GUIDELINES

to **AmpliSens® *HPV* 6/11-FRT** PCR kit

for qualitative detection and differentiation of genotypes 6 and 11

of *human papillomavirus* (*HPV*) DNA in clinical material by the

polymerase chain reaction (PCR) with real-time hybridization-

fluorescence detection

AmpliSens®

EC | REP

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens®** *HPV* **6/11-FRT** PCR kit for qualitative detection and differentiation of genotypes 6 and 11 of *human papillomavirus* (*HPV*) DNA in the clinical material (urogenital swabs) 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, iCycler iQ5 (Bio-Rad, USA),

– CFX96 (Bio-Rad, USA),

– Mx3000P, Mx3005P (Stratagene, 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 6.1 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 0.2 ml PCR tubes with flat caps (detection through the bottom of the tube), or 0.1 ml PCR tubes.

Programming the thermocycler

1. Turn on the instrument.

2. Insert the tubes into the rotor of the instrument so that the first tube is in the first well, insert the rotor into the instrument, and close the lid (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler).

NOTE: Balance the rotor if it is not loaded entirely. Fill unused wells with empty tubes. Do not use tubes from previous runs.
Well 1 must be filled with any test tube except for an empty one.

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

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

5. In the opened window select **36-Well Rotor** (or **72-Well Rotor**) and tick the **No Domed Tubes/Locking Ring Attached**. Click **Next**.

6. In the opened window enter the operator name, select the reaction volume: ***Reaction volume*** is **30 µl** for variant **FRT** (or **25 µl** for variant **FRT-100 F**). Tick the ***15 µl oil layer volume*** option. Click ***Next***.

7. In the opened window, set the temperature profile of the experiment. To do this click the ***Edit profile*** button and set the amplification program:

AmpliSens-1 amplification program for rotor-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling1	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling2	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange	
	72	15 s	–	

NOTE: The unified amplification program for rotor-type instruments can be used in case of simultaneous use of this PCR kit and AmpliSens® *HPV* HCR screen-FRT PCR kit.

Step	Temperature, °C	Time	Number of cycles
Hold	95	15 min	1
Hold2	65	2 min	1
Cycling	95	20 s	5
	64 Touchdown: 1 deg. per cycle	25 s	
	65	55 s	
Cycling2	95	15 s	40
	60	25 s	
	65	25 s fluorescence detection	

8. After setting up the temperature profile click the ***OK*** button.

9. Click the ***Calibrate/Gain Optimisation…*** button in the ***New Run Wizard*** window.
   – for detection in FAM/Green, JOE/Yellow, and ROX/Orange channels select ***Calibrate Acquiring/Optimise Acquiring***;
   – click the ***Edit*** button in the ***Auto gain calibration channel settings*** window and set ***Min Reading*** as ***4*** and ***Max Reading*** as ***8*** for FAM/Green, JOE/Yellow, and ROX/Orange channels.
   – tick the ***Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition***. Click ***Close***.

10. Click ***Next***. Select the ***Start run*** button for amplification to run.

11. Name the experiment and save it to 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). Define names/numbers of the test samples in the **Name** column. Define the Negative Control of amplification as **NCA** and the Positive Control of amplification as **C+**. Set the type **Unknown** opposite all the test samples, the type **Positive control** – for the Positive control of amplification, the type **Negative control** – for the Negative control of amplification. Set the **None** type for the cells matching with the corresponding empty tubes.

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

Data analysis

Amplified DNA products corresponding to the specific targets are detected in the FAM/Green and JOE/Yellow channels; the amplified Internal Control (endogenous internal control that is the fragment of β-globin gene) is detected in the ROX/Orange channel. The results are interpreted according to the crossing (or not-crossing) of the **S-shaped** fluorescence curve with the threshold line, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value of the DNA sample in the corresponding column of the results table.

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

2. Cancel the automatic choice of the threshold line level **Threshold** for each of the opened main windows (FAM/Green, JOE/Yellow, ROX/Orange).

3. Select the **Linear scale**.

4. Activate the **Dynamic tube** and **Slope Correct** buttons in the main window menu (**Quantitation analysis**).

5. In the **CT Calculation** (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box**.**

6. Select the **More Settings/Outlier Removal** parameter and set the **NTC threshold** value as **10 %.**

7. *Ct* values will appear in the results grid (**Quantitation Results** window).

8. The *Ct* values of test samples are considered reliable only if the results obtained for the control samples are correct:

   – Negative Control of extraction (C–) **– Negative Control (C–) –** no *Ct* value is detected;

   – Negative Control of amplification (NCA) **– DNA-buffer –** no *Ct* value is detected;

   – Positive Control of amplification **– Positive Control DNA *HPV* genotypes 6, 11

and human DNA (C+$_{HPV\,6,11}$) – *Ct* values are detected in all the channels.

9. Interpretation of result is carried out in accordance with boundary *Ct* values specified in the *Important Product Information Bulletin*. Samples are considered positive if their *Ct* values do not exceed values specified in the *Important Product Information Bulletin*.

TROUBLESHOOTING

Possible problems	Reason	How to identify	Ways to eliminate
Negative samples are analyzed as positive samples by Rotor-Gene software	Incorrect mathematical processing of negative samples in the presence of the fluorescence decrease section at the initial cycles (Fig. 2a)	A typical positive sample has a characteristic S-shaped curve of fluorescence accumulation (Fig. 1, 3-5). Incorrectly processed negative samples are viewed as quite straight bottom-up lines (Fig. 7)	One must use the ***Ignore First*** by selecting the value 5 cycles. If it does not lead to a proper result, try to increase this value by 1 - 5
	The threshold line crosses descending fluorescence curves at the initial cycles (Fig. 6)	The red threshold line crosses (or touches) fluorescence curves at the left (initial cycles) on the chart of processed fluorescence curves (Fig. 6)	Use the ***Eliminate cycles before…*** option: select 5 (crossing the threshold and the fluorescence curve at the first 5 cycles is ignored)
Sensitivity decrease due to impurity of the instrument lenses	Impurity of lenses leads to reduction of effectiveness of fluorescence excitation and detection. It primarily affects to samples with small quantity of specific DNA, which show low fluorescence growth	Low values of the background signal in all 4 detection channels (<1) at the maximum value of the ***gain*** parameter (10)	Clean the horizontal and vertical lenses of the instrument by a dry disposable cotton disc at least once a month
Sensitivity decrease due to deterioration of probes	Incorrect storage or utilizing kit components (high temperature, multiple opening reagent tubes, foul working conditions) can cause oligonucleotides destruction	Probe destruction can be find out as a result of comparison of experimental data obtained at the first use of the reagents and after a period of time or in comparison with the reagents of the <u>same lot</u> stored properly. It can be revealed as a reduction of value of the ***gain*** multiplying coefficient (selected automatically) by more than 2 units in different	Use mixtures stored under stated conditions until labeled expire date (see part "Stability and Storage" in the Instruction Manual)

		experiments (in case the <u>same instrument</u> is used). Note! Effect of the *gain* parameter increase can appear after cleansing the instrument lenses from sever contamination	
Sensitivity decrease due to decrease of polymerase (TaqF) activity	Incorrect storage or contamination cause enzyme deterioration	It appears as a fail of the positive control or if a boundary *Ct* value of the positive control is greater than the threshold of weak samples	Use the reagent stored under required condition (see the section "Stability and Storage" in the Instruction Manual) or use a new reagent

Note – A report on all parameters specified as well as a report on auto-calibration is available if the *Settings* button is activated. In particular, the *Messages* tab, the *Autocalibration Log Messages* option.

| Possible mistakes	Feature	Ways to eliminate
Contamination of a specific DNA	Detection of a signal of the negative control in any channel	Repeat the experiment. Take measures to detect and eliminate the source of contamination
The volume of a DNA sample added to a reaction tube is less than required/DNA samples is not added	Background signal of a sample is much stronger than the others (see raw curves, Fig. 2b). The sample is negative	Repeat PCR for this sample
The volume of the reaction mixture added to a reaction tube is less than required/reaction mixture is not added/the volume of a DNA sample added to a reaction tube is greater than required	Background signal of a sample is much lower than the others (see raw curves, Fig. 2b).	If the sample is negative, repeat the test
The autocalibration parameter *from 4Fl to 8Fl* is not set or it is a failure of the first tube in the rotor (the No.1 well is empty; a volume of DNA sample or reaction mixture added to the tube is incorrect)	Most of the fluorescence background signals are less than 1 or greater than 20	Set the parameter in the next run. In case of off-scale reading or if the signals are too weak (no positive signals after processing; a background is less than 0.5) the experiment should be repeated beginning with PCR
Polymerase (TaqF) was not added to a reaction mixture	Positive signals are absent for all samples including a positive control	Repeat the experiment beginning with PCR. Ensure appropriate preparation of reaction mixtures

Unprocessed data
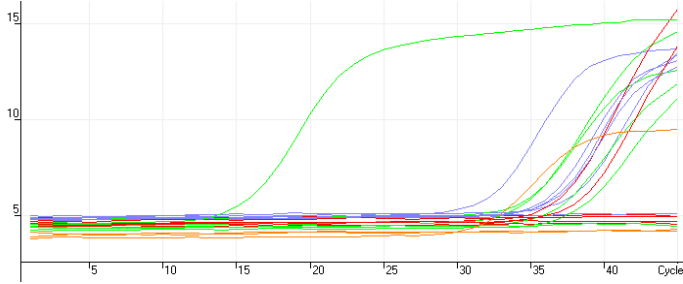
Fig. 1. Raw curves are normal
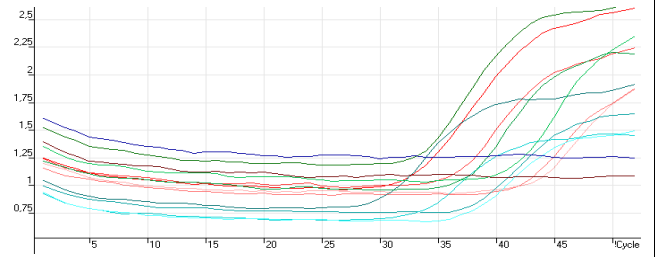
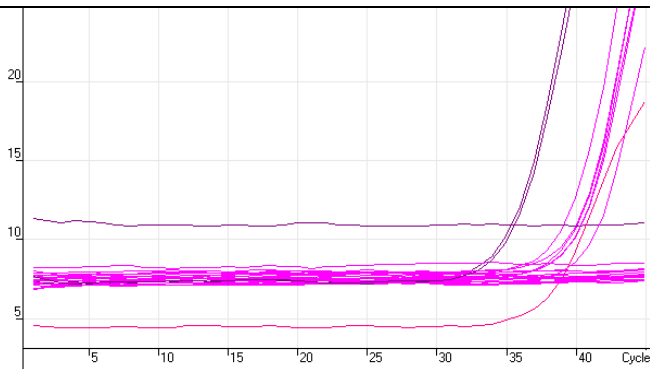Fig. 2a. Raw curves have a bend (drop of fluorescence at the initial cycles)

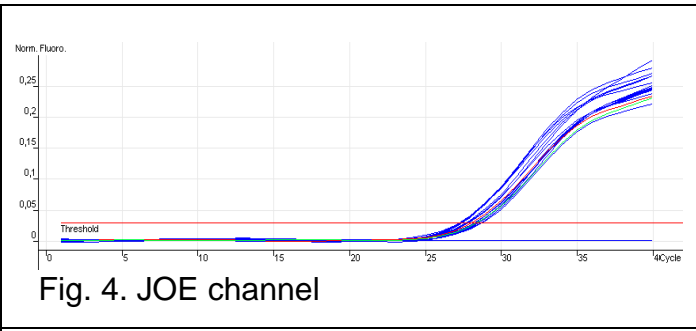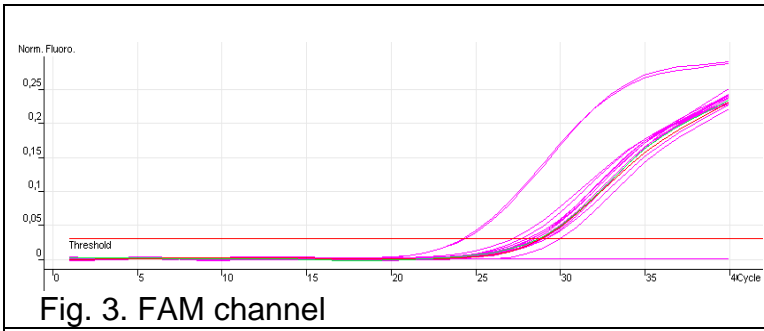Fig. 2b. Level of background signal points on the possible error:
DNA sample was not added to the tube 1;
double volume of DNA sample was added to the tube 2

Processed data

a) processed curves are normal (S-shaped, threshold line crosses curves at the area of growth of fluorescence)

Fig. 3. FAM channel

Fig. 4. JOE channel

Fig. 5. ROX channel – internal control (β-globin gene)
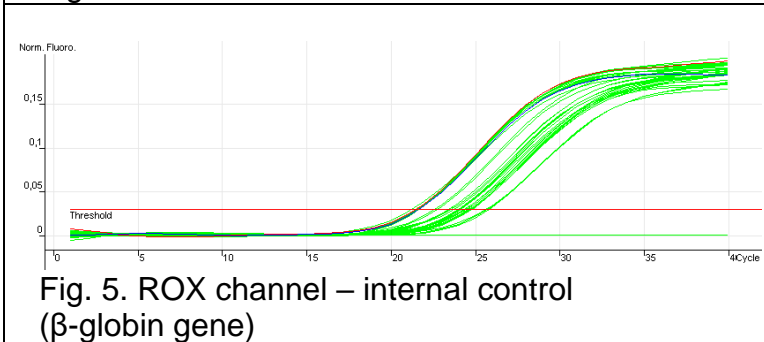
b) curves are processed incorrectly

Fig. 6. Threshold line crosses fluorescence curves twice

Fig. 7. Incorrect processing of the curves with bend (from Fig. 2)
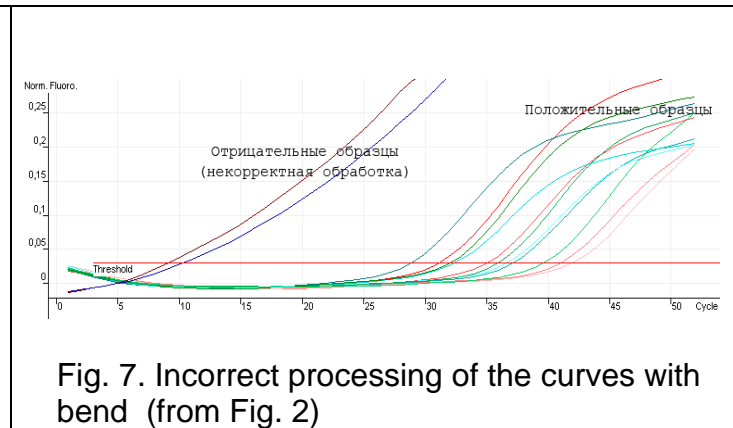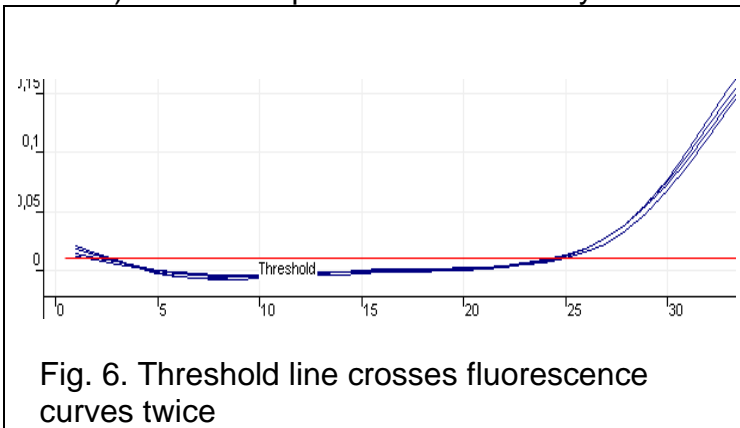
AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and iCycler iQ5 (Bio-Rad, USA) INSTRUMENTS

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 caps (detection through the cap of the tube).

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. Open the iCycler/iQ5 program.

3. Insert the tubes or strips into the reaction module of the 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 tubes (strips) upside down while inserting them into the instrument.

Program the instrument as specified in the manufacturer's instruction manual:

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

   – **iCycler iQ5**. Click the ***Create New*** or ***Edit*** button in the ***Selected Plate Setup*** window of the ***Workshop*** module. The plate setup can be edited in the ***Whole Plate loading*** mode. Set the ***Sample Volume*** as **30 µl** for variant **FRT** (or **25 µl** for variant **FRT-100 F)**, indicate ***Seal Type*** as ***Domed Cap***, and ***Vessel Type*** as ***Tubes***. Indicate detection of fluorescent signal in **FAM**, **JOE/HEX**, and **ROX** channels. Click the ***Save &Exit Plate Editing*** button.

   – **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*** option 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 ***Plate Setup*** used previously. In order to do this, open the ***View Plate Setup*** in the ***Library*** window, select an appropriate ***Plate Setup*** (a file with .pts extension) and activate the ***Edit*** button at the right. Save the edited file prior to use. Select ***Run with selected protocol*** to enable the created plate setup.

2. Set all the clinical samples as ***Unknown***, positive controls as "**+**", and negative controls

as "**–**".

3. Set the amplification program.

AmpliSens-1 amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycle repeats
Hold	95	15 min	–	1
Cycling 1	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	30 s	FAM/FAM-490, JOE/HEX/JOE-530, ROX/ROX-575	
	72	15 s	–	

NOTE: The unified amplification program for plate-type instruments can be used in case of simultaneous use of this PCR kit and AmpliSens® *HPV* HCR screen-FRT PCR kit.

Step	Temperature, °C	Time	Cycle repeats
Hold	95	15 min	1
Cycling	95	15 s	6
	65 Touchdown: 1 deg. per cycle	55 s	
	65	25 s	
Cycling2	95	15 s	41
	60	55 s *fluorescence detection*	
	65	25 s	

–   **iCycler iQ5**. Click the **Create New** or **Edit** button in the **Selected Protocol** window of the **Workshop** module. Set amplification parameters and save the protocol by clicking the **Save&Exit Protocol Editing** button. The file with the created program can be selected from the **Protocol** unit for further runs (it is saved by default in the **Users** folder).

–   **iCycler iQ**. Select the **Edit Protocol** option in the **Workshop** module. Set the amplification program in the bottom window; indicate **Cycle 2 – Step 2** in the window at the right. Save the protocol: name the file in the **Protocol Filename** window (file with .tmo extension) and click the **Save this protocol** button (at the top of the screen). The file with the created program can be selected from the **View Protocol** tab of the **Library** module for further runs. Enable the selected or edited program by clicking the **Run with selected plate setup** button.

4. Prior to run the program:

–   **iCycler iQ5**. Ensure the correctness of the **Selected Protocol** and **Selected Plate**

Setup. Click the ***Run*** button to start the program. For detection of the well factor select the ***Collect Well Factors from Experimental Plate*** option. Click the ***Begin Run*** button, name the experiment (the results of the experiment will be automatically saved to 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 reaction mixture volume as **30 µl** for variant **FRT** (or **25 µl** for variant **FRT-100 F)**. Press ***Begin Run*** to start. Name the experiment (the results of the experiment will be automatically saved to this file) and click ***OK***.

5. Proceed to analysis of results by the end of the run.

Data analysis

Amplified DNA products corresponding to the specific targets are detected in the FAM/FAM-490 and HEX/HEX-530 channels; amplified Internal Control (endogenous internal control that is the fragment of β-globin gene) is detected in the ROX/ROX-575 channel. The results are interpreted according to the crossing (or not-crossing) of **S-shaped** fluorescence curve with the threshold line set at the specific level (the middle of the area of linear growth of fluorescence of the positive control sample in logarithmic scale) that corresponds with the presence (or absence) of the *Ct* value in the corresponding column of the results grid.

1. Start the software and open the saved file.

– **iCycler iQ5.** Select the needed data file from the ***Data File*** window of the ***Workshop*** module and click the ***Analyze*** button;

– **iCycler iQ.** Activate the ***View Post-Run Data*** window in the ***Library*** module. Select the needed file from the ***Data File*** window and click the ***Analyze Data*** button.

2. To browse obtained data:

– **iCycler iQ5.** Select the ***Analysis Mode: PCR Base Line Subtracted Curve Fit*** (selected by default);

– **iCycler iQ.** Select the icon of the required channel in the ***PCR Quantification*** tab of the ***Select a Reporter*** menu. Make sure that the ***PCR Base Line Subtracted Curve Fit*** mode is activated (selected by default).

3. Browse obtained data in one channel at a time.

4. Set the threshold line. To do this:

– **iCycler iQ5.** In the ***Base Line Threshold*** window set the ***Base Line Cycles – Auto Calculated*** parameter (if cut, set this parameter in the ***User Defined, 2 through***

10 cycles mode) and set the ***Crossing Threshold – Auto Calculated*** parameter. Normally, a threshold line should cross only S-shaped curves of positive samples and controls and should not cross a base line. Otherwise, the threshold line should be raised: click the ***Log View*** button and set the threshold line at the level where fluorescence curves are linear and do not cross graphs of negative samples.

- **iCycler iQ.** In the ***Threshold Cycle Calculation*** menu indicate that threshold line adjusting and base line calculation are carried out in automatic mode. To do this, select ***Auto Calculated*** in the ***Baseline Cycles*** submenu and select ***Auto Calculated*** in the ***Threshold Position*** submenu. Normally, a threshold line should cross only S-shaped curves of signal accumulation of positive samples and controls and should not cross the base line. Otherwise, select ***User Defined*** in the ***Threshold Position*** submenu, click the ***Log View*** button, and set the threshold (use the left mouse button) at the level where fluorescence curves are linear and do not cross curves of negative samples.

5. Click the ***PCR Quant*** button (iCycler iQ) or activate the ***Results*** button (iCycler iQ5) to analyze results.

6. The *Ct* values of test samples are considered reliable only if the results obtained for the control samples are correct:

   - Negative Control of extraction (C–) **– Negative Control (C–) –** no *Ct* value is detected;

   - Negative Control of amplification (NCA) **– DNA-buffer –** no *Ct* value is detected;

   - Positive Control of amplification **– Positive Control DNA *HPV* genotypes 6, 11 and human DNA (C+*HPV* 6,11) –** *Ct* values are detected in all channels.

7. Interpretation of result is carried out in accordance with boundary *Ct* values specified in the *Important Product Information Bulletin*. Samples are considered positive if their *Ct* values do not exceed values specified in the *Important Product Information Bulletin*.
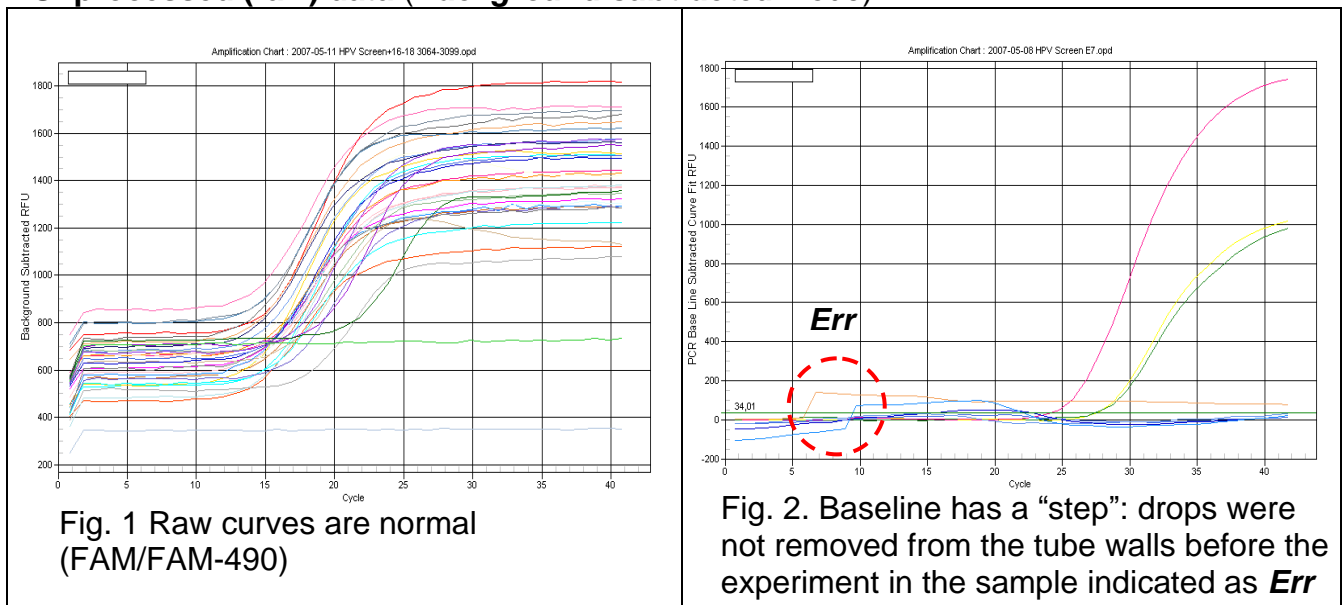
TROUBLESHOOTING

Possible problems	Reason	How to identify	Ways to eliminate
Drop of sensitivity due to deterioration of probes	Incorrect storage or utilizing kit components (high temperature, multiple opening reagent tubes, foul working conditions) can cause destroy of oligonucleotides	Probe destruction can be find out as a result of comparison of experimental data obtained at the first use of the reagents and after a period of time or in comparison with the reagents of the <u>same lot</u> stored properly. It is appeared as an increase of background fluorescence (fluorescence is assessed in the ***Background subtracted*** mode at the beginning of the experiment) twice as much in different experiments (in case the <u>same instrument</u> is used). Note! Effect of fluorescence increase can appear in case the lamp was changed, optical system was cleaned, or calibration was done)	Use mixtures stored under stated conditions until labeled expire date (see part "Stability and Storage" in the Instruction Manual)
Drop of sensitivity due to decrease of activity of polymerase (TaqF)	Incorrect storage or contamination cause enzyme deterioration	It appears as a fail of the positive control or if a boundary *Ct* value of the positive control is much greater than the normal one	Use the reagent stored under required condition (see part "Stability and Storage" in the Instruction Manual) or use a new reagent

Possible mistakes	Feature	Ways to eliminate
Contamination of a specific DNA	Detection of a signal for a negative control in any channel	Repeat the experiment. Take measures to detect and eliminate the source of contamination
The volume of a DNA sample added to a reaction tube is less than required/DNA samples is not added	Background signal of the sample is much stronger than the others (see raw curves – ***Background subtracted*** mode) The sample is negative	Repeat PCR for this sample
The volume of a reaction mixture added to a reaction tube is less than required/reaction mixture is not added **or** the volume of a DNA sample added to a reaction	Background signal of the sample is much lower than the others (see raw curves **-** ***Background subtracted*** mode)	If the sample is negative, repeat the test

tube is greater than required		
Incorrect adjustment of the threshold level	Threshold line is located along with negative samples or above some or all positive curves (S-shaped)	Set the threshold line so that it crosses only sigmoid curves of fluorescence accumulation or at the level of ¼ between final values of negative and positive samples
Drops were not removed from the tube walls before experiment started	Curves of fluorescence accumulation have negative or positive "steps" (Fig. 2)	Select the **BaseLine Threshold** window(the right mouse button on the graph of fluorescence) and indicate that base line is to be calculated beginning with the first cycle after the "step"
Polymerase (TaqF) was not added to a reaction mixture	Positive signals are absent for all samples including a positive control	Repeat the experiment beginning with PCR. Ensure appropriate preparation of reaction mixtures

Unprocessed (raw) data (***Background subtracted*** mode).

Fig. 1 Raw curves are normal (FAM/FAM-490)

Fig. 2. Baseline has a "step": drops were not removed from the tube walls before the experiment in the sample indicated as ***Err***

Processed data

Processed curves are normal; S-shaped; thresholds are located correctly (*PCR Base Line Subtracted Curve Fit* mode)
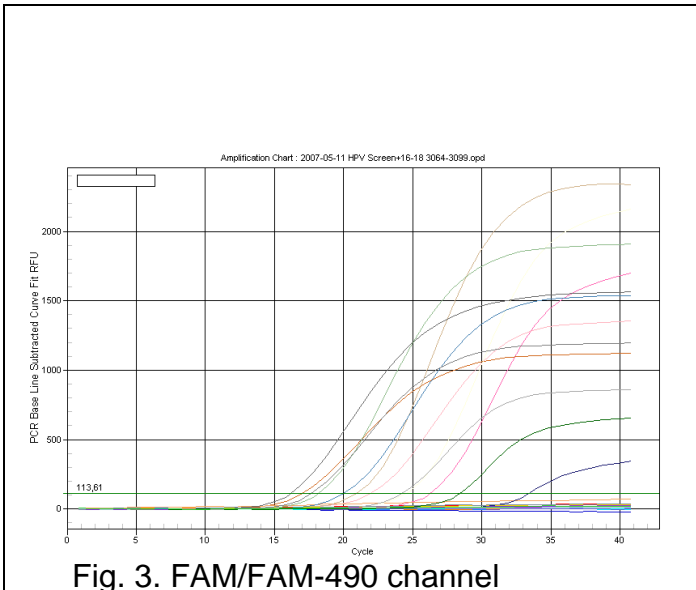

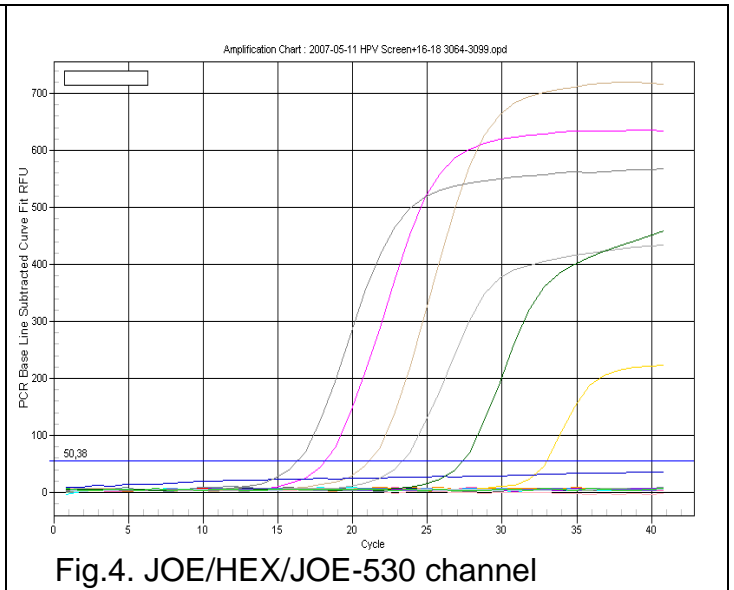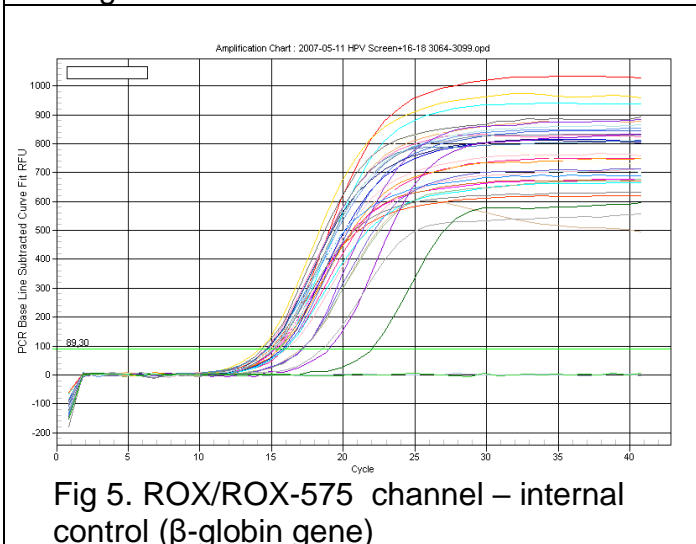Fig. 3. FAM/FAM-490 channel

Fig.4. JOE/HEX/JOE-530 channel

Fig 5. ROX/ROX-575 channel – internal control (β-globin gene)

AMPLIFICATION AND DATA ANALYSIS WITH Mx3000P, Mx3005P (Stratagene, 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 caps (detection through the cap of the tube).

1. Switch on the instrument and open the Mx3000P/Mx3005P program.
2. Select **Quantitative PCR (Multiple Standards)** in the **New Experiment Options** window and check the **Turn lamp on for warm-up** box.

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

3. Insert the tubes in the instrument; lock the fixing arm and the door of the instrument.
4. Select **Optics Configuration** in the **Options** menu. In the **Dye Assignment** tab set **JOE** parameter next to the **HEX/JOE filter set** option; set **FAM** parameter next to the **FAM filter set** option; set **ROX** parameter next to the **ROX filter set** option.

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/plate upside down while inserting them into the instrument.

5. Set the fluorescence detection parameters in the **Plate Setup** menu. To do this:
    a) hold **Ctrl** and use the mouse to select all cells in which test tubes are inserted;
    b) define all selected cells as **Unknown** in the **Well type** window. In the **Collect fluorescence data** option tick **FAM**, **JOE**, and **ROX**. Double click each cell and enter a sample name (**Well Information** window). Sample names can be entered during or after the amplification run in the **Plate Setup** menu.
6. Set the amplification program. To do this, use one of the following methods:
    Using a template file (recommended)
    Proceed to the **Thermal Profile Setup** tab. Click the **Import…** button at the right of the thermocycling profile image. Proceed to the folder with the previous experiment file and open it. Required thermocycling profile will appear in the **Thermal Profile** window.
    Individual programming
    − in the **Plate Setup** tab select all cells in which test tubes are inserted. Proceed to the **Thermal Profile Setup** menu and set the amplification program:

AmpliSens-1 amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycle repeats
Hold	95	15 min	–	1
Cycling 1	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	30 s	FAM, JOE/HEX, ROX	
	72	15 s	–	

NOTE: The unified amplification program for plate-type instruments can be used in case of simultaneous use of this PCR kit and AmpliSens® *HPV* HCR screen-FRT PCR kit.

Step	Temperature, °C	Time	Cycle repeats
Hold	95	15 min	1
Hold 2	65	2 min	1
Cycling	95	20 s	5
	64 Touchdown: 1 deg. per cycle	25 s	
	65	55 s	
Cycling 2	95	20 s	40
	60	25 s	
	65	55 s *fluorescence detection*	

− to set the parameter for detection of fluorescence signal at a specific temperature, select the **All points** option for the **Data collection marker by dragging** parameter and pull it from the right side to the shelf with the required temperature;

− click **Run** to launch amplification, then **Start**, and name the experiment.

Data analysis

Amplified DNA products corresponding to the specific targets are detected in the FAM and JOE channels; the amplified Internal Control (endogenous internal control that is the fragment of β-globin gene) is detected in the ROX channel. The results are interpreted according to the crossing (or not-crossing) of **S-shaped** fluorescence curve with the threshold line set at the specific level (the middle of the area of linear growth of fluorescence of the positive control sample in logarithmic scale) that corresponds with the presence (or absence) of Ct value in the corresponding cell of the results grid.

1. Select **Analysis** by clicking the corresponding button of the tool bar.

2. The **Analysis Selection/Setup** tab will open. Make sure that all the test samples are active (the cells corresponding to the samples should be of a different colour). Otherwise select all the test samples by holding down the **Ctrl** button and selecting the needed range with the mouse.

3. Proceed to the **Results** tab.

4. Ensure that all three fluorescence channels are active (the **JOE**, **FAM**, and **ROX** buttons are activated in the **Assay Shown** field at the bottom of the program window).

5. Select the **Threshold fluorescense** field and make sure that tick marks are put against three fluorescence channels: JOE/HEX, FAM and ROX. Ensure that the threshold line is set correctly. Normally, a threshold line should cross S-shaped curves of signal accumulation of positive samples and controls and should not cross the base line. Otherwise, the threshold should be raised. By default fluorescence curves are displayed in linear scale. To switch to logarithmic scale double click one of the axes (X or Y) with the left mouse button and tick off **Log** option for Y axis of in the **Scale** field of the opened **Graph properties** window.

6. The *Ct* values are considered reliable only if the results obtained for the control samples are correct:

   – Negative Control of extraction (C–) **– Negative Control (C–) –** no *Ct* value is detected;

   – Negative Control of amplification (NCA) **– DNA-buffer –** no *Ct* value is detected;

   – Positive Control of amplification **– Positive Control DNA *HPV* genotypes 6, 11 and human DNA (C+*HPV 6,11*) –** *Ct* values are detected in all channels.

7. Interpretation of result is carried out in accordance with boundary *Ct* values specified in the *Important Product Information Bulletin*. Samples are considered positive if their *Ct* values do not exceed values specified in the *Important Product Information Bulletin*.
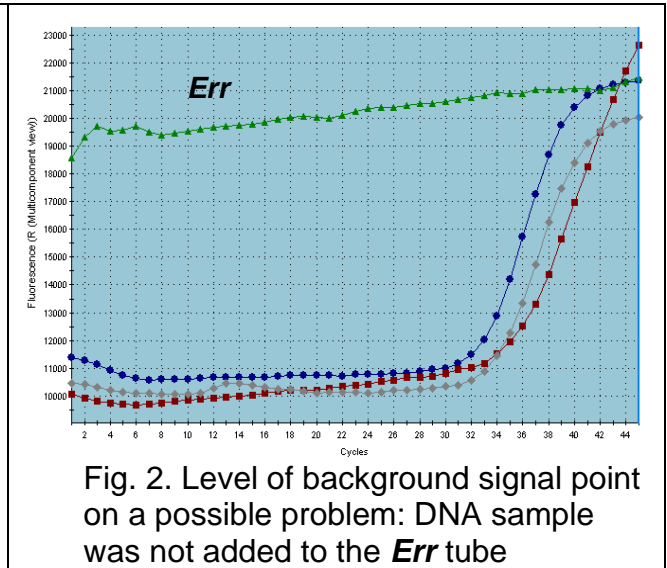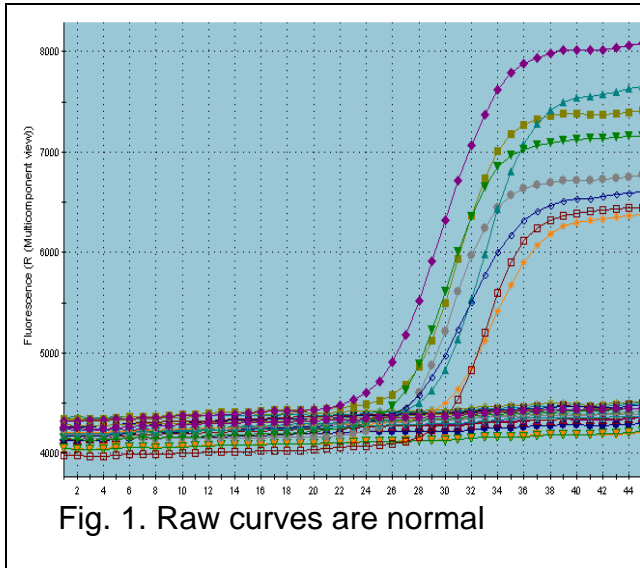
TROUBLESHOOTING

Possible problems	Reason	How to identify	Ways to eliminate
Drop of sensitivity due to deterioration of probes	Incorrect storage or utilizing kit components (high temperature, multiple opening reagent tubes, foul working conditions) can cause destroy of oligonucleotides	Probe destruction can be find out as a result of comparison of experimental data obtained at the first use of the reagents and after a period of time or in comparison with the reagents of the <u>same lot</u> stored properly. It is appeared as an increase of background fluorescence (fluorescence is assessed in the **R** mode at the beginning of the experiment) twice as much in different experiments (in case the <u>same instrument </u>is used). Note! Effect of fluorescence increase can appear after cleansing the optical system	Use mixtures stored under stated conditions until labeled expire date (see part "Stability and Storage" in the Instruction Manual)

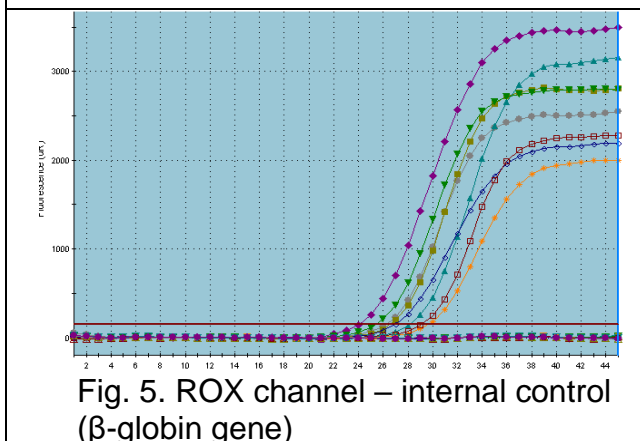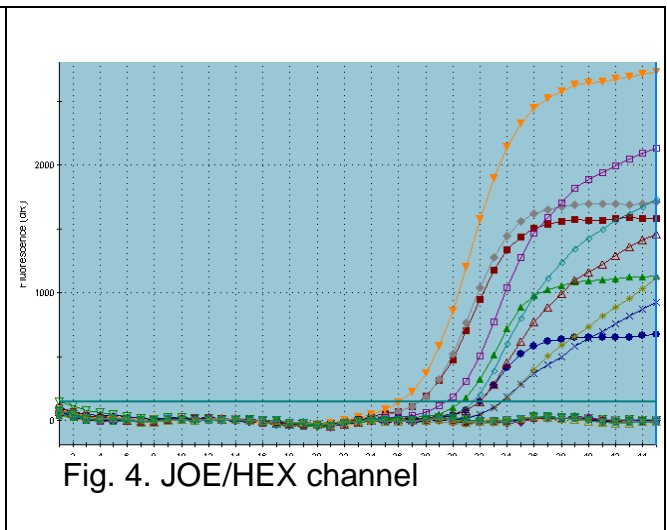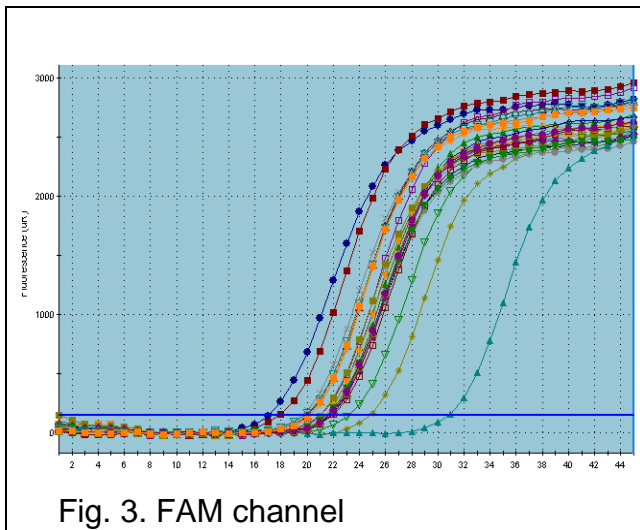|			
Drop of sensitivity due to decrease of polymerase (TaqF) activity	Incorrect storage or contamination cause enzyme deterioration	It appears as a fail of the positive control or if a boundary *Ct* value of the positive control is greater than the threshold of weak samples	Use the reagent stored under required condition (see part "Stability and Storage" in the Instruction Manual) or use a new reagent

Possible mistakes	Feature	Ways to eliminate
Contamination of a specific DNA	Detection of a signal for a negative control in any channel	Repeat the experiment. Take measures to detect and eliminate the source of contamination
The volume of a DNA sample added to a reaction tube is less than required/DNA samples is not added	Background signal of a sample is much stronger than the others (see raw curves – ***R-multicomponent view*** mode) The sample is negative	Repeat PCR for this sample
The volume of a reaction mixture added to a reaction tube is less than required/reaction mixture is not added **or** the volume of a DNA sample added to a reaction tube is greater than required	Background signal of a sample is much lower than the others (see raw curves *-* – ***R-multicomponent view*** mode)	If the sample is negative, repeat the test
Incorrect adjustment of the threshold level	Threshold line is located along with negative samples or above some or all positive curves (S-shaped)	Set the threshold line so that it crosses only sigmoid curves of fluorescence accumulation or at the level of ¼ between final values of negative and positive samples
Polymerase (TaqF) was not added to a reaction mixture	Positive signals are absent for all samples including a positive control	Repeat the experiment beginning with PCR. Ensure appropriate preparation of reaction mixtures

Unprocessed data (R mode)

Fig. 1. Raw curves are normal

Fig. 2. Level of background signal point on a possible problem: DNA sample was not added to the *Err* tube

Processed data

Normal curves that were processed, **dR** mode (S-shaped)

Fig. 3. FAM channel

Fig. 4. JOE/HEX channel

Fig. 5. ROX channel – internal control (β-globin gene)

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

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).

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/plate upside down while inserting them into the instrument.

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). Set ***Sample Volume –25 µl*** for **FRT-100 F** variant and ***30 µl*** for **FRT** variant.

HPV Genotypes 6 and 11 DNA amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	95	15 min	–	1
Cycling 1	95	20 s	–	45
	60	1 min	FAM, HEX, ROX	

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

NOTE! This amplification program is unified with amplification program for AmpliSens® *HPV* 16/18-FRT PCR kit

NOTE: Universal **AmplSens-1** amplification program can be used (see table below). With the use of the **AmplSens-1** amplification program can be carried out simultaneously in the same instrument any combination of tests on the general program (for example together with tests for the detection of STIs DNA). With the use *HPV* HCR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 DNA amplification program (for the plate-type instruments: iCycler iQ5) can be carried out simultaneously in the same instrument combination of tests for the detection of *HPV* HCR DNA and *HPV* types 6 and 11 DNA.

The analytic parameters of AmpliSens® *HPV* 6/11-FRT PCR kit by using AmplSens-1 amplification program and AmpliSens® *HPV* HCR screen-titre-FRT by using *HPV* HCR

types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 DNA amplification program are not changed.

AmplSens-1 amplification program for the plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	95	15 min	–	1
Cycling1	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	30 s	FAM, HEX, ROX	
	72	15 s	-	

4. Save protocol, selected *File*, then *Save As* in the *Protocol Editor New* window and name the file. This protocol can be used for further runs by clicking the *Select Existing…* button in the *Protocol* tab.

5. Click *OK*.

6. Set the tube order in the opened *Plate Editor – New* window. In the *Sample type* menu select *Unknown*; click the *Select Fluorophores…* button and indicate the required fluorophores with a checkmark; click *OK*; then indicate with a checkmark the fluorescence signal acquiring for the selected wells in the required channels. Define sample names in the *Sample name* window

7. In the *Plate Editor New* window select *File*, then *Save As*, and name the plate. 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.

Analysis of results

Amplified DNA products corresponding to the specific targets are detected in the FAM and JOE channels; the amplified Internal Control (endogenous internal control that is the fragment of β-globin gene) is detected in the ROX channel. The results are interpreted according to the crossing (or not-crossing) of **S-shaped** fluorescence curve with the threshold line set at the specific level (the middle of the area of linear growth of fluorescence of the positive control sample in logarithmic scale) that corresponds with the presence (or absence) of *Ct* value in the appropriate cell of the results grid.

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

<u>Variant 1</u>

For each channel at a time set the threshold line (drag it with a cursor while pressing the left mouse button) at the level of 10-20 % of maximum fluorescence obtained for the Positive Controls in the last amplification cycle. Make sure that fluorescence curve of the Positive Control crosses the threshold line at the zone of exponential growth of fluorescence passing onto linear growth.

<u>Variant 2</u>

For each channel at a time tick off *Log Scale.* Set the threshold line (the left mouse button) at the level, where the fluorescence curves are linear f and above the noise level.

Click the *View/Edit Plate* button and set in the opened window the name of samples and calibrator concentrations.

The *Ct* values are considered reliable only if the results obtained for the control samples are correct:

– Negative Control of extraction (C–) **– Negative Control (C–) –** no *Ct* value is detected;

– Negative Control of amplification (NCA) **– DNA-buffer –** no *Ct* value is detected;

– Positive Control of amplification **– Positive Control DNA** *HPV* **genotypes 6, 11 and human DNA (C+**$_{HPV\,6,11}$**) –** *Ct* values are detected in all channels.

<u>Interpolation of results</u>

Interpretation of result is carried out in accordance with boundary *Ct* values specified in the *Important Product Information Bulletin*. Samples are considered positive if their *Ct* values do not exceed values specified in the *Important Product Information Bulletin*.

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

TROUBLESHOOTING

1. If a *Ct* value appears for the Negative Control of amplification (NCA) and/or for the Negative Control of extraction (C–) in the FAM/Green, JOE/Yellow/HEX, and/or ROX/Orange channels, this indicates contamination of samples or reagents. The result of the analysis is invalid for all samples. It is necessary to repeat the analysis (starting with DNA extraction stage) of all samples that showed the presence of *HPV* DNA and to eliminate the source of contamination.

2. If a *Ct* value of the Positive Control of amplification (C+) in the FAM/Green, JOE/Yellow/HEX, and/or ROX/Orange is absent or exceeds the boundary value in the results grid, PCR should be repeated for all samples that did not show the presence of *HPV* DNA.

3. If a *Ct* value of a sample is not detected or it exceeds the boundary *Ct* value specified for *HPV* genotypes 6 and 11 in the FAM/Green, and/or JOE/Yellow/HEX channels, whereas the *Ct* value detected in the ROX/Orange channel exceeds the boundary *Ct* value specified for the Internal Control, the analysis of this sample should be performed in two repeats starting with DNA extraction stage. The result is considered positive if the reproducible positive result is obtained. The result is considered equivocal if the irreproducible result is received in two repeats.

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
04.04.14 SA	Throughout the text	Section Amplification and data analysis with CFX96 (Bio-Rad, USA) instrument was added
29.12.15 ME	Throughout the text	Corrections according to the template
14.10.19 PM	Footer	**REF** R-V11-100(RG)-CE, **REF** R-V11-100(iQ,Mx,Dt)-CE were changed to **REF** R-V11-100-CE
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
24.03.21 EM	Front page	The name, address and contact information for Authorized representative in the European Community was changed