

# **GUIDELINES**

## **to AmpliSens<sup>®</sup> Genoscreen-*IL28B*-FRT**

### **PCR kit**

for qualitative detection of the single-nucleotide polymorphism (SNP) rs8099917 and rs12979860 in the Interleukin-28B gene (*IL28B*) in the biological material (whole blood or swabs collected from the internal cheek surface (buccal epithelium)) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

# **AmpliSens<sup>®</sup>**



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## INTENDED USE

The guidelines describe the procedure of using **AmpliSens® Genoscreen-IL28B-FRT** PCR kit for qualitative detection of the single-nucleotide polymorphism (SNP) rs8099917 and rs12979860 in the Interleukin-28B gene (*IL28B*) in the biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using the following instruments:

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

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

When working with Rotor-Gene 3000 one should use the Rotor-Gene 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 thermocycler

1. Switch the instrument on.
2. Insert the tubes into the carousel of the Rotor-Gene 3000/6000/Q instrument. First tube should be inserted into well 1. Inert the carousel into the instrument, close the cap (the carousel cells are numbered; the numbers are used for the further programming of the samples' position in the thermocycler). Program the instrument.

**NOTE:** Well 1 must be filled with tube from current run containing reaction mixture "rs17".

3. Click the **New** button in the main menu.
4. In the opened window, select the template of the experiment start-up **Advanced** and mark **Dual Labeled Probe/Hydrolysis probes**. Click the **New** button.
5. In the opened window, select **36-Well Rotor** (or **72-Well Rotor**) and **No Domed 0.2 ml Tubes/Locking ring attached**. Click **Next**.

6. In the opened window, set the operator and select the reaction mixture volume: **Reaction volume – 25 µl**. For Rotor-Gene 6000/Q set check in front of **15 µl oil layer volume**. Click **Next**.
7. In the opened window, set the temperature profile of experiment: click the **Edit profile** button and set the following parameters (see Table 1).

Table 1

### The amplification program for rotor-type instruments

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

8. Click the **OK** button.
9. Select the **Calibrate/Gain Optimisation** button in the **New Run Wizard** window:
  - perform the calibration in FAM/Green, JOE/Yellow and ROX/Orange channels (activate the **Calibrate Acquiring/Optimise Acquiring** button);
  - perform the calibration in FAM/Green, JOE/Yellow and ROX/Orange channels before the first detection (activate the **Perform Calibration Before 1<sup>st</sup> Acquisition/ Perform Optimisation Before 1<sup>st</sup> Acquisition** button);
  - to set channels calibration, indicate **5** in the **Min Reading** box and **10** in the **Max Reading** box for all dyes (activate **Edit...**, the window **Auto gain calibration channel settings**).
10. Click the **Next** button. Start the amplification program by activating the **Start Run** button.
11. Name the experiment and save it to the disc (the experiment results 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**. Set the type **None** for the cells matching with the corresponding empty tubes.

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

### Data analysis in the FAM/Green channel

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 by activating the **Threshold** button.

3. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the menu of main window (***Quantitation analysis***).
4. In the ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level **0.03** in the ***Threshold*** box.
5. Choose the parameter ***More settings/Outlier Removal*** and set **30 %** for the value of negative samples threshold (***NTC/Threshold***).
6. In the results grid (the ***Quant. Results*** window) one will be able to see the *Ct* values.

#### **Data analysis in the JOE/Yellow channel**

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 by activating the ***Threshold*** button.
3. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the menu of main window (***Quantitation analysis***).
4. In the ***CT Calculation*** menu (in the right part of the window) indicate the threshold line level **0.03** in the ***Threshold*** box.
5. Choose the parameter ***More settings/Outlier Removal*** and set **30 %** for the value of negative samples threshold (***NTC/Threshold***).
6. In the results grid (the ***Quant. Results*** window) one will be able to see the *Ct* values.

#### **Data analysis in the ROX/Orange channel**

1. Activate the button ***Analysis*** in the menu, select the mode of the analysis ***Quantitation***, activate the button ***Cycling A. ROX/Cycling A. Orange, Show***.
2. Cancel the automatic choice of the threshold line level by activating the ***Threshold*** button.
3. Activate the ***Dynamic tube*** and ***Slope Correct*** buttons in the menu of main window (***Quantitation analysis***).
4. In the ***Calculation*** menu (in the right part of the window) indicate the threshold line level **0.03** in the ***Threshold*** box.
5. Choose the parameter ***More settings/Outlier Removal*** and set **30 %** for the value of negative samples threshold (***NTC/Threshold***).
6. In the results grid (the ***Quant. Results*** window) one will be able to see the *Ct* values.

## Results interpretation of test samples

### A. Interpretation of results, obtained with the use of reaction mixture “rs17”

1. If in the results grid for the sample with given reaction mixture the *Ct* value is defined only in the **FAM/Green and ROX/Orange** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX/Orange** channel, then the result for SNP **rs8099917** is given as “**Genotype TT is detected**”.
2. If in the results grid for the sample with given reaction mixture the *Ct* value is defined only in the **JOE/Yellow and ROX/Orange** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX/Orange** channel, then the result for SNP **rs8099917** is given as “**Genotype GG is detected**”.
3. If in the results grid for the sample with given reaction mixture the *Ct* value is defined in the **FAM/Green, JOE/Yellow and ROX/Orange** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX/Orange** channel, then the result is given as “**Genotype TG is detected**” only if the *Ct* value in the **FAM/Green** channel exceeds *Ct* value in the channel for the **JOE/Yellow** fluorophore. If the *Ct* value in the **FAM/Green** channel is less than the *Ct* value in the channel for the **JOE/Yellow** fluorophore then the result in the **JOE/Yellow** channel is not interpreted and the result is given as “**Genotype TT is detected**”.
4. If in the results grid for the sample with given reaction mixture the *Ct* value is not defined in the **FAM/Green** and **JOE/Yellow** channels then it is necessary to repeat the PCR analysis for this sample beginning from the DNA extraction stage.
5. If in the results grid for the sample with given reaction mixture the *Ct* value in the **ROX/Orange** channel exceeds the value specified in the *Important Product Information Bulletin* (regardless of the results obtained in the **FAM/Green** and **JOE/Yellow** channels) then it is necessary to repeat the PCR analysis for this sample beginning from the DNA extraction stage.

### B. Interpretation of results, obtained with the use of reaction mixture “rs60”

1. If in the results grid for the sample with given reaction mixture the *Ct* value is defined only in the **FAM/Green and ROX/Orange** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX/Orange** channel, then the result for SNP **rs12979860** is given as “**Genotype TT is detected**”.
2. If in the results grid for the sample with given reaction mixture the *Ct* value is defined only in the **JOE/Yellow and ROX/Orange** channels and the *Ct* value does not exceed

the value specified in the *Important Product Information Bulletin* in the **ROX/Orange** channel, then the result for SNP **rs12979860** is given as “**Genotype CC is detected**”.

3. If in the results grid for the sample with given reaction mixture the *Ct* value is defined in the **FAM/Green, JOE/Yellow and ROX/Orange** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX/Orange** channel, then the result is given as “**Genotype CT is detected**” only if the *Ct* value in the **FAM/Green** channel exceeds *Ct* value in the **JOE/Yellow** channel. If the *Ct* value in the **FAM/Green** channel is less than the *Ct* value in the **JOE/Yellow** channel then the result in the **JOE/Yellow** channel is not interpreted and the result is given as “**Genotype TT is detected**”.
4. If in the results grid for the sample with given reaction mixture the *Ct* value is not defined in the **FAM/Green** and **JOE/Yellow** channels then it is necessary to repeat the PCR analysis for this sample beginning from the DNA extraction stage.
5. If in the results grid for the sample with given reaction mixture the *Ct* value in the **ROX/Orange** channel exceeds the value specified in the *Important Product Information Bulletin* (regardless of the results obtained in the **FAM/Green** and **JOE/Yellow** channels) then it is necessary to repeat the PCR analysis for this sample beginning from the DNA extraction stage.

## **AMPLIFICATION AND DATA ANALYSIS USING iCycler 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 optically transparent domed or 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 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. Enter the mode of new amplification protocol creation, activating the **Create new** button in the **Selected Plate Setup** window of the **Workshop** module.
2. Set the amplification program in the opened window (see Table 2).

Table 2

**The amplification program for plate-type instruments**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Number of cycles</b>
Hold	95	15 min	–	1
Cycling 1	95	5 s	–	5
	60	20 s	–	
Cycling 2	95	5 s	–	40
	60	50 s	FAM, JOE/HEX, ROX	

3. Name the new protocol and save it by activating the **Save&Exit Protocol Editing** button. Later, for further runs one may select the file containing this program in the **Protocol** box (the protocol files are saved in the **Users** folder on default).
4. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal). To do this click the **Create New** buttons in the **Selected Plate Setup** window of the **Workshop** module. Edit the plate setup in the **Whole Plate loading** mode.
5. Set all the test samples as **Unknown** in opened window. Select the fluorescent signal detection through the FAM, JOE/HEX and ROX channels in the **Select and load Fluorophores** option.
6. Set the reaction volume (**Sample Volume**) as **25 µl**, the caps type (**Seal Type**) as **Domed Cap**, and the tubes type (**Vessel Type**) as **Tubes**. The amplification should be carried out with the use of the same plastic type with which the instrument calibration was carried out. Save the set plate setup by clicking the **Save&Exit Plate Editing** button.
7. Click the **Run** button. Mark **Use Persistent Well Factors** in opened window. Click the **Begin Run** button and save the experiment.
8. Proceed to the results analysis at the end of the program.

**Data analysis:**

1. Start the program and open the saved file. To do this, select the needed file for the analysis in the **Data File** window of the **Workshop** module, and click the **Analyze** button.
2. Select the **PCR Base Line Subtracted Curve Fit** mode of data analysis (selected on

default).

3. The data for each channel are to be browsed separately.

Data analysis for each PCR-mix-1 is to be carried out **individually (!)** by selecting area of the tubes corresponding to the given PCR-mix. To do this,

**NOTE:** activate the **Display Wells** button, the samples studied with the use of PCR-mix-1 leave as active. Make the samples studied with the use of another mix inactive by pressing the left mouse button. Click the **OK** button.

4. For the **FAM, JOE/HEX** and **ROX** channels at a time set the level of the threshold line at the level **30 %** of maximum fluorescence level at the last amplification cycle.
5. Activate the **Results** button which is situated under the buttons with the fluorophores' names for the results analysis with the given PCR-mix-1.
6. Start browsing the results for another PCR-mix-1.

## Results interpretation of test samples

### A. Interpretation of results, obtained with the use of reaction mixture “rs17”

1. If in the results grid for the sample with given reaction mixture the *Ct* value is defined only in the **FAM and ROX** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX** channel, then the result for SNP **rs8099917** is given as “**Genotype TT is detected**”.
2. If in the results grid for the sample with given reaction mixture the *Ct* value is defined only in the **JOE/HEX and ROX** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX** channel, then the result for SNP **rs8099917** is given as “**Genotype GG is detected**”.
3. If in the results grid for the sample with given reaction mixture the *Ct* value is defined in the **FAM, JOE/HEX and ROX** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX** channel, then the result for SNP **rs8099917** is given as “**Genotype TG is detected**” only if the *Ct* value in each **FAM** or **JOE/HEX** channel exceeds the *Ct* value in the **ROX** channel **not more than 10 cycles**. If in one of the **FAM** or **JOE/HEX** channels the *Ct* value exceeds the *Ct* value in the **ROX** channel **more than 10 cycles**, then the result in this channel is not interpreted and the result is given as “**Genotype GG is detected**” or “**Genotype TT is detected**”.
4. If in the results grid for the sample with given reaction mixture the *Ct* value is not defined in the **FAM** and **JOE/HEX** channels then it is necessary to repeat the PCR analysis for this sample beginning from the DNA extraction stage.

5. If in the results grid for the sample with given reaction mixture the *Ct* value in the **ROX** channel exceeds the value specified in the *Important Product Information Bulletin* (regardless of the results obtained in the **FAM** and **JOE/HEX** channels) then it is necessary to repeat the PCR analysis for this sample beginning from the DNA extraction stage.

**B. Interpretation of results, obtained with the use of reaction mixture “rs60”**

1. If in the results grid for the sample with given reaction mixture the *Ct* value is defined only in the **FAM and ROX** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX** channel, then the result for SNP **rs12979860** is given as “**Genotype TT is detected**”.
2. If in the results grid for the sample with given reaction mixture the *Ct* value is defined only in the **JOE/HEX and ROX** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX** channel, then the result for SNP **rs12979860** is given as “**Genotype CC is detected**”.
3. If in the results grid for the sample with given reaction mixture the *Ct* value is defined in the **FAM, JOE/HEX and ROX** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX** channel, then the result is given as “**Genotype CT is detected**” only if the *Ct* value in each **FAM** or **JOE/HEX** channel exceeds the *Ct* value in the **ROX** channel **not more than 10 cycles**. If in one of the **FAM** or **JOE/HEX** channels the *Ct* value exceeds the *Ct* value in the **ROX** channel **more than 10 cycles**, then the result in this channel is not interpreted and the result is given as “**Genotype CC is detected**” or “**Genotype TT is detected**”.
4. If in the results grid for the sample with given reaction mixture the *Ct* value is not defined in the **FAM** and **JOE/HEX** channels then it is necessary to repeat the PCR analysis for this sample beginning from the DNA extraction stage.
5. If in the results grid for the sample with given reaction mixture the *Ct* value in the **ROX** channel exceeds the value specified in the *Important Product Information Bulletin* (regardless of the results obtained in the **FAM** and **JOE/HEX** channels) then it is necessary to repeat the PCR analysis for this sample beginning from the DNA extraction stage.

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

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

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

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

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

Table 3

**Amplification program for plate-type instruments**

<b>Step</b>	<b>Temperature, °C</b>	<b>Time</b>	<b>Fluorescence detection</b>	<b>Number of cycles</b>
Hold	95	15 min	–	1
Cycling 1	95	5 s	–	5
	60	20 s	–	
Cycling 2	95	5 s	–	40
	60	50 s	FAM, HEX, ROX	

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

4. In the ***Protocol Editor New*** window select ***File***, then ***Save As***, and name the protocol. This protocol can be used for further runs by clicking the ***Select Existing...*** button in the ***Protocol*** tab.

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

1. Run the program and open the saved file.
2. The fluorescence curves, plate setup, and results grid with *Ct* values are displayed in the **Quantification** tab (opens on default).
3. Browse data separately for each channel for each PCR-mix-1.

Data analysis for each PCR-mix-1 is to be carried out **individually (!)** by selecting area of the tubes corresponding to the given PCR-mix. To do this, the

**NOTE:** samples studied with the use one of PCR-mix-1 leave as active in the area representing tubes order in the module. Make the samples studied with the use of another mix inactive by pressing the left mouse button.

4. For **FAM**, **HEX** and **ROX** channel at a time set the threshold line at the level of 30 % of maximum fluorescence level obtained in the last amplification cycle with the analyzed PCR-mix-1 in the corresponding channel. For setting the threshold line at the corresponding level drag it with a cursor while pressing the left mouse button.
5. Click the **View/Edit Plate** button on the toolbar and enter sample names in the opened window, if required.
6. Click **Tools** on the toolbar, then **Reports...**, and then save the generated report about run with analyzed PCR-mix-1.
7. Start browsing the results for another PCR-mix-1.

### **A. Interpretation of results, obtained with the use of reaction mixture “rs17”**

1. If in the results grid for the sample with given reaction mixture the *Ct* value is defined only in the **FAM** and **ROX** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX** channel, then the result for SNP **rs8099917** is given as “**Genotype TT is detected**”.
2. If in the results grid for the sample with given reaction mixture the *Ct* value is defined only in the **HEX** and **ROX** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX** channel, then the result for SNP **rs8099917** is given as “**Genotype GG is detected**”.
3. If in the results grid for the sample with given reaction mixture the *Ct* value is defined in the **FAM**, **HEX** and **ROX** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX** channel, then the result for SNP **rs8099917** is given as “**Genotype TG is detected**” only if the *Ct* value in each **FAM** or **HEX** channel exceeds the *Ct* value in the **ROX** channel **not more than 10 cycles**. If in one of the **FAM** or **HEX** channels the *Ct* value exceeds the *Ct* value in the **ROX** channel **more than 10 cycles**, then the result in this channel is not

interpreted and the result is given as “**Genotype GG is detected**” or “**Genotype TT is detected**”.

4. If in the results grid for the sample with given reaction mixture the *Ct* value is not defined in the **FAM** and **HEX** channels then it is necessary to repeat the PCR analysis for this sample beginning from the DNA extraction stage.
5. If in the results grid for the sample with given reaction mixture the *Ct* value in the **ROX** channel exceeds the value specified in the *Important Product Information Bulletin* (regardless of the results obtained in the **FAM** and **HEX** channels) then it is necessary to repeat the PCR analysis for this sample beginning from the DNA extraction stage.

#### **B. Interpretation of results, obtained with the use of reaction mixture “rs60”**

1. If in the results grid for the sample with given reaction mixture the *Ct* value is defined only in the **FAM** and **ROX** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX** channel, then the result for SNP **rs12979860** is given as “**Genotype TT is detected**”.
2. If in the results grid for the sample with given reaction mixture the *Ct* value is defined only in the **HEX** and **ROX** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX** channel, then the result for SNP **rs12979860** is given as “**Genotype CC is detected**”.
3. If in the results grid for the sample with given reaction mixture the *Ct* value is defined in the **FAM**, **HEX** and **ROX** channels and the *Ct* value does not exceed the value specified in the *Important Product Information Bulletin* in the **ROX** channel, then the result is given as “**Genotype CT is detected**” only if the *Ct* value in each **FAM** or **HEX** channel exceeds the *Ct* value in the **ROX** channel **not more than 10 cycles**. If in one of the **FAM** or **HEX** channels the *Ct* value exceeds the *Ct* value in the **ROX** channel **more than 10 cycles**, then the result in this channel is not interpreted and the result is given as “**Genotype CC is detected**” or “**Genotype TT is detected**”.
4. If in the results grid for the sample with given reaction mixture the *Ct* value is not defined in the **FAM** and **HEX** channels then it is necessary to repeat the PCR analysis for this sample beginning from the DNA extraction stage.
5. If in the results grid for the sample with given reaction mixture the *Ct* value in the **ROX** channel exceeds the value specified in the *Important Product Information Bulletin* (regardless of the results obtained in the **FAM** and **HEX** channels) then it is necessary to repeat the PCR analysis for this sample beginning from the DNA extraction stage.