IVD

For Professional Use Only

GUIDELINES

to **AmpliSens® *HPV* 16/18-FRT** PCR kit

for qualitative and quantitative detection and differentiation of genotypes 16 and 18 of *Human Papillomavirus* (*HPV*) DNA in the clinical 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* 16/18-FRT** PCR kit for qualitative and quantitative detection and differentiation of genotypes 16 and 18 of *Human Papillomavirus* (*HPV*) DNA in the clinical material (urogenital swabs) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia),
- Rotor-Gene Q (QIAGEN, Germany),
- iCycler iQ, iQ5 (Bio-Rad, USA),
- Mx3000P, Mx3005P (Stratagene, USA),
- CFX96 (Bio-Rad, USA)

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

When working with Rotor-Gene 3000 one should use the Rotor-Gene Version 6 software and the Rotor-Gene 6000 versions 1.7 (build 67) software or higher for Rotor-Gene 6000 and Rotor-Gene Q instruments.

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

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual.* When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with flat caps (e.g. Axygen, USA), or 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 instrument

1. Switch the instrument on.
2. Insert the tubes into the carousel of the Rotor-Gene 3000/6000/Q instrument (the carousel cells are numbered, the numbers are used for the further programming of the samples' position in the thermocycler). Program the instrument.

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

3. Click the ***New*** button in the program main menu.
4. In the opened window select ***Advanced*** and click ***Dual Labeled Probe/Hydrolysis probes***. Activate the ***New*** button.

5. Select ***36-Well Rotor*** (or ***72-Well Rotor***) and ***No Domed Tubes/Locking ring attached***. Click the ***Next*** button.
6. In the opened window define the operator and set the reaction volume: ***Reaction volume*** is **30 µl** for variant **FRT** (or **25 µl** for variant **FRT-100 F**). Mark the ***15 µl oil layer volume*** option. Click the ***Next*** button.
7. In the opened window click the ***Edit profile*** button and set the temperature profile of the experiment as follows:

DNA HPV 16-18 amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	95	15 min	–	1
Cycling	95	15 s	–	45
	60	35 s	FAM/Green, JOE/Yellow, ROX/Orange	

AmpliSens-1 amplification program for rotor-type instruments

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	**95**	15 min	–	1
Cycling 1	**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	
	72	15 s	–	

8. Activate the ***OK*** button.
9. Activate the ***Calibrate/Gain Optimisation*** button in the ***New Run Wizard*** window.
   - for detection in FAM/Green, JOE/Yellow, and ROX/Orange channels select ***Calibrate Acquiring/Optimise Acquiring***;
   - activate the ***Edit*** button in the ***Auto gain calibration channel settings*** window and set ***Min Reading*** as ***4*** and ***Max Reading*** as ***8*** for FAM/Green, JOE/Yellow, and ROX/Orange channels.
   - mark ***Perform Calibration Before 1st Acquisition***/***Perform Optimisation Before 1st Acquisition***. Activate the ***Close*** button.
10. Activate the ***Next*** button. Select the ***Start run*** button to start the amplification program.
11. Name the experiment and save it to disk (results of the run will be automatically saved in this file).
12. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Indicate the names/numbers of the test samples in the box ***Name***. Define the Negative Control of amplification as ***NCA***, define the calibrators as ***C1***, ***C2***,

and ***C3***. In the ***Type*** column enter ***Unknown*** for all clinical samples and calibrators. Indicate the ***Negative Control*** type for the Negative Control of amplification. Set the type ***None*** for the cells matching with the corresponding empty tubes

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

Data analysis

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

1. Activate the button ***Analysis*** in the menu; select the mode of the analysis ***Quantitation***. Activate ***Cycling A. FAM/Cycling A. Green***, ***Cycling A. JOE/Cycling A. Yellow***, ***Cycling A. ROX/Cycling A. Orange***; ***Show*** buttons***.***
2. Cancel the automatic choice of the threshold line level for each of the main open windows (FAM/Green, JOE/Yellow и ROX/Orange) by activating the ***Threshold*** button.
3. Select ***Linear scale***.
4. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the main window menu (***Quantitation analysis***).
5. In the ***CT Calculation*** menu set ***Threshold = 0.03*****.**
6. Select the ***More Settings/Outlier Removal*** parameter and set ***NTC threshold*** value ***10 %.***
7. Select the ***Name*** column in the results grid (the ***Quant. Results*** window). To do this, click on the headline with the left mouse button. Copy the column by selecting ***Copy*** from the context menu (right mouse button).
8. Open the Microsoft Excel **AmpliSens *HPV* 16-18 Result Matrix** program enclosed to the PCR kit. Agree to run macro.

NOTE: If micro is disabled in the opened Excel file (the ***Result*** button is inactive, an appropriate message is displayed) the Microsoft Excel security level should be changed. To do this, select ***Service>Macro>Security*** and set the medium security level.

9. Point the cursor on the ***Name*** cell of the ***Identifier*** column and select ***Paste*** from the context menu by clicking the right mouse button.
10. Select and copy data from the ***Ct*** column of the result grid (the ***Quant. Results*** table) in similar way. Point the cursor on the ***Ct*** cell of Excel file below the appropriate fluorescence dye and select ***Paste*** from the context menu by clicking the right mouse

button.

11. Repeat the same procedure for other fluorescence dyes.

Qualitative analysis

12. Set the ***Qualitative analysis*** mode.
13. Data analysis is carried out automatically. When all data entered in Excel, click the ***Results*** button. Detected *HPV* genotypes will appear in the ***Genotype*** column; results of the analysis will appear in the ***Qual.*** column: ***pos*** (positive), ***neg*** (negative), or ***N/V*** (invalid) (see the ***Instruction*** sheet of Microsoft Excel **AmpliSens *HPV* 16-18 Result Matrix** program).
14. Save the Microsoft Excel file with another name.

Quantitative analysis

15. Set the ***Quantitative analysis*** mode, ***Internal calibration***.
16. In the ***Calibrator value*** table enter the concentration values of the calibrators specified in the *Important Product Information Bulletin* enclosed in the PCR kit of the given lot.
17. Make sure that C1 calibrator is defined as ***C1***, C2 calibrator is defined as ***C2***, C3 calibrator is defined as ***C3*** (no space between the letter and digit), negative control is defined as ***NCA*** or "**–**" in the ***Identifier*** column.
18. Data analysis is carried out automatically. When all data are entered in Excel, activate the ***Results*** button. Detected *HPV* genotypes will appear in the ***Genotype*** column; results of the analysis will appear in the ***Qual.*** column: ***pos*** (positive), ***neg*** (negative), ***weak*** (weak positive), or ***N/V*** (invalid) (see the ***Instruction*** sheet of Microsoft Excel **AmpliSens *HPV* 16-18 Result Matrix**). Quantity of 2n human genomes (represent cell quantity) per reaction (used for sample validity assessment) is displayed next. Calculated concentration values of *HPV* DNA for each genotype expressed in log per 10^5 cells and total viral load are displayed further. The last column outlines possible interpretation of clinical significance of result in accordance with the table below:

Interpretation of result calculated in log (*HPV* per 100,000 cells)

log(*HPV* per 100,000 cells)	Interpretation
<3	Clinically insignificant
3 – 5	Clinically significant. Dysplasia cannot be excluded. Risk of dysplasia development
>5	Clinically significant, increased. Dysplasia is highly expectable

19. Save the Microsoft Excel file with another name.

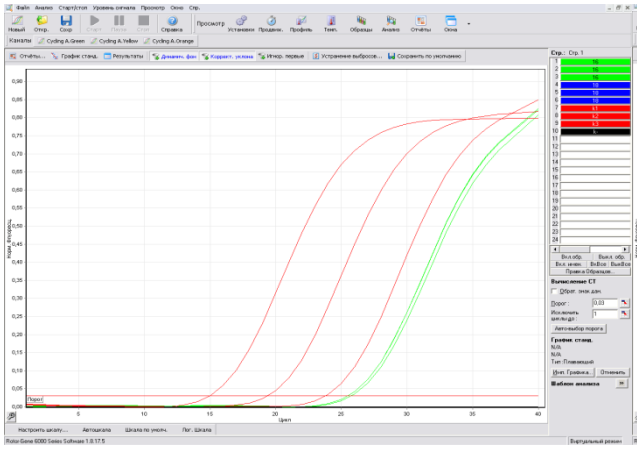
Results of analysis

The results of analysis are considered reliable only if the results obtained for the DNA calibrators, the Negative Control of amplification as well as for the Negative Controls of extraction are correct:

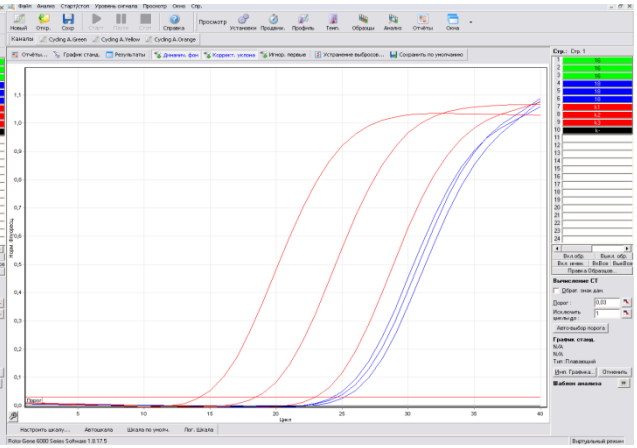
– Negative Control of extraction (C–) **– Negative Control –** no *Ct* value is detected;
– Negative Control of amplification (NCA) **– DNA-buffer –** no *Ct* value is detected;
– DNA calibrators (C1, C2, C3) **– C1 *HPV* 16, 18; C2 *HPV* 16, 18; C3 *HPV* 16, 18 –** *Ct* values are detected in all channels.

Examples of results

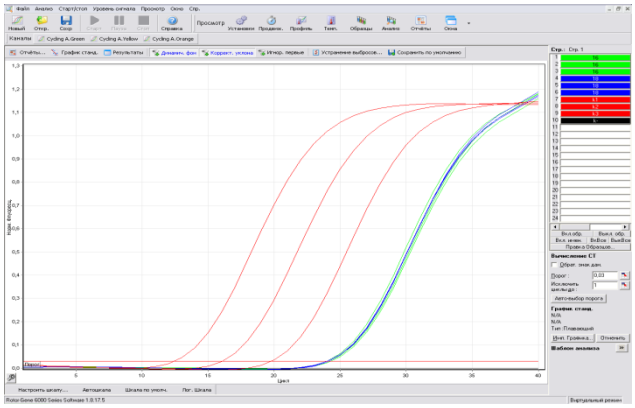
Data in FAM/Green channel



Data in JOE/Yellow channel



Data in ROX/Orange channel



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

Carry out the sample pretreatment and reaction mixture preparation stages according to the PCR kit *Instruction Manual.* When carrying out the amplification it is recommended to use thin-walled PCR tubes (0.2 ml) with round or optically transparent flat caps, or tubes (0.1 ml) with transparent caps from the eight-pieces-strips (e.g. Axygen, USA) (detection through the cap of the tube).

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

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

2. Start the program iCycler iQ/iQ5.
3. Insert the tubes or strips into the reaction module of the amplifier (thermocycler) and program the instrument.

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

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

1. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal).
   - For **iCycler iQ5**. Click the ***Create New*** or ***Edit*** buttons in the ***Selected Plate Setup*** window of the ***Workshop*** module. One can edit the plate setup in the ***Whole Plate loading*** mode. Set the reaction volume (***Sample Volume***) as **30 µl** for variant **FRT** (or **25 µl** for variant **FRT-100 F)**, 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** and **ROX** channels. Save the set plate setup by clicking the ***Save&Exit Plate Editing*** button.
   - For **iCycler iQ**. Edit the plate setup in the ***Edit Plate Setup*** window of the ***Workshop*** module. To do this, set the order of the tubes in the reaction chamber in the ***Samples: Whole Plate Loading*** option. Name each sample in the ***Sample Identifier*** window. Set the fluorescent signal detection in all the tubes through **FAM-490**, **HEX-530**, and **ROX-575** channels in the ***Select and Load Fluorophores*** option. Save the plate setup by naming the file in the ***Plate Setup Filename*** window (with .pts extension) and activate the ***Save this plate setup*** button (at the top of the screen). It is possible to edit the ***Plate Setup*** used previously. To do this, choose the ***View Plate Setup*** in the

Library window, select an appropriate ***Plate Setup*** (a file with .pts extension) and activate the ***Edit*** button at the right. It is necessary to save the edited file before using. Set the using of the given plate setup by clicking the ***Run with selected protocol*** button.

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

DNA HPV 16-18 amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	95	15 min	–	1
2	95	20 s	–	45
	60	1 min	FAM/FAM-490, JOE/HEX/JOE-530, ROX/ROX-575	

AmpliSens-1 amplification program for plate-type instruments

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

- For **iCycler iQ5**. Click the ***Create New*** or ***Edit*** button in the ***Selected Protocol*** window of the ***Workshop*** module. Set amplification parameters and save the protocol by clicking the ***Save&Exit Protocol Editing*** button. The file with the created program can be selected from the ***Protocol*** unit for further runs (it is saved by default the ***Users*** folder).
- For **iCycler iQ**. Select the ***Edit Protocol*** option in the ***Workshop*** module. Set the amplification program in the bottom window; indicate ***Cycle 2 – Step 2*** in the window at the right. Save the protocol: name the file in the ***Protocol Filename*** window (file with .tmo extension) and click the ***Save this protocol*** button (at the top of the screen). The file with the created program can be selected from the ***View Protocol*** tab of the ***Library*** module for further runs. Enable the selected or edited program by clicking the ***Run with selected plate setup*** button.

4. Prior to run the program:
   - For **iCycler iQ5**. Check if the selected protocol (***Selected Protocol***) and the plate scheme (***Selected Plate Setup***) in the ***Run Prep*** window are correct. Click the ***Run***

button to start the program. For detection of the well factor select the ***Collect Well Factors from Experimental Plate*** option. Click the ***Begin Run*** button, name the experiment (the results of the experiment will be automatically saved to this file), and click the ***OK*** button.

- For **iCycler iQ**. Check if the selected protocol (***Selected Protocol***) and the plate scheme (***Selected Plate Setup***) in the ***Run Prep*** window are correct. For the detection of the well factor select ***Experimental Plate*** in the ***Select well factor source*** menu. Set the reaction mixture volume as **30 µl** for variant **FRT** (or **25 µl** for variant **FRT-100 F)**. Activate the ***Begin Run*** button to start. Name the experiment (the results of the experiment will be automatically saved to this file) and click the ***OK*** button..

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

Data analysis:

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

1. Start the program and open the saved file. To do this:
   - for **iCycler iQ5.** Select the needed data file for the analysis in the ***Data File*** window of the ***Workshop*** module and click the ***Analyze*** button;
   - for **iCycler iQ.** Activate the ***View Post-Run Data*** window in the ***Library*** module. Select the needed file from the ***Data File*** window and click the ***Analyze Data*** button.
2. To review obtained data:
   - for **iCycler iQ5.** Select the ***Analysis Mode: PCR Base Line Subtracted Curve Fit*** (selected by default);
   - for **iCycler iQ.** Select the icon of the required channel in the ***PCR Quantification*** tab of the ***Select a Reporter*** menu. Make sure that the ***PCR Base Line Subtracted Curve Fit*** mode is activated (selected by default).
3. Review obtained data in one channel at a time.
4. Set the level of the threshold line. To do this:
   - for **iCycler iQ5.** Set the ***Base Line Cycles – Auto Calculated*** parameter in the ***Base Line Threshold*** window (in case of the curves excursion set this parameter in

the ***User Defined, 2 through 10 cycles*** mode), set the ***Crossing Threshold – Auto Calculated*** parameter. Normally, the threshold line is to intersect only with S-shaped 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 raise the threshold level by clicking the ***Log View*** button and setting the threshold line at the level (with the left mouse button) so that the fluorescence curves should be of a linear character and not intersect with the curves of the negative samples.

- for **iCycler iQ.** choose the mode of the threshold line automatic setting and the automatic calculation of the base line in the ***Threshold Cycle Calculation*** menu. To do this, select ***Auto Calculated*** in the ***Baseline Cycles*** menu and select ***Auto Calculated*** in the ***Threshold Position*** menu. Normally, the threshold line is to intersect only with S-shaped 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 choose ***User Defined*** in the ***Threshold Position*** submenu and raise the threshold level by clicking the ***Log View*** button and setting the threshold lines level (with the left mouse button) so that the fluorescence curves should be of a linear character and not intersect with the curves of the negative samples.

5. Click on the results grid displayed with the right mouse button. Select ***Export to Excel*** in the drop-down menu. Agree that the file will be saved. The file will be opened by default if Microsoft Excel is installed (otherwise, carry out data analysis on a computer where Microsoft Excel is installed). Results are displayed in the following order: results in the FAM/FAM-490 channel, then in the JOE/HEX/JOE-530 channel, and then in the ROX/ROX-575 channel.
6. Open the Microsoft Excel ***AmpliSens HPV 16-18 Result Matrix*** software (program for result calculation) enclosed to the PCR kit. Agree to run macro.

NOTE: If micro is disabled when Excel is opened (the ***Result*** button is inactive, an appropriate message is displayed) the Microsoft Excel security level should be changed. To do this, select ***Service>Macro>Security*** and set the medium security level.

7. Select and copy the cells of the ***Threshold Cycle (Ct)*** column referring to the FAM/FAM-490 channel. Proceed to the program for result calculation and paste these data in the FAM column beginning from the first cell.
8. Copy the cells of the ***Threshold Cycle (Ct)*** column referring to the JOE/HEX/JOE-530 and ROX/ROX-575 channels in the similar way and paste in the JOE and ROX

columns of the program for result calculation, respectively.

9. Copy names of the samples from the ***Identifier*** column (if they were named) to the ***Name*** column of the program for result calculation.

Qualitative analysis

10. Set the ***Qualitative analysis*** mode.
11. Data analysis is carried out automatically. When all are data entered in Excel, click the ***Results*** button. Detected *HPV* genotypes will appear in the ***Genotype*** column; results of the analysis will appear in the ***Qual.*** column: ***pos*** (positive), ***neg*** (negative), or ***N/V*** (invalid) (see the ***Instruction*** sheet of Microsoft Excel AmpliSens HPV 16-18 Result Matrix program).
12. Save the Microsoft Excel file under a different name.

Quantitative analysis

13. Set the ***Quantitative analysis*** mode, ***Internal calibration***.
14. In the ***Calibration value*** table enter concentration values of calibrators specified in the *Important Product Information Bulletin* enclosed in the PCR kit of the given lot.
15. Make sure that C1 calibrator is defined as ***C1***, C2 calibrator is defined as ***C2***, C3 calibrator is defined as ***C3*** (no space between a letter and digit), negative control is defined as ***NCA*** or **"–"** in the ***Identifier*** column.
16. Data analysis is carried out automatically. When all data are entered in Excel, activate the ***Results*** button. Detected *HPV* genotypes will appear in the ***Genotype*** column; results of the analysis will appear in the ***Qual.*** column: ***pos*** (positive), ***neg*** (negative), ***weak*** (weak positive), or ***N/V*** (invalid) (see the ***Instruction*** sheet of Microsoft Excel ***AmpliSens HPV 16-18 Result Matrix***). Quantity of 2n human genomes (represent cell quantity) per reaction (used for sample validity assessment) is displayed next. Calculated concentration values of *HPV* DNA for each genotype expressed in log per 10^5 cells and total viral load are displayed further. The last column outlines possible interpretation of clinical significance of result in accordance with the table below:

Interpretation of result calculated in log (*HPV* per 100,000 cells)

log(*HPV* per 100,000 cells)	Interpretation
<3	Clinically insignificant
3-5	Clinically significant. Dysplasia cannot be excluded. Risk of dysplasia development
>5	Clinically significant, increased. Dysplasia is highly expectable

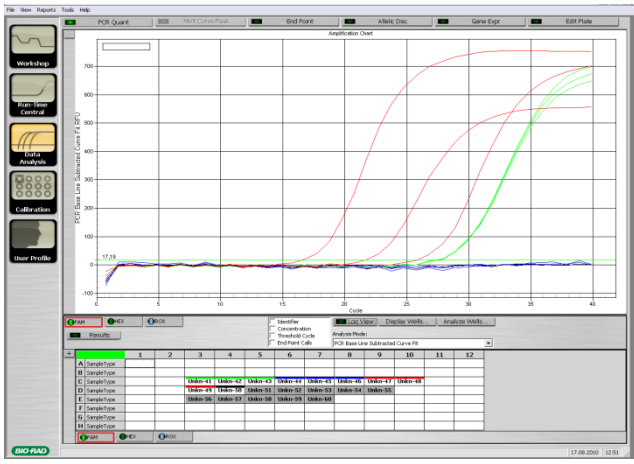
17. Save the Microsoft Excel file under a different name.

The results of analysis are considered reliable only if the results obtained for the DNA calibrators, the Negative Control of amplification as well as for the Negative Controls of extraction are correct:

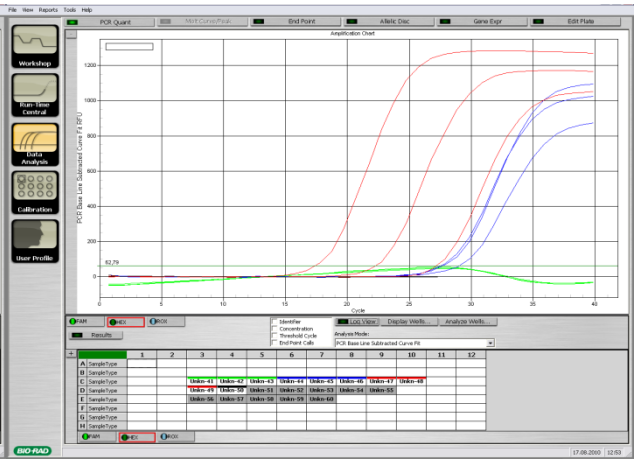
– Negative Control of extraction (C–) **– Negative Control –** no *Ct* value is detected;
– Negative Control of amplification (NCA) **– DNA-buffer –** no *Ct* value is detected;
– DNA calibrators (C1, C2, C3) **– C1 *HPV* 16, 18; C2 *HPV* 16, 18; C3 *HPV* 16, 18 –** *Ct* values are detected in all channels.

Examples of results

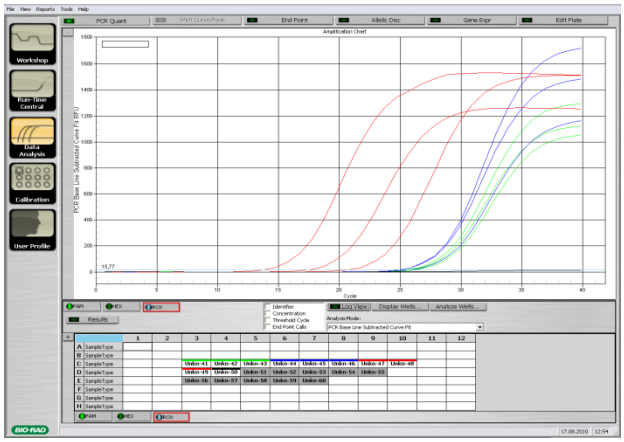
Data for FAM/FAM-490 channel



Data for JOE/HEX/JOE-530



Data for ROX/ROX-575 channel



AMPLIFICATION AND DATA ANALYSIS WITH Mx3000P, Mx3005P (Stratagene, USA) INSTRUMENTS

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

1. Switch on the instrument and open the Mx3000P/Mx3005P program.
2. In the ***New Experiment Options*** window select ***Quantitative PCR (Multiple Standards)*** and tick off ***Turn lamp on for warm-up***.

NOTE: The lamp should be warmed up for at least 15 min before the experiment starts.

3. Place the tubes in the instrument, secure the locking tool, and close the lid.
4. Select ***Optics Configuration*** in the ***Options*** menu. In the ***Dye Assignment*** tab indicate ***JOE*** next to the ***HEX/JOE filter set*** option; indicate ***FAM*** next to the ***FAM filter set*** option; indicate ***ROX*** next to the ***ROX filter set*** option.

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

5. Define parameters of fluorescence acquiring in the ***Plate Setup*** menu. To do this:
   a) hold ***Ctrl*** and use the mouse to select all cells in which test tubes are inserted.
   b) define all selected cells as ***Unknown*** in the ***Well type*** window. In the ***Collect fluorescence data*** option tick off ***FAM***, ***JOE***, and ***ROX***. Double click each cell and enter a sample name (***Well Information*** window). Sample names can be entered during or after the amplification run in the ***Plate Setup*** menu.
6. Set amplification program. To do this, use one of the following methods:

   <u>Using a template file (recommended)</u>

   Proceed to the ***Thermal Profile Setup*** tab. Click the ***Import…*** button at the right of the thermocycling profile image. Proceed to the folder with the previous experiment file and open it. Required thermocycling profile will appear in the ***Thermal Profile*** window.

   <u>Self-programming</u>

   – in the ***Plate Setup*** tab select all cells in which test tubes are inserted. Proceed to the ***Thermal Profile Setup*** menu and set amplification program;

DNA HPV 16-18 amplification program

Step	Temperature, °C	Time	Fluorescent signal detection	Cycles
1	95	15 min	–	1
2	95	20 s	–	45
	60	1 min	FAM, JOE/HEX, ROX	

AmpliSens-1 amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescent signal detection	Cycles
Hold	**95**	15 min	–	1
Cycling 1	**95**	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling 2	**95**	5 s	–	40
	60	30 s	FAM, JOE/HEX, ROX	
	72	15 s	–	

- to set the parameter for detection of fluorescence signal at a specific temperature, select the ***All points*** option for the ***Data collection marker by dragging*** parameter and pull it from the right side to the shelf with the required temperature;
- to launch amplification click ***Run,*** then ***Start***, and name the experiment.

Data analysis

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

1. Proceed to the ***Analysis*** item by clicking the button on the toolbar.
2. Ensure that all test samples are enabled (the cells are tinted) in the opened ***Analysis Selection/Setup*** tab. Otherwise, hold ***Ctrl*** and select all test samples with the mouse.
3. Proceed to the ***Results*** tab.
4. Ensure that all three fluorescence channels are enabled (the ***JOE***, ***FAM***, and ***ROX*** buttons are activated in the ***Assay Shown*** field at the bottom of the program window).
5. Ensure that ***JOE***, ***FAM***, and ***ROX*** fluorescence channels are ticked off in the ***Threshold fluorescence*** field. Ensure that the threshold line is set correctly. Normally, a threshold line should cross S-shaped curves of signal accumulation of positive samples and controls and should not cross the base line. If the automatically selected threshold line crosses negative samples, the threshold should be raised so that it crosses only positive samples. (By default fluorescence curves are displayed in linear

scale. To switch to logarithmic scale double click one of the axes (X or Y) with the left mouse button and tick off ***Log*** option for Y axis of in the ***Scale*** field of the opened ***Graph properties*** window).

6. View the results in the ***Text Report*** field and export data for further calculation (for example to Excel. To do this, click the right mouse button and select ***Export Text Report>Export Text Report to Excel*** from the drop-down menu).
7. Open the Microsoft Excel **AmpliSens *HPV* 16-18 Result Matrix** software (program for result calculation) enclosed in the PCR kit. Agree to run macro.

NOTE: If micro is disabled when Excel is opened (the ***Result*** button is inactive, an appropriate message is displayed), the Microsoft Excel security level should be changed. To do this, select ***Service>Macro>Security*** and set the medium security level.

8. Copy sample names from the ***Well name*** column of the ***Text Report*** window and paste them in the ***Identifier*** column of the program for result calculation.
9. Copy *Ct* values for all three channels and paste them in the corresponding cells of the program for automatic result calculation.

Qualitative analysis

10. Set the ***Qualitative analysis*** mode.
11. Data analysis is carried out automatically. When all data are entered in Excel, click the ***Results*** button. Detected *HPV* genotypes will appear in the ***Genotype*** column; results of the analysis will appear in ***Qual.*** column: ***pos*** (positive), ***neg*** (negative), or ***N/V*** (invalid) (see the Instruction sheet of Microsoft Excel **AmpliSens *HPV* 16-18 Result Matrix**).
12. Save the Microsoft Excel file under a different name.

Quantitative analysis

13. Set the ***Quantitative analysis*** mode, ***Internal calibration***.
14. In the ***Calibrator value*** table, enter concentration values of the calibrators specified in the *Important Product Information Bulletin* enclosed in the PCR kit of the given lot.
15. Make sure that C1 calibrator is defined as ***C1***, C2 calibrator is defined as ***C2***, C3 calibrator is defined as ***C3*** (no space between a letter and digit) in the ***Identifier*** column.
16. Data analysis is carried out automatically. When all data are entered in Excel, click the ***Results*** button. Detected *HPV* genotypes will appear in the ***Genotype*** column; results of the analysis will appear in the ***Qual.*** column: ***pos*** (positive), ***neg*** (negative), ***weak*** (weak positive), or ***N/V*** (invalid) (see the Instruction sheet of Microsoft Excel

AmpliSens *HPV* 16-18 Result Matrix). The quantity of 2n human genome equivalents (represent cell quantity) per reaction (used for sample validity assessment) is displayed next. Calculated concentration values of *HPV* DNA for each genotype expressed in log per 10^5 cells and the total viral load are displayed further. The last column outlines possible interpretation of clinical significance of the result in accordance with the table below:

Interpretation of result calculated in log (*HPV* per 100,000 cells)

log(*HPV* per 100,000 cells)	Interpretation
<3	Clinically insignificant
3-5	Clinically significant. Dysplasia cannot be excluded. Risk of dysplasia development
>5	Clinically significant, increased. Dysplasia is highly expectable

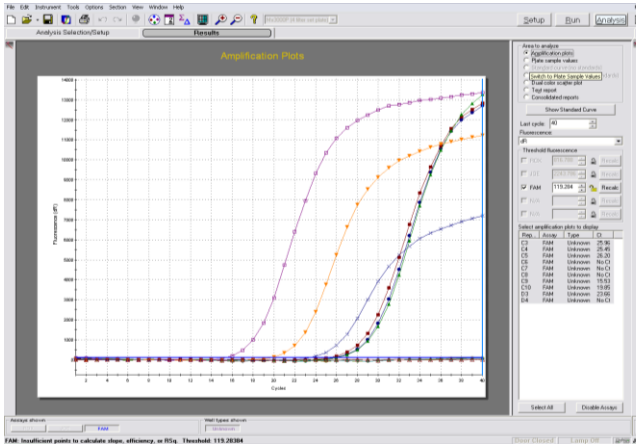
17. Save the Microsoft Excel file under a different name.

The results of analysis are considered reliable only if the results obtained for the DNA calibrators, Negative Control of amplification as well as for the Negative Controls of extraction are correct:

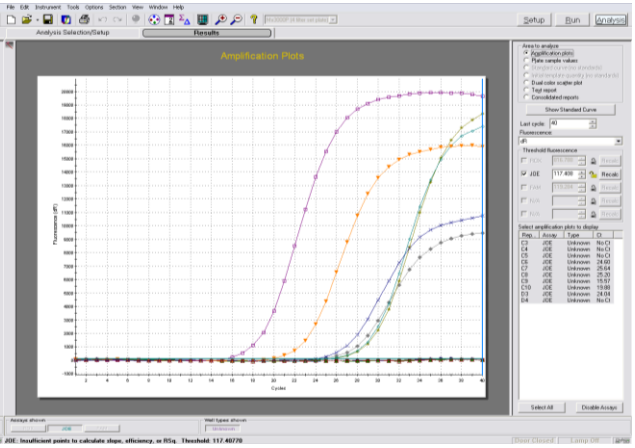
– Negative Control of extraction (C–) **– Negative Control –** no *Ct* value is detected;
– Negative Control of amplification (NCA) **– DNA-buffer –** no *Ct* value is detected;
– DNA calibrators (C1, C2, C3) **– C1 *HPV* 16, 18; C2 *HPV* 16, 18; C3 *HPV* 16, 18 –** *Ct* values are detected in all channels.

Examples of results

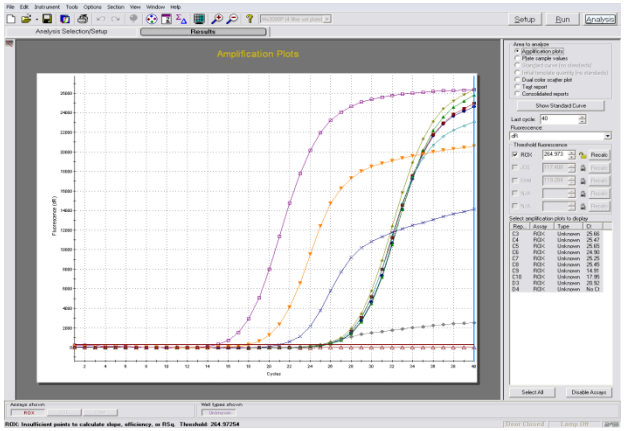
Data for FAM channel



Data for JOE/HEX channel



Data for ROX channel



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

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

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

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, cycles, and fluorescence acquiring cycle)

in the opened ***Protocol Editor – New*** window (see table 7). Set ***Sample Volume* – 25 µl** (in case of **variant FRT-100 F**) or **30 µl** (in case of **variant FRT**).

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

DNA HPV 16-18 amplification program

Step	Temperature, °C	Time	Fluorescent signal detection	Cycles
1	95	15 min	–	1
2	95	20 s	–	45
	60	1 min	FAM, HEX, ROX	

AmpliSens-1 amplification program for plate-type instruments

Step	Temperature, °C	Time	Fluorescentsignal 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	
	72	15 s	–	

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. This file can be selected for further runs from the ***Protocol*** tab by clicking the ***Select Existing…*** button. When the required program is entered or edited, click ***OK*** at the bottom of the window.
5. 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.
6. 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.
7. Place the reaction tubes in the wells of the instrument in accordance with the entered plate setup. In the ***Start Run*** tab click the ***Start Run*** button then save the file of the experiment.
8. Proceed to the analysis of results after the end of the run.

Data analysis

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

Curves of accumulation of fluorescence signals are analyzed in three channels:

- the signal of accumulation of the Internal Control (fragment of β-globin gene) amplification product is detected in the ROX channel;
- the signals of accumulation of the specific target amplification products are detected in the FAM and HEX channel.

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

Variant 1

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

Variant 2

For each channel indicate ***Log Scale*** with a checkmark. Set the threshold line at the level where fluorescence curves are linear (use the left mouse button).

Click the ***View/Edit Plate*** button on the toolbar and enter sample names and calibrator concentrations in the opened window.

Data analysis

1. Click on the appeared results grid using the right mouse button. Select Export to Excel from the drop-down menu. Agree to save the file. In case Microsoft Excel program is installed on the computer, this file will open automatically (following data analysis can be performed on the computer with installed Microsoft Excel program). Results are open in the following order: FAM channel, then HEX and then ROX.
2. Open the Microsoft Excel **AmpliSens *HPV* 16-18 Results Matrix** file enclosed to the PCR kit. Agree to run macro.

Note! If micro is disabled when Excel is opened (the ***Result*** button is inactive, an appropriate message is displayed), the Microsoft Excel security level should be changed. To do this, select ***Service>Macro>Security*** and set the medium security level.

3. Copy threshold cycle values (***Cq***) obtained in the FAM channel from the result grid, proceed to the program of automatic result calculation, and paste them in the corresponding cells of the FAM column.

4. Similarly, copy threshold cycle values (Cq) obtained in the HEX and ROX channels and paste in the corresponding cells of JOE and ROX columns of the program of automatic result calculation.
5. Copy sample names from the ***Sample*** column (if they were indicated) and paste them in the ***Identifier*** column of the program of automatic result calculation.

Qualitative analysis

- Set the ***Qualitative analysis*** mode.
- Data analysis is carried out automatically. When all data are entered in Excel, click the ***Results*** button. Detected *HPV* genotypes will appear in the ***Genotype*** column; results of the analysis will appear in the ***Qual.*** column: ***pos*** (positive), ***neg*** (negative), or ***N/V*** (invalid) (see the ***Instruction*** sheet of Microsoft Excel **AmpliSens *HPV* 16-18 Result Matrix** program).
- Save the Microsoft Excel file under a different name.

Quantitative analysis

- Set the ***Quantitative analysis*** mode, internal calibration.
- In the ***Calibration value*** table enter concentration values of calibrators specified in the *Important Product Information Bulletin* enclosed in the PCR kit of the given lot.
- Make sure that C1 calibrator is defined as ***C1***, C2 calibrator is defined as ***C2***, C3 calibrator is defined as ***C3*** (no space between a letter and digit), negative control is defined as ***NCA*** or **"–"** in the ***Identifier*** column.
- Data analysis is carried out automatically. When all data are entered in Excel, click the ***Results*** button. Detected *HPV* genotypes will appear in the ***Genotype*** column; results of the analysis will appear in the ***Qual.*** column: ***pos*** (positive), ***neg*** (negative), ***weak*** (weak positive), or ***N/V*** (invalid) (see the ***Instruction*** sheet of Microsoft Excel AmpliSens HPV 16-18 Result Matrix). Quantity of 2n human genomes (represent cell quantity) per reaction (used for sample validity assessment) is displayed next. Calculated concentration values of *HPV* DNA for each genotype expressed in log per 10^5 cells and total viral load are displayed further. The last column outlines possible interpretation of clinical significance of result in accordance with the table below:

Interpretation of result calculated in log (*HPV* per 100,000 cells)

log(*HPV* per 100,000 cells)	Interpretation
<3	Clinically insignificant
3-5	Clinically significant. Dysplasia cannot be excluded. Risk of dysplasia development
>5	Clinically significant, increased. Dysplasia is highly expectable

- Save the Microsoft Excel file under a different name.

TROUBLESHOOTING

1. If a *Ct* value appears for the Negative Control of amplification (NCA) and/or for the Negative Control of extraction (C–) in the FAM/Green/FAM-490, JOE/HEX/Yellow/JOE-530, and/or ROX/Orange/ROX-575 channels, this indicates contamination of samples or reagents. The result of the analysis is invalid for all samples. Repeat the analysis (starting from DNA extraction stage) of all samples that shown the presence of *HPV* DNA and to eliminate the source of contamination.
2. If *Ct* values of DNA calibrators (C1 *HPV* 16, 18, C2 *HPV* 16, 18, and C3 *HPV* 16, 18) in the FAM/Green/FAM-490, JOE/HEX/Yellow/JOE-530, and/or ROX/Orange/ROX-575 are absent in the results grid, PCR should be repeated for all samples that did not show the presence of *HPV* DNA.
3. If a *Ct* value of a sample is not detected or exceeds the boundary *Ct* value specified for FAM/Green/FAM-490, and/or JOE/HEX/Yellow/JOE-530 channels, whereas the *Ct* value detected in the ROX/Orange/ROX-575 channel exceeds the boundary *Ct* value specified for the Internal Control, the analysis should be repeated starting from the DNA extraction stage. This may be caused by the presence of PCR inhibitors or by the loss of DNA during clinical sample handling.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
02.04.14 SA	Cover page	Address of European representative was added
21.04.14 ME	Intended Use	Information about the real-time instrument, CFX96 (Bio-Rad, USA), was added
	Throughout the text	"DNA HPV 16-18" amplification program was added for all types of applied real-time PCR instruments
		The section "Amplification and data analysis with CFX96 (Bio-Rad, USA) instrument" was added
		The channels names are corrected in accordance with the template
18.06.14 PM	Intended use	PCR kit intended use was specified ("qualitative detection" was changed to "qualitative and quantitative detection")
03.02.16 ME	Cover page	The type of clinical material was deleted from the title
	Intended use	The type of clinical material was indicated
13.11.19 PM	Footer	**REF** R-V12-Mod(RG,iQ,Mx)-CE was changed to **REF** R-V12-F-CE **REF** R-V12-100(iQ,Mx,Dt)-CE, **REF** R-V12-100(RG)-CE were changed to **REF** R-V12-100-CE
28.12.20 MM	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase "Not for use in the Russian Federation" was added
24.03.21 EM	Front page	The name, address and contact information for Authorized representative in the European Community was changed