

GUIDELINES
to AmpliSens[®] HPV HCR genotype-titre-FRT
PCR kit

for qualitative and quantitative detection and differentiation of DNA of *human papillomaviruses* of high carcinogenic risk (HPV HCR) in the 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® HPV HCR genotype-titre-FRT** PCR kit for qualitative and quantitative detection and differentiation of DNA of *human papillomaviruses* of high carcinogenic risk (**HPV HCR**) in the biological material (urogenital swabs, biopsy material of cervical mucous membrane) 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 iQ5 (Bio-Rad, USA);
- Mx3000P, Mx3005P (Stratagene, USA);
- CFX96 (Bio-Rad, USA).

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

When working with Rotor-Gene 3000 one should use the Rotor-Gene 6 Version 6.1 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 Rotor-Gene PCR tubes (0.1 ml) with caps from the four-pieces-strips (e.g. Corbett Research, Australia; QIAGEN, Germany) (detection through the bottom of the tube).

Programming the thermocycler

1. Switch the instrument on.
2. Place the tubes so that the first tube (with **PCR-mix-FL HPV 16,18,31 / Glob**) of the first strip into the first well (number 1 or A1 depending on the rotor type). Then, place all other tubes in the “head-to-tail” order. Thus, the first tube of each strip is in positions Nos. 1, 5, 9, 13, etc. (or A1, A5, B1, B5, C1, etc.). Attach locking ring and, place the rotor in the instrument, close the cap.

NOTE: Do not rotate strips.

NOTE: Do not leave gaps between strips.

NOTE: Well 1 must be filled with any studied tube except for an empty one.

3. Select the **New** item from the menu or click the **New** button on the toolbar to open the **New Run** window.

Note – The **New Run** window can be activated automatically when the program starts.

4. In the opened window select the template of experiment run **Advanced** and select **Empty Run**. Then select the **New** button.

Note – To use the settings of previous run one can select **Perform Last Run**.

5. Set **72-Well Rotor** in the opened window. Select **Locking ring attached** and click **Next**.

6. Set the operator and select the **Reaction volume** – 25 µl. Click **Next**.

7. Program the temperature profile of the experiment in the opened window by clicking **Edit profile** button:

Table 1

AmpliSens-1 amplification program for rotor-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s	–	

8. Click **OK** button.

9. Select the **Calibrate/Gain Optimisation** button in the **New Run Wizard** window:

- perform the fluorescence detection in FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red channels (activate the **Calibrate Acquiring/Optimise Acquiring** button);
- for signal measurement optimisation for the selected channels set calibration of the ROX/Orange channel – from **4FI** to **8FI**, the FAM/Green, the JOE/Yellow, the Cy5/Red channels – from **5FI** to **10FI**;
- perform the calibration in FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red channels before the first detection (activate the **Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition** button). Click **Close**.

10. Click the **Next** button. Start the amplification program by activating the **Start Run** button.

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

12. During the work of the amplifier or after it has stopped working it is necessary to program the position of the tubes in the rotor. To do this use the **Edit samples** button (in the lower right part of the window). Set the type **Unknown** opposite all the test samples and controls. Set the type **None** for the cells matching with the corresponding empty tubes.

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

Data analysis:

1. Make sure that all the test samples are activated in the legend rightward.
2. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the buttons **Cycling A. FAM/Cycling A. Green, Show, Cycling A. JOE/Cycling A. Yellow, Show, Cycling A. ROX/Cycling A. Orange, Show, Cycling A. Cy5/Cycling A. Red, Show**.
3. Cancel the automatic choice of the threshold line level for each of the main open windows (FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red) by activating the **Threshold** button.
4. Select the linear type of scale (**Linear scale**).
5. Activate the **Dynamic tube** and **Slope Correct** buttons in the main window menu (**Quantitation analysis**).
6. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box.
7. Choose the parameter **More settings/Outlier Removal** and set 15 % in the FAM/Green, JOE/Yellow, ROX/Orange channels and 12 % in the Cy5/Red channel for the value of negative samples threshold (**NTC/Threshold**).
8. In the results grid (**Quantitation Results** window), select the **Name** column by clicking on its title. Copy the column using the **Copy** command from the context menu (activated by the right mouse button) (see figure 9).
9. Open the AmpliSens® HPV HCR genotype-titre Microsoft Excel file provided with the reagent kit and agree to activate macro.
Note – If macro is not enabled (the **Results** button is not activated) when an Excel document is opened, security level must be changed. To do this, select **Service>Macro>Security** and set the middle security level.
10. Point the cursor on the **Name** cell and select the **Paste** command from the context menu (see figure 10).
11. Similarly, select the **Ct** column from the results grid (**Quant. Results**). Set the cursor in the Excel table in the **Ct** cell under the name of corresponding fluorescent dye.
12. Repeat the procedure for all other channels.
13. Save the Microsoft Excel file under a different name.
14. To ensure correct data processing, check that data in the **Name** column are set correctly. Mark the calibrators as C1 16-18-31, C1 39-45-59, C1 33-35-56-68, C1 51-52-58-66, C2 16-18-31, C2 39-45-59, C2 33-35-56-68, C2 51-52-58-66, and the negative controls as **C-**.

15. Click the **Results** button. The genotype of *HPV* detected in a sample will appear in the **Result (detected genotype)** column.

Results interpretation

Principles of automatic data analysis:

Signal in the tube in a given channel is considered positive if the corresponding fluorescence accumulation curve crosses the threshold line. The characteristic of a given signal is the threshold cycle – the cycle that corresponds to the crossing point of fluorescence curve and threshold line. The threshold cycle values (and also its presence or absence) are analyzed by the software of automatic result interpretation.

NOTE: In rare cases, crossing of the threshold line with fluorescent curves corresponding to negative samples is possible. However, such cases are identified simply by form of fluorescence curves: see “Possible problems and errors”, as well as figures 7 and 8

The experiment is valid if:

- for the negative controls *Ct* value is absent in all channels (FAM/Green, JOE/Yellow, ROX/Orange and Cy5/Red).
- For the positive controls all 14 genotypes of *HPV* are detected.

NOTE: In case of invalid experiment all obtained data are considered unreliable. The experiment is to be repeated.

The result of qualitative detection and genotyping for the sample is considered:

- *invalid*, if in one of two tubes or in two tubes of strip (1st and 2^d tubes) the signal of internal control is not registered (Cy5/Red channel) or the signal of internal control is registered only and the calculated value is less than 500 cells (10³ GE human DNA per reaction).
- *negative* if in two tubes of strip (1st and 2^d tubes) the signal of internal control is present (Cy5/Red channel) and in all four tubes the signals are absent in other channels (FAM/Green, JOE/Yellow, ROX/Orange), and in last two tubes of strip (3th and 4th) the signal is absent in all four detection channels.
- *positive* in all other cases.

Possible problems and errors

It is recommended to read this chapter before PCR kit using.

Possible problems	Reason	How to identify?	Suggested solutions
Negative samples are analyzed by Rotor-Gene software as positive	Incorrect mathematical processing of negative samples in the presence of the fluorescence decrease section at the initial cycles (see figures 2a, 8)	Typical positive sample has a characteristic S-shaped curve of fluorescence accumulation (see figures 1, 3-6). Incorrect processed negative samples are viewed as pretty straight lines going upward (Fig. 8)	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 to 1-5
	Crossing the threshold line with downstream fluorescence curves in the initial cycles (see figure 7)	In the graph of processed fluorescence curves red threshold line (Threshold) crosses or "touches" fluorescence curves in the left side of the graph (first cycles) (see figure 7)	Use the function Eliminate cycles before ... by setting 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 lower efficiency of the excitation and fluorescence detection, which primarily affects to samples with small quantity of specific DNA, giving a small fluorescence increase	Low values of the background signal in all 4 detection channels (<1) at the maximum value of the multiplier gain (10)	Clean the instrument lenses at least 1 time per month
Sensitivity decrease due to the destruction of probes	Incorrect storage or use of the reagents (high temperature, repeated opening of the tubes with the mixes, work in "dirty" conditions) may lead to the destruction of oligonucleotides	Destruction of the probes may be identified only by comparing the experimental data at the beginning and after a certain time of reagents using or by comparing with <u>the same lot</u> of reagents stored adequately. It can be identified by decrease of automatically chosen multiplier gain coefficient value more than 2 units in different experiments (using <u>the same instrument</u>) WARNING! Effect of increasing the multiplier gain can also be observed after cleaning the instrument lenses from heavy dirt	Use mixes stored in adequate conditions and with unexpired date (see the chapter "Stability and storage" in <i>Instruction Manual</i>)
Sensitivity decrease due to the decrease of polymerase (TaqF) activity	Incorrect storage of polymerase or impurity leads to the enzyme destruction	It can be identified by incorrect running of positive control	Use adequately stored (see the chapter "Stability and storage" in <i>Instruction Manual</i>) or a new enzyme

Possible error	Characters	Way to eliminate
Contamination of the specific DNA	The signal is registered in any channel in negative control	Repeat the experiment. Take measures to detect and eliminate the source of contamination
Less of DNA sample is added or not added at all to the tube	Background signal greatly exceeds other signals (seen on untreated curves). The sample is negative (See figure 2b)	Repeat the sample analysis
Less of reaction mixture is added or not added at all or more of DNA sample is added to the tube	Background signal is greatly less than other signals (seen on untreated curves). (figure 2b)	Repeat the analysis if the sample is negative
Autocalibration parameter is not set from 4FI to 8FI for ROX/Orange channel, from 5FI to 10FI for FAM/Green, JOE/Yellow, Cy5/Red channels or an error in first tube in rotor is present (tube is absent, DNA sample or reaction mixture is added incorrectly)	Most of fluorescence background signals is less than 1 or more than 20	Set the parameter at the next running. Repeat the experiment if there is "overshoot" or the signal are too weak (there is no positive signals at processing, background is less than 0.5)
Polymerase (TaqF) is not added during the reaction mixture preparation	Any positive signal including the positive control is not registered in any sample	Repeat the analysis with correctly prepared mixes

EXAMPLES OF OBTAINED DATA

Raw data

Figure 1 – Normal initial curves

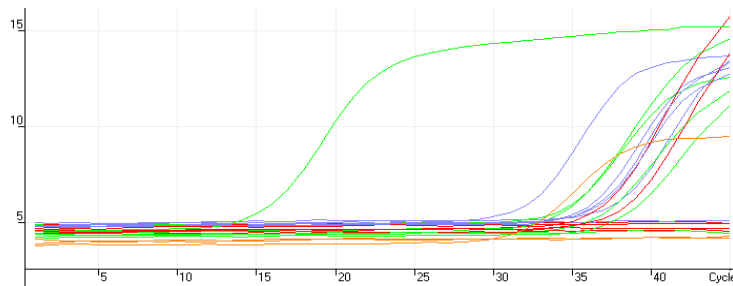


Figure 2a – Initial curves with "bend" and low level of background signal (range 0.75-1.5) – *impurity of the instrument lenses*

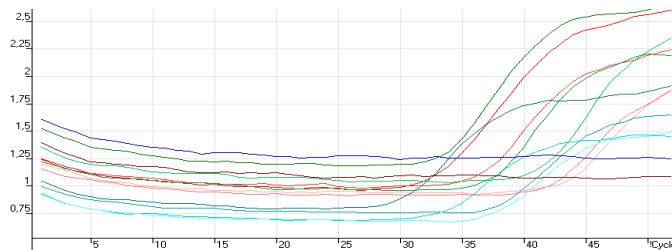
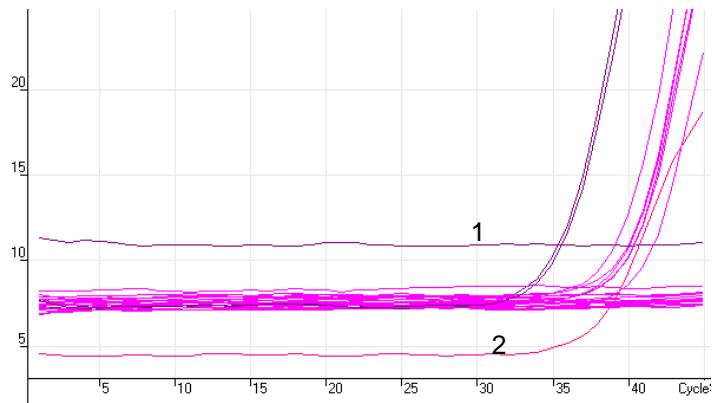


Figure 2b – One of possible errors is viewed by background signal level: DNA sample was not added to tube **1**, double quantity of DNA sample was added to the tube **2**.



Processed data

a) normal processed curves (typical S-shape, threshold line crosses the curves only in the area of fluorescence accumulation)

Figure 3 – Data on Cy5/Red channel – internal control (β -globin gene)

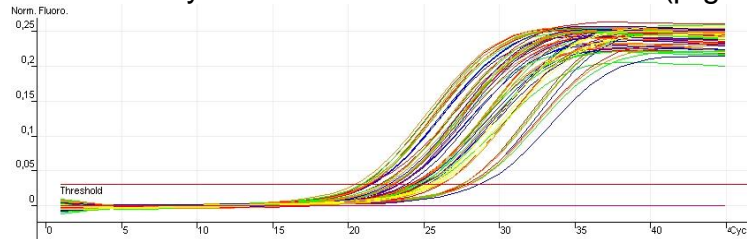


Figure 4 – Data on FAM/Green channel

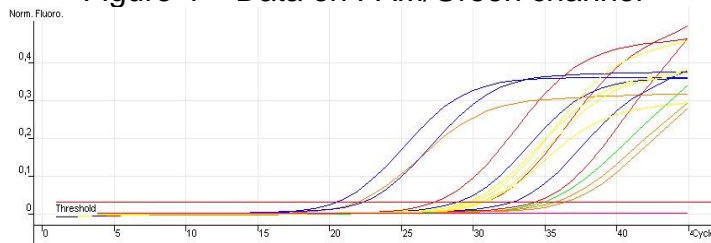


Figure 5 – Data on JOE/Yellow channel

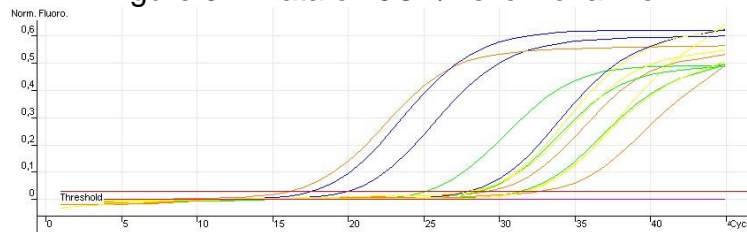
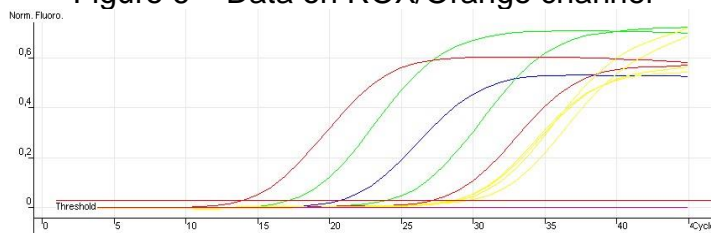


Figure 6 – Data on ROX/Orange channel



b) incorrect curves processing

Figure 7 – Threshold line crosses twice the fluorescence curves

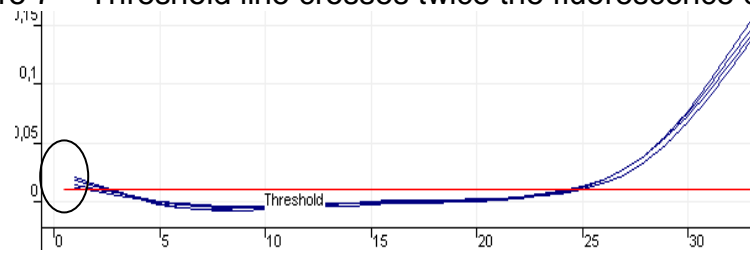
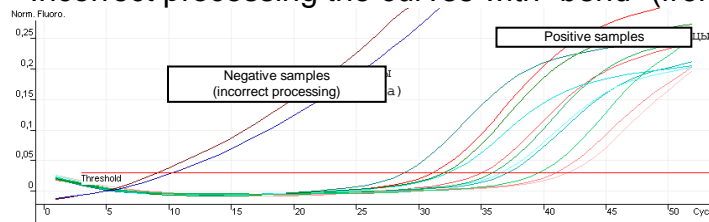


Figure 8 – Incorrect processing the curves with “bend” (from figure 2)



Results analysis

Figure 9 – Results grid **Quant.Results**

Name	Type	Ct	Given Conc (cop)	Cali
842t	Unknown	20,06		
	Unknown			
	Unknown	31,60		
	Unknown			
690kt	Unknown			
	Unknown	25,33		
	Unknown			
	Unknown	28,82		
+	Positive Control	28,66		
+	Positive Control	31,89		
+	Positive Control	31,86		
+	Positive Control	28,19		
-	Negative Control			
-	Negative Control			
-	Negative Control			
-	Negative Control			

Figure 10 – Software for data analysis in Microsoft® Excel format

Date	Matrix for comparison	Ct values	Result (detected genotype)	number of cells	Ig HPV 16,39,33, 58 / 10 ⁵ cells	Ig HPV 31,45,35, 52 / 10 ⁵ cells	Ig HPV 18,59,68, 66 / 10 ⁵ cells	Ig HPV 56, 51 / 10 ⁵ cells	Total Ig HPV / 10 ⁵ cells	Clinical significance
NN	FAM	JOE	ROX	Cy5	FAM	JOE	ROX	Cy5		
Well	Well Name				Ct	Ct	Ct	Ct		
1	1	16	31	18	IC	20,0		17,0		
2	1	39	45	59	IC	25,0		17,1		
3	1	33	35	68	56			19,0		
4	1	58	52	66	51					
					31					
					39				6,31E+03	
					68					
									4,41	
									2,81	
									4,53	
										4,78
										Significant

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ5 (Bio-Rad, USA)

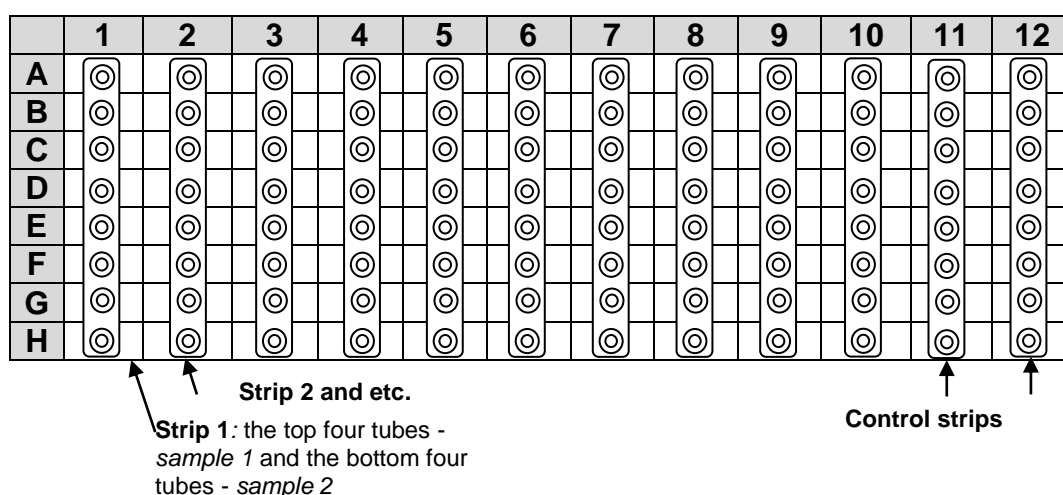
INSTRUMENT

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 optically transparent flat caps, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

1. Switch on the instrument and the power supply unit of the optical part of the instrument.

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

2. Start the program iCycler iQ5.
3. Insert the tubes or strips into the reaction module of the amplifier (thermocycler) according the plate scheme below and program the instrument.



NOTE: Do not leave gaps between strips while inserting (even if the number of strips is less than specified on the scheme). Insert the control strip last.

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

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

1. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal).
 - Click the **Create New** or **Edit** buttons in the **Selected Plate Setup** window of the **Workshop** module. One can edit the plate setup in the **Whole Plate loading** mode. Set the reaction volume (**Sample Volume**) as **25 µl**, the caps type (**Seal Type**) as **Domed Cap**, and the tubes type (**Vessel Type**) as **Tubes**. Select the fluorescent signal detection through the FAM, JOE/HEX, ROX, Cy5 channels. Save the set

plate setup by clicking the **Save&Exit Plate Editing** button.

2. Set all the samples including controls as **Unknown**.
3. Click the **Cancel & Exit Plate Editing** button.
4. Set the amplification program (see Table 2).

Table 2

AmpliSens-1 amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	30 s	FAM, JOE/HEX, ROX, Cy5	
	72	15 s	–	

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).

5. Before starting the program execution it is **obligatory** to check if the selected protocol (**Selected Protocol**) and the plate scheme (**Selected Plate Setup**) are correct. Start the instrument (**Run**), select **Collect Well Factors from Experimental Plate**. Click the **Begin Run button**, name the experiment (the experiment results will be automatically saved in this file).
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 *C_t* (threshold cycle) value in the corresponding column of the results table.

1. Start the program and open the needed file with data of the analysis in the **Data File** window of the **Workshop** module. Click the **Analyze** button.
2. Select the **Analysis Mode: PCR Base Line Subtracted Curve Fit** (is set by default).
3. 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, click the **Log View** (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 C2 sample in the last amplification cycle. Make sure that the fluorescence curve of the Positive control has the typical exponential growth of fluorescence.

4. In order to analyze the results click the **Results** button which is situated under the buttons with the fluorophores' names.
5. To continue work with data, click the right mouse button on the appeared table with results. In the drop-down menu select Export to Excel. Save the file to the needed folder. Open the AmpliSens® HPV HCR genotype-titre Microsoft Excel file provided with the reagent kit and agree to activate macro.

Note – If macro is not enabled (the **Results** button is not activated) when an Excel document is opened, security level must be changed. To do this, select **Service>Macro>Security** and set the middle security level.

6. Copy the samples names and the threshold cycles values for each channel from the instrument file to the software AmpliSens® HPV HCR genotype-titre file. Check the values set in the software for calibrators. They must match the values in the *Important Product Information Bulletin*. Check the correctness and supplement the samples names. To ensure correct data processing, check that data in the **Name** column are set correctly. Mark the calibrators as C1 16-18-31, C1 39-45-59, C1 33-35-56-68, C1 51-52-58-66, C2 16-18-31, C2 39-45-59, C2 33-35-56-68, C2 51-52-58-66, and the negative controls as C–.
7. Save the Microsoft Excel file under a different name.
8. Click the **Results** button. The genotype of HPV detected in a sample will appear in the **Result (detected genotype)** column.

Results interpretation

Principles of automatic data analysis:

Signal in the tube in a given channel is considered positive if the corresponding fluorescence accumulation curve crosses the threshold line. The characteristic of a given signal is the threshold cycle – the cycle that correspond to the crossing point of fluorescence curve and threshold line. The threshold cycles values (and also its presence or absence) are analyzed by the software of automatic result interpretation.

The experiment is valid if:

- for the negative controls *Ct* value is absent in all channels (FAM, JOE/HEX, ROX, Cy5).
- for the positive controls all 14 genotypes of *HPV* and human DNA are detected.

NOTE: In case of invalid experiment all obtained data are considered unreliable. The experiment is to be repeated.

The result of qualitative detection and genotyping *HPV* DNA for the sample is considered:

- *invalid*, if in one of two tubes or in two tubes of strip (1st and 2^d tubes) the signal of internal control is not registered (Cy5 channel) or the signal of internal control is registered only and the calculated value is less than 500 cells (10^3 GE human DNA per reaction).
- *negative* if in two tubes of strip (1st and 2^d tubes) the signal of internal control is present (Cy5 channel) and in all four tubes the signals are absent in other channels (FAM, JOE/HEX, ROX), and in last two tubes of strip (3th and 4th) the signal is absent in all four detection channels.
- *positive* in all other cases.

EXAMPLES OF OBTAINED DATA

Figure 11 – Data in the FAM channel

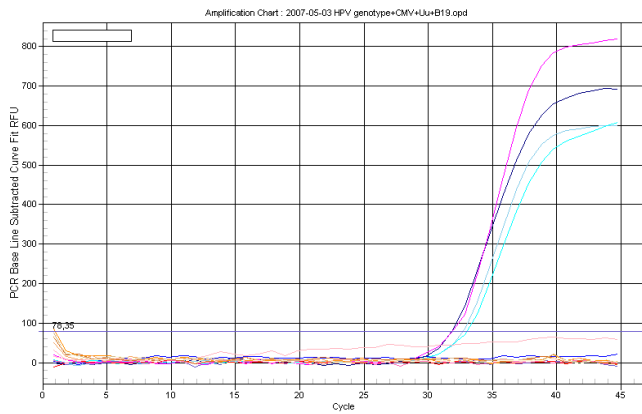


Figure 12 – Data in the JOE/HEX channel

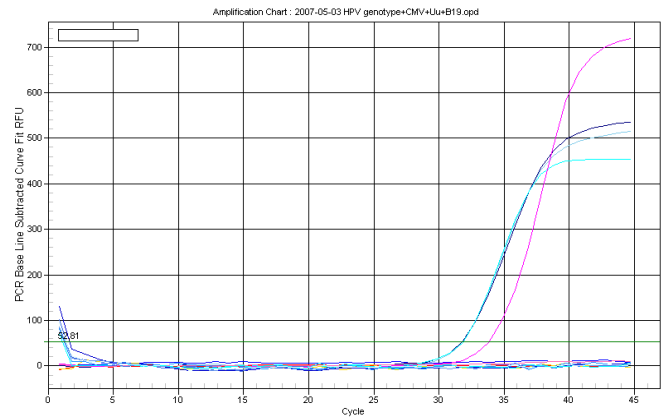


Figure 13 – Data in the ROX channel

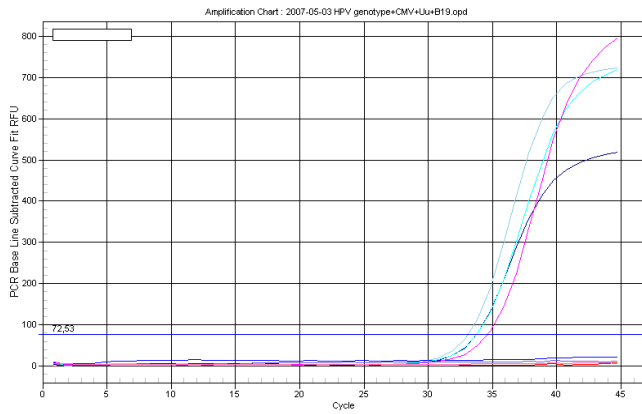
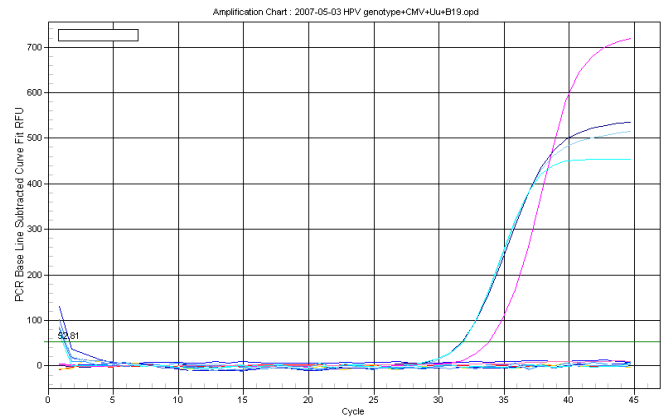


Figure 14 – Data in the Cy5 channel



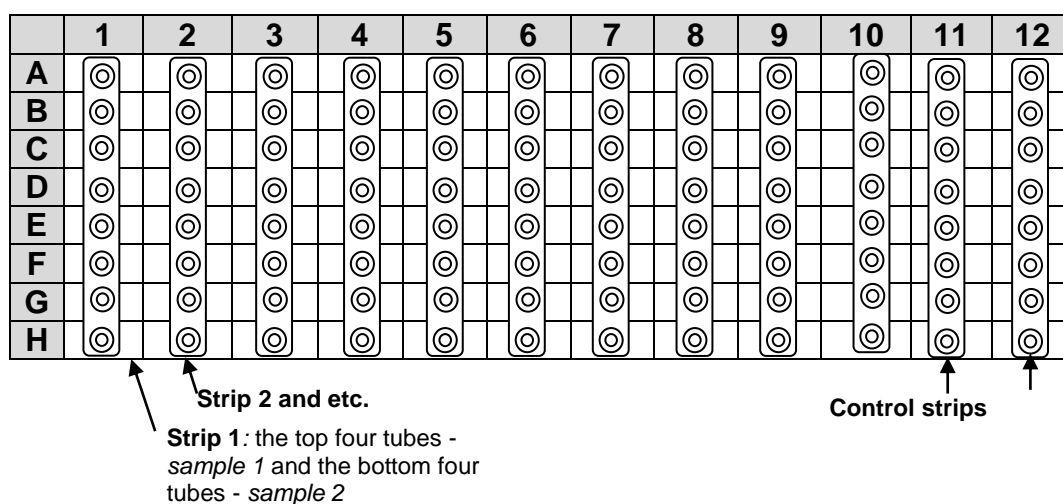
AMPLIFICATION AND DATA ANALYSIS USING Mx3000P, Mx3005P (Stratagene, USA)

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with optically transparent domed or flat caps, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

1. Switch the instrument on, start the program Mx3000P/Mx3005P.
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 into the instrument according the scheme below, lock the fixing arm and the door of the instrument.



4. Select **Optics Configuration** in the **Options** menu and in the **Dye Assignment** tab set FAM parameter next to the **FAM filter set** item, JOE parameter next to the **HEX/JOE filter set**, ROX parameter next to the **ROX filter set**, Cy5 parameter next to the **Cy5 filter set**.

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.

NOTE: Do not leave gaps between strips while inserting (even if the number of strips is less than specified on the scheme). Insert the control strip last.

5. Set the fluorescence detection parameters in the **Plate Setup** menu. To do this, select all the cells with the test tubes or strips (by holding down the **Ctrl** button and selecting the needed range with the mouse) and mark them as **Unknown** in the **Well type** field. Select FAM, JOE, ROX and Cy5 fluorophores in the **Collect fluorescence data** option.

Set the name for each test sample (**Well Information** window) by double click on each cell. One can also name the samples during amplification or after it returning to **Plate Setup** menu.

6. Select all the cells in the **Plate Setup** tab where the test tubes are set. Select the **Thermal Profile Setup** menu and set the amplification program (see Table 3).

Table 3

AmpliSens-1 amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	30 s	FAM, JOE/HEX, ROX, Cy5	
	72	15 s	–	

NOTE: For setting the parameter of the fluorescence signal detection at the set temperature it is necessary to select the **All points** option for the **Data collection marker for dragging** parameter and drag it with a mouse from the right part of the box to the field with the necessary temperature

7. Start the amplification by clicking the **Run** and **Start** buttons, then name the experiment.

Data analysis:

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. Select the **Results** tab.
4. Make sure that four fluorescence channels are active (the **Cy5, ROX, JOE/HEX, FAM** buttons are activated in the **Assays Shown** field in the lower part of the program's window).
5. Select the **Threshold fluorescence** field and make sure that tick marks are put against four fluorescence channels:
 - Cy5 – 100
 - ROX – 200
 - JOE/HEX – 50
 - FAM – 300
6. Select the **Text Report** point in the **Area to analyze** field. Make sure visually that all data are sorted by the wells names in ascending order (**Well** column). Otherwise, click

once on the name of the **Well** column.

7. Open the AmpliSens® HPV HCR genotype-titre Microsoft Excel file provided with the reagent kit and agree to activate macro.

Note – If macro is not enabled (the **Results** button is not activated) when an Excel document is opened, security level must be changed. To do this, select **Service>Macro>Security** and set the middle security level.

8. Copy the samples names and the threshold cycles values for each channel from the instrument file to the software AmpliSens® HPV HCR genotype-titre file. Check the values set in the software for calibrators. They must match the values in the *Important Product Information Bulletin*.
9. To ensure correct data processing, check that data in the **Name** column are set correctly. Mark the calibrators as C1 16-18-31, C1 39-45-59, C1 33-35-56-68, C1 51-52-58-66, C2 16-18-31, C2 39-45-59, C2 33-35-56-68, C2 51-52-58-66, and the negative controls as C–.
10. Save the Microsoft Excel file under a different name.
11. Click the **Results** button. The genotype of HPV detected in a sample will appear in the **Result (detected genotype)** column.

Results interpretation

Principles of automatic data analysis:

Signal in the tube in a given channel is considered positive if the corresponding fluorescence accumulation curve crosses the threshold line. The characteristic of a given signal is the threshold cycle – the cycle that correspond to the crossing point of fluorescence curve and threshold line. The threshold cycles values (and also its presence or absence) are analyzed by the software of automatic result interpretation.

The experiment is valid if:

- for the negative controls Ct value is absent in all channels (FAM, JOE/HEX, ROX, Cy5).
- For the positive controls all 14 genotypes of HPV are detected.

NOTE: In case of invalid experiment all obtained data are considered unreliable. The experiment is to be repeated.

The result of qualitative detection and genotyping HPV DNA for the sample is considered:

- *invalid*, if in one of two tubes or in two tubes of strip (1st and 2^d tubes) the signal of internal control is not registered (Cy5 channel) or the signal of internal control is registered only and the calculated value is less than 500 cells (10³ GE human DNA per reaction).

- *negative* if in two tubes of strip (1st and 2^d tubes) the signal of internal control is present (Cy5 channel) and in all four tubes the signals are absent in other channels (FAM, JOE/HEX, ROX), and in last two tubes of strip (3th and 4th) the signal is absent in all four detection channels.
- *positive* in all other cases.

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

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual*. When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with optically transparent domed or flat caps, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

Program the instrument according to the *Instruction Manual* provided by the manufacturer:

1. Turn on the instrument and start the **Bio-Rad CFX Manager** program.
2. Select **Create a new Run** (or select **New** and then **Run...** in the **File** menu).
3. In the **Run Setup** window, select **Protocol** and click the **Create new...** button. Set amplification parameters (time, temperature, number of cycles, and step of reading fluorescent signal) in the opened **Protocol Editor – New** window (see Table 4). Set **Sample Volume – 25 µl**.

Table 4

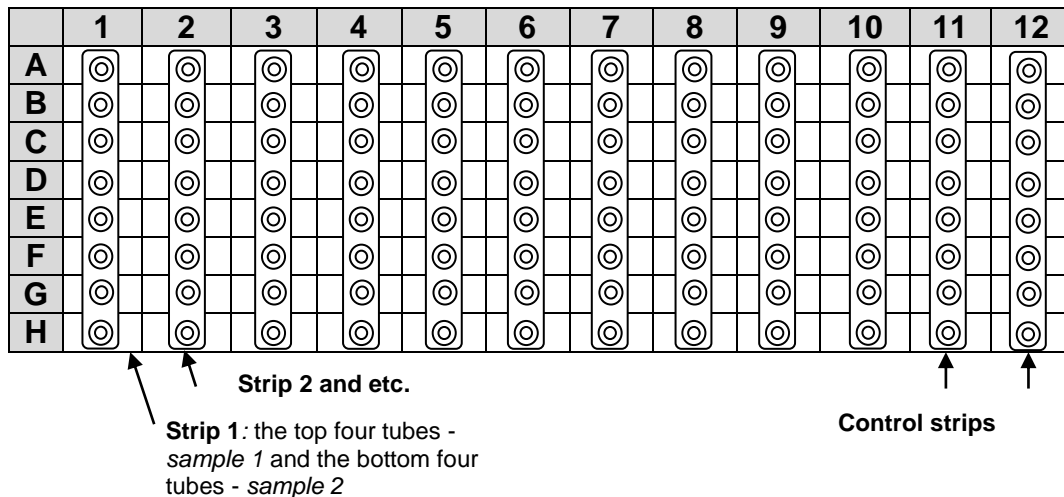
AmpliSens-1 amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	95	5 s	–	40
	60	30 s	FAM, HEX, ROX, Cy5	
	72	15 s	–	

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

4. In the **Protocol Editor New** window select **File**, then **Save As**, and name the protocol. This protocol can be used for further runs by clicking the **Select Existing...** button in the **Protocol** tab.
5. When the required program is entered or edited, click **OK** at the bottom of the window.
6. In the **Plate** tab click the **Create new...** button. Set the tube order in the opened **Plate Editor – New** window. In the **Sample type** menu select **Unknown**; click the **Select Fluorophores...** button and indicate the required fluorophores 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 scheme below.



NOTE: Do not leave gaps between strips while inserting (even if the number of strips is less than specified on the scheme). Insert the control strip last.

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.

9. In the **Start Run** tab click the **Start Run** button then save the file of the experiment.

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

Data analysis

The obtained results are analyzed by the software of the CFX96 instrument. The results are interpreted according to the crossing (or not-crossing) of the S-shaped (sigmoid) fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *Ct* (threshold cycle) value in the corresponding column of the results table.

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.
3. 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 C2 sample 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 C2 sample (or another one appropriate sample), set the cursor to the plate setup or to the results grid.

4. Click the **View/Edit Plate** button on the toolbar and enter sample names in the opened window.
5. Click **Tools** on the toolbar, then **Reports...**, and then save the generated report.
6. Click by right mouse button on the **Well** cell in the results grid.
7. 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**. Open the AmpliSens® HPV HCR genotype-titre Microsoft Excel file provided with the reagent kit and agree to activate macro.

Note – If macro is not enabled (the **Results** button is not activated) when an Excel document is opened, security level must be changed. To do this, select **Service>Macro>Security** and set the middle security level.

6. Copy the samples names and the threshold cycles values for each channel from the instrument file to the software AmpliSens® HPV HCR genotype-titre file. Check the values set in the software for calibrators. They must match the values in the *Important Product Information Bulletin*. Check the correctness and supplement the samples names. To ensure correct data processing, check that data in the **Name** column are set correctly. Mark the calibrators as C1 16-18-31, C1 39-45-59, C1 33-35-56-68, C1 51-52-58-66, C2 16-18-31, C2 39-45-59, C2 33-35-56-68, C2 51-52-58-66, and the negative controls as C–.
7. Save the Microsoft Excel file under a different name.
8. Click the **Results** button. The genotype of HPV detected in a sample will appear in the **Result (detected genotype)** column.

Results interpretation

Principles of automatic data analysis:

Signal in the tube in a given channel is considered positive if the corresponding fluorescence accumulation curve crosses the threshold line. The characteristic of a given signal is the threshold cycle – the cycle that correspond to the crossing point of fluorescence curve and threshold line. The threshold cycles values (and also its presence or absence) are analyzed by the software of automatic result interpretation.

The experiment is valid if:

- for the negative controls *Ct* value is absent in all channels (FAM, HEX, ROX, Cy5);
- for the positive controls all 14 genotypes of *HPV* are detected.

NOTE: In case of invalid experiment all obtained data are considered unreliable. The experiment is to be repeated.

The result of qualitative detection and genotyping *HPV* DNA for the sample is considered:

- *invalid*, if in one of two tubes or in two tubes of strip (1st and 2^d tubes) the signal of internal control is not registered (Cy5 channel) or the signal of internal control is registered only and the calculated value is less than 500 cells (10^3 GE human DNA per reaction).
- *negative* if in two tubes of strip (1st and 2^d tubes) the signal of internal control is present (Cy5 channel) and in all four tubes the signals are absent in other channels (FAM, HEX, ROX), and in last two tubes of strip (3th and 4th) the signal is absent in all four detection channels.
- *positive* in all other cases.

TROUBLESHOOTING

1. If for Negative Control of extraction (C–) any *Ct* value appears in the FAM/Green, JOE/HEX/Yellow, ROX/Orange and/or Cy5/Red channels, it indicates the contamination of the reagents or samples. In this case results of the analysis for all samples are considered to be invalid. It is necessary to repeat the analysis of all samples, in which *HPV* DNA was detected, and to take measures to detect and eliminate the source of contamination.
2. If for PCR calibrators the *Ct* value is absent or exceeds the boundary values appears in the FAM/Green, JOE/HEX/Yellow, ROX/Orange and/or Cy5/Red channels, it is necessary to repeat the amplification of all samples, in which *HPV* DNA was not detected.
3. If for the sample the *Ct* value is not defined or exceeds the boundary value in the FAM/Green, JOE/HEX/Yellow, ROX/Orange and/or Cy5/Red channels in 3th and 4th tubes, and the *Ct* value exceeds the boundary value in the channels for the Cy5 fluorophore in 1st and 2^d tubes, then the PCR analysis should be repeated beginning with the DNA extraction stage. Possible reason is a mistake of biological material preparing that leads to DNA loss, or the presence of PCR inhibitors.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
20.03.24 BA	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) instruments	Calibrate/Gain Optimisation for ROX/Orange channel was changed from «from 5FI to 10FI» to «from 4FI to 8FI»
		Outlier Removal for Cy5/Red channel was changed from 15% to 12%