

GUIDELINES
to AmpliSens[®] *Influenza virus*
A-type-H5, H7, H9-FRT PCR kit

for typing (identification) of *Influenza virus* A subtypes H5, H7, H9 in *Influenza virus* cultures and biological material containing *Influenza virus* A RNA by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection



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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® Influenza virus A-type-H5, H7, H9-FRT** PCR kit for typing (identification) of *Influenza virus A* subtypes H5, H7, H9 in *Influenza virus* cultures and biological material containing *Influenza virus A* RNA 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 (four channels or more), iCycler iQ5 (Bio-Rad, USA);
- CFX96 (Bio-Rad, USA).

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/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 0.2 ml PCR tubes with flat caps (e.g. Axygen, USA) or 0.1 ml PCR tubes (detection through the bottom of the tube).

Programming the thermocycler

1. Switch the instrument on.
2. Insert the tubes into the carousel of the Rotor-Gene 3000/6000/Q instrument. First tube should be inserted into well 1. Inert the carousel into the instrument, close the cap (the carousel cells are numbered; the numbers are used for the further programming of the samples' position in the thermocycler). Program the instrument.

NOTE: Balance the rotor of the instrument if it is not loaded entirely. Fill the spare wells with empty tubes (don't use the tubes left after previous experiments). Well 1 must be filled with any studied tube except for an empty one.

3. Click the **New** button in the main menu.
4. In the opened window, select the template of the experiment start-up **Advanced** and mark **Dual Labeled Probe/Hydrolysis probes**. Click the **New** button.
5. In the opened window, select **36-Well Rotor** (or **72-Well Rotor**) and **No Domed 0.2 ml Tubes/Locking ring attached**. Click **Next**.

6. In the opened window, set the operator and select the reaction mixture volume: **Reaction volume – 25 µl**. Set check in front of **15 µl oil layer volume**. Click **Next**.
7. In the opened window, set the temperature profile of experiment in accordance to the amplification program (see Table 1).

Table 1

The 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	–	

8. Click the **OK** button.
9. 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 before the first detection (activate the **Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition** button);
 - set the calibration of FAM/Green, JOE/Yellow and ROX/Orange channels (activate **Edit...**, the window **Auto gain calibration channel settings**). Click the **Close** button.
10. Click the **Next** button. Start the amplification program by activating the **Start Run** button.
11. Name the experiment and save it to the disc (the experiment results 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). Indicate the names/numbers of the test samples in the box **Name**. Set the type **None** for the cells matching with the corresponding empty tubes. Use the **Edit samples** button (in the right bottom corner of the main window) for samples names editing. Mark all samples and controls as **Unknown** in the **Samples** menu.

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

Data analysis:

The results of *Influenza virus A H5* cDNA amplification are detected in the FAM/Green channel, *Influenza virus A H7* cDNA – in the JOE/Yellow channel, *Influenza virus A H9* cDNA – in the ROX/Orange channel.

Analysis of results of Influenza virus A H5 cDNA amplification:

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the button **Cycling A. FAM/Cycling A. Green, Show**.
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 **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.1** in the **Threshold** box.
5. Choose the parameter **More settings/Outlier Removal** and set **10 %** 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.

Analysis of results of Influenza virus A H7 cDNA amplification:

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the button **Cycling A. JOE/Cycling A. Yellow, Show**.
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 **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.1** in the **Threshold** box.
5. Choose the parameter **More settings/Outlier Removal** and set **10 %** 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.

Analysis of results of Influenza virus A H9 cDNA amplification:

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the button **Cycling A. ROX/Cycling A. Orange, Show**.
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 **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.1** in the **Threshold** box.
5. Choose the parameter **More settings/Outlier Removal** and set **10 %** 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 *C_t* values.

AMPLIFICATION AND DATA ANALYSIS WITH USING iCycler iQ and iCycler iQ5 (Bio-Rad, USA) INSTRUMENTS

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with 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 the power supply unit of the optical part of the instrument.

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

2. Start the program iCycler iQ/iQ5.

3. Insert the tubes or strips into the reaction module of the amplifier (thermocycler) and program the instrument.

NOTE: Monitor the tubes. There must not be drops left on the walls of the tubes as falling drops during the amplification process may lead to the signal failure and complicate the results analysis. Don't turn the strips upside down while inserting them into the instrument.

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

1. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal in the **FAM, JOE** и **ROX** 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**. Select the fluorescent signal detection through the FAM, JOE and ROX channels. 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 through **FAM-490, JOE-530** and **ROX-575** channels in the **Select and load Fluorophores** option. Save the plate setup by naming the file in the **Plate Setup Filename** window (with .pts filename suffix) and clicking the **Save this plate setup** button (in the upper part of the screen). One can edit the plate setup which was used before. To do this, choose **View Plate Setup** in the **Library** window, select the needed setup in **Plate**

Setup (the file with .pts filename suffix) and click the **Edit** button to the right. It is necessary to save the edited file before using. Set the using of the given plate setup by clicking the **Run with selected protocol** button.

2. Set the amplification program (see Table 2).

Table 2

The amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling	95	10 s	–	10
	54	25 s	–	
	72	25 s	–	
Cycling 2	95	10 s	–	35
	54	25 s	FAM, JOE, ROX	
	72	25 s	–	

- For **iCycler iQ5** 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).
 - For **iCycler iQ** 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.
- For **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**.
 - 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** 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.

Data analysis:

The results of *Influenza virus A H5* cDNA amplification are detected in the FAM channel, *Influenza virus A H7* cDNA – in the JOE channel, *Influenza virus A H9* cDNA – in the ROX channel.

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.

Analysis of results of *Influenza virus A H5* cDNA amplification:

For **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). To set the threshold line level, drag it with a cursor while pressing the left mouse button. Activate the **Results** button to display the results grid.

For **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. Set the threshold line (drag it with a cursor while pressing the left mouse button) at the level of 10 % 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. Press the **Recalculate Threshold Cycles** button. *Ct* values will appear in the results grid.

Analysis of results of *Influenza virus A H7* cDNA amplification:

For **iCycler iQ5** select data in the **JOE** channel turning off the **FAM** button. The **PCR Base Line Subtracted Curve Fit** mode should be activated (set by default). To set the threshold line level, drag it with the mouse holding the left mouse button. Activate the **Results** button to display the results grid.

For **iCycler iQ** activate the **View Post-Run Data** window in the **Library** module. Select the needed file with analysis data in the **Data Files** window and click the **Analyze Data** button. In the **PCR Quantification** option (**Select a Reporter** menu) select the **JOE-530** 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. Set the threshold line (drag it with a cursor while pressing the left mouse button) at the level of 10 % 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. Press the **Recalculate Threshold Cycles** button. Ct values will appear in the results grid.

Analysis of results of Influenza virus A H9 cDNA amplification:

For **iCycler iQ5** select data in the **ROX** channel turning off the **JOE** button. The **PCR Base Line Subtracted Curve Fit** mode should be activated (set by default). To set the threshold line level, drag it with the mouse holding the left mouse button. Activate the **Results** button to display the results grid.

For **iCycler iQ** activate the **View Post-Run Data** window in the **Library** module. Select the needed file with analysis data in the **Data Files** window and click the **Analyse Data** button. In the **PCR Quantification** option (**Select a Reporter** menu) select the **ROX-575** 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. Set the threshold line (drag it with a cursor while pressing the left mouse button) at the level of 10 % 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. Press the **Recalculate Threshold Cycles** button. Ct values will appear in the results grid.

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (see the *Instruction manual*) and boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

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

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

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with optically transparent 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).

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** (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 program in the opened **Protocol Editor – New** window (see Table 3). Set **Sample Volume – 25 µl**.

Table 3

Amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling	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/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.
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 (FAM, HEX, ROX) with a checkmark; click **OK**; then indicate with a checkmark the fluorescence signal acquiring for the selected tubes in the required channels. Define sample names in the **Sample name** window, confirming name of each sample clicking the **Load** button.
7. In the **Plate Editor New** window select **File**, then **Save As**, and name the plate. Define the file name. When the required plate is entered or edited, click **OK** at the bottom of the window.

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

Data analysis

Obtained data are interpreted by the real-time PCR instrument software by the crossing of a fluorescence curve with the threshold line set at the specific level (that corresponds to the presence of *Ct* value in the results grid).

The fluorescence curves, plate setup, and results grid with *Ct* values are displayed in the **Quantification** tab. Make sure that the threshold line was set correctly for each channel. Use one of the following variants:

For all channels (FAM, HEX and ROX) set the threshold line (drag it with a cursor while pressing the left mouse button) at the level of 10 % 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.

RESULTS INTERPRETATION

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification are correct (in accordance with the table with results for controls in the *Instruction manual* and boundary *Ct* values specified in the *Important Product Information Bulletin*).

Results interpretation of test samples is carried out in accordance with *Instruction manual* and *Important Product Information Bulletin*.

- **Influenza virus A subtype H5 is identified** in the test sample if the *Ct* value determined in the results grid for this sample in the FAM channel is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.
- **Influenza virus A subtype H7 is identified** in the test sample if the *Ct* value determined in the results grid for this sample in the JOE/HEX channel is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.
- **Influenza virus A subtype H9 is identified** in the test sample if the *Ct* value determined in the results grid for this sample in the ROX channel is less than the

boundary *Ct* value specified in the *Important Product Information Bulletin*. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.

- The given *Influenza virus A* subtype is not identified (not detected) if the *Ct* values in the specified detection channel are absent.
- The result is **equivocal** if for this sample the *Ct* value determined in the respective channel is greater than the boundary *Ct* value specified in the *Important Product Information Bulletin*. In this case, the PCR analysis of respective sample should be repeated. If the same result is obtained or the *Ct* value is determined less than threshold cycle, the sample is considered positive.

TROUBLESHOOTING

The absence of positive signal for the Positive Control of amplification (C+) can indicate incorrectly selected amplification program and other mistakes made at the stage of PCR. In this case it is necessary to repeat the PCR once more.

If the *Ct* value is determined for the Negative Control of Amplification (NCA) in any channel, it indicates the contamination of the reagents or samples. In this case the results of analysis of all samples are considered invalid. It is necessary to repeat the analysis of all samples and to take measures to detect and eliminate the source of contamination.