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For Professional Use Only

GUIDELINES

to AmpliSens® Genoscreen HLA B*5701-FRT PCR kit

for qualitative detection of B locus 5701 allele of human major histocompatibility complex (HLA B*5701) in the clinical material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

AmpliSens®

EC REP

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Not for use in the Russian Federation

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® Genoscreen HLA B*5701-FRT** PCR kit for qualitative detection of B locus 5701 allele of human major histocompatibility complex (HLA B*5701) in the clinical material (whole blood, oropharyngeal 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);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA);
- Mx3000P, Mx3005P (Stratagene, USA).

AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) 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 0.2-ml clear tubes with flat caps (detection through the bottom of the tube) or 0.1-ml tubes.

Insert the tubes into the rotor of the Rotor-Gene 3000/6000 instrument beginning from the first well, insert the rotor into the instrument, and close the lid (the rotor wells are numbered, the numbers are used for the further programming of the samples' order in the thermocycler).

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 Rotor-Gene 3000/6000 instrument

1. Click the ***New*** button in the software main menu.
2. In the opened window, select the template of the experiment start-up ***Advanced*** and mark ***Dual Labeled Probe/Hydrolysis probes***. Click the ***New*** button.
3. In the opened window, select the ***36-Well Rotor*** (or ***72-Well Rotor***) and tick ***No Domed 0.2 ml Tubes*** (for Rotor-Gene 3000) ***/ Locking ring attached*** (for Rotor-Gene 6000). Click ***Next***.
4. In the opened window, set the operator and select the reaction mixture volume: ***Reaction volume*** – **25 µl**. For Rotor-Gene 6000, tick the ***15 µl oil layer volume*** option. Click ***Next***.
5. In the opened window, set the temperature profile of the experiment. To do this click the ***Edit profile*** button and set the following parameters:

Amplification program for rotor-type instruments

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycles*
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
Cycling	95	5 s	–	40
	60	40 s	FAM/Green, JOE/Yellow	

6. Click the ***OK*** button.
7. In the ***New Run Wizard*** window, click the ***Calibrate/Gain Optimisation…*** button.
   - perform calibration in the FAM/Green and JOE/Yellow channels (click the ***Calibrate Acquiring/Optimise Acquiring*** button);
   - perform calibration before the first measurement (***Perform Calibration Before 1st Acquisition/Perform Optimisation Before 1st Acquisition***);
   - set channel calibration for all dyes from 5Fl to 10Fl (the ***Edit…*** button, ***Auto gain calibration channel settings*** window). Click the ***Close*** button.
8. Click the ***Next*** button, start the amplification by the ***Start run*** button.
9. Name the experiment and save it on a disc (the results of the experiment will be automatically saved in this file).
10. Enter the data into the grid of the samples (it opens automatically after the amplification has been started). Enter the names/numbers of test and control samples in the ***Name*** column. For empty wells, set the type ***None***.

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

Data analysis

The amplification results of the HLA B*5701 DNA fragment and IC are analyzed. Accumulation of the amplification product of the HLA B*5701 DNA fragment is detected in the JOE/Yellow channel; accumulation of the IC amplification product is detected in the FAM/Green channel.

Amplification data analysis of IC (endogenous internal control):

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the button ***Cycling A. FAM/Cycling A. Green***, ***Show***.
2. Cancel the automatic choice of the threshold line level ***Threshold***.
3. Activate the ***Dynamic tube*** and the ***Slope Correct*** buttons in the main window menu (***Quantitation analysis***).
4. In the ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level ***Threshold* = 0.03**.
5. Choose the parameter ***More settings/Outlier Removal*** and set **10 %** for the value of

negative samples threshold (***NTC/Threshold***).

6. In the results grid (the ***Quantitation Results*** window) one will be able to see the *Ct* values.

Amplification data analysis of HLA B*5701 DNA:

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the button ***Cycling A. JOE/Cycling A. Yellow, Show***.
2. Cancel the automatic choice of the threshold line level ***Threshold***.
3. Activate the ***Dynamic tube*** and the ***Slope Correct*** buttons in the main window menu (***Quantitation analysis***).
4. In the ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level ***Threshold* = 0.03**.
5. Choose the parameter ***More settings/Outlier Removal*** and set **20 %** for the value of negative samples threshold (***NTC/Threshold***).
6. In the results grid (the ***Quantitation Results*** window) one will be able to see the *Ct* values.

Results interpretation of control samples

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see Table 1).

Table 1

Results for controls for different steps of PCR-analysis (Rotor-Gene)

Control	Stage for control	Ct in channel	
		FAM /Green	JOE/Yellow
C–	DNA extraction	> boundary value or absent	absent
NCA	PCR	absent	absent
C+	PCR	< boundary value	< boundary value

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

Results interpretation of test clinical samples

1. The sample is considered to be **positive** if a positive result is obtained in the JOE/Yellow channel and the *Ct* value exceeds the value in the FAM/Green channel not more than 5 cycles.
2. The sample is considered to be **negative** if either a negative result is obtained in the

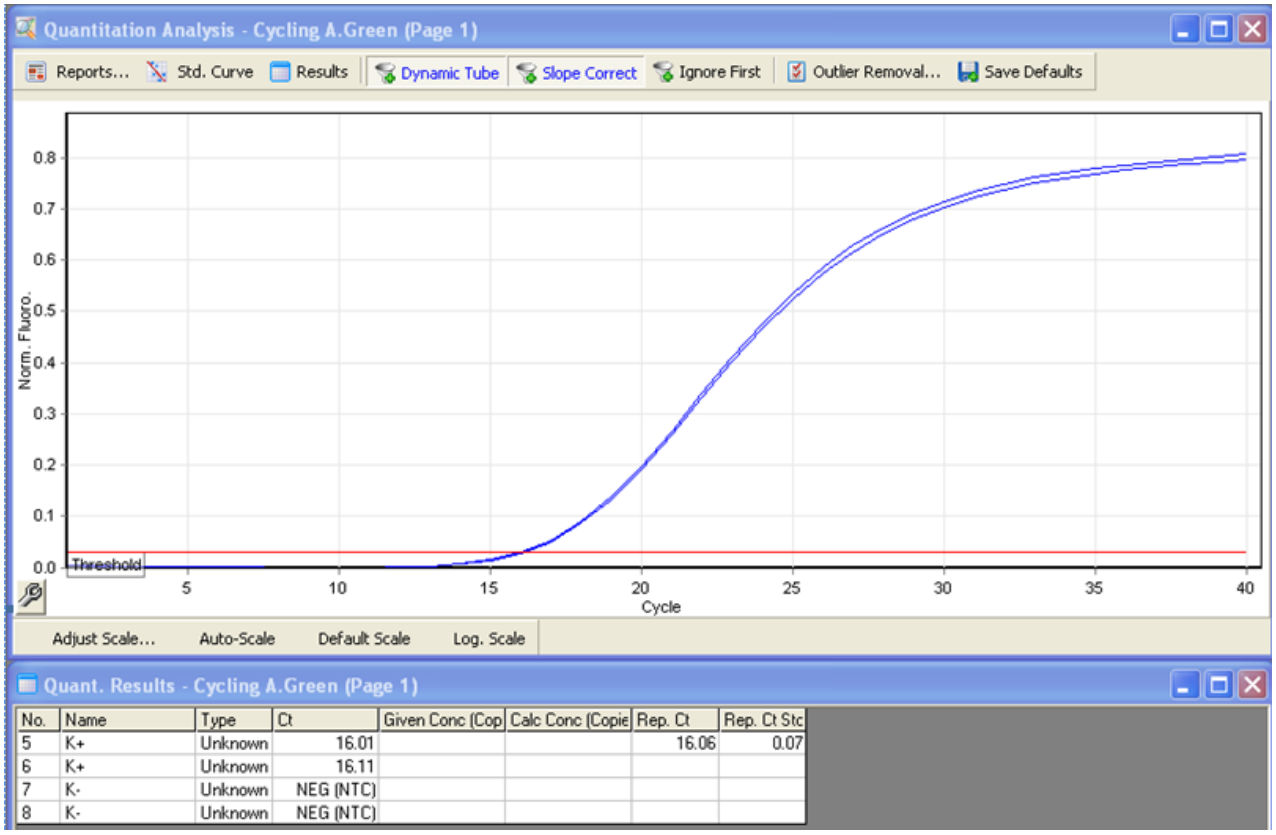
JOE/Yellow channel or the *Ct* value exceeds the value in the FAM/Green channel more than 5 cycles.

Troubleshooting

1. If the positive signal is absent in any channel for the Positive Control of amplification (C+), this may be due to errors in the PCR run. PCR should be repeated.
2. If the *Ct* value is absent for the clinical sample in the channel for IC detection, this may be due to incorrect clinical material pretreatment and subsequent loss of DNA or inhibition of PCR. The analysis should be repeated starting from the DNA extraction stage.
3. If *Ct* value determined for the clinical sample in the channel for IC detection is greater than the *Ct* value specified in the *Important Product Information Bulletin*, the sample is considered **equivocal**. This may be due to incorrect clinical material pretreatment and subsequent loss of DNA or inhibition of PCR. The analysis should be repeated starting from the DNA extraction stage.
4. If the positive signal is detected for the Negative Control of amplification (NCA) in any channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples are considered **invalid**. It is necessary to repeat the analysis and to take measures to detect and eliminate the source of contamination.
5. If the positive signal is detected for the Negative Control of extraction (C–) in the JOE/Yellow channel (HLA B*5701) or the Ct value determined in the FAM/Green channel is less than the value specified in the *Important Product Information Bulletin*, the results of analysis of all samples are considered **invalid**. It is necessary to repeat the analysis and to take measures to detect and eliminate the source of contamination.

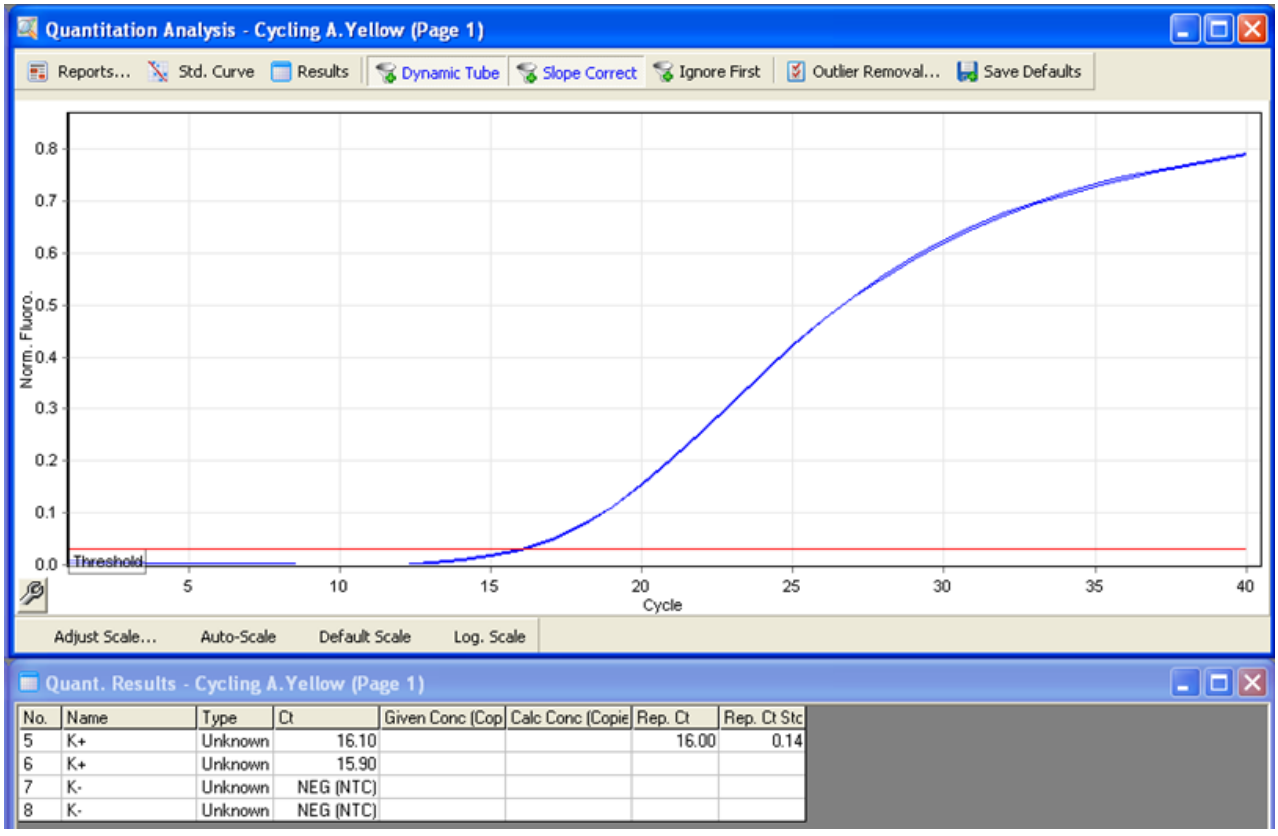
Example of obtained results

Data in the FAM/Green channel (IC):



No.	Name	Type	Ct	Given Conc (Cop	Calc Conc (Copie	Rep. Ct	Rep. Ct Stc
5	K+	Unknown	16.01			16.06	0.07
6	K+	Unknown	16.11				
7	K-	Unknown	NEG (NTC)				
8	K-	Unknown	NEG (NTC)				

Data in the JOE/Yellow channel (samples containing HLA B*5701 DNA):



No.	Name	Type	Ct	Given Conc (Cop	Calc Conc (Copie	Rep. Ct	Rep. Ct Stc
5	K+	Unknown	16.10			16.00	0.14
6	K+	Unknown	15.90				
7	K-	Unknown	NEG (NTC)				
8	K-	Unknown	NEG (NTC)				

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

1. Switch on the instrument, start the iCycler/iQ5 program.

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

2. Insert the tubes or strips (part of the plate) or the plate into the reaction module of the thermocycler and program the instrument

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

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

1. Click the ***Create New*** button in the ***Workshop*** module in order to create a new protocol.
2. Set the amplification parameters in the opened window.

Amplification program for plate-type instruments

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Cycle repeats***
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
3	95	5 s	–	40
	60	50 s	FAM, JOE/HEX	

3. Name the new protocol and save it.
4. Create a new plate of samples (***Plate Setup***). Set the order of the tubes in the plate.
5. In the opened window set all the clinical samples as ***Unknown.*** For all samples, set the fluorescence measurement in 2 channels: FAM and JOE/HEX.
6. Set the reaction volume *(**Sample Volume**)* **25 µl,** type of caps *(**Domed Caps**),* and the type of tubes *(**Vessel Type***). It is necessary to perform amplification with the use of the same type of plastics as plastics for calibration. Save the scheme of the plate.
7. Click the ***Run*** button. In the opened window click the ***Use Persistent Well Factors*** button, then click the ***Begin Run*** button and save the experiment.

Data analysis

The amplification results of the HLA B*5701 DNA fragment and IC are analyzed. Accumulation of the amplification product of the HLA B*5701 DNA fragment is detected in the JOE/HEX channel; accumulation of the IC amplification product is detected in the FAM channel. It is recommended to analyze the results obtained with the iCycler iQ instrument using the iQ5 instrument software.

Data processing

1. Start the software and open the saved file: select ***Data file*** in the ***Workshop*** module and select data file. Proceed to the ***Data Analysis*** mode.
2. View data separately for each channel.
3. Ensure that the automatic selection of threshold level is correct. The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline. Otherwise the threshold level should be raised. To do this, select ***Log View*** and set threshold line by left mouse button on level, where fluorescence curves are linear and do not cross curves of negative samples.
4. To analyze the results, select the ***Results*** button (it is above the buttons with fluorophores names).

Results interpretation of control samples

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see Table 2).

Table 2

Results for controls for different steps of PCR-analysis (Bio-Rad)

Control	Stage for control	Ct in channel	
		FAM	JOE /HEX
C–	DNA extraction	> boundary value or absent	absent
NCA	PCR	absent	absent
C+	PCR	< boundary value	< boundary value

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

Interpretation of results for clinical samples

1. The sample is considered to be **positive** if a positive result is obtained in the JOE/HEX channel and the *Ct* value exceeds the value in the FAM channel not more than 5 cycles.
2. The sample is considered to be **negative** if either a negative result is obtained in the JOE/HEX channel or the *Ct* value exceeds the value in the FAM channel more than 5 cycles.

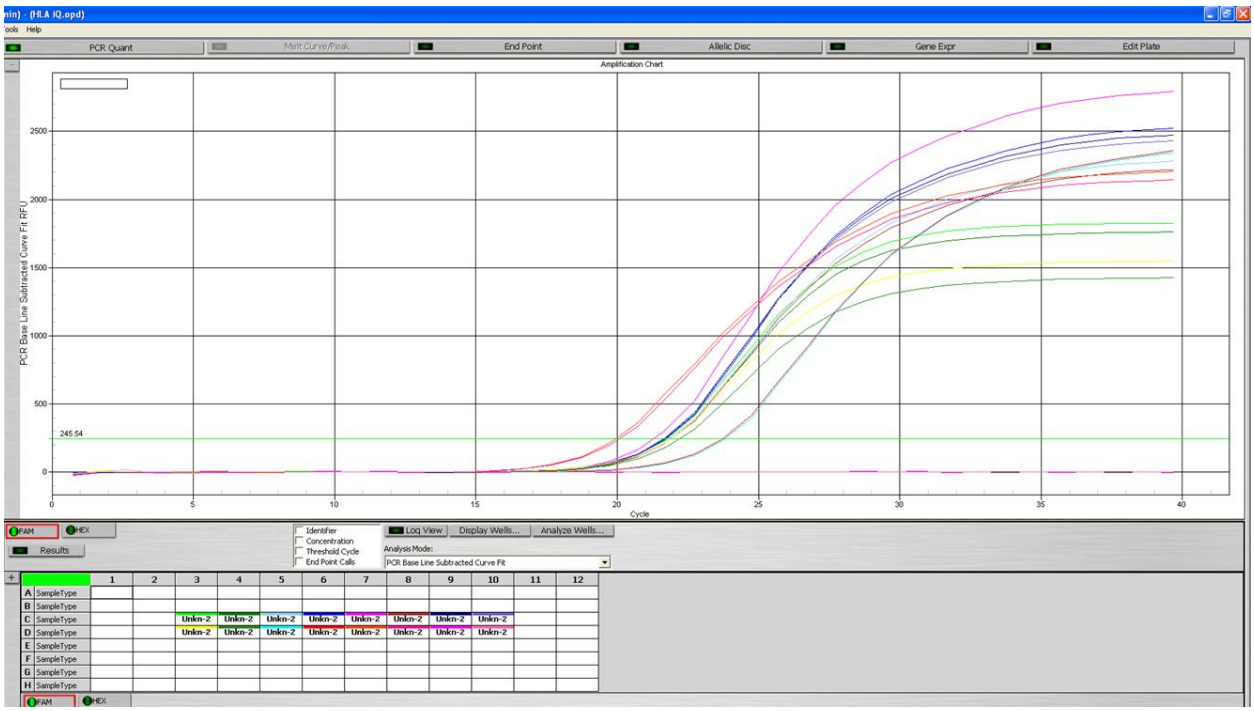
Troubleshooting

1. If the positive signal is absent in any channel for the Positive Control of amplification (C+), this may be due to errors in the PCR run. PCR should be repeated.

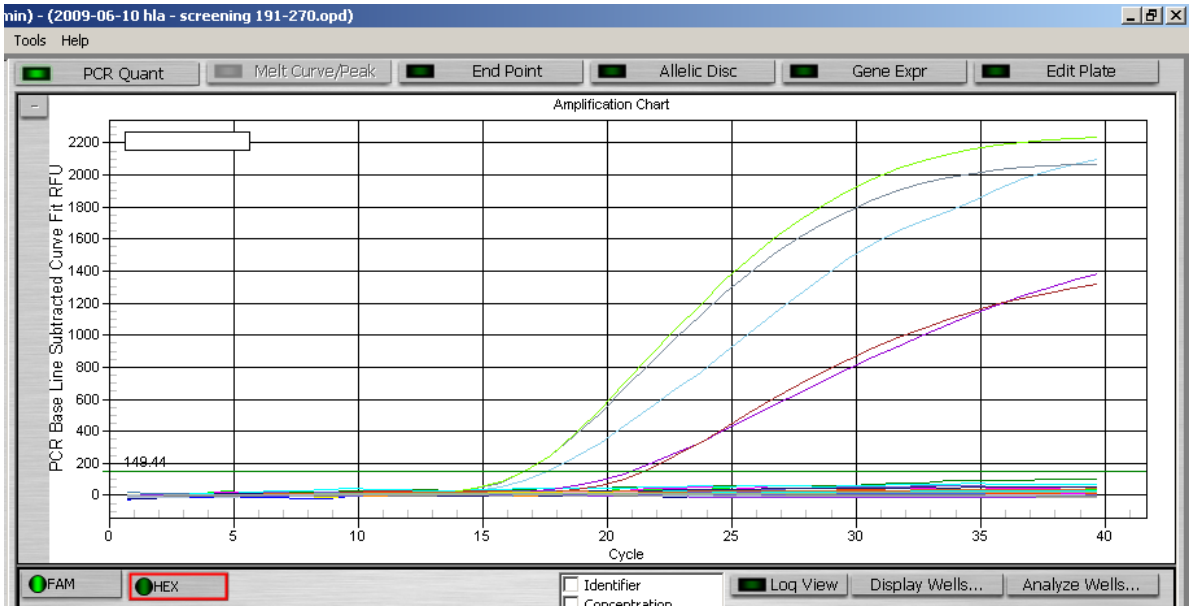
2. If the *Ct* value is absent for the clinical sample in the channel for IC detection, this may be due to incorrect clinical material pretreatment and subsequent loss of DNA or inhibition of PCR. The analysis should be repeated starting from the DNA extraction stage.
3. If *Ct* value determined for the clinical sample in the channel for IC detection is greater than the *Ct* value specified in the *Important Product Information Bulletin*, the sample is considered **equivocal**. This may be due to incorrect clinical material pretreatment and subsequent loss of DNA or inhibition of PCR. The analysis should be repeated starting from the DNA extraction stage.
4. If the positive signal is detected for the Negative Control of amplification (NCA) in any channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples are considered **invalid**. It is necessary to repeat the analysis and to take measures to detect and eliminate the source of contamination.
5. If the positive signal is detected for the Negative Control of extraction (C–) in the JOE/HEX channel (HLA B*5701) or the *Ct* value determined in the FAM channel is less than the value specified in the *Important Product Information Bulletin*, the results of analysis of all samples are considered **invalid**. It is necessary to repeat the analysis and to take measures to detect and eliminate the source of contamination.

Example of obtained results

Data in the FAM channel – IC



Data in the JOE/HEX channel for the samples containing HLA B*5701 DNA:



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

1. Switch the instrument on, start the program 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, lock the fixing arm and the door of the instrument.
4. Select ***Optics Configuration*** in the ***Options*** menu and in the ***Dye Assignment*** tab set FAM parameter next to the ***FAM filter set*** item and JOE parameter next to the ***HEX/JOE filter set***.
5. Set the fluorescence detection parameters in the ***Plate Setup*** menu. To do this:
   - select all the wells with the test tubes or strips;
   - mark all selected wells as ***Unknown*** in the ***Well type*** window. Select FAM and JOE/HEX fluorophores for the ***Collect fluorescence data*** option.
6. Name each sample in the ***Well Information*** window.
7. Set amplification program in ***Thermal Profile Setup*** tab.

Amplification program for plate-type instruments

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Cycle repeats*
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
3	95	5 s	–	40
	60	50 s	FAM, JOE/HEX (all points)	

8. Select the ***Run*** button, then the ***Start*** button, and name the file.

Data analysis

The amplification results of the HLA B*5701 DNA fragment and IC are analyzed. Accumulation of the amplification product of the HLA B*5701 DNA fragment is detected in the JOE/HEX channel; accumulation of the IC amplification product is detected in the FAM channel.

Data processing

1. Proceed to the ***Analysis*** mode.
2. Make sure that all samples in the ***Analysis Selection/Setup*** tab are active (wells corresponding to samples should have another color).
3. Proceed to the ***Results*** tab.
4. Make sure that the automatic selection of the threshold level for each channel is correct. Normally, the threshold line should cross only the sigmoid curves of signal accumulation of positive samples and controls and should not cross the baseline. Otherwise, the threshold level should be raised. To do this, activate each channel image separately in the ***Dyes shown*** lower panel. Look at the threshold line location and change it if necessary.

Results interpretation of control samples

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see Table 3).

Table 3

Results for controls for different steps of PCR-analysis

Control	Stage for control	Ct in channel	
		FAM	JOE /HEX
C–	DNA extraction	> boundary value or absent	absent
NCA	PCR	absent	absent
C+	PCR	< boundary value	< boundary value

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

Interpretation of results for clinical samples

1. The sample is considered to be **positive** if a positive result is obtained in the JOE/HEX channel and the *Ct* value exceeds the value in the FAM channel not more than 5 cycles.
2. The sample is considered to be **negative** if either a negative result is obtained in the

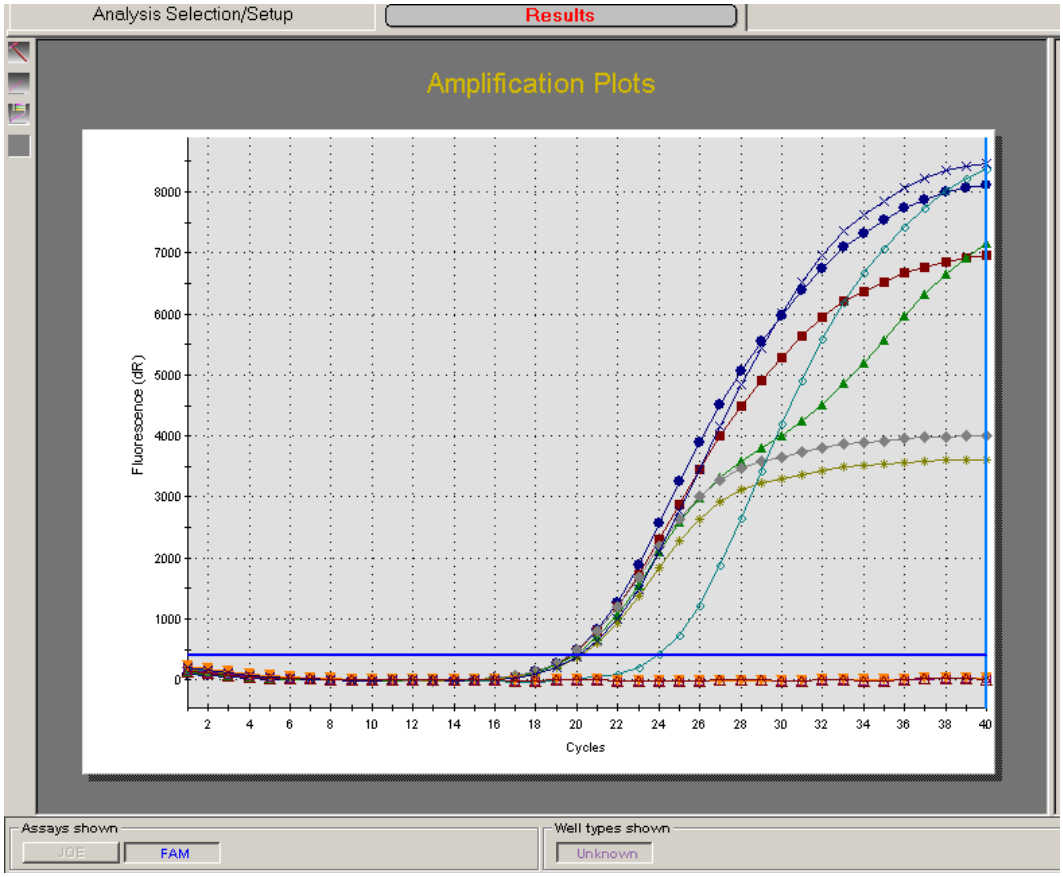
JOE/HEX channel or the *Ct* value exceeds the value in the FAM channel more than 5 cycles.

Troubleshooting

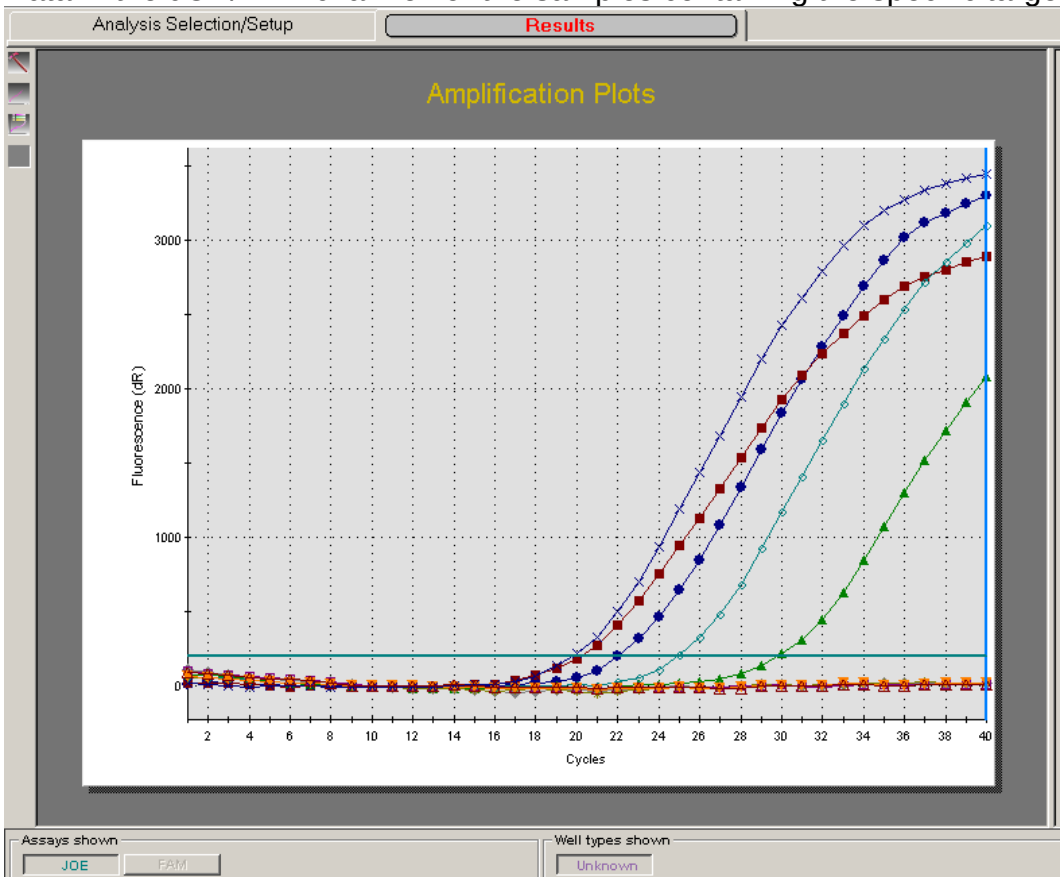
1. If the positive signal is absent in any channel for the Positive Control of amplification (C+), this may be due to errors in the PCR run. PCR should be repeated.
2. If the *Ct* value is absent for the clinical sample in the channel for IC detection, this may be due to incorrect clinical material pretreatment and subsequent loss of DNA or inhibition of PCR. The analysis should be repeated starting from the DNA extraction stage.
3. If *Ct* value determined for the clinical sample in the channel for IC detection is greater than the *Ct* value specified in the *Important Product Information Bulletin*, the sample is considered **equivocal**. This may be due to incorrect clinical material pretreatment and subsequent loss of DNA or inhibition of PCR. The analysis should be repeated starting from the DNA extraction stage.
4. If the positive signal is detected for the Negative Control of amplification (NCA) in any channel, this indicates the contamination of reagents or samples. In this case, the results of analysis of all samples are considered **invalid**. It is necessary to repeat the analysis and to take measures to detect and eliminate the source of contamination.
5. If the positive signal is detected for the Negative Control of extraction (C–) in the JOE/HEX channel (HLA B*5701) or the *Ct* value determined in the FAM channel is less than the value specified in the *Important Product Information Bulletin*, the results of analysis of all samples are considered **invalid**. It is necessary to repeat the analysis and to take measures to detect and eliminate the source of contamination.

Example of obtained results

Data in the FAM channel (Internal Control):



Data in the JOE/HEX channel for the samples containing the specific target:



List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
23.06.11 RT	Cover page	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”
19.03.12 LA	Amplification and data analysis using Rotor-Gene 3000 and Rotor-Gene 6000 (Corbett Research, Australia)	Fig. “Example of obtained result” is corrected
		The *NTC threshold* parameter is changed from 10% to 15% in “Amplification data analysis of HLA B*5701 DNA” item
04.07.13 ME	Footer	**REF** R-O2(RG,iQ)-CE-B was deleted
18.09.15 ME	Text	Corrections according to the template. Information about result interpretation and troubleshooting for C– was corrected. The example of obtained results for the FAM/Green channel was added for the Rotor-Gene 3000/6000 anf iCycler iQ and iCycler iQ5 instruments
27.02.20 PM	Front page	The phrase “Not for use in the Russian Federation” was added
29.12.20 KK	Through the text	The symbol  was changed to NOTE:
01.03.21 EM	Front page	The name, address and contact information for Authorized representative in the European Community was changed