the type ***None*** for the cells matching with the corresponding empty tubes.

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

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 presence (absence) of the *Ct* (cycle threshold) value in 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. Activate the ***Dynamic tube*** 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 parameter ***More settings/Outlier Removal*** and set **0 %** for the value of negative samples threshold (***NTC/Threshold***).
6. 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. Activate the ***Dynamic tube*** button in the main window menu (***Quantitation analysis***).
4. In ***CT Calculation*** menu (in the right part of the window) set
   - ***Threshold = 0.1*** for H1N1 test;
   - ***Threshold=0.05*** for H3N2 test.
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.

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.

Analysis of results for control samples

The results of the analysis are considered reliable only if the results obtained for both positive and negative controls of amplification as well as for the positive and negative controls of extraction are correct. These results should not be greater than the boundary *Ct* values specified for Rotor-Gene instruments in the Table 3.

Table 3

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

Control	Stage for control	*Ct* value in channel		
		FAM/Green	**JOE/Yellow**	**ROX/Orange**
		IC detection	**H1/H3 detection**	**N1/N2 detection**
C–	RNA extraction	**< 28**	Absent	Absent
NCA	Amplification	Absent	Absent	Absent
CS+	Amplification	**< 26**	Absent	Absent
C+$_{A\ H1N1}$	Amplification	Absent	**< 25**	**< 25**
C+$_{A\ H3N2}$	Amplification	Absent	**< 25**	**< 25**

Analysis of results for clinical samples

- *AH1 (or A/H3) Influenza virus* is **detected** in a sample if the *Ct* value is detected in the JOE/Yellow channel.
- *AN1 (or A/N2) Influenza virus* is **detected** in a sample if the *Ct* value is detected in the ROX/Orange channel.
- If *Ct* value is not detected in one or both channels (JOE/Yellow or ROX/Orange) and the *Ct* value for the Internal Control is less than the *Ct* value specified for the IC in the FAM/Green channel (see Table 4), the analyzed A/H1N1 (or A/H3N2) subtype of the epidemic *Influenza virus* is not found.

Table 4

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

PCR-mix-1	*Ct* value in channel		
	FAM/Green	**JOE/Yellow**	**ROX/Orange**
	Internal Control detection	**H1H3 detection**	**N1N2 detection**
PCR-mix-1-FEP/FRT (F) *Influenza virus* A H1N1	IC<28	H1≤35	N1≤35
PCR-mix-1-FEP/FRT (F) *Influenza virus* A H3N2	IC<28	H3≤35	N2≤35

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

1. The samples 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 clinical material. Negative result is normally detected 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 clinical 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 the

nucleic acid extraction stage to rule out possible contamination.

AMPLIFICATION AND DATA ANALYSIS USING SmartCycler II (Cephied, 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 0.025-ml PCR tubes. Spin the reaction mixture tubes using the microcentrifuge of SmartCycler II instrument. Transfer the tubes to the wells of the instrument and close the lids of the wells.

Programming the SmartCycler II

1. 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	900 s	–	1
Stage 2 2-Temperature Cycle	95	15 s	–	42
	54	25 s	FAM, Cy3, Texas Red	
	72	25 s	–	

2. Turn on the detection acquiring on the second step (54 ºC). To do this, click the ***Optics*** box of the second step and select ***ON***.
3. Select the ***Save Protocol*** button at the bottom of the window**.**
4. 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.
5. 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***.
6. 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.
7. Click ***Start Run*** button at the bottom of the monitor to run the program.

Analysis of results for control samples

The results of the analysis are considered reliable only if the results obtained for both positive and negative controls of amplification as well as for the positive and negative

controls of extraction are correct. These results should not be greater than the boundary *Ct* values specified for SmartCycler II instruments in the Table 6.

Table 6

Results for controls for SmartCycler II

Control	**Stage for control**	***Ct* value in channel**		
		FAM	**Cy3**	**Texas Red**
		IC detection	**H1/H3 detection**	**N1/N2 detection**
C–	RNA extraction	**< 38**	Absent	Absent
NCA	Amplification	Absent	Absent	Absent
CS+	Amplification	**< 36**	Absent	Absent
C+*A H1N1*	Amplification	Absent	**< 35**	**< 35**
C+*A H3N2*	Amplification	Absent	**< 35**	**< 35**

Analysis of results for clinical samples

- AH1 (or A/H3) *Influenza virus* is **detected** in a sample if the *Ct* value of a sample is detected in the JOE/Yellow/Cy3 channel.
- AN1 (or A/N2) *Influenza virus* is **detected** in a sample if the *Ct* value of a sample is detected in the ROX/Orange/Texas Red channel.
- If *Ct* value is not detected in one or both channels (JOE/Yellow/Cy3 or ROX/Orange/Texas Red) and the *Ct* value for the Internal Control is less than the *Ct* value specified for the IC in the FAM/Green channel (see Table 7), the analyzed A/H1N1 (or A/H3N2) subtype of the epidemic *Influenza virus* is not found.

Table 7

Results for clinical samples for SmartCycler II

PCR-mix-1	***Ct* value in channel**		
	FAM	**JOE/Yellow/Cy3**	**ROX/Orange/Texas Red**
	Internal Control detection	***H1H3* detection**	***N1N2* detection**
PCR-mix-1-FEP/FRT (F) *Influenza virus* A H1N1	IC<38	H1≤42	N1≤42
PCR-mix-1-FEP/FRT (F) *Influenza virus* A H3N2	IC<38	H3≤42	N2≤42

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

1. The samples 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 clinical material. Negative result is normally detected 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 clinical 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 the nucleic acid extraction stage to rule out possible contamination.

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 (detection through the cap of the tube).

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 30 min before starting the experiment.

2. Start the program iCycler iQ/iQ5.
3. 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.
4. 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.

5. 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 µl**. Press ***Begin Run*** to start. Name the experiment (the results of the experiment will be automatically saved to this file) and click ***OK*** button
6. 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.

7. Proceed to the results analysis at the end of the program.

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.

Analysis of results for control samples

The result of the analysis is considered reliable only if the results obtained for both positive and negative controls of amplification as well as for the positive and negative controls of extraction are correct. These results should not be greater than the boundary *Ct* values specified for iCycler iQ and iQ5 instruments in Table 9.

Table 9

Results for controls for iCycler iQ or iQ5

Control	**Stage for control**	***Ct* value in channel**		
		FAM	**HEX/JOE**	**ROX**
		IC detection	**H1/H3 detection**	**N1/N2 detection**
C–	RNA extraction	**< 28**	Absent	Absent
NCA	Amplification	Absent	Absent	Absent
CS+	Amplification	**< 26**	Absent	Absent
C+*A H1N1*	Amplification	Absent	**< 25**	**< 25**
C+*A H3N2*	Amplification	Absent	**< 25**	**< 25**

Analysis of results for clinical samples

- AH1 (or A/H3) *Influenza virus* is **detected** in a sample if the *Ct* value of the sample is detected in the JOE/HEX channel.
- AN1 (or A/N2) *Influenza virus* is **detected** in a sample if the *Ct* value of a sample is detected in the ROX channel.
- If *Ct* value is not found in one or both channels (JOE/HEX or ROX) and the Ct value for

the Internal Control is less than the *Ct* value specified for the IC in the FAM channel (see Table 10), the analyzed A/H1N1 (or A/H3N2) subtype of the epidemic *Influenza virus* is not found.

Table 10

Results for clinical samples for iCycler iQ or iQ5

PCR-mix-1	*Ct* value in channel		
	FAM	**JOE/HEX**	**ROX**
	Internal Control detection	**H1H3 detection**	**N1N2 detection**
PCR-mix-1-FEP/FRT (F) *Influenza virus* A H1N1	IC<28	H1≤35	N1≤35
PCR-mix-1-FEP/FRT (F) *Influenza virus* A H3N2	IC<28	H3≤35	N2≤35

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

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 clinical 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 clinical 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.

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.

Analysis of results for control samples

The result of the analysis is considered reliable only if the results obtained for both positive and negative controls of amplification as well as for the positive and negative controls of extraction are correct. These results should not be greater than the boundary *Ct* values specified for CFX96 instrument in Table 11.

Table 11

Results for controls for CFX96

Control	**Stage for control**	***Ct* value in channel**		
		FAM	**HEX**	**ROX**
		IC detection	**H1/H3 detection**	**N1/N2 detection**
C–	RNA extraction	**< 28**	Absent	Absent
NCA	Amplification	Absent	Absent	Absent
CS+	Amplification	**< 26**	Absent	Absent
C+*A H1N1*	Amplification	Absent	**< 25**	**< 25**
C+*A H3N2*	Amplification	Absent	**< 25**	**< 25**

Analysis of results for clinical samples

– A/H1 (or A/H3) *Influenza virus* is **detected** in a sample if the *Ct* value of the sample is detected in the HEX channel.

– A/N1 (or A/N2) *Influenza virus* is **detected** in a sample if the *Ct* value of a sample is

detected in the ROX channel.

– If *Ct* value is not found in one or both channels (HEX or ROX) and the Ct value for the Internal Control is less than the *Ct* value specified for the IC in the FAM channel (see Table 12), the analyzed A/H1N1 (or A/H3N2) subtype of the epidemic *Influenza virus* is not found.

Table 12

Results for clinical samples for CFX96

PCR-mix-1	***Ct* value in channel**		
	FAM	**HEX**	**ROX**
	Internal Control detection	**H1H3 detection**	**N1N2 detection**
PCR-mix-1-FEP/FRT (F) *Influenza virus* A H1N1	IC<28	H1≤35	N1≤35
PCR-mix-1-FEP/FRT (F) *Influenza virus* A H3N2	IC<28	H3≤35	N2≤35

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

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 clinical 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 clinical 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
27.06.11 RT	Cover page	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
11.10.11 VV	Data analysis in the JOE/Yellow channel (for Rotor-Gene instruments)	The *Threshold* for H3N2 test was changed from 0.03 to 0.05
		The name of section was corrected
11.01.12 VV	Data analysis Results of analysis are not taken into account in the following cases:	“If the *Ct* value for the Negative Control of extraction (C–) and/or Negative Control of amplification (NCA) is less than the boundary *Ct* value of the positive result …” changed to “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…”
	Text	Names of sections were changed
02.04.14 SA	Cover page	Address of European representative was added
12.09.14 ME	Text	The text was corrected according to the template
	Intended use	The CFX96 (Bio-Rad, USA) instrument was added
	Amplification and data analysis using iCycler iQ and iQ5 (Bio-Rad, USA) instruments	The subtitle “Analysis of results for control samples” was added, the section was transferred before the section “Analysis of results for clinical samples”
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	The chapter was added
18.03.15 ME	Cover page	Corrections according to the tamplate
	Intended use	The NucliSENS easyMAG instrument was added. Types of clinical material was added
	WORK with the NucliSENS easyMAG automated nucleic acid extraction system	The chapter was added
08.11.18 DV	Amplification and data analysis using iCycler iQ5 and iCycler iQ (Bio-Rad, USA) instruments	Information about typical exponential growth of fluorescence was added in Data analysis
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	Information about typical exponential growth of fluorescence was added in Data analysis
13.11.19 PM	Through the text	Variant FRT (**REF** R-V54(RG)-CE; **REF** R-V54(iQ,Dt)-CE) was deleted
28.12.20 MM	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase “Not for use in the Russian Federation” was added
11.03.21 MA	Front page	The name, address and contact information for Authorized representative in the European Community was changed