



For Professional Use Only

## GUIDELINES

### to AmpliSens<sup>®</sup> HPV HCR screen-titre-FRT PCR kit

for qualitative and quantitative detection of high carcinogenic risk (HCR) *human papillomaviruses (HPV)* types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 DNA in the clinical material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

# AmpliSens<sup>®</sup>



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## INTENDED USE

Guidelines describe the procedure of the use of **AmpliSens® HPV HCR screen-titre-FRT** PCR kit for qualitative and quantitative detection of high carcinogenic risk (HCR) *human papillomaviruses (HPV)* types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 DNA in the clinical material (cervical and urethral scrapes) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Two- and four-channel Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA);
- Smart Cycler II (Cepheid, USA);
- Mx3000P (Stratagene, USA).

## AMPLIFICATION AND DATA ANALYSIS USING TWO-CHANNEL Rotor-Gene 3000 and Rotor-Gene 6000 (Corbett Research, Australia) 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.

### Programming the thermocycler

1. Turn on the instrument, run the Rotor-Gene software.
2. For further automated result processing, select one of the variants of tube placement in the rotor of the device:
  - Insert sequentially all tubes with *HPV FRT A9* mixture from the 1<sup>st</sup> one, then in the same order insert tubes with *HPV FRT A7+* mixture;

**NOTE:** If less than 14 samples are studied (incomplete load of the carousel) insert tubes with *HPV FRT A9* mixture from the 19 well of the rotor for providing adequate analysis of results.

- Insert tubes in pairs: *HPV FRT A9* mixture and *HPV FRT A7+* mixture for the first sample, *HPV FRT A9* mixture and *HPV FRT A7+* mixture for the second sample, etc... *HPV FRT A9* mixture and *HPV FRT A7+* mixture for **DNA calibrator C1 HPV**, *HPV FRT A9* mixture and *HPV FRT A7+* mixture for **DNA calibrator C2 HPV**, *HPV FRT A9* mixture and *HPV FRT A7+* mixture for **DNA calibrator C3 HPV**, *HPV FRT A9* mixture and *HPV FRT A7+* mixture for NCA (Negative Control of Amplification).

If the carousel is not filled completely, balance it. To do this, fill the free wells with **NOTE:** empty tubes (*don't use tubes from previous experiments*). Place any study tube (*not an empty tube*) to the well 1.

3. Insert the rotor into the instrument, close the lid.
4. Start amplification program. Use one of variants:

## Using the ready template for the run

**NOTE:** Usage of the template file **AmpliSens FRT HR HPV Screen RG2x.ret** during amplification is highly recommended.

For usage of the template file for the PCR kit, copy previously **AmpliSens FRT HR HPV Screen RG2x.ret** file, enclosed to the kit, to the **Templates** folder of the RotorGene program. The default location of this folder for the RotorGene is the following: **C:\Program Files\RotorGene 6\Templates** for Rotor-Gene 3000, and **C:\Program Files\RotorGene 6000 Software\Templates** for Rotor-Gene 6000. After that, in the window **New Run** (called by the **New** button on the toolbar) the **AmpliSens FRT HR HPV Screen RG2x** item will appear. Select it to start new experiment.

Note: Template file can be opened from another folder as well. To do this, in the **New Run** window select **Advanced** tab, and there select the **Open A Template In Another Folder...** item, then switch to folder with the **AmpliSens FRT HR HPV Screen RG2x.ret** file.

Template file contains all the needed parameters of amplification; therefore one has only to enter the experiment data and designations of samples.

## Individual programming

1. Run the new run wizard in the **Advanced** tab.
2. Select the reaction volume – **25 µl** and tick the **15 µl oil layer volume** option in the window opened after the window of rotor selecting.
3. Set the amplification program:

### **DNA amplification program for HCR HPV types 16, 18, 31, 33, 35; 39, 45, 51, 52, 56, 58, and 59**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Cycles</b>
<i>Hold</i>	95	15 min	–	1
<i>Hold2</i>	65	2 min	–	1
<i>Cycling</i>	95	20 s	–	5
	64 <u>Touchdown:</u> 1 deg. per cycle / Lower temperature of each step on 1 °C	25 s	–	
	65	55 s	–	
<i>Cycling2</i>	95	15 s	–	40
	60	25 s	–	
	65	40 s	FAM/Green, JOE/Yellow	

**Note** - *AmpliSens FRT HR HPV Screen-titr RG2x Program.pro* file in the RotorGene software format can be used for setting the amplification program.

**NOTE:** The “**AmpliSens-1 RG**” universal program for DNA amplification and detection can also be used (see below). Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, combined with tests for STI pathogen DNA detection).

The analytical performance characteristics of the reagent kit do not change when the universal amplification program is used.

**Amplification program “AmpliSens-1 RG”**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Cycles</b>
<i>Hold</i>	95	15 min	–	1
<i>Cycling</i>	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
<i>Cycling2</i>	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s	–	

**Note** – The ROX/Orange and Cy5/Red channels are enabled when required if the “multiprime” format tests are performed.

4. Click the **Calibrate/Gain Optimisation...** button in the **New Run Wizard** window. In the opened window:
  - a) Perform the calibration in the FAM/Green, JOE/Yellow channels (click the **Calibrate Acquiring/Optimise Acquiring** button);
  - b) perform the calibration in the selected channels before the first detection (tick the **Perform Calibration Before 1<sup>st</sup> Acquisition/ Perform Optimisation Before 1<sup>st</sup> Acquisition** option);
  - c) for signal measurement optimisation for the selected channels set calibration from **4FI to 8FI** for all the channels (the **Edit** button, the **Auto gain calibration channel settings** window).
5. Select the **Start run** button for amplification run.
6. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Enter the names/numbers of the test samples in the **Name** column. Analysis is performed with two tubes; therefore, for correct automatic analysis of results the both tubes of the sample must have the same name. Set the type **Unknown** opposite all the test samples including control samples. Set the type **None** for the cells matching with the corresponding empty tubes. Name calibrators as **K1, K2, K3**, Negative control of amplification as **K-**.

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

## **Data processing and analysis**

Browse raw data of the device in two channels (FAM/Green, JOE/Yellow) selecting **Cycling A...** buttons on **Channels** panel. Take into account:

- Among all signals of samples on the channel at least one must be positive (positive signal have specific S-shaped fluorescence accumulation curve, see figure 1). If experiment is organized and performed correctly, signals of **DNA calibrator C1 HPV**, **DNA calibrator C2 HPV**, **DNA calibrator C3 HPV** must be obtained.

**NOTE:** If positive signals on fluorescence channel are completely absent, analysis of data on this channel may provide incorrect result.

- Presence of samples with background signal that differs significantly from majority of signals means the mistake of insertion of reaction mixture or DNA sample into the tube (see figure 2b and the “Possible problems and errors” section).

**Difference in background signals of HPV FRT A9 mixture and HPV FRT A7+ mixture is allowed**

### **Data processing**

1. Make sure that all the test samples are active in the legend at the right.
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**.
3. After opening the **Quantitation analysis** window set the threshold manually: Threshold = 0,03.
4. Select the **Linear scale**. Select the **Dynamic tube** and **Slope Correct** buttons.
5. Choose the parameter **More settings/Outlier Removal** and set **15 %** for the value of negative samples threshold (**NTC/Threshold**).

**NOTE:** In some rare cases due to the descend-type curves at the beginning of the experiment the threshold line can cross the fluorescence curves at the first cycles, and RotorGene program can record the crossing data as positive signal (see figure 7). To block this effect, use the **Eliminate cycles before...** option by setting 5 (crossing the threshold and the fluorescence curve at the first 5 cycles is ignored).

6. In the results grid (**Quant. Results** window); select the **Name** column by single click on the title. Copy the column selecting the **Copy** button from the right-click menu (figure 9).
7. Open Microsoft® Excel **AmpliSens FRT HR HPV Screen RG2x Results Matrix.xls** file, enclosed to the reagent kit. Agree to enable macros.

**Note** – If macros is disable when opening Excel worksheet (the **Results** button is not activated), then security level should be changed. Select

**Tools>Macro>Security>Medium.**

8. Set cursor on **Name** cell of **Samples labeling** column of **Device data** table, and select **Insert** command from the context menu (figure 10).
9. Similarly, select and copy **Ct** column from the results grid (**Quant.Results**). Set cursor in Excel table into **Ct** cell under the name of fluorescent dye FAM/Green.
10. Repeat the procedure for fluorescent dye JOE/Yellow.
11. Set **Quantitative analysis** regimen with internal calibration.
12. Insert data on concentration of calibrators into the table **Calibrator value** according to *Important Product Information Bulletin* enclosed to the given lot of the PCR kit.
13. Check in the **Samples labelling** column, that **DNA calibrator C1 HPV** is named as **K1**, **DNA calibrator C2 HPV** is named as **K2**, **DNA calibrator C3 HPV** is named as **K3** (without blank between letter K and number), and negative control is named as **K-** or **“-**“.
14. Save Microsoft® Excel file under different name.

**Data analysis**

Data are analyzed automatically. When data are inserted into the Excel matrix, press **Results** button. In the **Results** column detected phylogenetic groups of *HPV* will appear, and the result: Positive (**pos**), negative (**neg**), weak positive (**weak**), non-valid (**N/V**). Then in the table number of human cells per reaction will appear; that is used for evaluation of validity of the sample. Then *HPV* DNA is calculated as log for 10<sup>5</sup> of cells for each group, and total viral load. The last column contains probable explanation of clinical evaluation of result according the following:

Result log ( <i>HPV</i> DNA copies per 100,000 cells)	Evaluation
<3	Clinically insignificant value
3-5	Clinically valuable. Dysplasia cannot be excluded; risk of dysplasia.
>5	Clinically valuable, of increased value. Dysplasia is highly suggestive.

To provide correct data processing, check that in the **Name** column:

- Tubes with different mixtures of the same sample have the same name;
- Positive controls are named as **“+”** or **K+**, and negative controls as **“-”** or **K-**.

## 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 <b>Ignore First</b> 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 <b>Eliminate cycles before ...</b> 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 <b>gain</b> (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 <b>gain</b> coefficient value more than 2 units in different experiments (using <u>the same instrument</u> ) <b>WARNING!</b> Effect of increasing the multiplier <b>gain</b> 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 violation of sterility conditions-leads to the enzyme destruction	It can be identified by signal absence of positive control or if <i>Ct</i> value for the positive control is greater than the threshold of the weak samples	Use adequately stored (see the chapter "Stability and storage" in <i>Instruction Manual</i> ) or a new enzyme



**Note** – The information about all set parameters of the experiment and the autocalibration report can be found by browsing the experiment settings (the **Settings** button). Particularly, **Autocalibration Log Messages** point in the **Messages** tab is the autocalibration report.

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 5FI to 10FI 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

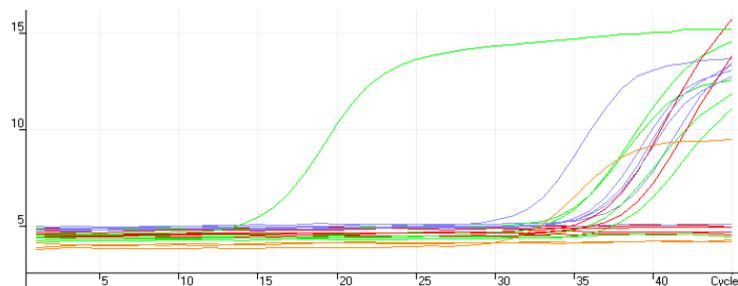


Figure 2a – Initial curves with “bend” (fluorescence decrease at the initial cycles)

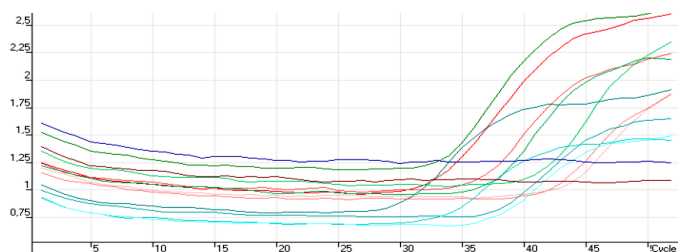
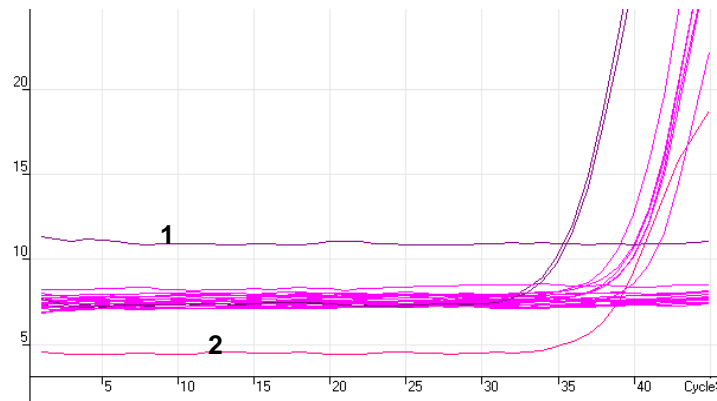


Figure 2b – One of possible errors is viewed by background signal level: DNA sample was not added to tube **1**, double quantity of DNA sample was added to the tube **2**.



**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 FAM/Green channel – internal control ( $\beta$ -globin gene)

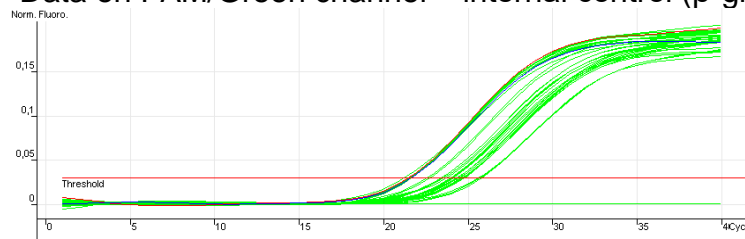


Figure 4 – Data on Cy5/Red channel

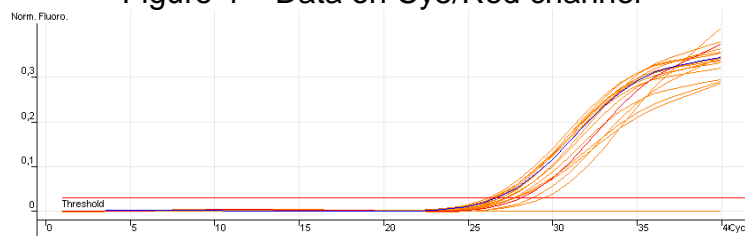


Figure 5 – Data on JOE/Yellow channel

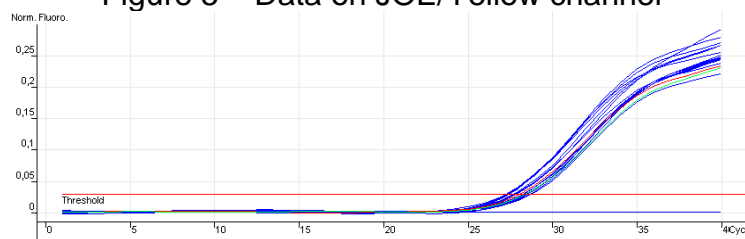
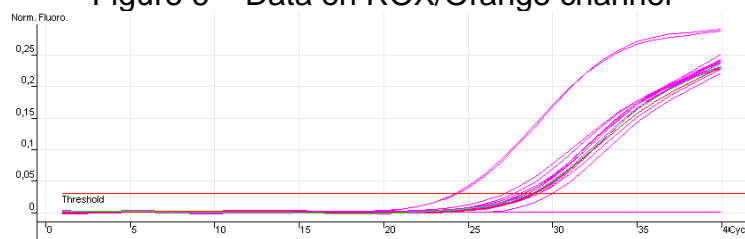


Figure 6 – Data on ROX/Orange channel



b) incorrect curves processing

Figure 7 – Threshold line crosses twice the fluorescence curves

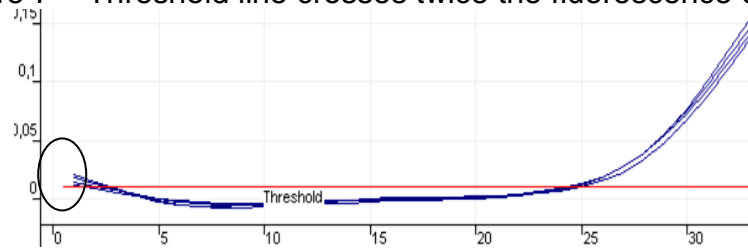
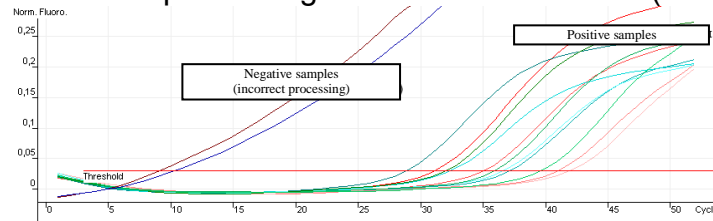


Figure 8 – Incorrect processing the curves with “bend” (from figure 2)



**Results analysis**

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

Name	Type	Ct	Given Conc (cop)	Cal
842t	Unknown	20,06		
	Unknown			
	Unknown	31,60		
	Unknown			
690kt	Unknown			
	Unknown	25,33		
	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 – AmpliSens FRT HR HPV Screen RG2x Results Matrix.xls software for data analysis in Microsoft® Excel format



**DNA amplification program for HCR HPV types 16, 18, 31, 33, 35; 39, 45, 51, 52, 56, 58, and 59**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Cycles</b>
1	95	15 min	–	1
2	65	2 min	–	1
3	95	20 s	–	5
	64	25 s	–	
	<b>Temp -:</b> <i>1.0 for a cycle</i>		–	
4	65	55 s	–	42
	95	20 s	–	
	60	25 s	–	
	65	55 s	FAM, HEX	

**Note** – If the instrument is not calibrated in the HEX channel, using the JOE channel is acceptable.

**NOTE:** The “**AmpliSens-1 iQ**” universal program for DNA amplification and detection can also be used. Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, combined with tests for STI pathogen DNA detection).

The analytical performance characteristics of the reagent kit do not change when the universal amplification program is used.

**Amplification program “AmpliSens-1 iQ”**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Cycles</b>
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	FAM, HEX, ROX, Cy5	
	72	15 s	–	

**Note** – The ROX and Cy5 channels are enabled when required if the “multiprime” format tests are performed.

5. Before start of the program, in the window of the **Run-Time Central** module set the measurement of wells factors by the test tubes — under the **Select well factor source** line select **Experimental Plate**. The standard program for determination of wells factors is used (file dynamicwf.tmo: **95 °C — 30 sec**, then 2 cycles with fluorescence measurement: **95 °C – 30 sec**)

**Insertion of tubes into the instrument**

For the further automatic result processing, select one of the variants of tube placement into the device:

- Variant 1: Insert into the device pair of tubes corresponding to one clinical sample: first with **HPV FRT A9** mixture, then with **HPV FRT A7+** mixture. Then insert pair

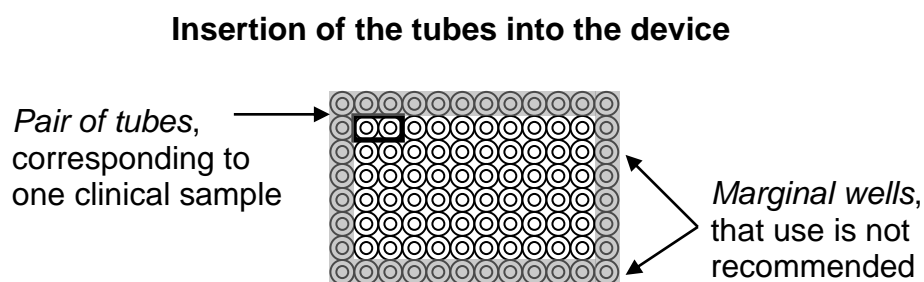
corresponding to the next sample, etc.

- Variant 2 (reversal): Insert into the device pair of tubes corresponding to one clinical sample: first with *HPV FRT A7+* mixture, then with *HPV FRT A9* mixture. Then insert pair corresponding to the next sample, etc.

**Pairs of tubes are inserted horizontally from left to right.**

**When inserting tubes, it is advisable not to use marginal wells, that is, to begin insertion of the tubes from the second well of the second row, and to finish with next to last well in next to last row. That means that one run will be performed with no more than 60 tubes.**

Scheme 1



6. When the tubes are inserted, close the device cover and press ***Begin Run***, then name the file of results.

### **Data processing and analysis**

After the experiment we recommend to control raw data of the device in two channels (FAM, HEX/JOE). To do this, select displaying of information on one of the channels (the ***Select a Reporter*** window), then set the regimen of displaying (***Select Analysis Mode***): ***Background subtracted***.

Among all signals of samples on the channel at least one must be positive (positive signal have specific S-shaped fluorescence accumulation curve). If experiment is organized and performed correctly, signals of positive control must be obtained.

**If positive signals of fluorescent channel are completely absent, processing of data on this channel can provide incorrect result.**

### **Data processing**

Return to the regimen of data displaying ***PCR Base Line Subtracted Curve Fit***.

1. Check displaying of all samples for analysis. (Images of all wells for analysis in the ***Select Well*** window must be blue).
2. Select the Fam-490 channel in the ***Select Reporter*** module.
3. Set the ***Baseline cycle*** parameter as ***User defined*** in the module ***Threshold Cycle***

**Calculations, Baseline cycles** “2” through “10”. Usually before this parameter is set, fluorescence curves on FAM channel are displayed somewhat incorrect, that is, are **thrown over** (initial and final parts are intensity descent, and not horizontal, see figure 15).

4. Set automatic selection of threshold: **Threshold Position – Auto Calculated**. If, with automatic selection of threshold, the threshold line locates too high (at the level where fluorescence curves reach plateau), select **Threshold Position – User Defined** regimen and set the threshold line as 1/3 of distance between base line and level where fluorescence curves reach plateau (see figure 13).
5. In the table of results click on the title once to select the **Threshold Cycle Ct** column. Press **Ctrl+C** to copy the column.
15. Open the Microsoft® Excel **AmpliSens FRT HR HPV Screen iQ Results Matrix.xls** file. Agree to enable macros.

**Note** – If macros is disable when opening Excel worksheet (the **Results** button is not activated), then security level should be changed. Select **Tools>Macro>Security>Medium**.

6. Place cursor into the *Ct* cell under name of fluorescent dye FAM of **Device data** table, and select **Insert** from the context menu.
7. Then similarly click on **Identifier** title and press **Ctrl+C** (in iQ iCycler program), Place cursor into the **Identifier** cell of **Sample labeling** column of **Device data** table, and select **Insert** from the context menu.
8. Select the Hex-530/Joe-530 channel in the **Select Reporter** module.
9. An automatic selection of the base line is usually correct, therefore, curves are not “**thrown over**”. Otherwise, set the **Baseline cycle parameter** as **User defined** in the **Threshold Cycle Calculations** module, **Baseline cycles** “2” through “10”.
10. Set an automatic selection of threshold: **Threshold Position – Auto Calculated**.
11. Similarly, select from the table of results and copy the **Threshold Cycle Ct** column. Insert cursor in the Excel table into the *Ct* column, named by the HEX dye.
12. In the **Order of insertion of tubes into the device** dropdown menu select your variant of tube insertion (see item **Insertion of tubes into the device**).
13. Insert data of thresholds for weak samples according to the **Important Product Information Bulletin** enclosed to the given lot of the PCR kit.
14. Save Microsoft® Excel files under a different name.

## Data analysis

Data are analyzed automatically. When data are inserted into the Excel matrix, click the **Results** button. In the **Results** column detected phylogenetic groups of *HPV* will appear, and the result: Positive (**pos**), negative (**neg**), weak positive (**weak**) and non-valid (**N/V**).

To process data correctly, set the following in the **Identifier** column:

- Mark tubes with different mixtures, but of one sample, with the same name, or mark only one tube and leave the second tube without mark.
- Mark positive controls as “+” or **K+**, and negative controls as “-” or **K-**.

## Possible problems and errors

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

Possible problems	Reason	How to identify?	Suggested solutions
Processed fluorescence curves ( <b>PCR Base Line Subtracted Curve Fit</b> mode) are “thrown over” (figure 15)	Incorrect mathematical processing of experiment data by the iCycler iQ program	Initial and final parts are intensity descent, and not horizontal (figure 15)	Set the <b>Baseline cycle</b> parameter as <b>User defined</b> in the module <b>Threshold Cycle Calculations</b> , and set <b>Baseline cycles</b> “2” through “10” for the calculation
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 increase of background fluorescence values (the fluorescence at the initial of the experiment is evaluated in the <b>Background subtracted</b> mode) in different experiments by more than 3 times (using <u>the same</u> instrument and similarly prepared solutions for wells factors calculation) WARNING! Effect of increasing the fluorescence can also be observed after cleaning the instrument optical part	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 violation of sterility conditions-leads to the enzyme destruction	It can be identified by signal absence of positive control or if the <i>Ct</i> value for the positive control is greater than the threshold of the weak samples	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 PCR analysis. Take measures to detect and eliminate the source of contamination
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 PCR analysis with correctly prepared mixes

## EXAMPLES OF OBTAINED DATA

### Raw data (*Background subtracted* mode)

Figure 11 – Initial curves (FAM)

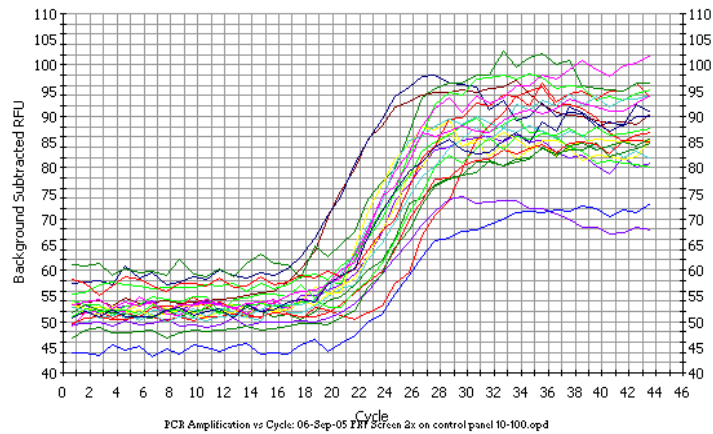
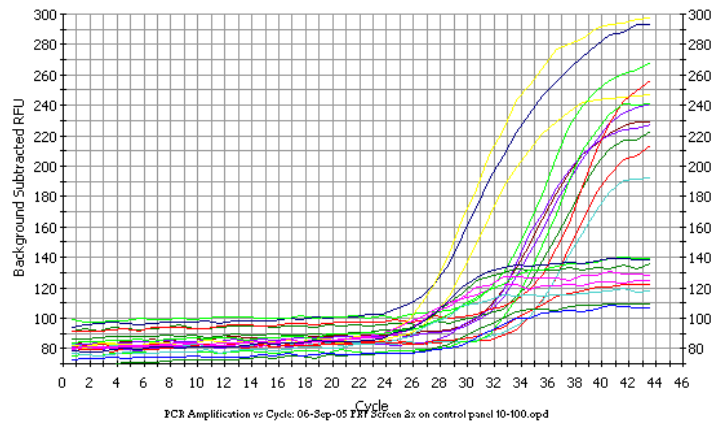


Figure 12 – Initial curves (JOE/HEX)



## Processed data

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

Figure 13 – Processed curves (FAM) – the parameter of user's calculation of base line is set (**User Defined**)

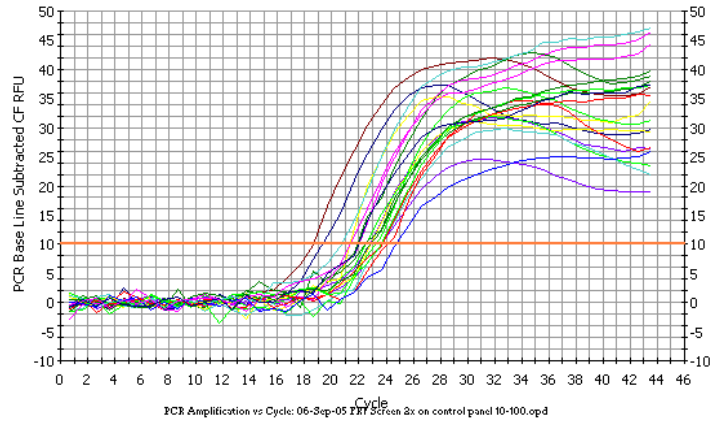
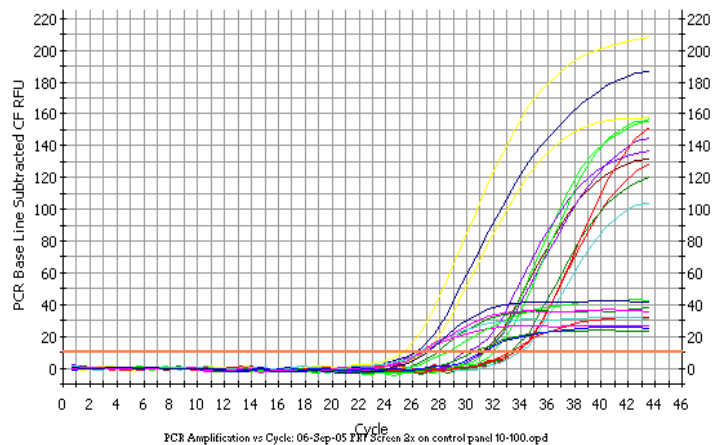
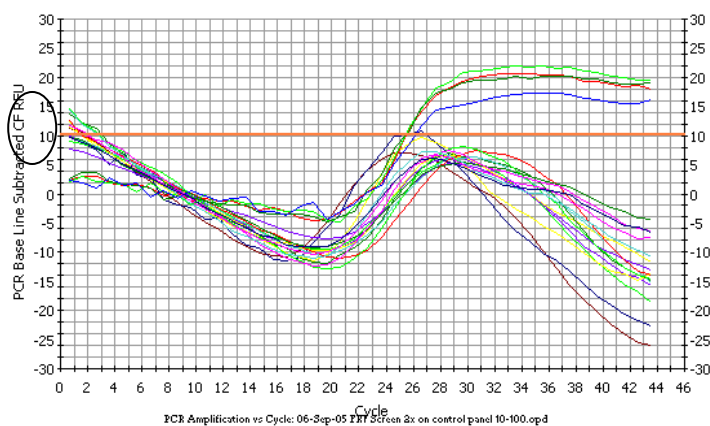


Figure 14 – Processed curves (JOE/HEX)



- b) incorrect curves processing

Figure 15 – Incorrectly processed curves (FAM) – curves are “thrown over” with automatic calculation of the base line (**Auto Calculated**)





## AMPLIFICATION AND DATA ANALYSIS USING Smart Cycler (Cepheid, USA)

### INSTRUMENT

#### Programming the thermocycler

1. Switch the device on and start the SmartCycler program.
2. Before placing the tubes into the instrument it is necessary to centrifuge the reaction tubes for 3 s on the supplied SmartCycler centrifuge.
3. Insert the tubes into the instrument.

#### Variants of tubes insertion

For further automatic results processing select one of the variants of tubes placement into the device:

- Variant 1: Insert sequentially into 2 adjacent (by number) modules a pair of tubes corresponding to one clinical sample: first with *HPV FRT A9* mixture, then with *HPV FRT A7+* mixture. Then, insert the pair corresponding to the next sample, etc.
- Variant 2 (reversal): Insert sequentially into 2 adjacent (by number) modules pair of tubes, corresponding to one clinical sample: first with *HPV FRT A9* mixture, then with *HPV FRT A9* mixture. Then, insert the pair corresponding to the next sample, etc.

**NOTE:** Pairs of tubes are inserted horizontally from left to right.

4. Select **Define Protocols** in the main menu of the program. In the opened window, select the **New Protocol** button in the left lower corner. Name the protocol and set the following parameters of the experiment (see table below):

#### Amplification program for HCR *HPV 16, 18, 31, 33, 35; 39, 45, 51, 52, 56, 58, 59* types DNA

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Cycles</b>
<i>Stage 1. Hold</i>	95	900 s	–	1
<i>Stage 2. Hold</i>	65	120 s	–	1
<i>Stage 3. 3-Temperature Cycle</i>	95	20 s	–	5
	63	30 s	–	
	65	60 s	–	
<i>Stage 4. 3-Temperature Cycle</i>	95	25 s	–	42
	60	30 s	–	
	65	60 s	<b>Switched on</b>	

5. Select the **Save Protocol** button in the lower segment of the window.
6. Click the **Create Run** button in the main menu.
7. Click the **Dye set** button in the left central segment of the window and select the combination *FTTC25*.
8. Click the **Add/Remove Sites** button in the center of the screen and in the window opened select needed protocol (program) and sites where analysis will be performed. Press the button **OK**.

9. Start the program of the experiment by pressing the **Start Run** button in the lower segment of the screen. In the dialog window opened set the file to save all data of the experiment.
10. In the next opened window the main parameters of the experiment will be displayed.
11. To name samples, press the **Results Table** button. In the table opened in the Sample ID column name the samples. Only one of the pair of samples can be named. According to the manual for the device, Control samples C1, C2, C3 must be named as Standard, and values of calibrators must be taken from the *Important Product Information Bulletin*, and the negative control must be named as “-”.
12. During the experiment intensity of fluorescence can be observed on TET/JOE channels for HPV DNA, and on FAM channel for internal control.

### **Data processing and analysis**

1. After amplification, HPV DNA concentrations are calculated for two tubes.
2. Select the **Analysis settings** item in menu.
3. In the opened window, set the threshold line level as 10 for FAM and TET/JOE channels, and click the **Update Analysis** button in the lower segment of the window. In the results table (window **Results Table**), values of the threshold cycles (Ct) and concentration for each sample will appear.
4. Click the **Export** button in the lower left segment of the program window. In the opened dialog window, mark only the **Results table and Analysis Settings** item. Click the **Export** button. Name the file.
5. The final result of HPV DNA concentration for two tubes normalized on number of human cells is calculated according to the formula:
  - a. The number of HPV DNA copies *for the first tube* is divided on number of human DNA copies for the first tube, and the result is multiplied by 200,000;
  - b. The number of HPV DNA copies *for the second tube* is divided by the number of human DNA copies for the second tube, and the result is multiplied by 200,000;
  - c. The result of calculation for the first tube is summarized with the result in the second tube;
  - d. The common logarithm of the resulting value is calculated;
  - e. the result is the concentration of HPV DNA in the log of HPV DNA copies per 10<sup>5</sup> cells:

$$\log \text{ HPV\_on\_100,000\_cells} = \log \left( \frac{\text{HPV\_DNA\_amount\_1}}{\text{human\_DNA\_amount\_1}} * 200,000 + \frac{\text{HPV\_DNA\_amount\_2}}{\text{human\_DNA\_amount\_2}} * 200,000 \right)$$

6. Save the Microsoft® Excel files under a different name.

## Data analysis

The run is **valid** if

- Negative controls have no signal in all the channels (FAM/Green, JOE/Yellow/HEX/TET);
- All calibrators have signals in all the channels (FAM/Green, JOE/Yellow/HEX/TET);
- The correlation coefficient for calibration curves for all the channels is no less than 0.98.

**NOTE:** If the run is invalid all obtained results are considered to be unreliable. The PCR run should be repeated.

The result of *HPV* DNA detection of a given sample is considered to be

**Negative**, if the signal of the Internal Control (IC; FAM/Green channel) is detected in both tubes for the sample and the quantity of human DNA cells per reaction exceeds 500.

**Positive**, if a signal in the channel for the JOE fluorophore is detected at least in one of the two tubes. Result:

- One or several types belonging to the phylogenetic group A9 (if the signal is detected in the tube with the *HPV* FRT A9 mixture);
- One or several types belonging to the phylogenetic group A7 or types 51/56 (if the signal is detected in the tube with the *HPV* FRT A7+ mixture).

The result is **invalid** in the following cases:

- If no positive signal in the JOE/Yellow/HEX/TET channel (A9, A7, A5/A6) is detected in any of the two tubes and the signal of the Internal Control (IC; the FAM/Green channel) is not detected or number of human DNA cells per reaction does not exceed 500.
- Weak positive signal(s) is/are detected in the JOE/Yellow/HEX/TET channel but the signal of the Internal Control (IC) in the FAM/Green channel is not detected or the number of human DNA cells does not exceed 500.

**NOTE:** In case of invalid result, the analysis for this sample should be repeated from the DNA extraction stage or from the material sampling.

Possible error	Characters	Way to eliminate
Contamination of the specific DNA	The signal is registered in any channel in negative control	Repeat the PCR analysis. Take measures to detect and eliminate the source of contamination
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 PCR analysis with correctly prepared mixes

## AMPLIFICATION AND DATA ANALYSIS USING FOUR-CHANNEL Rotor-Gene 3000 and Rotor-Gene 6000 (Corbett Research, Australia) 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.

### Programming the thermocycler

1. Turn on the instrument, run the Rotor-Gene software.
2. Insert tubes into the device. Ensure that the 1<sup>st</sup> tube is placed in the 1<sup>st</sup> well. Insert the rotor into the instrument, close the lid.

**NOTE:** If the carousel is not filled completely, balance it. To do this, fill the free wells with empty tubes (*don't use tubes from previous experiments*). Place any study tube (*not an empty tube*) to the well 1.

3. Start amplification program. Use one of variants:

### Using the ready template for the run

**NOTE:** Usage of the template file ***AmpliSens FRT HR HPV Screen RG4x.ret*** during amplification is highly recommended.

For usage of the template file for the PCR kit, copy previously ***AmpliSens FRT HR HPV Screen RG4x.ret*** file, enclosed to the kit, to the ***Templates*** folder of the RotorGene program. The default location of this folder for the RotorGene is the following: ***C:\Program Files\RotorGene 6\Templates*** – for Rotor-Gene 3000, and ***C:\Program Files\RotorGene 6000 Software\Templates*** - for Rotor-Gene 6000. After that, in the ***New Run*** window (called by the ***New*** button on the toolbar), the ***AmpliSens FRT HR HPV Screen RG4x*** item will appear. Select it to start new experiment.

**Note** – Template file can be opened from another folder as well. Therefore, in the ***New Run*** window select tab ***Advanced***, and there select the item ***Open A Template In Another Folder....***, then switch to folder with the file ***AmpliSens FRT HR HPV Screen RG4x.ret***.

The template file contains all the needed parameters of amplification; therefore, only the type data on the experiment and the designations of samples should be entered.

### Individual programming

1. Run the new run wizard in the ***Advanced*** tab.
2. Select the reaction volume – **25 µl** and tick the ***15 µl oil layer volume*** option in the window opened after the window of rotor selecting.
3. Set the amplification program:

**Amplification program for HCR HPV 16, 18, 31, 33, 35; 39, 45, 51, 52, 56, 58, 59 types  
DNA**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Cycles</b>
<i>Hold</i>	95	15 min	–	1
<i>Hold2</i>	65	2 min	–	1
<i>Cycling</i>	95	20 s	–	5
	64	25 s	–	
	<u>Touchdown:</u> 1 deg. per cycle / Lower temperature of each step on 1 °C			
	65	55 s	–	
<i>Cycling2</i>	95	15 s	–	40
	60	25 s	–	
	65	25 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	

**Note** - *AmpliSens FRT HR HPV Screen RG4x Program.pro* file in the RotorGene software format can be used for setting the amplification program.

**NOTE:** The “**AmpliSens-1 RG**” universal program for DNA amplification and detection can also be used (see below). Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, combined with tests for STI pathogen DNA detection).

The analytical performance characteristics of the reagent kit do not change when the universal amplification program is used.

**Amplification program “AmpliSens-1 RG”**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Cycles</b>
<i>Hold</i>	95	15 min	–	1
<i>Cycling</i>	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
<i>Cycling2</i>	95	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s	–	

**Note** – The ROX/Orange and Cy5/Red channels are enabled when required if the “multiprime” format tests are performed.

4. Click the **Calibrate/Gain Optimisation...** button in the **New Run Wizard** window. In the opened window:
  - a) Perform the calibration in the FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red channels (click the **Calibrate Acquiring/Optimise Acquiring** button);
  - b) perform the calibration in the selected channels before the first detection (tick the **Perform Calibration Before 1<sup>st</sup> Acquisition/ Perform Optimisation Before 1<sup>st</sup> Acquisition** option);
  - c) for signal measurement optimization for the selected channels set calibration from



4FI to 8FI for all the channels (the **Edit** button, the **Auto gain calibration channel settings** window).

5. Select the **Start run** button for amplification run. Name the file.
6. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Enter the names/numbers of the test samples in the **Name** column. Analysis is performed with two tubes; therefore, for correct automatic analysis of results the both tubes of the sample must have the same name. Set the type **Unknown** opposite all the test samples including control samples. Set the type **None** for the cells matching with the corresponding empty tubes. Name calibrators as **K1, K2, K3**, Negative control of amplification as **K-**.

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

### **Data processing and analysis**

Browse raw data of the device in four channels (FAM/Green, JOE/Yellow ROX/Orange, Cy5/Red)) selecting **Cycling A...** buttons on **Channels** panel. Take into account:

- Among all signals of samples on the channel, at least one must be positive (positive signal have specific S-shaped fluorescence accumulation curve, see figure 18). If experiment is organized and performed correctly, signals of **DNA calibrator C1 HPV, DNA calibrator C2 HPV, DNA calibrator C3 HPV** must be obtained.

**NOTE:** If positive signals on fluorescence channel are completely absent, analysis of data on this channel may provide incorrect result.

- Presence of samples with background signal that differs significantly from majority of signals means the mistake of insertion of reaction mixture or DNA sample into the tube (see figure 19b and the "Possible problems and errors" section).

### **Data processing**

1. Make sure that all the test samples are active in the legend at the right.
2. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the **Cycling A. FAM/Cycling A. Green, Show** buttons.
3. After opening the **Quantitation analysis** window set the threshold manually: **Threshold = 0,03**.
4. Select the **Linear scale**. Select the **Dynamic tube** and **Slope Correct** buttons.
5. Choose the parameter **More settings/Outlier Removal** and set **20 %** for the value of negative samples threshold (**NTC/Threshold**).

**NOTE:** In some rare cases due to the descend-type curves at the beginning of the experiment the threshold line can cross the fluorescence curves at the first cycles, and RotorGene program can record the crossing data as positive signal (see figure 24). To block this effect, use the **Eliminate cycles before...** option by

setting 5 (crossing the threshold and the fluorescence curve at the first 5 cycles is ignored).

6. In the results grid (**Quant. Results** window); select the **Name** column by single click on the title. Copy the column selecting the **Copy** button from the right-click menu (figure 26).
  7. Open file Microsoft® Excel **AmpliSens FRT HR HPV SCREEN Quant Results Matrix.xls**, enclosed to the reagent kit. Agree to enable macros.
- Note** – If macros is disable when opening Excel worksheet (the **Results** button is not activated), then security level should be changed. Select **Tools>Macro>Security>Medium**.
8. Set cursor on **Name** cell of **Samples labeling** column of **Device data** table and select **Insert** command from the context menu.
  9. Similarly, select and copy column **Ct** column from the results grid (**Quant.Results**). Set cursor in Excel table into **Ct** cell under the name of fluorescent dye FAM/Green
  - 10.Repeat the procedure for fluorescent dye JOE/Yellow ,ROX/Orange, Cy5/Red
  - 11.Set **Quantitative analysis** regimen with internal calibration.
  - 12.Insert data on concentration of calibrators into the table **Calibrator value** according to *Important Product Information Bulletin* enclosed to the given lot of the PCR kit.
  - 13.Check in the **Samples labelling** column, that **DNA calibrator C1 HPV** is named as **K1**, **DNA calibrator C2 HPV** is named as **K2**, **DNA calibrator C3 HPV** is named as **K3** (without blank between letter K and number), and negative control is named as **K-** or **“-**“.
  - 14.Save Microsoft® Excel file under different name.

### Data analysis

Data are analyzed automatically. When data are inserted into the Excel spreadsheet, press the **Results** button. In the **Results** column detected phylogenetic groups of **HPV** will appear, and the result: Positive (**pos**), negative (**neg**), weak positive (**weak**), non-valid (**NV**). Then in the table number of human cells per reaction will be shown; that is used for evaluation of validity of the sample. Then **HPV** DNA is calculated as log for 10<sup>5</sup> cells for each group, and total viral load. The last column contains probable explanation of the clinical value of the result according to the following:

Result Ig (HPV DNA copies per 100 000 cells)	Value
<3	Clinically insignificant value
3-5	Clinically valuable. Dysplasia cannot be excluded; risk of dysplasia.
>5	Clinically valuable, of increased value. Dysplasia is highly suggestive.

To provide correct data processing, check that in the **Name** column:

- Tubes with different mixtures of the same sample have the same name;
- Positive controls are named as “+” or **K+**, and negative controls as “-“ or **K-**.

### 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 19a, 25)	Typical positive sample has a characteristic S-shaped curve of fluorescence accumulation (see figures 18, 20 - 23). Incorrect processed negative samples are viewed as pretty straight lines going upward (Fig. 25)	One must use the <b>Ignore First</b> 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 24)	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 24)	Use the function <b>Eliminate cycles before ...</b> 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 <b>gain</b> (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 <b>gain</b> coefficient value more than 2 units in different	Use mixes stored in adequate conditions and with unexpired date (see the chapter "Stability and storage" in <i>Instruction Manual</i> )

Possible problems	Reason	How to identify?	Suggested solutions
		experiments (using <u>the same instrument</u> ) WARNING! Effect of increasing the multiplier <b>gain</b> can also be observed after cleaning the instrument lenses from heavy dirt	
Sensitivity decrease due to the decrease of polymerase (TaqF) activity	Incorrect storage of polymerase or violation of sterility conditions leads to the enzyme destruction	It can be identified by signal absence of positive control or if <i>Ct</i> value for the positive control is greater than the threshold of the weak samples	Use adequately stored (see the chapter “Stability and storage” in <i>Instruction Manual</i> ) or a new enzyme

**Note** – The information about all set parameters of the experiment and the autocalibration report can be found by browsing the experiment settings (the **Settings** button). Particularly, **Autocalibration Log Messages** point in the **Messages** tab is the autocalibration report.

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 19b)	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 19b)	Repeat the analysis if the sample is negative
Autocalibration parameter is not set from 5FI to 10FI 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 18 – Normal initial curves

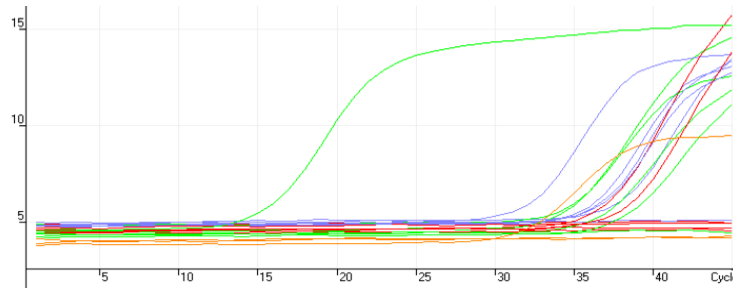


Figure 19a – Initial curves with “bend” (fluorescence decrease at the initial cycles)

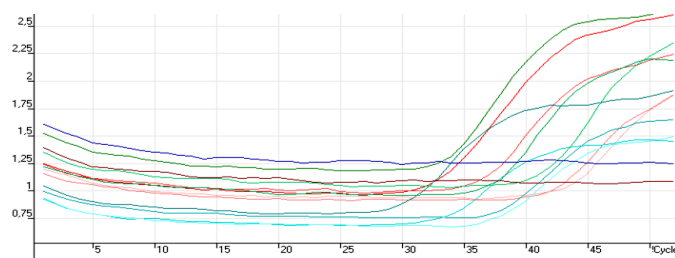
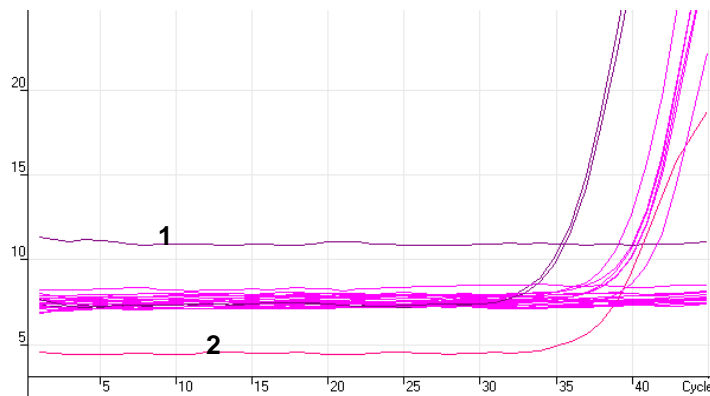


Figure 19b – One of possible errors is viewed by background signal level: DNA sample was not added to tube 1, double quantity of DNA sample was added to the tube 2.



### Processed data

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

Figure 20 – Data on FAM/Green channel – internal control ( $\beta$ -globin gene)

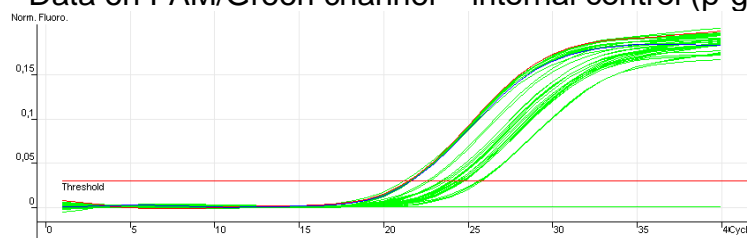


Figure 21 – Data on Cy5/Red channel

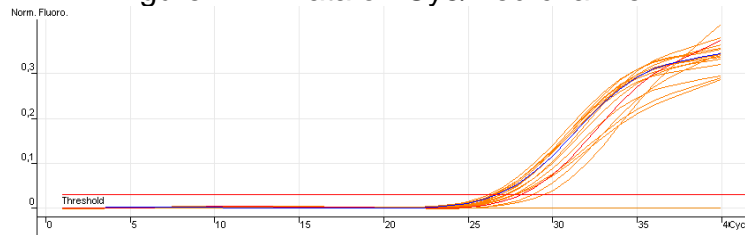


Figure 22 – Data on JOE/Yellow channel

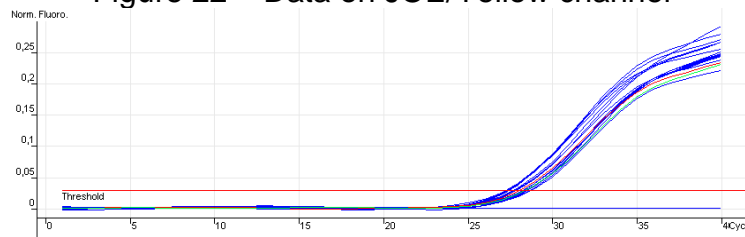
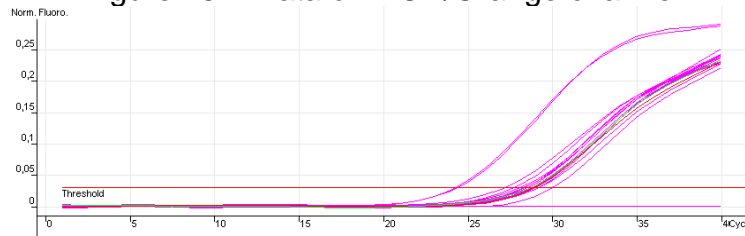


Figure 23 – Data on ROX/Orange channel



d) incorrect curves processing

Figure 24 – Threshold line crosses twice the fluorescence curves

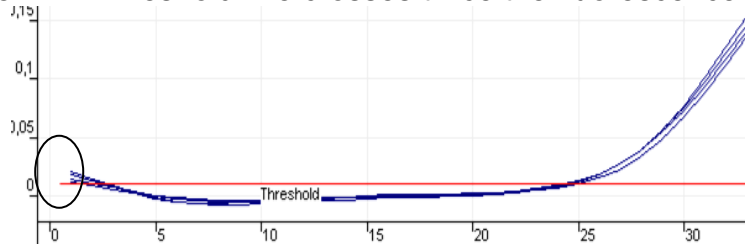
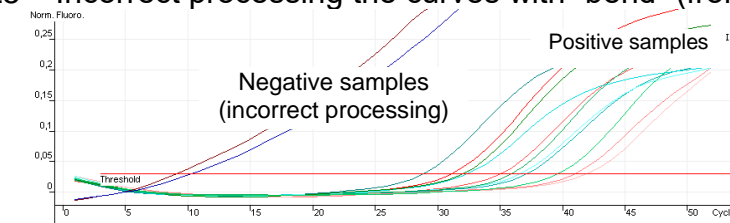


Figure 25 – Incorrect processing the curves with “bend” (from figure 2)



**Results analysis**

Figure 26 – Results grid **Quant.Results**

Name	Type	Ct	Given Conc (cop	Cal
842t	Unknown	20,06		
	Unknown			
	Unknown	31,60		
	Unknown			
690kt	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 27 – AmpliSens FRT HR HPV Screen Quant Results Matrix.xls software for data analysis in Microsoft® Excel format

**AmpliSens® HPV HCR screen-titre-FRT**

Date: \_\_\_\_\_ Device: \_\_\_\_\_ Description: \_\_\_\_\_

Qualitative analysis
  Quantitative analysis
   
 Internal calibration (calibrators in current setting)
   
 External calibration (set parameters K and B)

Calibrator value	GLOBAL Fam/Green	HPV16 Joe/Yellow	HPV18 Rox/Orange	HPV51 Cy5/Red
K1				
K2				
K3				

№№	Samples labelling	Fam Green (IC)	Joe Yellow (A9)	Rox Orange (A7)	Cy5 Red (A5/A6)	Results							
		Ct	Ct	Ct	Ct	phylogenetic group	Qual.	quantity of cells	Ig HPV A9/ 10 <sup>5</sup> cells	Ig HPV A7/ 10 <sup>5</sup> cells	Ig HPV A5A6/ 10 <sup>5</sup> cells	Total Ig HPV / 10 <sup>5</sup> cells	Clinical significance
1	Name												
2													
3													
4													
5													
6													
7													
8													
9													
10													
11													
12													
13													
14													
15													
16													

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

### INSTRUMENT

#### Programming the thermocycler

1. Turn on the instrument, start iQ5 iCycler program.

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

2. Set and save the amplification program in the **Workshop** module:

#### DNA amplification program for HCR HPV types 16, 18, 31, 33, 35; 39, 45, 51, 52, 56, 58, and 59

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	15 s	–	6
	65	55 s	–	
	<u>Temp -:</u> 1.0 for a cycle			
65	25 s	–		
3	95	15 s	–	41
	60	55 s	Real-time	
	65	25 s	–	

For installation of amplification program the included file **AmpliSens FRT HR HPV Screen iQ5.tmo** can be used.

**NOTE:** The “AmpliSens-1 iQ” universal program for DNA amplification and detection can also be used (see below). Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, combined with tests for STI pathogen DNA detection).

The analytical performance characteristics of the reagent kit do not change when the universal amplification program is used.

#### “AmpliSens-1 iQ” amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	FAM, HEX, ROX, Cy5	
	72	15 s	–	

**Note** – The ROX and Cy5 channels are enabled when required if the “multiprime” format tests are performed.

3. Select **Plate Setup** to mark the new samples. Set the fluorescent measurement on four channel: FAM, HEX, ROX, Cy5
4. Click **Run**. Select the mode of wells factor measurement (**Well factor**). Both the mode with measurement of well factors by experimental tubes and the mode with fixed well factors (recommended) can be used. Start the experiment.



## Data processing and analysis

### Data Processing

1. Proceed to the **Data Analysis** mode.
2. Browse the data separately in each of four channels.
3. Ensure that automatic selection of threshold level is correct for each channel. Normally, the threshold line should cross only the sigmoid curves of signal accumulation of positive samples and controls and should not cross the base line. Otherwise, the threshold level should be raised.
4. Click the **Results** button (situated under the buttons with the fluorophores' names).
5. Right-click in the displayed results grid. Select **Export to Excel** from the drop-down menu. Agree to save the file. If the Microsoft Excel program is installed on the computer then this file will open automatically (if the program is not installed, then use the computer with installed Microsoft Excel). The structure of this file is that the results are displayed sequentially for the FAM, HEX, ROX and Cy5 channels.
6. Open the Microsoft® Excel **AmpliSens FRT HR HPV Screen iQ Results Matrix.xls** file and agree to enable macros.

**Note** – If macros is disable when opening Excel worksheet (the **Results** button is not activated), then security level should be changed. Select **Tools>Macro>Security>Medium**.

7. In the file with results select and copy the cells of **Threshold Cycle (Ct)** column corresponding to the FAM channel. Proceed to the results calculation file and paste the copied cells into the **FAM** column beginning from the first cell.
8. Similarly, copy the cells of **Threshold Cycle (Ct)** column corresponding to the HEX, ROX and Cy5 channels channel into the **HEX, ROX** and **Cy5** columns of the results calculation program.
9. Copy the samples names from the **Identifier** column into the **Name** column of the results calculation program.
10. Mark in the **Name** column, the **DNA calibrators** as **K1, K2, K3**, and the negative controls as **K-** or “-“. Mark the wells which are not corresponding to the test tubes by the # symbol.
11. Save Microsoft® Excel files under a different name.
12. Click the **Results** button.

## Data analysis

Data are analyzed automatically. When data are inserted into the Excel spreadsheet, click the **Results** button. In the **Results** column the detected phylogenetic groups of HPV, and the result (positive (**pos**), negative (**neg**), weak positive (**weak**), non-valid (**N/V**)) will appear. Then in the table number of human cells per reaction will appear; that is used for evaluation of validity of the sample. Then HPV DNA is calculated as log for 10<sup>5</sup> cells for each group, and total viral load. The last column contains probable explanation of the clinical value of the result according to the following:

Result lg (HPV DNA copies per 100,000 cells)	Value
<3	Clinically insignificant value
3-5	Clinically valuable. Dysplasia cannot be excluded; risk of dysplasia.
>5	Clinically valuable, of increased value. Dysplasia is highly suggestive.

## Possible problems and errors

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

Possible problems	Reason	How to identify?	Suggested solutions
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 increase of background fluorescence values (the fluorescence at the initial of the experiment is evaluated in the <b>Background subtracted</b> mode) in different experiments by more than 2 times (using <u>the same</u> instrument and similarly prepared solutions for wells factors calculation) WARNING! Effect of increasing the fluorescence can also be observed after cleaning the instrument optical part	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 violation of sterility conditions leads to the enzyme destruction	It can be identified by signal absence of positive control or if the Ct value for the positive control is greater than the threshold of the weak samples	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 PCR analysis. 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 – <b>Background subtracted</b> mode). The sample is negative	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 – <b>Background subtracted</b> mode)	Repeat the analysis if the sample is negative
The threshold level is set incorrectly	The threshold line is drawn with the negative samples or is higher than some or all positive curves (S-shaped)	Set the threshold line so that it crosses only the sigmoid curves of fluorescence accumulation or at the level of $\frac{1}{4}$ of height between the final fluorescence value of negative and positive samples
The drops are not sedimented from the walls of the tubes before the run	The appearance of negative or positive “steps” in the curves of the fluorescence accumulation	Open the <b>BaseLine Threshold</b> window by clicking the right mouse button on the fluorescence curve. Set the range for calculation of the base line beginning from the first cycle after the step.
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 PCR analysis with correctly prepared mixes

## EXAMPLES OF OBTAINED DATA

### Raw data (**Background subtracted** mode)

Figure 28 – Normal initial curves (FAM)

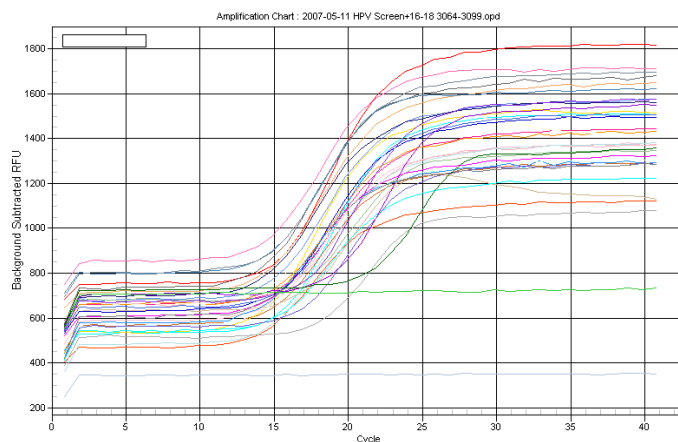
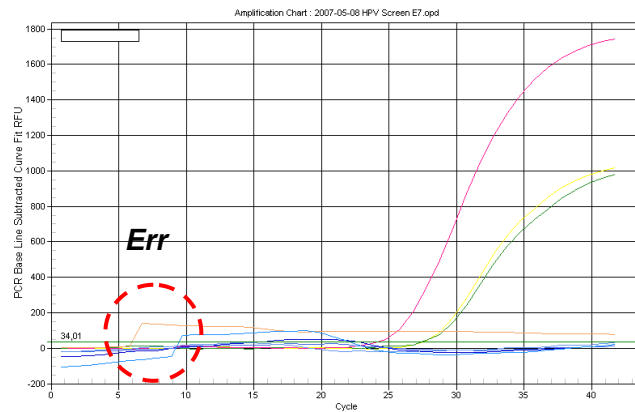


Figure 29 – One of possible errors – “step” of base line: In the **Err** samples the drops are not sedimented from the walls of the tubes before the run, the mixes “fell in” during the amplification



**Processed data**

- c) normal processed curves, typical S-shape, correct placement of thresholds (**PCR Base Line Subtracted Curve Fit** mode)

Figure 30 – FAM channel – internal control ( $\beta$ -globin gene)

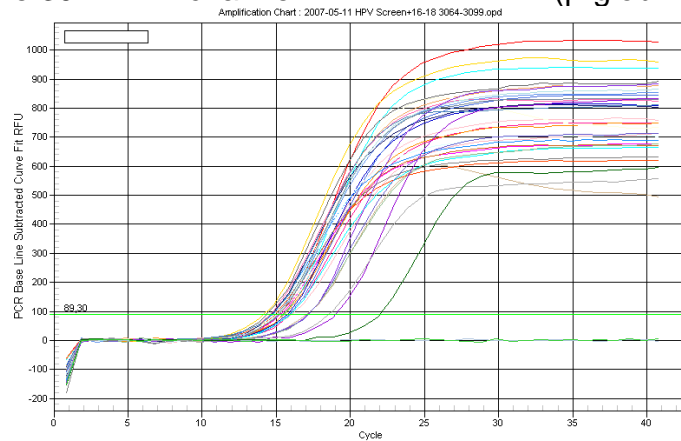


Figure 31 – Cy5 channel

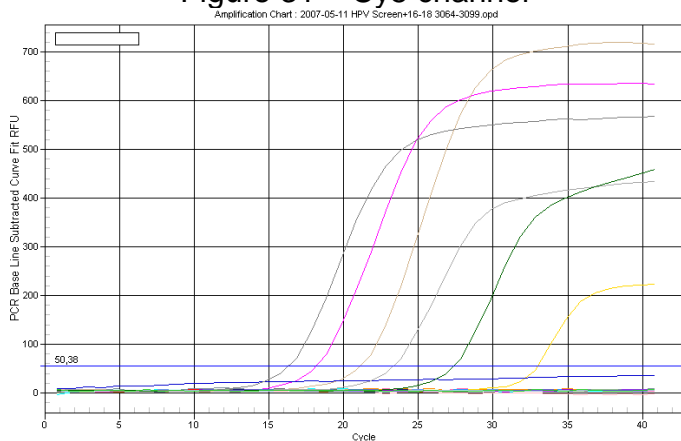


Figure 32 – JOE channel

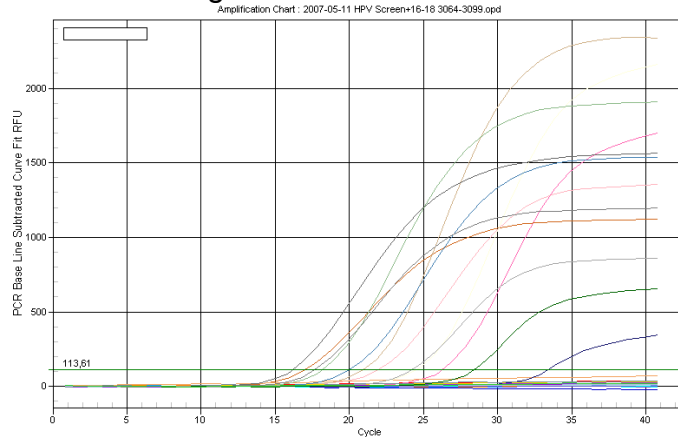
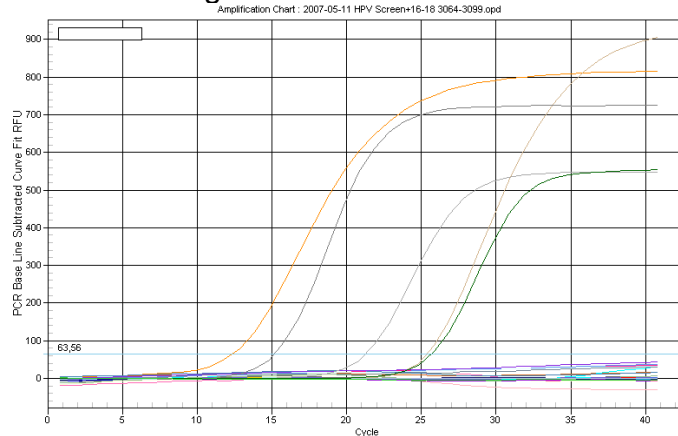


Figure 33 – ROX channel



## AMPLIFICATION AND DATA ANALYSIS USING Mx3000P (Stratagene, USA) INSTRUMENT

### Programming the thermocycler

1. Switch the instrument on, start the program Stratagene Mx3000P.
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 and close the lid.
4. Select **Optics Configuration** in the **Options** menu. In the **Dye Assignment** window set **FAM** next to the **FAM filter set**, set **JOE** next to the **HEX/JOE filter set**.
5. In **instrument** menu, select **Filter Set Gain Settings...** In the opened window, place the following parameters of the multiplication factor:

<b>Cy5</b>	<b>x4</b>
<b>ROX</b>	<b>x1</b>
<b>HEX/JOE</b>	<b>x4</b>
<b>FAM</b>	<b>x4</b>

6. Set fluorescence detection parameters in the **Plate Setup** menu. For this:
  - Select all cells with the test tubes (holding **Ctrl** and selecting the needed range)

- Mark all selected cells as **Unknown** in **Well type** window. Set **FAM, HEX, ROX** and **Cy5** in the **Collect fluorescence data** option.
- Name each sample by double clicking on each cell (**Well Information** window). Mark positive control as “+”, negative control as “-“ (samples names can also be entered during the amplification or after it returning to this tab).

7. In **Thermal Profile Setup** tab, set the amplification program. This may be performed by one of two methods:

**Using of the template file for setting the amplification program (is recommended).**

Click the **Import...** button which is to the right of the depicted thermocycling profile. Select the fold containing *AmpliSens FRT HR HPV Screen Mx.mxp* file enclosed to the PCR kit and open it. The needed thermocycling profile will appear in the **Thermal Profile** window.

**Individual programming**

**DNA amplification program for HCR HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Cycles</b>
<i>Segment 1</i>	95	15 min	–	1
<i>Segment 2</i>	65	2 min	–	1
<i>Segment 3 (Cycling)</i>	95	20 s	–	5
	64 <u>Touchdown:</u> <i>1 deg. per cycle</i>	25 s	–	
	65	55 s	–	
<i>Segment 4 (Cycling)</i>	95	20 s	–	40
	60	25 s	–	
	65	55 s	Cy5, FAM, JOE, ROX	

**NOTE:** The “**AmpliSens-1 Mx**” **universal program** for DNA amplification and detection can also be used (see below). Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, combined with tests for STI pathogen DNA detection).

The analytical performance characteristics of the reagent kit do not change when the universal amplification program is used.

### Amplification program “AmpliSens-1 Mx”

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Cycles</b>
Segment 1.	95	15 min	–	1
Segment 2. (Cycling)	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Segment 3. (Cycling)	95	5 s	–	40
	60	30 s	FAM, JOE, ROX, Cy5	
	72	15 s	–	

**Note** – The ROX and Cy5 channels are enabled when required if the “multiprime” format tests are performed.

8. Start the amplification selecting the **Run** button, then **Start**. Name the experiment.

#### Data processing and analysis

##### Data Processing

- In Mx3000P software, select **Analysis** by clicking the corresponding button of the tool bar.
- 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.
- Proceed to the **Results** tab.
- Activate Cy5 channel and do not activate other channels in the **Dyes Shown** panel. Set in the **Threshold fluorescence** 150 for the Cy5 channel. Then, sequentially activate other channels and set the threshold values as follows:

Cy5	150
ROX	150
HEX/JOE	150
FAM	150

- Then activate all four channels (click **Cy5**, **ROX**, **HEX** and **FAM** buttons in the **Dyes Shown** field).
- Make sure that all four fluorescence channels are ticked.
- In the **Area to analyze** field, select the **Text Report** item. Ensure that all data are sorted by dye name (**Dye** column). For this click once on the **Dye** column name (figure 40).
- Proceed to the **File** menu, then to **Export Text Report** and **Export Text Report to Excel**. Microsoft Excel window will be opened.
- Open the **AmpliSens FRT HR HPV Screen Quant 96 Results Matrix.xls** Microsoft® Excel file. Agree to enable macros.

**Note** – If macros is disable when opening Excel worksheet (the **Results** button is not activated), then security level should be changed. Select **Tools>Macro>Security>Medium**.

10. Copy the samples names from the **Text Report** window into the **Samples labeling** column of the automatic result analysis software.
11. Copy the *Ct* values for all four channels into the corresponding cells of automatic result analysis software.
12. Set the **Quantitative analysis** mode, **Internal calibration**.
13. Insert data on concentration of calibrators into the table **Calibrator value** according to *Important Product Information Bulletin* enclosed to the given lot of the PCR kit.
14. Check in the **Samples labelling** column, that **DNA calibrator C1 HPV** is named as **K1**, **DNA calibrator C2 HPV** is named as **K2**, **DNA calibrator C3 HPV** is named as **K3** (without blank between letter K and number), and negative control is named as **K-** or **“-“**.
15. Save Microsoft® Excel files under a different name.
16. Click the **Results** button.

### Data analysis

Data are analyzed automatically. When data are inserted into the Excel matrix, click **Results** button. In the **Results** column, the detected phylogenetic groups of *HPV* will appear, and the result: Positive (**pos**), negative (**neg**), weak positive (**weak**), non-valid (**NV**). Then in the table number of human cells per reaction will appear; that is used for evaluation of validity of the sample. Then *HPV* DNA is calculated as log for 10<sup>5</sup> cells for each group, and total viral load. The last column contains probable explanation of the clinical value of the result according to the following:

Result Ig ( <i>HPV</i> DNA copies per 100 000 cells)	Value
<3	Clinically insignificant value
3-5	Clinically valuable. Dysplasia cannot be excluded; risk of dysplasia.
>5	Clinically valuable, of increased value. Dysplasia is highly suggestive.



## Possible problems and errors

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

Possible problems	Reason	How to identify?	Suggested solutions
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 increase of background fluorescence values (the fluorescence at the initial of the experiment is evaluated in the <b>Background subtracted</b> mode) in different experiments by more than 2 times (using <u>the same</u> instrument and similarly prepared solutions for wells factors calculation) WARNING! Effect of increasing the fluorescence can also be observed after cleaning the instrument optical part	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 violation of sterility conditions-leads to the enzyme destruction	It can be identified by signal absence of positive control or if the <i>Ct</i> value for the positive control is greater than the threshold of the weak samples	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 PCR analysis. 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 – <b>R – multicomponent view</b> mode). The sample is negative	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 – <b>R – multicomponent view</b> mode)	Repeat the analysis if the sample is negative

Possible error	Characters	Way to eliminate
The threshold level is set incorrectly	The threshold line is drawn with the negative samples or is higher than some or all positive curves (S-shaped)	Set the threshold line so that it crosses only the sigmoid curves of fluorescence accumulation or at the level of $\frac{1}{4}$ of height between the final fluorescence value of negative and positive samples
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 PCR analysis with correctly prepared mixes

## EXAMPLES OF OBTAINED DATA

### Raw data (R mode)

Figure 34 – Normal initial curves

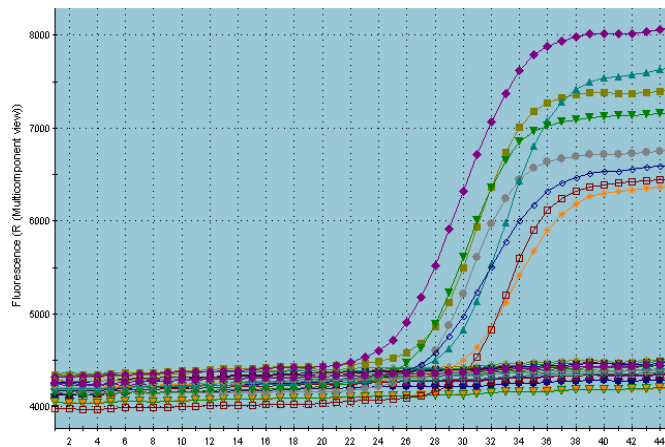
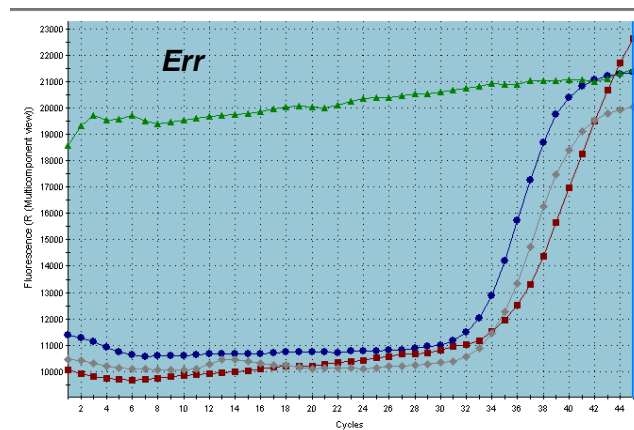


Figure 35 – One of possible errors is seen by the level of background signal: In the DNA sample was not added into the **Err** tube



**Processed data**

d) normal processed curves, **dR** mode (typical S-shape)

Figure 36 – FAM channel – internal control ( $\beta$ -globin gene)

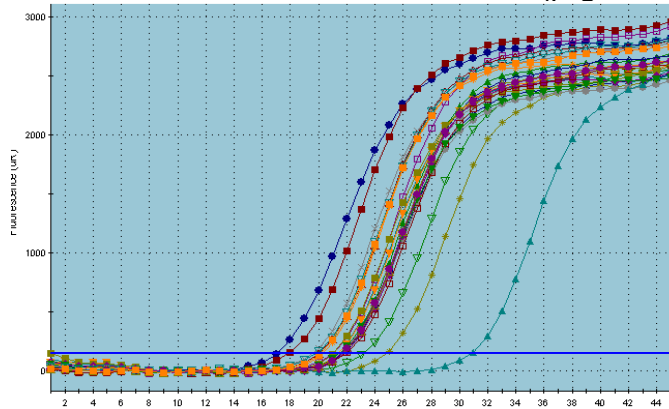


Figure 37 – Cy5 channel

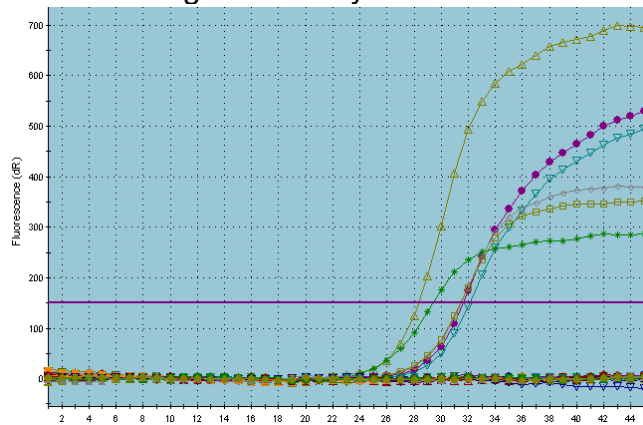


Figure 38 – JOE channel

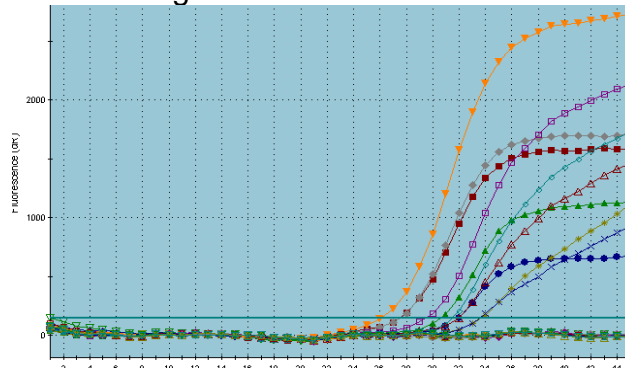
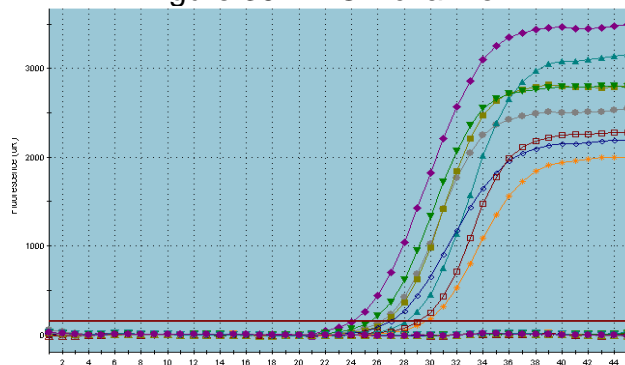


Figure 39 – ROX channel



**Results analysis**

Figure 40 – **Text Report** window. All data are sorted by the well name (**Well**) in ascending order

Well	Dye	Well Type	Threshold (dR)	Ct (dR)
C3	CY5	Unknown	150.000	No Ct
C3	ROX	Unknown	150.000	No Ct
C3	JOE	Unknown	150.000	31.99
C3	FAM	Unknown	150.000	17.08
C4	CY5	Unknown	150.000	No Ct
C4	ROX	Unknown	150.000	No Ct
C4	JOE	Unknown	150.000	27.46
C4	FAM	Unknown	150.000	17.90
C5	CY5	Unknown	150.000	No Ct
C5	ROX	Unknown	150.000	No Ct
C5	JOE	Unknown	150.000	30.62
C5	FAM	Unknown	150.000	21.76
C6	CY5	Unknown	150.000	No Ct
C6	ROX	Unknown	150.000	No Ct
C6	JOE	Unknown	150.000	27.51
C6	FAM	Unknown	150.000	21.74
C7	CY5	Unknown	150.000	No Ct
C7	ROX	Unknown	150.000	No Ct
C7	JOE	Unknown	150.000	33.71
C7	FAM	Unknown	150.000	24.81
C8	CY5	Unknown	150.000	No Ct
C8	ROX	Unknown	150.000	34.02
C8	JOE	Unknown	150.000	31.48
C8	FAM	Unknown	150.000	21.38
C9	CY5	Unknown	150.000	No Ct
C9	ROX	Unknown	150.000	No Ct
C9	JOE	Unknown	150.000	33.53
C9	FAM	Unknown	150.000	21.08
C10	CY5	Unknown	150.000	No Ct
C10	ROX	Unknown	150.000	No Ct
C10	JOE	Unknown	150.000	32.54
C10	FAM	Unknown	150.000	21.41
D3	CY5	Unknown	150.000	No Ct
D3	ROX	Unknown	150.000	No Ct

Figure 41 – AmpliSens FRT HR HPV Screen Quant Results Matrix.xls software

**AmpliSens® HPV HCR screen-titre-FRT**

Date: \_\_\_\_\_ Device: \_\_\_\_\_ Description: \_\_\_\_\_

Quantitative analysis

Qualitative analysis

Internal calibration (calibrators in current setting)

External calibration (set parameters K and B)

Calibrators table:

Calibrat or value	GLOB Fam/Green	HPV16 Joe/Yellow	HPV18 Rox/Orange	HPV51 Cy5/Red
K1	71756	42380	26488	125188
K2	8400	943	1222	1347
K3	913	47	46	139

Buttons: **Clear table** **Mark unnamed**

№№	Samples labelling	Fam Green (IC)	Joe Yellow (A9)	Rox Orange (A7)	Cy5 Red (A5/A6)	Results								
		Ct	Ct	Ct	Ct	phylogenetic group	Qual.	quantity of cells	Ig HPV A9/ 10 <sup>5</sup> cells	Ig HPV A7/ 10 <sup>5</sup> cells	Ig HPV A5A6/ 10 <sup>5</sup> cells	Total Ig HPV / 10 <sup>5</sup> cells	Clinical significance	
1	Name													
2														
3														
4														
5														
6														
7														
8														
9														
10														
11														
12														
13														
14														
15														
16														

## 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).

### Programming the thermocycler

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**.

#### Amplification program for HCR HPV 16, 18, 31, 33, 35; 39, 45, 51, 52, 56, 58, 59 types DNA

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	95	15 min	–	1
2	95	15 s	–	6
	65	55 s	–	
	<u>Touchdown:</u> 1 deg. per cycle			
65	25 s	–		
3	95	15 s	–	41
	60	55 s	FAM, HEX, ROX, Cy5	
	65	25 s	–	

**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:** The “**AmpliSens-1**” universal program for DNA amplification and detection can also be used (see below). Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, combined with tests for STI pathogen DNA detection).

The analytical performance characteristics of the reagent kit do not change when the universal amplification program is used.

### AmpliSens-1 amplification program for plate-type instruments

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Number of cycles</b>
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	FAM, HEX, ROX, Cy5	
	72	15 s	–	

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...** to mark appropriate fluorophores for this run and click **OK**. 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 *C<sub>t</sub>* (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 *C<sub>t</sub>* 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 *C<sub>t</sub>* 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

1. Click the right mouse button at the A1 position of appeared results grid. Due to the structure of this file, the results for Cy5, FAM, a then for HEX and ROX channels successively are shown.
2. Open the program for results interpretation in the Microsoft® Excel format (**AmpliSens FRT HR HPV SCREEN Quant 2x Results Matrix** for PCR kit variant screen-titre-

FRT 2x or **AmpliSens FRT HR HPV SCREEN Quant 4x Results Matrix** for PCR kit variant screen-titre-FRT 4x) provided on the disc. Agree to enable the 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.

3. Paste data in the results grid of the software in the columns with *Ct* values for appropriate channels.
4. Save the Microsoft® Excel file under another name.
5. Click the **Results** button.
6. In the **Results** column the phylogenetic groups of HPV detected in the samples will appear, as well as the result: positive (**pos**), negative (**neg**), weakly positive (**weak**), invalid (**N/V**).


The interpretation of the test samples is to be carried out in accordance with the *Instruction Manual*.

#### **Principles which underlie automatic data analysis:**

Signal in a tube in the channel is considered to be positive, if corresponding fluorescence accumulation curve cross the threshold line. The signal is characterized by the threshold cycle, the cycle corresponding to the crossing of the fluorescence curve and the threshold line. The *Ct* values (as well as their absence or presence) are analyzed by the software of automated recording of results of analysis.



### List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
03.07.13 FN	Footer	Catalogue numbers <b>REF</b> R-V31-T-2x(RG,iQ,SC)-CE-B, <b>REF</b> R-V31-T-4x(RG,iQ,Mx)-CE-B were deleted
02.04.14 SA	Cover page	Address of European representative was added
16.08.16 ME	Text	Corrections according to the template. The amplification programs, possible problems and errors, examples of obtained results were added
02.03.18 PM	Amplification and data analysis using CFX96 (Bio-Rad, USA)	The section was added
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
25.03.21 EM	Front page	The name, address and contact information for Authorized representative in the European Community was changed