



For Professional Use Only

GUIDELINES

to **AmpliSens[®] HPV HCR screen-14-titre-FRT** PCR kit
for detection of the DNA of *human papillomaviruses (HPV)*
of high carcinogenic risk (HCR) genotypes 16, 18, 31, 33, 35, 39,
45, 51, 52, 56, 58, 59, 66, 68 in the biological material by
polymerase chain reaction (PCR) with real-time hybridization-
fluorescence detection

AmpliSens[®]



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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® HPV HCR screen-titre-14-FRT** PCR kit for quantitation of DNA of *human papillomaviruses (HPV)* of high carcinogenic risk (HCR) in biological material (vaginal swab, epithelial scrape from the cervical mucous membrane (ectocervix and endocervix)) 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);
- CFX96 (Bio-Rad, USA).

Nucleic acid extraction kits recommended for DNA extraction

The Federal Budget Institute of Science “Central Research Institute for Epidemiology” recommends the following nucleic acid extraction kits for DNA extraction and further PCR analysis with the use of **AmpliSens® HPV HCR screen-titre-14-FRT** PCR kit:

- **DNA-sorb-AM** **REF** K1-12-100-CE for DNA extraction from vaginal swabs and cervical scrapes in accordance with the Instruction manual for the nucleic acid extraction kit.
- **AmpliSens® MAGNO-sorb-URO**, **REF** K1-19-100-CE for DNA extraction from vaginal swabs and cervical scrapes in accordance with the Instruction manual for the nucleic acid extraction kit.
- **AmpliSens® DNA-sorb-D** **REF** K1-8-100-CE for DNA extraction from vaginal swabs and cervical scrapes in accordance with the Instruction manual for the nucleic acid extraction kit

Correspondence of targets and detection channels

Fluorophore	FAM	JOE	ROX	Cy5	Cy5.5
Detection channel name for different instrument models ¹	FAM/ Green	JOE/ HEX/ R6G/ Yellow/ Cy3	ROX/ Orange/ TxR	Cy5/ Red	Cy5.5/ Crimson/ Quasar705
DNA-target	DNA of HPV HCR genotype 16	DNA of HPV HCR genotype 18	genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	DNA fragment of β -globin gene (IC Glob)	DNA of HPV HCR genotype 45
Target gene	E6 gene	E6 gene	E1 gene (for genotypes 16, 31, 33, 35, 52, 58)/ E2 gene (for genotypes 18, 39, 45, 56, 59, 66, 68)/ E7 gene (for genotype 51)	β -globin gene	E6 gene

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

When working with Rotor-Gene 3000 one should use the Rotor-Gene version 6.1 and higher software and the Rotor-Gene 6000 versions 1.7 (build 67) software or higher for Rotor-Gene 6000 and Rotor-Gene Q instruments.

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

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

Programming the thermocycler

1. Turn on the instrument, run the Rotor-Gene software.
2. Insert the tubes or strips into the rotor of the Rotor-Gene 3000/6000/Q instrument beginning from the first well (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler). Insert the rotor into the instrument, close the lid.

Well 1 must be filled with any test tube except for an empty one. If the tubes with reagents from different PCR kits or with different PCR-mixes are inserted into the rotor then the tubes' numbers for calibration in each detection channel should be indicated in the Rotor-Gene software (item 7). Recommendations about the calibration are described in the information list "Calibration priorities for the AmpliSens kits for Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) thermocyclers".

NOTE:

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

Creating the template for the run

1. Click the **New** button in the software main menu. To create the template select the **Advanced** tab in the opened window **New run**.
2. Select the **TwoStep/Hidrolysis Probes** template in the tab for edition and click The **New** button.
3. In the newly opened window select rotor for 36 wells, **36-Well Rotor** (or rotor for 72 wells, **72-Well Rotor**), and tick the **No Domed 0,2ml Tubes / Locking Ring Attached** position. Press the **Next** button.
4. In the newly opened window set the name of the operator and choose the reaction mix volume: **Reaction volume – 25 µl**. Press the **Next** button.
5. In the **New Run Wizard** window set the temperature profile of the experiment. To do this click the **Edit profile** button and set the amplification program:

AmpliSens unified amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
Hold	50	15 min	-	1
Hold	95	15 min	-	1
Cycling	95	10 s	-	45
	60	20 cs	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson	

NOTE: Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program. If in one instrument only the tests for the pathogen DNA detection are carried out simultaneously, the first step of reverse transcription (50 °C – 15 min) can be omitted for time saving.

AmpliSens-1 amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	95	15 min	-	1
2	95	5 s	-	5
	60	20 s	-	
	72	15 s	-	
3	95	5 s	-	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson	
	72	15 s	-	

NOTE: **AmpliSens-1** is an universal program for conducting tests for identifying *human papillomaviruses (HPV HCR)* and detection of STIs and other infections of reproductive system with AmpliSens PCR kits. Therefore, any combination of these tests can be carried out simultaneously in the same instrument.

6. After setting up the temperature profile click the **OK** button.
7. Click the **Calibrate/Gain Optimisation...** button in the **New Run Wizard** window. In the opened window:

- a) for signal measurement optimisation for the selected channels set calibration from **5FI** to **10FI** for all the channels (FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson).

To do this, click the **Calibrate Acquiring/Optimise Acquiring** button. In the opened window for first channel (**Auto Gain Optimisation Channel Settings/Auto Gain Calibration Channel Settings**) indicate the values of minimum and maximum signal in the **Target Sample Range** line. Click the **OK** button. The window for the next channel will open automatically. The selected values for all the channels can be checked in the **Min Reading, Max Reading** boxes.

NOTE: The additional requirements for setting the channels' calibration ranges are specified in the information list "Calibration priorities for the AmpliSens kits for Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) thermocyclers"

- b) perform the calibration in the selected channels before the first detection (tick the **Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition** option). Click the **Close** button.
8. Click the **Next** button. For saving the programmed template it is necessary to click the **Save Template** button and enter the template file name, corresponding to the amplification program – **HPV HCR screen-titre-14**. Save the file into a proposed folder: **Templates\Quick Start Templates**; close the **New Run Wizard window**. After that the programmed template will appear in the template list in the **New Run** window.

Using the ready template for the run

1. Click the **New** button in the software main menu. In the opened **New Run** window select the **Advanced** tab. Then select the **HPV HCR screen-titre-14** template (which is programmed as described in the **Creating the template for the run** section) in the template list.
2. In the opened window select the **36-Well Rotor (or 72-Well Rotor)** and tick the **No Domed 0,2ml Tubes / Locking Ring Attached** option. Click the **Next** button.
3. In the newly opened window choose the reaction mix volume: **Reaction volume – 25 µl**. Press the **Next** button.
4. In the next window the correctness of the amplification program and signal level auto-optimization parameters can be checked. Go to the next window clicking the **Next** button. Start the amplification by the **Start run** button. Herewith, the rotor with the samples should be already fixed and the lid should be closed. Name the experiment

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

5. 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. Define the Negative control of Extraction as **C-**, DNA-calibrators as **C1**, **C2**. Set the type **Unknown** opposite all the test samples, the type **Negative control** – for the Negative control of amplification. Set the type **None** for the cells matching with the corresponding empty tubes. Click the **Finish/OK** button.

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

Note – To edit the table of samples before the start it is needed previously to select the **Edit Samples Before Run Started** option in the **User preferences** submenu of the **File** menu.

Data analysis:

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

1. Activate the button **Analysis** in the menu, select the mode of the analysis **Quantitation**, activate the **Cycling A. FAM/Cycling A. Green**, **Show** buttons.
2. Cancel the automatic choice of the threshold line level **Threshold**.
3. Select the **Linear scale**.
4. Activate the **Dynamic tube** and **Slope Correct** buttons in the menu of main window (**Quantitation analysis**).
5. In the **CT Calculation** menu (in the right part of the window) indicate the threshold line level **0.03** in the **Threshold** box.
6. Choose the parameter **More settings/Outlier Removal** and set **10%** for the value of negative samples threshold (**NTC/Threshold**).
7. In the results grid (the **Quantitation Results** window) one will be able to see the *Ct* values.

Results analysis in the JOE/Yellow, ROX/Orange, Cy5/Red, Cy5.5/Crimson is carried out similarly to results analysis in the FAM/Green channel in accordance with the settings in the table below.

Channel	Threshold	Dynamic tube/	Slope Correct	More Settings/Outlier Removal
FAM/Green	0.03	on	on	10%
JOE/Yellow	0.03	on	on	10%
ROX/Orange	0.03	on	on	7%
Cy5/Red	0.03	on	on	10%
Cy5.5/Crimson	0.03	on	on	15%

If the fluorescence curves in the FAM/Green, JOE/Yellow, ROX/Orange, **NOTE:** Cy5/Red channels do not correspond to the exponential growth, then **NTC threshold** value can be increased up to 15 %.

8. In the results grid (**Quantitation Results** window), select the **Name** column by clicking on its title. Copy the column using the **Copy** command from the context menu (activated by the right mouse button).
9. Open the **AmpliSens® HPV HCR screen-14-titre** Microsoft® Excel file provided on the disc with the reagent kit and agree to activate macro.
Note – If macro is not enabled (the **Results** button is not activated) when an Excel document is opened, security level must be changed. To do this, select **Service>Macro>Security** and set the middle security level.
10. Point the cursor on the **Name** cell and select the **Paste** command from the context menu.
11. Similarly, select the **Ct** column from the results grid (**Quant. Results**). Set the cursor in the Excel table in the **CT** cell under the name of corresponding fluorescent dye in the **Threshold cycle** column.
12. Repeat the procedure for all other channels.
13. Save the Microsoft® Excel file under another name.
14. To ensure correct data processing, check that data in the **Name** column are set correctly. Mark the calibrators as **C1, C2** and the negative controls as **C-, NCA**.
15. Click the **Results** button after entering all the data in the Excel data matrix. The results of data processing will appear in the **Results** column.

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (see the *Instruction manual*) and boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

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

Manual and the *Important Product Information Bulletin* enclosed to the PCR kit.

Possible problems and special aspects of results analysis with the use of Rotor-Gene 3000/6000/Q software

Possible problems	Reason	Characters	Ways to eliminate
Negative samples are analyzed as positive by Rotor-Gene software	Incorrect mathematical processing of negative samples in the presence of the fluorescence decrease section at the initial cycles	A typical positive sample has a characteristic S-shaped curve of fluorescence accumulation. Incorrectly processed negative samples are viewed as quite straight bottom-up lines	One must use the Ignore First by selecting the value 5 cycles. If it does not lead to a proper result, try to increase this value by 1 - 5.
	The threshold line crosses descending fluorescence curves at the initial cycles	The red threshold line crosses (or touches) fluorescence curves at the left (initial cycles)	Use the Eliminate cycles before ... option by setting 5 (crossing the threshold and the fluorescence curve at the first 5 cycles is ignored)
The absence of the fluorescence curves when the fluorescence values are less than 1 or more than 100	The autocalibration parameter from 5FI to 10FI is not set or it is a failure of the first tube in the rotor (tube is absent, DNA sample or reaction mixture is added incorrectly)	Most of fluorescence background signals are less than 1 or greater than 20	Repeat the PCR after setting the parameters according to the information list "Calibration priorities for the AmpliSens kits for Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN, Germany) thermocyclers"

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.

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 start window it is necessary to select the **Create a new Run** (or select **New** and then **Run...** in the **File** menu). Click **OK**.
2. In the **Run Setup** window, select **Protocol** tab and click the **Create new....** Set the amplification parameters (time, temperature, cycles, and fluorescence acquiring cycle) in the opened **Protocol Editor – New** window. Set **Sample Volume – 25 µl**

AmpliSens unified amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of cycles
1	50	15 min	-	1
2	95	15 min	-	1
3	95	10 s	-	45
	60	20 s	FAM, HEX, ROX, Cy5, Quasar705	

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**.



1	50,0	C for 15:00
		Slow Ramp Rate to 2,5 C per second
2	95,0	C for 15:00
		Slow Ramp Rate to 2,5 C per second
3	95,0	C for 0:10
		Slow Ramp Rate to 2,5 C per second
4	60,0	C for 0:20
		+ Plate Read
		Slow Ramp Rate to 2,5 C per second
5	GOTO 3	, 44 more times

Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program. If only the tests for pathogen agent DNA detection are performed in one instrument then the first step of reverse transcription (50 °C – 15 minutes) can be deleted from the program for time saving.

NOTE:

AmpliSens-1 amplification program

Step	Temperature, °C	Time	Fluorescence detection	Number of 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, Quasar705	
	72	15 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**.

1	95,0 C for 15:00
2	95,0 C for 0:05
	Slow Ramp Rate to 2,5 C per second
3	60,0 C for 0:20
	Slow Ramp Rate to 2,5 C per second
4	72,0 C for 0:15
	Slow Ramp Rate to 2,5 C per second
5	GOTO 2 , 4 more times
6	95,0 C for 0:05
	Slow Ramp Rate to 2,5 C per second
7	60,0 C for 0:30
	+ Plate Read
	Slow Ramp Rate to 2,5 C per second
8	72,0 C for 0:15
	Slow Ramp Rate to 2,5 C per second
9	GOTO 6 , 39 more times
	END

NOTE: **AmpliSens-1** is an universal program for conducting tests for identifying *human papillomaviruses (HPV HCR)* and detection of STIs and other infections of reproductive system with AmpliSens PCR kits. Therefore, any combination of these tests can be carried out simultaneously in the same instrument.

3. Save the protocol: in the **Protocol Editor New** window select **File**, then **Save As**, name the file and click **Save**.
4. Set the plate setup: in the **Plate** tab click the **Create new...** button. Set the tube order in the opened **Plate Editor – New** window. Click the **Select Fluorophores...** button and click the **Selected** checkbox next to the **FAM, HEX, ROX, Cy5, Quasar 705** fluorophores and click **OK**. In the **Sample type** menu select **Unknown** for all the samples. Then in the **Load** column (in the right part of the window) tick the fluorescence signal acquiring for all the samples in the required channels. Define sample names in the **Sample name** window, moreover the **Load** function is to be ticked
5. Save the plate setup: select **File** and then **Save as** in the **Plate Editor New** window. Enter the file name, click **Save**.
6. Select the **Start Run** tab. Open the lid of the instrument by the **Open Lid** button. Insert

the reaction tubes in the wells of the instrument in accordance with the entered plate setup. Close the lid by the **Close Lid** button.

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

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

Using the ready template for the run

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

- click the **Select Existing...** button in the **Run Setup** window of the **Protocol** tab. Select the needed file with the amplification program in the **Select Protocol** window. Click **Open**.
- go to the **Plate** tab in the **Run Setup** window. Click the **Select Existing...** button. Select the needed file with the plate setup in the **Select Plate** window. Click **Open**. Click the **Edit selected** button to edit the plate setup.

Data analysis

The obtained results are analyzed by the software of the CFX96 instrument. The results are interpreted according to the crossing (or not-crossing) of the S-shaped (sigmoid) fluorescence curve with the threshold line set at the specific level, that corresponds to the presence (or absence) of the *C_t* (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_t* 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 C2 sample in the last amplification cycle. Make sure that the fluorescence curve of the Positive control has the typical exponential

growth of fluorescence. To select the curve of C1 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. Click by right mouse button on the **Well** cell in the results grid.
5. Select all the content of the results grid and copy it.
6. Open the **AmpliSens® HPV HCR screen-titre14** Microsoft Excel file provided on the disc with the reagent kit and agree to activate macro.

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

7. Copy the samples names from the instrument' file into the file of **AmpliSens® HPV HCR screen-titre-14** software. Copy the *Ct* values the instrument' file into the file of the software. Check the calibrators values indicated in the software. Those values must be the same as indicated in the *Important Product Information Bulletin*. Check the correctness and add letterings of the samples. Check the correctness and supplement the samples names. To ensure correct data processing, check that data in the **Name** column are set correctly. Mark the calibrators as **C1**, **C2** and the negative controls as **C-**, **NCA**.
8. Save the Microsoft® Excel file under another name.
9. Click the **Results** button after entering all the data in the Excel data matrix. The results of data processing will appear in the **Results** column.

Results interpretation

The result of the PCR analysis is considered reliable only if the results for the controls of the amplification and the extraction are correct in accordance with the table of assessment of results for controls (see the *Instruction manual*) and boundary values specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The interpretation of the test samples is to be carried out in accordance with the *Instruction Manual* and the *Important Product Information Bulletin* enclosed to the PCR kit.

Possible problems and special aspects of results analysis with the use of Bio-Rad

CFX Manager software

Possible problems	Characters	Ways to eliminate
The threshold level is set incorrectly	The threshold line passes with the negative samples or above some or all positive curves (curves are S-shaped)	Set the threshold line so that it crosses only the sigmoid curves of the fluorescence accumulation or at the level of $\frac{1}{4}$ of height between the final fluorescence values of positive and negative 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	Repeat the amplification for the sample

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
06.03.18 PM	Footer	REF R-V72-F-CE was changed to REF H-2311-1-13-CE
21.03.19 SK	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) instruments	The information about Dynamic tube option was added to the table with settings
25.07.19 DV	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) instruments	The value of Outlier Removal for channel Cy5.5/Crimson was changed to 15%
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