GUIDELINES

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

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

EC | REP

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *Influenza virus* A/B-FRT** PCR kit for qualitative detection of RNA of *Influenza virus* A and *Influenza virus* B in the biological material (nasopharyngeal and oropharyngeal swabs; sputum; aspirate of trachea; bronchoalveolar lavage, bronchial washing fluid, autopsy material, viral culture) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

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

− Rotor-Gene Q (QIAGEN, Germany),

− iCycler iQ, iCycler iQ5 (Bio-Rad, USA);

− CFX96 (Bio-Rad, USA);

− 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

Variant 1

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

This method of extraction allows reducing the consumption of NucliSens lysis buffer. It is preferred for working with clinical samples which contain 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 – *Other*
   - *Volume* – *0.1 ml*
   - *Eluate* – *25 µl*
   - *Type* – *Lysed*
   - *Priority* – *Normal*.

3. Create a new protocol of RNA 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 (including a negative control of extraction) of the tubes intended for RNA extraction in the NucliSENS easyMAG instrument. Add **10 µl of**

Internal Control STI-rec (IC) and **550 µl** of **NucliSens lysis buffer** per each tube.

NOTE: If there are some clots in extraction material, use 1.5-ml tubes for lysis. After incubation (see p. 8) is done, spin the tubes at 10,000 rpm for 1 min and transfer supernatant into the tubes intended for RNA extraction in the NucliSENS easyMAG instrument

6. Add **100 µl of test samples** into each tube with NucliSens lysis buffer and Internal Control STI-rec (IC) using disposable filter tips and thoroughly mix by pipetting. Avoid adding clots and large particles into the tubes.

7. To prepare **Negative Control of Extraction (C–)** add **100 µl** of **Negative Control (C–)** to the tube intended for C–.

8. Incubate the tubes for 10 min at room temperature to ensure lysis.

9. Thoroughly vortex the tube containing **NucliSens magnetic silica**. Add **25 µl** of **NucliSens magnetic silica** per each tube using a new disposable filter tip for each sample. Mix by pippeting. Make sure that magnetic silica is evenly disseminated in a tube content.

10. Place the tubes with samples into the instrument, insert aspiration tip sets and start the RNA extraction program selecting the ***off board*** mode for lysis.

11. After the extraction procedure is completed, remove the tubes from the instrument and carry out the RT-PCR just after the completion of RNA extraction.

If extracted RNA is intended for storage it should be transferred into sterile tubes within 30 min after extraction. Store at 2–8 °C for up to 4 hours, at ≤–16°C for up to 1 month, or at ≤–68°C for a long-term storage.

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 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 – ***Other***
   - ***Volume*** – ***0.1 ml***
   - ***Eluate*** – ***25 µl***
   - ***Type*** – ***Primary***
   - ***Priority*** – ***Normal***.

3. Create a new protocol of RNA extraction and save it. In the protocol, select: ***On-board Lysis Buffer Dispensing – Yes, On-board Lysis Incubation – Yes.***

4. Relocate sample table into the created protocol.

5. Take the required number (including negative control of extraction) of the tubes intended for RNA extraction in the NucliSENS easyMAG instrument. Add **10 µl of Internal Control STI-rec (IC)** per each tube.

6. Add **100 µl of test samples** into each tube with internal control using disposable filter tips. Avoid adding clots and large particles into the tubes.

7. To prepare **Negative Control of Extraction (C–)** add **100 µl** of **Negative Control (C–)** to the tube intended for C–.

8. Place the tubes with samples into the instrument, insert aspiration tip sets and start the RNA extraction program with lysis of samples by selecting the ***on board*** mode.

9. Wait until the NucliSENS easyMAG instrument stop working in ***Instrument State-Idle*** position.

10. Thoroughly vortex the tube containing **NucliSens magnetic silica**. Open the lid of the instrument and add **25 µl of NucliSens magnetic silica** per each tube using a new disposable filter tip for each sample. Mix by pippeting. Make sure that magnetic silica is evenly disseminated through the tube content.

11. Close the lid of the instrument and continue RNA extraction.

12. After the extraction procedure is completed, remove the tubes from the instrument and carry out the RT-PCR just after the completion of RNA extraction.

If extracted RNA is intended for storage it should be transferred into sterile tubes within 30 min after extraction. Store at 2–8 °C for up to 4 hours, at ≤–16°C for up to 1 month, or at ≤–68°C for a long-term storage.

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

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

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

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

Programming the thermocycler

1. Switch on the instrument.

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.

3. Click the **New** button in the software main menu.

4. In the opened window select **Advanced** tab and **Dual Labeled Probe/Hydrolysis probes**. Click the **New** button.

5. In the opened window select the **36-Well Rotor** and **No Domed 0.2 ml Tubes**/**Locking ring attached**. Click the **Next** button.

6. Set an operator and specify the **Reaction volume** as **25 µl**. For Rotor-Gene 6000 select **15 µl oil layer volume** with a checkmark. Click the **Next** button.

7. Select the **Edit profile** button and set the amplification program (see table 1).

Table 1

Influenza virus A/B cDNA amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	95	**15 min**	–	1
Cycling1	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	-	

8. Click **OK**.

9. In the **New Run Wizard** window select the **Calibrate**/**Gain Optimisation**.

   – For calibration in FAM/Green, JOE/Yellow, and ROX/Orange channels select **Calibrate Acquiring/Optimise Acquiring**.

   – Check the **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition**.

   – For all channels set calibration from **5Fl** to **10Fl (Edit…** button, **Auto gain calibration channel settings** window). Press the **Close** button.

10. Click the **Next** button. Select the **Start run** button.

11. Name the experiment and save it to the disk (results of the run will be automatically saved in this file).

12.Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Enter the names/numbers of test samples in the **Name** column. For empty wells indicate **None**.

NOTE: Samples indicated as **None** will not be analyzed.

Data analysis:

Results are analyzed by the software of the used real time PCR instrument. Results are interpreted by the crossing (or not-crossing) of the fluorescence curve with the threshold line and shown as the presence (or absence) of the *Ct* (threshold cycle) value in the result grid.

Data analysis of the Internal Control amplification (FAM/Green channel)

1. Click the **Analysis** button in the menu, select **Quantitation**, and then select **Cycling A.FAM/Cycling A.Green**. Click **Show**.
2. Cancel the **Threshold** automatic choice.
3. Activate the **Dynamic tube** button in the menu of the main window (**Quantitation analysis**).
4. In the **CT Calculation** menu, set **Threshold = 0.1.**
5. Select the **More Settings**/**Outlier Removal** parameter and set **NTC threshold** value **0%.**
6. In the results grid (**Quant. Results** window) the *Ct* (cycle threshold) values will appear.

Data analysis of the *Influenza virus* B amplification (JOE/Yellow channel)

1. Click the **Analysis** button in the menu, select **Quantitation**, and then select **Cycling A.JOE/Cycling A.Yellow**. Click **Show**.
2. Cancel the **Threshold** automatic choice.
3. Activate the **Dynamic tube** button in the menu of the main window (**Quantitation analysis**).
4. In the **CT Calculation** menu, set **Threshold = 0.1.**
5. Select the **More Settings**/**Outlier Removal** parameter and set **NTC threshold** value **5%.**
6. In the results grid (**Quant. Results** window) the *Ct* (cycle threshold) values will appear.

Data analysis of the *Influenza virus* A amplification (ROX/Orange channel)

1. Click the **Analysis** button in the menu, select **Quantitation**, and then select **Cycling A.ROX/Cycling A.Orange**. Click **Show**.
2. Cancel the **Threshold** automatic choice.
3. Activate the **Dynamic tube** button in the menu of the main window (**Quantitation analysis**).
4. In the **CT Calculation** menu, set **Threshold = 0.1.**

5. Select the **More Settings**/**Outlier Removal** parameter and set **NTC threshold** value **5%.**

6. In the results grid (**Quant. Results** window) the *Ct* (cycle threshold) values will appear.

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 the "Results for control" table in the Instruction manual and the *Important Product Information Bulletin*).

Interpretation of test samples should be carried out in accordance with the Instruction Manual and the *Important Product Information Bulletin* enclosed to the PCR kit.

– **Influenza virus A** RNA is **detected** if *Ct* value determined in the result grid in the ROX/Orange channel is less than 33. Moreover, the fluorescence curve of the sample should cross the threshold line at the area of typical exponential growth of fluorescence.

– **Influenza virus B** RNA is **detected** if *Ct* value determined in the result grid in the JOE/Yellow channel is less than 33. Moreover, the fluorescence curve of the sample should cross the threshold line at the area of typical exponential growth of fluorescence.

– **Influenza virus A** RNA and **Influenza virus B** RNA are **not detected** in a sample if *Ct* value is not determined (absent) in the ROX/Orange and JOE/Yellow channels, whereas *Ct* value determined in the FAM/Green channel is less than 28.

– The result is **invalid** if *Ct* value is not determined (absent) in the JOE/Yellow or ROX/Orange channel, whereas *Ct* value in the FAM/Green channel is not determined (absent) or greater than 28. In such cases PCR analysis (beginning with RNA extraction) should be repeated. If the same result is obtained in the second run, re-sampling of material is recommended.

– The result is **equivocal** if the *Ct* value determined in the ROX/Orange or JOE/Yellow channel is greater than 33, whereas the *Ct* value determined in the FAM/Green channel is less than 28. In such cases PCR analysis should be repeated (beginning with RNA extraction). If the same result is obtained the sample is considered to be positive.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ5 and iCycler iQ (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).

1. Switch on the instrument and power unit of the optical module.

NOTE: The lamp should be warmed up for at least 30 min before the experiment starts.

2. Open iCycler iQ5 / iQ software, depends on the instrument used.

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.

Program the instrument in accordance with manufacturer operation manual:

1. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal in FAM, JOE/HEX and ROX channels for all tubes).

   – **iCycler iQ5**. Click the ***Create New*** or ***Edit*** button in the ***Selected Plate Setup*** window of the ***Workshop*** module. Edit plate setup in the ***Whole Plate loading*** mode. Set the ***Sample Volume*** as ***25 µl***, ***Seal Type*** as ***Domed Cap***, and ***Vessel Type*** as ***Tubes***. Click the ***Save &Exit Plate Editing***.

   – **iCycler iQ**. Edit the plate setup in the ***Edit Plate Setup*** window of the ***Workshop*** module. To do this, set the order of the tubes in the reaction chamber in the ***Samples: Whole Plate Loading*** module and indicate the name of each sample in the ***Sample Identifier*** window. Set fluorescent signal detection in FAM-490, JOE-530, and ROX-575 channels for all tubes in the ***Select and Load Fluorophores*** option. Save the plate setup. Name the file in the ***Plate Setup Filename*** window (use .pts extension) and activate the ***Save this plate setup*** button (at the top of the screen). It is possible to edit the earlier used plate and in order to do this, open the ***View Plate Setup*** in the ***Library*** window, select the appropriate ***Plate Setup*** (a file with .pts extension) and activate the ***Edit*** button. Prior to using, save the edited file. Select ***Run with selected protocol*** in order to use the plate setup.

2. Set the amplification program (see the table below).

Table 2

Influenza virus A/B cDNA amplification program

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

– **iCycler iQ5**. To create the protocol, click the **Create New** or **Edit** button in the **Selected Protocol** window of the **Workshop** module. Enter amplification parameters and click the **Save&Exit Protocol Editing**. For further runs the file with this program can be selected from the **Protocol** unit (protocol files are saved in the **Users** folder by default).

– **iCycler iQ**. To create the protocol, select the **Edit Protocol** option in the **Workshop** module. Enter the amplification parameters (cycles, time, temperature) at the bottom window and indicate **Cycle 3 – Step 2** in the window at the right. Name the file in the **Protocol Filename** window and click the **Save this protocol** button (at the top of the display). For further runs, the file with this program can be selected from the **View Protocol** tab of the **Library** module. Click **Run with selected plate setup**.

3. Place tubes into the module of the instrument in accordance with the defined order and start the selected protocol with the selected plate setup.

– **iCycler iQ5**. Ensure the correctness of the **Selected Protocol** and **Selected Plate Setup** before starting the program. Click **Run** to start. For detection of the well factor select the **Collect Well Factors from Experimental Plate** option and click the **Begin Run** button. Name the experiment (the results of the experiment will be automatically saved in this file) and click **OK**.

– **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** in the **Select well factor source** menu. Set the **Sample 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.

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

<u>**Data analysis:**</u>

The obtained results are analyzed by the 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, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value in the corresponding column of the results table. For JOE/HEX and ROX channels set the threshold (drag it with the cursor while holding the left mouse button) at 10 % level of the maximal fluorescence of the positive control sample in the last amplification cycle while for FAM channel – at 20 % level of the maximal fluorescence of the positive control sample in the last amplification cycle. Make sure that the fluorescence curve of the Positive control has the typical exponential growth of fluorescence.

<u>**Amplification data analysis for Internal Control:**</u>

− **iCycler iQ5.** Select the required data file (**Data File** window of the **Workshop** module) and click the **Analyze** button. Select data in the **FAM** channel. The **PCR Base Line Subtracted Curve Fit** mode should be activated (set by default). The threshold line is set manually. Press the **Results** button to display the results grid.

− **iCycler iQ.** In the **PCR Quantification** option (**Select a Reporter** menu) select the **FAM-490** icon. The **PCR Base Line Subtracted Curve Fit** mode should be activated (set by default). In the **Threshold Cycle Calculation** menu select manual setting of the threshold line and automatic baseline calculation. To do this, 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.

<u>**Amplification data analysis for cDNA *Influenza virus* B:**</u>

− **iCycler iQ5.** Select *JOE/HEX* channel data from the module window. The **PCR Base Line Subtracted Curve Fit** mode should be activated (set by default). The threshold line is set manually. Press the **Results** button to display the results grid.

− **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 **JOE-530** icon. Make sure that the **PCR Base Line Subtracted Curve Fit** mode is activated (set by default). In the **Threshold Cycle Calculation** menu select manual setting of the threshold line and automatic baseline calculation. To do this, 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.

Amplification data analysis for cDNA *Influenza virus* A:

– **iCycler iQ5.** Select **ROX** channel data in the module window. Disable **FAM** and **JOE** buttons. The **PCR Base Line Subtracted Curve Fit** mode should be activated (set by default). The threshold line is set manually. Press the **Results** button to display the results grid.

– **iCycler iQ**. In the **PCR Quantification** option (**Select a Reporter** menu), select the **ROX-575** icon. Make sure that the **PCR Base Line Subtracted Curve Fit** mode is activated (set by default). In the **Threshold Cycle Calculation** menu select manual setting of the threshold line and automatic baseline calculation. To do this, 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 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 the "Results for control" table in the Instruction Manual and the *Important Product Information Bulletin*).

Interpretation of test samples should be performed in accordance with the Instruction Manual and the *Important Product Information Bulletin* enclosed to the PCR kit.

– ***Influenza virus* A** RNA is **detected** if *Ct* value determined in the result grid in the ROX channel is less than 33. Moreover, the fluorescence curve of the sample should cross the threshold line at the area of typical exponential growth of fluorescence.

– ***Influenza virus* B** RNA is **detected** if *Ct* value determined in the result grid in the JOE/HEX channel is less than 33. Moreover, the fluorescence curve of the sample should cross the threshold line at the area of typical exponential growth of fluorescence.

– ***Influenza virus* A** RNA and ***Influenza virus* B** RNA are **not detected** in a sample if *Ct* value is not determined (absent) in the ROX and JOE/HEX channels, whereas *Ct* value determined in the FAM channel is less than 30.

– The result is **invalid** if *Ct* value is not determined (absent) in the JOE/HEX or ROX channel, whereas *Ct* value in the FAM channel is not determined (absent) or greater than 30. In such cases PCR analysis (beginning with RNA extraction) should be

repeated. If the same result is obtained in the second run, re-sampling of material is recommended.

– The result is **equivocal** if the *Ct* value determined in the ROX or JOE/HEX channel is greater than 33, whereas the Ct value determined in the FAM channel is less than 30. In such cases PCR analysis should be repeated (beginning with RNA extraction). If the same result is obtained the sample is considered to be positive.

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 caps (detection through the cap of the tube).

Program the instrument in accordance with the Operation Manual provided by the manufacturer.

Programming the instrument:

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

Table 3

Influenza virus A/B cDNA amplification program

Step	Temperature, ℃	Time	Fluorescence detection	Cycles
Hold	95	**15 min**	–	1
Cycling 1	95	10 s	–	10
	54	25 s	–	
	72	25 s	–	
Cycling 2	95	10 s	–	35
	54	25 s	FAM, HEX, ROX	
	72	25 s	–	

NOTE! Set **Ramp Rate 2,5 °C/sec** 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. This file can be selected for further runs from the **Protocol** tab by clicking the **Select Existing…** button. 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. 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 and click **Load** after entering name of each sample.

6. 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.

7. Place the reaction tubes in the wells of the instrument in accordance with the entered plate setup. In the **Start Run** tab click the **Start Run** button then save the file of the experiment.

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

Data analysis

The obtained results are analyzed by the software of the CFX96 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.

Fluorescence curves, plate setup, and the results grid with *Ct* values are displayed in the **Quantification** tab.

For each channel at a time (FAM, HEX, ROX) 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.

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 the "Results for control" table in the Instruction Manual and the *Important Product Information Bulletin*).

Interpretation of test samples should be performed in accordance with the Instruction Manual and the *Important Product Information Bulletin* enclosed to the PCR kit.

Click **Tools** on the toolbar, then **Reports...**, and then save the generated report.

–   ***Influenza virus* A** RNA is **detected** if *Ct* value determined in the result grid in the ROX channel is less than 33. Moreover, the fluorescence curve of the sample should cross

the threshold line at the area of typical exponential growth of fluorescence.

- – **Influenza virus B** RNA is **detected** if *Ct* value determined in the result grid in the HEX channel is less than 33. Moreover, the fluorescence curve of the sample should cross the threshold line at the area of typical exponential growth of fluorescence.
- – **Influenza virus A** RNA and **Influenza virus B** RNA are **not detected** in a sample if *Ct* value is not determined (absent) in the ROX and HEX channels, whereas *Ct* value determined in the FAM channel is less than 30.
- – The result is **invalid** if *Ct* value is not determined (absent) in the HEX or ROX channel, whereas *Ct* value in the FAM channel is not determined (absent) or greater than 30. In such cases PCR analysis (beginning with RNA extraction) should be repeated. If the same result is obtained in the second run, re-sampling of material is recommended.
- – The result is **equivocal** if the *Ct* value determined in the ROX or HEX channel is greater than 33, whereas the Ct value determined in the FAM channel is less than 30. In such cases PCR analysis should be repeated (beginning with RNA extraction). If the same result is obtained the sample is considered to be positive.

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

Carry out the pretreatment and reaction mixture preparation stages according to the PCR kit instruction manual. It is recommended that 0.025-ml PCR tubes (Cepheid, USA) are used. Spin the reaction mixture tubes with the use of Mini-Centrifuge provided with SmartCycler II instrument.

Programming the instrument

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

Table 4

Influenza virus A/B cDNA amplification program (for SmartCycler II only)

Step	Temperature, ℃	Time	Fluorescence detection	Cycles
Hold	95	900 s	–	1
Stage1	95	15 s	–	42
	54	25 s	**FAM, Cy3**, **Texas Red**	
	72	25 s	–	

Note – Enable the signal detection by clicking the **Optics** cell of the second step and selecting **ON**.

2. Click the **Save Protocol** button at the bottom of the window**.**
3. Select **Create Run** in main 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 monitor and select the **FCTC25** dye combination from the drop-down menu.

4. Click the **Add/Remove Sites** button at the centre of the monitor. Select the required protocol (program) and select the analyzed wells in the opened window. Click **OK**.

5. 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

6. Click **Start Run** button at the bottom of the monitor to run the program.

Data analysis

1. Select **Analysis settings** in menu. Make sure that threshold line is set correctly for each channel (FAM, Cy3 and Texas Red). For each channel at a time set threshold line at the level (use the left mouse button) where fluorescence curves are linear. Otherwise, set the threshold level in the **Manual Thresh Fluor Units** column and click the **Update Analysis** button at the bottom of the window.

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

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

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 the "Results for control" table in the Instruction Manual and the *Important Product Information Bulletin*).

Interpretation of test samples should be performed in accordance with the Instruction Manual and the *Important Product Information Bulletin* enclosed to the PCR kit.

– **Influenza virus A** RNA is **detected** if *Ct* value determined in the result grid in the Texas Red channel is less than 40. Moreover, the fluorescence curve of the sample should cross the threshold line at the area of typical exponential growth of fluorescence.

– **Influenza virus B** RNA is **detected** if *Ct* value determined in the result grid in the Cy3 channel is less than 40. Moreover, the fluorescence curve of the sample should cross the threshold line at the area of typical exponential growth of fluorescence.

– **Influenza virus A** RNA and **Influenza virus B** RNA are **not detected** in a sample if *Ct* value is not determined (absent) in the Texas Red and Cy3 channels, whereas *Ct* value

determined in the FAM channel is less than 37.

– The result is **invalid** if *Ct* value is not determined (absent) in the Texas Red or Cy3 channel, whereas *Ct* value in the FAM channel is not determined (absent) or greater than 37. In such cases PCR analysis (beginning with RNA extraction) should be repeated. If the same result is obtained in the second run, re-sampling of material is recommended.

– The result is **equivocal** if the *Ct* value determined in the Texas Red or Cy3 channel is greater than 40, whereas the *Ct* value determined in the FAM channel is less than 37. In such cases PCR analysis should be repeated (beginning with RNA extraction). If the same result is obtained the sample is considered to be positive.

TROUBLESHOOTING

1. *Ct* value determined for the Positive Control of Amplification (C+) in the **JOE/Yellow/HEX/Cy3** or **ROX/Orange/Texas Red** channels is greater than the boundary value or absent. Check the correctness of amplification parameters and repeat amplification and detection for all samples in which *Influenza virus* cDNA was not detected in the corresponding channel.

2. *Ct* value determined for the Negative Control of Amplification (NCA) and/or Negative Control of Extraction (C–) in the **JOE/Yellow/HEX/Cy3** or **ROX/Orange/Texas Red** channels. This indicates contamination of reagent or samples. Measures to detect and eliminate the source of contamination are to be taken. PCR analysis (beginning with RNA extraction stage) should be repeated for all samples in which *Influenza virus* cDNA was detected in the corresponding channel.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
02.04.14 SA	Cover page	Address of European representative was added
02.06.15 ME	Footer	**REF** R-V36-50-Mod(RG)-CE; **REF** R-V36-50-Mod(iQ)-CE were changed to **REF** R-V36-50-Mod-CE
15.08.16 ME	Text	Corrections according to the template
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
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