# GUIDELINES

## to AmpliSens® *Influenza virus* A H5N1-FRT PCR kit

for qualitative detection of *Influenza virus* A RNA and identification of H5N1 subtype in biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

# AmpliSens®

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## INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *Influenza virus* A H5N1-FRT** PCR kit for qualitative detection of *Influenza virus* A RNA and identification of H5N1 subtype in the biological material (nasal and oropharyngeal swabs or washes; tracheal aspirate; feces; autopsy material; animal material (bird droppings, cloak, pharyngeal, tracheal swabs, tracheal wash samples, visceral organs from fallen animals) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

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

− iCycler iQ5 (Bio-Rad, USA);

− SmartCycler II (Cepheid, USA)

− CFX96 (Bio-Rad, USA).

### Correspondence of PCR-mix-FL, targets and detection channels

| Fluorophore | FAM | JOE |
|---|---|---|
| **Detection channel name for different instrument models[1]** | FAM / Green | JOE / HEX / R6G / Yellow / Cy3 |
| **PCR-mix-FL** | **Detectable cDNA target** | |
| **PCR-mix-1-FRT (SC) *Influenza virus* A H5N1** | *Influenza virus* A H5 cDNA | *Influenza virus* A N1 cDNA |
| **PCR-mix-1-FRT (SC) *Influenza virus* A** | *Influenza virus* A cDNA | Internal Control cDNA |

## AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) 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 instrument.

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

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

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[1] The detection channels names in each section of the guidelines are specified in accordance with the described instrument.

**Programming the thermocycler**

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

2. In opened window select the **Advanced** menu and the **Dual Labeled Probe** or **Hydrolysis probes** template. Click the **New** button.

3. In the opened window select the **36-Well Rotor** and tick the **No Domed 0,2ml Tubes / Locking Ring Attached** option. Click the **Next** button.

4. Select the reaction volume - **25 µl**. Tick the **15 µl oil layer volume** option. Click the **Next** button.

5. In the **New Run Wizard** window set the temperature profile of the experiment. To do this click the **Edit profile** button and set the amplification program:

Table 1

**Amplification program for Rotor-Gene 3000/6000 instruments**

| Step | Temperature, °C | Time | Fluorescence detection | Cycles |
|------|-----------------|------|------------------------|--------|
| Hold | **95** | **15 min** | – | 1 |
| Cycling | **95** | 10 s | – | 10 |
| | **54** | 20 s | – | |
| | **72** | 10 s | – | |
| Cycling2 | **95** | 10 s | – | 35 |
| | **54** | 20 s | FAM/Green, JOE/Yellow | |
| | **72** | 10 s | – | |

7. Click twice the **OK** button.

8. Click the **Calibrate/Gain Optimisation…** button in the bottom of the window. Tick **Perform Calibration Before 1ˢᵗ Acquisition/Perform Optimisation Before 1ˢᵗ Acquisition** option.

   **In case of detection of _Influenza virus_ A RNA:**

   Indicate **Min Reading** - **10Fl**, **Max Reading** - **20Fl** for the FAM/Green and click the **Edit** button in the right panel of the window. Indicate **Min Reading - 5Fl, Max Reading - 10Fl** for the JOE/Yellow channel. Click the **Close** button.

   **In case of identifying of _Influenza virus_ A H5 N1 subtype:**

   Indicate **Min Reading** - **5Fl**, **Max Reading** - **10Fl** for the FAM/Green and click the **Edit** button in the right panel of the window. Indicate **Min Reading - 5Fl, Max Reading - 10Fl** for the JOE/Yellow channel. Click the **Close** button.

9. Click the **Next** button. Start the amplification by clicking the **Start run** button.

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

During the work of the thermocycler or at the end of it, it's necessary to set the order of the tubes in the carousel of the Rotor-Gene instrument. To do this, click the **Edit samples** button. Define all samples and controls in the **Samples** menu as **Unknown**.

**Data analysis:**

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.

**I. Detection of *Influenza virus* A cDNA.**

**Amplification data analysis for IC**

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 **Threshold**.
3. Select the **Linear scale**
4. Activate **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
5. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.1** in the **Threshold** box.
6. Choose the **More settings/Outlier Removal** parameter and set **20%**. for the value of negative samples' threshold (**NTC/Threshold**).
7. In the results grid (the **Quantitation Results** window) one will be able to see *Ct* values.

**Amplification data analysis for *Influenza virus* A cDNA**

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 **Threshold**.
3. Select the **Linear scale.**
4. Activate **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
5. In the **CT Calculation** menu (in the right part of мthe window) indicate the threshold line level **0.1** in the **Threshold** box.
6. Choose the **More settings/Outlier Removal** parameter and set **10%**. for the value of negative samples' threshold (**NTC/Threshold**).
7. In the results grid (the **Quantitation Results** window) one will be able to see *Ct* values.

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the

threshold line set at the specific level that corresponds to the presence (or absence) of the *Ct* value in the corresponding column of the results grid.

1. The sample is positive if the *Ct* value determined in FAM/Green channel is less than or equal to the specified boundary *Ct* value.

2. The sample is negative if the *Ct* value is not determined (absent) in FAM/Green channel, whereas the *Ct* value determined in the JOE/Yellow channel is less than the specified boundary *Ct* value.

3. If the *Ct* value in FAM/Green channel is greater than the specified boundary value, the PCR analysis (beginning with the RNA extraction stage) should be repeated. If the same result has been obtained or the *Ct* value in FAM/Green channel is less than or equal to the boundary value, the sample is considered positive. If the negative result has been obtained in the FAM/Green channel the sample is considered equivocal.

Table 1

**Boundary *Ct* values for control and test samples**

| Sample | FAM/Green channel | JOE/Yellow channel |
|---|---|---|
| C- | Absent | 31 |
| NCA | Absent | Absent |
| C+$_A$ | 33 | Absent |
| Test samples | 33 | 31 |

**Troubleshooting**

1. If the *Ct* value is determined for the Negative Control of Extraction (C-) in the FAM/Green channel and for the Negative Control of Amplification (NCA) 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.

2. If the *Ct* value determined for the Positive Control of Amplification (C+) in the respective channel is greater than the boundary *Ct* value or absent, the amplification should be repeated for all negative samples.

3. If the *Ct* value determined in the JOE/Yellow channel is greater than the boundary value or absent, the PCR should be repeated for this sample beginning from the RNA extraction stage.

**II. Identification of the _Influenza virus_ A H5N1 subtype**

**Amplification data analysis for _Influenza virus_ A N1 cDNA**

1. Activate the **_Analyses_** 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 **_Threshold_**.
3. Select the **_Linear scale_**
4. Activate the **_Dynamic tube_** and **_Slope Correct_** buttons in the menu of main window (**_Quantitation analysis_**).
5. In the **_CT Calculation_** menu (in the right part of the window) indicate the threshold line level **0.1** in the **_Threshold_** box.
6. Choose the **_More settings/Outlier Removal_** parameter and set **_10%_**. for the value of negative samples threshold (**_NTC/Threshold_**).
7. In the results grid (the **_Quantitation Results_** window) one will be able to see the _Ct_ values.
8. The sample is positive if the _Ct_ value determined in the JOE/Yellow channel is less than or equal to the specified boundary _Ct_ value.
9. The sample is negative if the _Ct_ value is not determined (absent) in the JOE/Yellow channel. If the _Ct_ value in the JOE/Yellow channel is greater than the specified boundary value, the PCR analysis (beginning with the RNA extraction stage) should be repeated. If the same result has been obtained or the _Ct_ value in the JOE/Yellow channel is less than or equal to the boundary value, the sample is considered positive. If the negative result has been obtained the sample is considered equivocal.

**Amplification data analysis for _Influenza virus_ A H5 cDNA**

1. Activate the **_Analysis_** button in the menu, select the mode of the analysis **_Quantitation_**, and activate **_Cycling A.FAM /Cycling A.Green_**, **_Show_** buttons.
2. Cancel the automatic choice of the threshold line level **_Threshold_**.
3. Activate **_Dynamic tube_** and **_Slope Correct_** buttons 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 **_More settings/Outlier Removal_** parameter and set **_10%_**. for the value of negative samples threshold (**_NTC/Threshold_**).
6. In the results grid (the **_Quantitation Results_** window) one will be able to see the _Ct_ values.

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

8. The sample is positive if the *Ct* value determined in the FAM/Green channel is less than or equal to the specified boundary *Ct* value.

9. The sample is negative if the *Ct* value is not determined (absent) in the FAM/Green channel. If the *Ct* value in the JOE/Yellow channel is greater than the specified boundary value, the PCR analysis (beginning with the RNA extraction stage) should be repeated. If the same result has been obtained or the *Ct* value in the FAM/Green channel is less than or equal to the boundary value, the sample is considered positive. If the negative result has been obtained the sample is considered equivocal.

Table 2

**Boundary *Ct* values for control and test samples**

| Sample | FAM/Green channel | JOE/Yellow channel |
|---|---|---|
| C– | Absent | Absent |
| NCA | Absent | Absent |
| C+ $_{N1}$ | 33 | Absent |
| C+ $_{H5}$ | Absent | 33 |
| Test samples | 33 | 33 |

If the positive signals are determined simultaneously in the sample in the FAM/Green and JOE/Yellow channels this indicates that **Influenza virus** **A H5N1** is present in the sample, or several *Influenza virus* subtypes with haemagglutinin 5 and neuraminidase 1 are present simultaneously.

**Troubleshooting**

1. If the *Ct* value is determined for the Negative Control of Extraction (C-) in the FAM**/**Green channel and for the Negative Control of Amplification (NCA) 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.

2. If the *Ct* value determined for the Positive Control of Amplification (C+) in the respective channel is greater than the boundary *Ct* value or absent, the amplification should be repeated for all negative samples.

**AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and iQ5 (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 flat optically transparent caps, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

## Programming the thermocycler

1. Turn on the instrument and the power supply unit of the optical block of the instrument.

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

2. Start the program iCycler iQ5 / iQ.

3. Insert the tubes or strips into the reaction module of the thermocycler and program the instrument according to the *Instruction Manual* given by the manufacturer of the instrument.

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

## Creating the template for the run

1. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal).

   – For iCycler iQ5.

   Click the **Create New** or **Edit** buttons in the **Selected Plate Setup** window of the **Workshop** module. Set the reaction volume (**Sample Volume**) as **25 µl**, the caps type (**Seal Type**) as **Domed Cap**, and the tubes type (**Vessel Type**) as **Tube.** *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** and **JOE-530** channels. Save the plate setup by naming the file in the **Plate Setup Filename** window (with .pts filename suffix) and clicking the **Save this plate setup** button (in the upper part of the screen). One can edit the plate setup which was used before. To do this, choose **View Plate Setup** in the **Library** window, select the needed setup in **Plate Setup** (the file with .pts filename suffix) and click the **Edit** button to

the right. It is necessary to save the edited file before using. Set the using of the given plate setup by clicking the **Run with selected protocol** button.

2. Set the amplification program.

**Gripp Amplification program**

| Step | Temperature, °C | Time | Fluorescence detection | Number of cycles |
|------|-----------------|------|------------------------|------------------|
| 1 | 95 | **15 min** | – | 1 |
| 2 | 95 | 10 s | – | 10 |
| | 54 | 25 s | – | |
| | 72 | 25 s | – | |
| 3 | 95 | 10 s | – | 35 |
| | 54 | 25 s | FAM, JOE/HEX | |
| | 72 | 25 s | – | |

   – For **iCycler iQ5:** In the **Selected Protocol** window of the **Workshop** module click the **Create New** or **Edit** buttons. Set the amplification parameters and save the protocol by activating the **Save&Exit Protocol Editing** button. Later, for further runs one may select the file containing this program in the **Protocol** box (the protocol files are saved in the **Users** folder on default).

   – **For iCycler iQ:** Select the **Edit Protocol** option in the **Workshop** window and set the amplification program. 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**.

4. Place tubes into the module of the instrument in accordance with the defined order.

5. Start the selected **Gripp** software with the set 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.

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

**Data analysis:**

The obtained results are analyzed by the iCycler iQ5 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 of the cDNA sample in the corresponding column of the results table. For FAM, JOE/HEX channels set the threshold (drag it with the cursor while holding the left mouse button) at 10-20 % level of the maximal fluorescence obtained for the Positive control in the last amplification cycle. Moreover, the fluorescence curve of the Positive control should cross the threshold line at the area of exponential fluorescence growth coming into the linear growth phase.

**I. Detection of *Influenza virus* A cDNA.**

**Amplification data analysis for Internal Control:**

− **iCycler iQ5.** Select the required data file in the **Data File** window of the **Workshop** module and click the **Analyze** button. Select the data for the **JOE/HEX** 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.** Activate the **View Post-Run Data** window in the **Library** module. Select the required data file in the **Data File** window and click the **Analyse Data** button. In the **PCR Quantification** option of the **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. The threshold line is set manually. Press the **Recalculate Threshold Cycles** button. *Ct* values will appear in the results grid.

**Amplification data analysis for *Influenza virus* A cDNA:**

− **iCycler iQ5.** Select **FAM** channel data from the module window. Disable the **JOE/HEX** button. 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 (the **Select a Reporter** menu) select the **FAM-490** 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. Click the **Recalculate Threshold Cycles** button. *Ct* values will appear in the results grid.

The interpretation of the control and test samples is to be carried out in accordance with the *Instruction Manual* and boundary *Ct* values (see Table 3).

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

1. The sample is positive if the *Ct* value determined in the FAM channel is less than or equal to the specified boundary *Ct* value.

2. The sample is negative if the *Ct* value is not determined (absent) in the FAM channel, whereas the *Ct* value determined in the JOE/Yellow channel is less than the specified boundary *Ct* value.

3. If the *Ct* value in the FAM channel is greater than the specified boundary value, the PCR analysis (beginning with the RNA extraction stage) should be repeated. If the same result has been obtained or the *Ct* value in the FAM channel is less than or equal to the boundary value, the sample is considered positive. If the negative result has been obtained in the FAM channel the sample is considered equivocal.

Table 3

**Boundary *Ct* values for control and test samples**

| Sample | FAM channel | JOE/HEX channel |
|---|---|---|
| C- | Absent | 31 |
| NCA | Absent | Absent |
| C+$_A$ | 33 | Absent |
| Test samples | 33 | 31 |

**Troubleshooting**

1. If the *Ct* value is determined for the Negative Control of Extraction (C-) in the FAM channel and for the Negative Control of Amplification (NCA) 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.

2. If the *Ct* value determined for the Positive Control of Amplification (C+) in the respective channel is greater than the boundary *Ct* value or absent, the amplification should be repeated for all negative samples.

3. If the *Ct* value in the JOE/HEX channel is absent or greater than the boundary *Ct* value, the PCR analysis (beginning with the RNA extraction stage) should be repeated.

**II. Identification of the *Influenza virus* A H5N1 subtype**

**Amplification data analysis for *Influenza virus* A N1 cDNA**

− **iCycler iQ5.** Select the required data file (***Data File*** window of the ***Workshop*** module) and click the ***Analyze*** button. Select data in the **JOE/HEX** 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.** 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.

− The sample is positive if the *Ct* value determined in the JOE/HEX channel is less than or equal to the specified boundary *Ct* value.

− The sample is negative if the *Ct* value is not determined (absent) in the JOE/HEX channel, If the *Ct* value in the JOE/HEX channel is greater than the specified boundary value, the PCR analysis (beginning with the RNA extraction stage) should be repeated. If the same result has been obtained or the *Ct* value in the JOE/HEX channel is less than or equal to the boundary value, the sample is considered positive. If the negative result has been obtained in the FAM channel the sample is considered equivocal

**Amplification data analysis for *Influenza virus* A H5 cDNA**

− **iCycler iQ5** Select **FAM** channel data in the module window. Disable the **JOE/HEX** button. 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. 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.

- The sample is positive if the *Ct* value determined in the FAM channel is less than or equal to the specified boundary *Ct* value.

- The sample is negative if the *Ct* value is not determined (absent) in the FAM channel,. If the *Ct* value in the FAM channel is greater than the specified boundary value, the PCR analysis (beginning with the RNA extraction stage) should be repeated. If the same result has been obtained or the *Ct* value in the FAM channel is less than or equal to the boundary value, the sample is considered positive. If the negative result has been obtained in the FAM channel the sample is considered equivocal.

The interpretation of the control and test samples is to be carried out in accordance with the *Instruction Manual* and boundary *Ct* values (see Table 4).

Table 4

**Boundary *Ct* values for control and test samples**

| Sample | FAM channel | JOE/HEX channel |
|---|---|---|
| C– | Absent | Absent |
| NCA | Absent | Absent |
| C+ N1 | 33 | Absent |
| C+ H5 | Absent | 33 |
| Test samples | 33 | 33 |

If the positive signals are determined simultaneously in the sample in the FAM and JOE/HEX channels this indicates that ***Influenza virus* A H5N1** is present in the sample, or several *Influenza virus* subtypes with haemagglutinin 5 and neuraminidase 1 are present simultaneously.

**Troubleshooting**

1. If the *Ct* value is determined for the Negative Control of Extraction (C-) in the FAM channel and for the Negative Control of Amplification (NCA) 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.

2. If the *Ct* value determined for the Positive Control of Amplification (C+) in the respective channel is greater than the boundary *Ct* value or absent, the amplification should be repeated for all negative samples.

**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*. When carrying out the amplification it is recommended to use disposable polypropylene 0.025-ml tubes.

**Programming the thermocycler**

1. Insert the tubes into the thermocycler wells. Close the wells' caps.

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

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

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

**SmartCycler II instrument amplification program**

| Step | Temperature, °C | Time | Fluorescence detection | Number of cycles |
|:---:|:---:|:---:|:---:|:---:|
| Stage1/Hold | 95 | 900 s | – | 1 |
| Stage2/<br>Temperature<br>Cycle | 95 | 15 s | – | 42 |
| | 54 | 25 s | FAM, Cy3 | |
| | 72 | 25 s | – | |

Note – Enable the signal detection on the second step (54 °C). Select the **Optics** cell of the second step and select **ON**.

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

5. Click the **Create Run** button in the main program menu. Enter the file name to save the experiment data in the **Run Name** window. Select **Dye set** in the central part of the monitor and select the **FCTC25** dye combination in a new menu.

6. 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**.

7. 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

8. Click **Start Run** button at the bottom of the monitor to start the run.

**Data analysis**

**I. Detection of the *Influenza virus* A cDNA**

**Amplification data analysis for Internal Control**

1. Select ***Analysis settings*** in the menu. In the ***Manual Tresh Fluor Units*** column, set the threshold level as 20 for the Cy3 channel. Select ***Update Analysis*** in the bottom part of the window.

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

**Amplification data analysis for *Influenza virus* A cDNA**

1. Select ***Analysis settings*** in the menu. In the ***Manual Tresh Fluor Units*** column, set the threshold level as 30 for the FAM channel. Select ***Update Analysis*** in the bottom part of the window.

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

3. The sample is positive if the *Ct* value determined in the FAM channel is less than or equal to the specified boundary *Ct* value.

4. The sample is negative if the *Ct* value is not determined (absent) in the FAM channel, whereas the *Ct* value determined in the Cy3channel is less than the specified boundary *Ct* value.

5. The sample is negative if the *Ct* value is not determined (absent) in the FAM channel. If the *Ct* value in the FAM channel is greater than the specified boundary value, the PCR analysis (beginning with the DNA/RNA extraction stage) should be repeated. If the same result has been obtained or the *Ct* value in the FAM channel is less than or equal to the boundary value, the sample is considered positive. If the negative result has been obtained in the FAM channel the sample is considered equivocal.

6. If the *Ct* value is not determined (absent) in the FAM and Cy3 channels, or the *Ct* value in the Cy3 channel is greater than the specified boundary *Ct* value the PCR analysis should be repeated. If the same result has been obtained, the PCR analysis (beginning with the DNA/RNA extraction stage) should be repeated for the appropriate test sample.

Table 5

**Boundary *Ct* values for control and test samples**

| Sample | FAM channel | Cy3 channel |
|---|---|---|
| C- | Absent | 37 |
| NCA | Absent | Absent |
| C+$_A$ | 39 | Absent |
| Test samples | 37 | 39 |

**Troubleshooting**

1. If the *Ct* value is determined for the Negative Control of Extraction (C-) in the FAM channel and for the Negative Control of Amplification (NCA) 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.

2. If the *Ct* value determined for the Positive Control of Amplification (C+) in the respective channel is greater than the boundary *Ct* value or absent, the amplification should be repeated for all negative samples.

## II. Identifying of *Influenza virus* A H5N1 subtype

**Amplification data analysis for *Influenza virus* A N1 cDNA**

1. Select **Analysis settings** in the menu. In **Manual Tresh Fluor Units** column, set the threshold level as 60 for the Cy3 channel. Select **Update Analysis** in the bottom part of the window.

2. *Ct* values in the Cy3 channel will appear in the **Results Table** window.

3. The sample is positive if the *Ct* value determined in the Cy3 channel is less than or equal to the specified boundary *Ct* value.

4. The sample is negative if the *Ct* value is not determined (absent) in the Cy3 channel. If the *Ct* value in the Cy3 channel is greater than the specified boundary value, the PCR analysis (beginning with the RNA extraction stage) should be repeated. If the same result has been obtained or the *Ct* value in the Cy3 channel is less than or equal to the boundary value, the sample is considered positive. If the negative result has been obtained in the Cy3 channel the sample is considered equivocal.

**Amplification data analysis for *Influenza virus* A H5 cDNA**

1. Select **Analysis settings** in the menu. In **Manual Tresh Fluor Units** column, set the threshold level as 30 for the FAM channel. Select **Update Analysis** in the bottom part of the window.

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

3. The sample is positive if the *Ct* value determined in the FAM channel is less than or equal to the specified boundary *Ct* value.

4. The sample is negative if the *Ct* value is not determined (absent) in the FAM channel. If the *Ct* value in the FAM channel is greater than the specified boundary value, the PCR analysis (beginning with the RNA extraction stage) should be repeated. If the same result has been obtained or the *Ct* value in the FAM channel is less than or equal to the boundary value, the sample is considered positive. If the negative result has been

obtained in the FAM channel the sample is considered equivocal.

The interpretation of the control and test samples is to be carried out in accordance with the *Instruction Manual* and boundary *Ct* values (see Table 6).

<div align="right">Table 6</div>

**Boundary *Ct* values for control and test samples**

| Sample | FAM channel | Cy3 channel |
|---|---|---|
| C– | Absent | Absent |
| NCA | Absent | Absent |
| C+ $_{N1}$ | 39 | Absent |
| C+ $_{H5}$ | Absent | 39 |
| Test samples | 39 | 39 |

If the positive signals are determined simultaneously in the sample in the FAM and Cy3 channels this indicates that ***Influenza virus* A H5N1** is present in the sample, or several *Influenza virus* subtypes with haemagglutinin 5 and neuraminidase 1 are present simultaneously.

**Troubleshooting**

1. If the *Ct* value is determined for the Negative Control of Extraction (C-) in the FAM channel and 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.

2. If the *Ct* value determined for the Positive Control of Amplification (C+) in the respective channel is greater than the boundary *Ct* value or absent, the amplification should be repeated for all negative samples.

**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 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).

<u>**Programming the thermocycler**</u>

1. Turn on the instrument and start the Bio-Rad CFX Manager software.
2. Program the instrument according to the *Instruction Manual* provided by the manufacturer.

**Creating the template for the run**

1. In the **Startup Wizard** window it is necessary to select the **Create a new Run/Experiment** (or select **New** in the **File** menu and then select **Run…/Experiment**…). Click **OK**.

2. In the **Run Setup** window, select **Protocol** tab and click the **Create new…**. Set the amplification parameters (time, temperature, cycles, and fluorescence acquiring cycle) in the opened **Protocol Editor – New** window. Set **Sample Volume – 25 µl.**

**AmpliSens unified amplification program**

| Step | Temperature, °C | Time | Fluorescence detection | Number of cycles |
|------|-----------------|------|------------------------|------------------|
| 1 | 95 | **15 min** | – | 1 |
| 2 | 95 | 10 s | – | 10 |
|   | 54 | 25 s | – |  |
|   | 72 | 25 s | – |  |
| 3 | 95 | 10 s | – | 35 |
|   | 54 | 25 s | FAM, HEX |  |
|   | 72 | 25 s | – |  |

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

3. Save the protocol: in the **Protocol Editor New** window select **File**, then **Save As**, name the file and click **Save**.

4. Set the plate setup: in the **Plate** tab click the **Create new…** button. Set the tube order in the opened **Plate Editor – New** window. Click the **Select Fluorophores…** button and click the **Selected** checkbox next to the **FAM, HEX** fluorophores and click **OK**. In the **Sample type** menu select **Unknown** for all the samples. Then in the **Load** column (in the right part of the window) tick the fluorescence signal acquiring for all the samples in the required channels. Define sample names in the **Sample name** window, moreover the **Load** function is to be ticked.

5. Save the plate setup: select **File** and then **Save as** in the **Plate Editor New** window. Enter the file name, click **Save**.

6. Select the **Start Run** tab. Open the lid of the instrument by the **Open Lid** button. Insert the reaction tubes in the wells of the instrument in accordance with the entered plate setup. Close the lid by the **Close Lid** button.

**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.

7. Click the **Start Run** button and start the program with the selected plate setup. Select the directory for the file saving, name the file, click **Save**.

## Using the ready template for the run

The test parameters and the plate setup set earlier can be used for the further runs. To do this:

– click the **Select Existing…** button in the **Run Setup** window of the **Protocol** tab. Select the needed file with the amplification program in the **Select Protocol** window. Click **Open**.

– go to the **Plate** tab in the **Run Setup** window. Click the **Select Existing…** button. Select the needed file with the plate setup in the **Select Plate** window. Click **Open**. Click the **Edit selected** button to edit the plate setup.

## 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.

1. Start the software and open the saved file with data of the analysis. To do this, select **File** in the menu, then **Open** and **Data file** and select the needed file.

2. The fluorescence curves, the tube order in the plate and the table with the *Ct* values are represented in the **Data Analysis** window of the **Quantification** tab.

   Check the correctness of threshold line automatic choice for each channel. The threshold line is to cross only with S-shaped (sigmoid) curves describing the accumulation of the signal detecting positive samples and controls. The threshold line is not to cross the base line. If it happens, it is necessary to set the threshold line level for each channel manually. To do this, tick the **Log Scale** item (logarithmic scale selection) and set (with the left mouse button) the threshold line at the level where the fluorescence curves have a linear character and do not cross with the curves of the negative samples. As a rule, the threshold line is set at the level of 10-20 % of maximum fluorescence obtained for the Positive control in the last amplification cycle. Make sure that the fluorescence curve of the Positive control has the typical exponential growth of fluorescence. To select the curve of C+ sample (or another one appropriate sample), set the cursor to the plate setup or to the results grid.

3. Click the **View/Edit Plate…** button on the toolbar and set the samples names in the opened window.

4. To continue work with data, the *Ct* values for each channel can be copied to the Excel from the results grid of the instrument software. To generate report of the run in **.pdf**

format it is necessary to select the **Tools** on the toolbar, then select **Reports…** Save the generated report: select **File** and then **Save as**, name the file and click **Save**.

## 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 specified boundary values.

1. ***Influenza virus* A cDNA** is **detected** if the *Ct* value determined in the results grid in the FAM channel is less than the specified boundary *Ct* value (see Table 7).

2. ***Influenza virus* A cDNA** is **not detected** in a sample if the *Ct* value is not determined (absent) in the FAM channel or greater than the specified boundary *Ct* value, whereas the *Ct* value determined in the HEX channel is less than the specified boundary *Ct* value (see Table 7).

3. The result is **invalid** if the *Ct* value is not determined (absent) in the FAM channel, whereas the *Ct* value in the HEX channel is not determined (absent) or greater than the specified boundary *Ct* value. In such cases, the PCR analysis should be repeated beginning with the RNA extraction stage.

4. *Influenza virus* A H5 cDNA is identified in a sample if the *Ct* value determined in the FAM channel is less than the specified boundary *Ct* value (see Table 8).

5. *Influenza virus* A N1 cDNA is identified in a sample if the *Ct* value determined in the HEX channel is less than the specified boundary *Ct* value (see Table 8).

6. The required H5N1 subtype of *Influenza virus* is not identified in a sample if the *Ct* value is not determined (absent) in the FAM and/or HEX channel, whereas the *Ct* value in the HEX channel (IC detection) is less than the specified boundary *Ct* value.

7. If the positive signals are determined simultaneously in the sample in the FAM and HEX channels this indicates that ***Influenza virus* A H5N1** is present in the sample, or several *Influenza virus* subtypes with haemagglutinin 5 and neuraminidase 1 are present simultaneously.

8. The result is **equivocal** for the presence of the pathogen or the target gene, if the *Ct* value determined in the channel for detection of the pathogen or the target-gene is less than the specified boundary *Ct* value (see Tables 7 and 8). In such cases, the PCR analysis (beginning with the DNA/RNA extraction stage) should be repeated. If the same result has been obtained, the sample is considered positive. If the negative result has been obtained the sample is considered equivocal.

Table 7

**Boundary *Ct* values for control and test samples
(detection of *Influenza virus* RNA)**

| Sample | FAM channel | HEX channel |
|---|---|---|
| C- | Absent | 31 |
| NCA | Absent | Absent |
| C+A | 33 | Absent |
| Test samples | 33 | 31 |

Table 8

**Boundary *Ct* values for control and test samples
(identifying of *Influenza virus* A H5N1 subtype)**

| Sample | FAM channel | HEX channel |
|---|---|---|
| C– | Absent | Absent |
| NCA | Absent | Absent |
| C+ N1 | 33 | Absent |
| C+ H5 | Absent | 33 |
| Test samples | 33 | 33 |

**Troubleshooting**

1. The *Ct* value determined for the Positive Control of amplification (C+) in the respective channels is greater than the boundary *Ct* value or absent. The amplification should be repeated for all negative samples.

2. The *Ct* value determined for the Negative Control of Extraction (C-) and/or Negative Control of Amplification (NCA) in the channel for the pathogen detection is less than the specified boundary *Ct* value. The PCR analysis (beginning with the RNA extraction stage) should be repeated for all samples in which specific RNA was detected to exclude the probable contamination.

**List of Changes Made in the Guidelines**

| VER | Location of changes | Essence of changes |
|---|---|---|
| 02.12.12 Ivl | Footer | **REF** R-V33(RG)-CE-B, **REF** R-V33(iQ)-CE-B, **REF** R-V33(SC)-CE-B catalogue numbers were added |
| 10.06.13 FN | Footer | **REF** R-V33(RG)-CE-B, **REF** R-V33(iQ)-CE-B, **REF** R-V33(SC)-CE-B catalogue numbers were deleted |
| 02.04.14 SA | Cover page | Address of European representative was added |
| 21.09.16 PM | Through the text | Corrections according to the template |
| | Intended use | The biological material was specified |
| | Data analysis | The data analysis was specified |
| | Amplification and data analysis using CFX96 (Bio-Rad, USA) | The chapter was added |
| 16.03.18 PM | Footer, 3. Content | **REF** R-V33(iQ)-CE was deleted |
| 10.10.19 PM | Through the text | Variant FRT (**REF** R-V33(RG)-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 |