GUIDELINES

to **AmpliSens® *Influenza virus* A/H1-swine-FRT** PCR kit

Studying of clinical material for the presence of *Influenza virus* A/H1N1(sw2009) RNA by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

Not for use in the Russian Federation

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens®** *Influenza virus* **A/H1-swine-FRT** PCR kit for qualitative detection of *Influenza virus* A/H1N1(sw2009) RNA in the clinical material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

– Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);

– Rotor-Gene Q (QIAGEN, Germany);

– iCycler iQ, iQ5 (Bio-Rad, USA);

– CFX96 (Bio-Rad, USA);

– SmartCycler II (Cepheid, USA).

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

WORK with the NucliSENS easyMAG AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM (bioMérieux, France)

Variant 1

RNA extraction from 100 µl - sample with lysis of sample outside of the instrument (off-board mode)

This method of extraction allows to reduce the consumption of NucliSens lysis buffer. It is preferred for working with clinical samples which contain clots (sputum, aspirates, bronchoalveolar lavage).

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 extraction (select *Other*);
   - *Volume* – 0.1 ml;
   - *Eluate* – 25 µl;
   - *Type* – Lysed;
   - *Priority* – Normal.

3. Create a new protocol of RNA 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 special disposable tubes intended for RNA/DNA extraction in the NucliSENS easyMAG instrument (include positive and negative controls of RNA 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 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 barriers and carefully mix by pipetting (avoid adding mucus clots and large particles to the tube).

7. Add **100 µl** of **Negative Control** 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 single tips with aerosol barriers and carefully mix by pipetting.

Magnetic silica should be distributed evenly throughout the tube volume.

10. Place 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 extraction take the tubes out of the instrument. **Carry out RT-PCR no later than 30 min after RNA extraction.**

Variant 2. RNA extraction with automated lysis of sample in the instrument (on-board mode)

1. Switch on the NucliSENS easyMAG instrument and prepare it for the RNA extraction according to the instruction manual.

2. In the window for input of test samples, enter the following parameters:

   - Sample name;
   - *Matrix* for RNA extraction (select *Other*);
   - *Volume* – 0.1 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 (include recommended positive and negative controls of RNA/DNA extraction). Add **10 µl of Internal Control STI-rec** to inner walls of each tube.

6. Add **100 µl** of the **prepared samples** into each tube with **Internal Control** using tips with aerosol barriers (avoid adding mucus clots and large particles to the tube).

7. Add **100 µl** of **Negative Control** 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 single tip with aerosol barrier (or using a multichannel pipettor with disposable tips with aerosol

barriers for 200 µl) 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 the extraction is completed, take the tubes out of the instrument. **Carry out the RT-PCR not later than 30 min after RNA extraction.**

If necessary to store, transfer purified RNA into sterile tubes within 30 min after RNA extraction (for both variants).

The purified RNA can be stored at 2-8 °C for 8 h, at ≤ –16 °C for 1 month, and at ≤ – 68 °C for a longer period of 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 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 Rotor-Gene PCR tubes (0.1 ml) with caps from the four-pieces-strips (e.g. Corbett Research, Australia; QIAGEN, Germany) (detection through the bottom of the tube).

Programming the thermocycler

1. Insert the prepared tubes into the rotor of instrument.
2. Click the **New** button in the main menu.
3. Select **Reaction volume** – **25 μl** and **Carousel** – **36-Well**.
4. Click the **Next** button.
5. At the top of window press the **Edit profile** button.
6. Set the following parameters of the experiment (see Table 1):

Table 1

The amplification program for rotor-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	95	**5 min** (variant FRT) **15 min** (variant FRT-50 F)	–	1
2	95	10 s	–	10
	54	25 s	–	
	72	25 s	–	
3	95	10 s	–	35
	54	25 s	FAM/Green, JOE/Yellow	
	72	25 s	–	

Fluorescent signal is detected in the channels designed for FAM/Green and JOE/Yellow fluorophores on the 2nd step (54°C) of stage 3.

7. Click the **OK** button twice.
8. At the bottom of the window press the **Calibrate/Gain Optimisation…** button, select **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition** option. For FAM/Green and JOE/Yellow channels set **Min reading** – **5Fl**, **Max reading** – **10 Fl**. To do this, mark **FAM/Green** line with the mouse and press the

Edit button on the right panel of the window. Press the *OK* button. Perform similar operations for other channels. Close the window by pressing the *Close* button.

9. Press the *Next* button and then *Start run* to start the amplification.

10. Name the experiment and save it on the hard drive (the results of the experiment will be automatically saved in this file). During or after amplification, it is necessary to program the placement of test samples in the rotor. To do this, use the *Edit samples* button (at the right bottom of the main window). In the *Sample* menu indicate all clinical samples and controls as *Unknown*.

NOTE:	If rapid analysis is required, a simultaneous run of **«AmpliSens® *Influenza virus* A/B-FRT»** and **«AmpliSens® *Influenza virus* A/H1-swine-FRT»** tests is possible. In this case, programming the device should be performed in accordance with the **AmpliSens® *Influenza virus* A/B-FRT** PCR kit Instruction Manual, and tubes with **PCR-mix-1-FEP/FRT *Influenza virus* A/B** should be placed into the 1st well of the rotor. After amplification, data analysis should be performed for each test separately in accordance with the *Instruction Manual* for each reagent kit.

Data analysis

Amplification data analysis for IC:

1. Activate the *Analysis* button in the menu, select the mode of the analysis *Quantitation*, activate *Cycling A. FAM/Cycling A. Green*, *Show* buttons.

2. Cancel the automatic choice of the threshold line level by activating the *Threshold* button.

3. Activate the *Dynamic tube* button in the menu of main window (*Quantitation analysis*).

4. In the main window menu *More settings/Outlier Removal* set the threshold value *NTC threshold* as *(NTC) 0 %.*

5. In the *CT Calculation* menu (in the right part of the window), set the threshold level *Threshold = 0.1*.

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

Amplification data analysis for cDNA *Influenza virus* A/H1-swine:

1. Activate the *Analysis* button in the menu, select the mode of the analysis *Quantitation*, activate *Cycling A. JOE/Cycling A. Yellow*, *Show* buttons.

2. Cancel the automatic choice of the threshold line level by activating the *Threshold* button.

3. Activate the *Dynamic tube* button in the menu of main window (*Quantitation analysis*).

4. In the main window menu *More settings/Outlier Removal* set the threshold value

NTC threshold as ***(NTC) 10 %.***

5. In the **CT Calculation** menu, set the threshold level **Threshold = 0.1**.

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

Interpretation of results

The result of the PCR analysis is considered reliable only if the results for the positive and negative controls of the amplification and the positive and negative controls of the RNA extraction are correct (see Table 2).

Table 2

Results for controls for different stages of PCR-analysis

Control	Stage for control	*Ct* value in channel	
		FAM/Green (IC)	**JOE/Yellow** (***Influenza virus* A/H1-swine**)
C–	RNA extraction and reverse transcription	< 28	absent
NCA	Amplification	absent	absent
C+	Amplification	absent	< 25
CS+	Amplification	< 26	absent

The interpretation of the test samples' results is to be carried out in accordance with the Table 3.

Table 3

The interpretation of the test samples' results

Ct value in channel		Result
FAM/Green (IC)	**JOE/Yellow** (***Influenza virus* A/H1-swine**)	
< 28	Absent or >35	*Influenza virus* A/H1-swine cDNA **is NOT detected**
< 28	≤ 35	*Influenza virus* A/H1-swine cDNA **Is detected**
Absent or >28	Absent or >35	**Invalid***

* If the result is invalid, the PCR-analysis for corresponding test sample should be repeated starting from the RNA extraction stage.

Troubleshooting

1. If no signal is detected for the Positive Control of Amplification, this may be due to wrong chosen amplification program and errors in the PCR run. In such cases PCR should be repeated.

2. If a signal is detected for the Negative Control of Amplification (TE-buffer) in any channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples are considered invalid. The analysis should be repeated for all samples as well as measures to detect and eliminate the source of contamination should be taken.

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 optically transparent flat caps, or tubes (0.1 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

Program the thermocycler only according to the *Instruction Manual* given by the manufacturer of the instrument:

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 iCycler/iQ5 program.

3. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal for all tubes in **FAM** and **HEX** channels).

    –   For **iCycler iQ5**, click the *Create New* or *Edit* buttons in the *Selected Plate Setup* window of the *Workshop* module. One can edit the plate setup in the *Whole Plate loading* mode. Set the reaction volume (*Sample Volume)* as **25 µl**, the caps type (*Seal Type*) as *Domed Cap,* and the tubes type (*Vessel Type)* as *Tubes.* Save the 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. In the *Select and load Fluorophores* option set fluorescence measurement for all tubes in **FAM-490** and **JOE-530** channels**.** Save the plate setup by naming the file name (with .pts extension) in the *Plate Setup Filename* window 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* (file with .pts extension), 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 (see Table 4).

Table 4

The amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	95	**5 min** (variant FRT) **15 min** (variant FRT-50 F)	–	1
2	95	10 s	–	
	54	25 s	–	10
	72	25 s	–	
3	95	10 s	–	
	54	25 s	FAM, JOE/HEX	35
	72	25 s	–	

– For **iCycler iQ5**, in order to create a protocol, click 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 **Users** folder by default).

– For **iCycler iQ**, 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, and press the **Save this protocol** button (in the upper part of the display). For further experiments, the file with this program can be selected in the **View Protocol** tab in the **Library** module. Press the **Run with selected plate setup** button after selecting or editing the required program to star it.

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

6. Start the selected **Influenza virus A/H1-swine** program with the required plate setup.

– For **iCycler iQ5** instrument, before starting the program, ensure that the protocol *(Selected Protocol)* and the plate setup *(Selected Plate Setup)* were selected correct. Press the **Run** button to start the program. For well factor measurement, 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 **iCycler iQ** instrument, before starting the program, ensure that the name of the protocol and the plate setup were selected correct 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* button to start the program, name the experiment (results of the experiment will be saved in this file automatically), and press the *OK* button.

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

Data analysis

For all FAM and JOE/HEX channels set the threshold line (drag it by cursor while holding the left mouse button) at the 10–20 % level of maximal fluorescence for the Positive Controls in the last amplification cycle. Make sure that the fluorescence curve of the Positive control has the typical exponential growth of fluorescence.

Amplification data analysis for Internal Control:

−   For **iCycler iQ5**, select data for the **FAM** channel in the module window by disabling the **JOE** channel. Data analysis mode *PCR Base Line Subtracted Curve Fit* should be selected (by default). The threshold line is set manually. Press the *Results* button to display the results grid.

−   For **iCycler iQ**, in the *PCR Quantification* option (*Select a Reporter* menu) select the icon of the **FAM-490** 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. For this purpose, select *Auto Calculated* in the *Baseline Cycles* submenu, and select *User Defined* in the *Threshold Position* submenu. The threshold line is set manually. Press the *Recalculate Threshold Cycles* button. *Ct* values will appear in the results grid.

Amplification data analysis for cDNA *Influenza virus* A/H1-swine:

−   For **iCycler iQ5** select the required file with the results of analysis (in the *Data File* window of the *Workshop* module*)* and press the *Analyze* button. Select data for the **JOE** channel. At the same time, data analysis mode *PCR Base Line Subtracted Curve Fit* should be selected (by default). The threshold line is set manually. Press the *Results* button to display the results grid.

−   For **iCycler iQ** activate the *View Post-Run Data* window in the *Library* module. In the *Data Files* window, select the required file with results of analysis and press the *Analyse Data* button. In the *PCR Quantification* option (*Select a Reporter* menu), select the icon of the **JOE-530** channel. At the same time, 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. For this purpose, select the *Auto Calculated* in the *Baseline Cycles* submenu and select *User Defined* in the *Threshold Position* submenu. The threshold line is set manually. Press the *Recalculate Threshold Cycles* button. *Ct* values will appear in the results grid.

Interpretation of results

The results are interpreted by the crossing (or not-crossing) of the fluorescence curve with the threshold line and are shown as the presence (or absence) of *Ct* (threshold cycle) in the results grid.

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 negative control of RNA extraction are correct (see Table 5).

Table 5

Results for controls for different stages of PCR-analysis

Control	Stage for control	*Ct* value in channel	
		FAM (IC)	**JOE/HEX (*Influenza virus* A/H1-swine)**
C–	RNA extraction and reverse transcription	< 30 positive	absent / negative
NCA	Amplification	absent	absent
C+	Amplification	absent	<25
CS+	Amplification	< 26	absent

The interpretation of the test samples' results is to be carried out in accordance with the Table 6.

Table 6

The interpretation of the test samples' results

Ct value in channel		Result
FAM (IC)	**JOE/HEX (*Influenza virus* A/H1-swine)**	
< 33	Absent or >35	*Influenza virus* A/H1-swine cDNA **is NOT detected**
< 33	≤ 35	*Influenza virus* A/H1-swine cDNA **Is detected**
Absent or >33	Absent or >35	**Invalid***

* If the result is **invalid**, the PCR-analysis for corresponding test sample should be repeated starting from the RNA extraction stage.

Troubleshooting

1. If no signal is detected for the Positive Control of Amplification, this may be due to wrong chosen amplification program and errors in the PCR run. In such cases PCR should be repeated.

2. If a signal is detected for the Negative Control of Amplification (TE-buffer) in any channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples are considered invalid. The analysis should be repeated for all samples as well as measures to detect and eliminate the source of contamination should be taken.

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 domed or optically transparent 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. In the start window **Startup Wizard** select **Create a new Run/Experiment** (or select **New** and then **Run…** 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 **7**). Set **Sample Volume – 25 µl.**

Table 7

Amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	95	**5 min** (variant FRT) **15 min** (variant FRT-50 F)	–	1
2	95	10 s	–	10
	54	25 s	–	
	72	25 s	–	
3	95	10 s	–	35
	54	25 s	FAM, HEX	
	72	25 s	–	

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

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.

When the required program is entered or edited, click **OK** at the bottom of the window.

5. In the **Plate** tab click the **Create new…** button. Set the tube order in the opened **Plate Editor – New** window.

6. Press the **Select Fluorophores** button, mark the **FAM, HEX** fluorophores in the

Selected column and press *OK.* In the *Sample type* menu select *Unknown* for all the samples. Then indicate with a checkmark in the *Load* column (in the right part of the window) the fluorescence signal acquiring for the selected wells in the required channels. Define sample names in the *Sample name* window (the *Load* parameter should be marked with a tick).

7. In the *Plate Editor New* window select *File*, then *Save As*, and name the plate. When the required plate is entered or edited, click *OK* at the bottom of the window.
    Select the *Start Run* tab*.* Press the *Open Lid* button to open the lid of the instrument. Place the reaction tubes in the wells of the instrument in accordance with the entered plate setup. Press the *Close Lid* button to close the lid of the instrument.

8. Start the amplification program by activating the *Start Run* button*,* and name the experiment

9. Proceed to the analysis of results after the end of the run.

Data analysis

Obtained data are interpreted by the CFX96 instrument software**.**

The results are interpreted according to the crossing (or not-crossing) of the S-shaped fluorescence curve with the threshold line (set in the middle of the linear fragment of fluorescence growth of the positive control in the log scale) 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 data file. To do this select *File* in the menu, then *Open* and *Data file* and choose the required file.

2. The fluorescence curves, the tubes order in the plate and the results grid are presented in the *Data Analysis* window (the *Quantification* tab).

For FAM and HEX channels 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 the fluorescence curve of the Positive control has the typical exponential growth of fluorescence.

To generate the report choose *Tools* in the toolbar**,** then *File* and *Save As,* name and save the file.

Interpretation of results

The result of the PCR analysis is considered reliable only if the results for the positive and negative controls of the amplification and the positive and negative controls of the RNA extraction are correct (see Table 8).

Table 8

Results for controls for different stages of PCR-analysis

Control	Stage for control	*Ct* value in channel	
		FAM (IC)	**JOE (*Influenza virus* A/H1-swine)**
C–	RNA extraction and reverse transcription	< 30 positive	Absent / negative
NCA	Amplification	Absent / negative	Absent / negative
C+	Amplification	Absent / negative	< 25 positive
CS+	Amplification	< 26 positive	Absent / negative

The interpretation of the test samples' results is to be carried out in accordance with the Table 9.

Table 9

The interpretation of the test samples' results

Ct value in channel		Result
FAM (IC)	**JOE (*Influenza virus* A/H1-swine)**	
< 33	Absent or >35	*Influenza virus* A/H1-swine cDNA **is NOT detected**
< 33	≤ 35	*Influenza virus* A/H1-swine cDNA **Is detected**
Absent or >33	Absent or >35	**Invalid***

* If the result is invalid, the PCR-analysis for corresponding test sample should be repeated starting from the RNA extraction stage.

Troubleshooting

1. If no signal is detected for the Positive Control of Amplification, this may be due to wrong chosen amplification program and errors in the PCR run. In such cases PCR should be repeated.

2. If a signal is detected for the Negative Control of Amplification (TE-buffer) in any channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples are considered invalid. The analysis should be repeated for all samples as well as measures to detect and eliminate the source of contamination should be taken.

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

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*.

NOTE: Work with PCR kit variant FRT-50 F only.

Programming the SmartCycler II instrument

1. Select *Define Protocols* in the main program menu. Select the *New Protocol* button at the right bottom of display. Name the protocol and set the following parameters of the experiment:

Table 10

Amplification program for SmartCycler II only

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	**95**	900 s	–	1
2	**95**	15 s	–	42
	54	25 s	FAM, Cy3	
	72	25 s	–	

2. Enable the signal detection on the 2nd step (54 °C). To do this, select the *Optics* cell of the second step and select *ON*.

3. Click the *Save Protocol* button at the bottom of the window**.**

4. Select *Create Run* in main program menu. Enter the file name to save the experiment data in the *Run Name* window. Select *Dye set* in the middle of the left panel of the screen and select the *FCTC25* dye combination from the dropdown menu.

5. Click the *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 specified 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 named in the *Sample ID* column.

7. Select *Start Run* at the bottom of the display to run the experiment program.

Data analysis

1. Select *Analysis settings* in the menu. Ensure that the automatic choice of threshold line is correct. Alternately for FAM and Cy3 channels set the threshold line (by left mouse button) at the level where fluorescence curves are linear. 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.

   Also it is possible to do by setting the threshold level In *Manual Tresh Fluor Units* column. Select *Update Analysis* in bottom of window.

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

Interpretation of results

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see Table 11).

Table 11

Results for controls for different stages of PCR-analysis

Control	Stage for control	*Ct* value in channel	
		FAM (IC)	**Cy3 (*Influenza virus* A/H1-swine)**
C−	RNA extraction and reverse transcription	< 37 positive	absent / negative
NCA	Amplification	absent / negative	absent / negative
C+	Amplification	absent / negative	< 31 positive
CS+	Amplification	< 31 positive	absent / negative

The interpretation of the test samples' results is to be carried out in accordance with the Table 12.

Table 12

The interpretation of the test samples' results

Ct value in channel		Result
FAM (IC)	**Cy3 (*Influenza virus* A/H1-swine**	
< 37	Absent or >37	*Influenza virus* A/H1-swine cDNA **is NOT detected**
< 37	≤ 37	*Influenza virus* A/H1-swine cDNA **Is detected**
Absent or >37	Absent or >37	**Invalid***

*   If the result is invalid, the PCR-analysis for corresponding test sample should be repeated starting from the RNA extraction stage.

Troubleshooting

1. If no signal is detected for the Positive Control of Amplification, this may be due to wrong chosen amplification program and errors in the PCR run. In such cases PCR should be repeated.

2. If a signal is detected for the Negative Control of Amplification (TE-buffer) in any channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples are considered invalid. The analysis should be repeated for all samples as well as measures to detect and eliminate the source of contamination should be taken.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
23.06.11 RT	Cover page	The name of Institute was changed to Federal Budget Institute of Science "Central Research Institute for Epidemiology"
24.07.12 lvl	Footer	A new catalogue number was added
02.04.14 SA	Cover page	Address of European representative was added
19.12.14 PM	Intended use	The CFX96 instrument was added
	Through the text	The amplification program was updated Corrections according to the template Troubleshooting have been changed
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	The chapter was added
15.03.18 PM	Footer, 3.Content	**REF** R-V55(iQ)-CE was deleted
09.10.18 PM	Cover page	The name of Guidelines was changed
	Intended use	«Biological material» was changed to «Clinical material»
	Text	Corrections according to the template
		Information about typical exponential growth of fluorescence was added in Data analysis
14.11.19 PM	Footer	**REF** R-V55-F(RG,iQ,Dt,SC)-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	Cover page	The name, address and contact information for Authorized representative in the European Community was changed