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GUIDELINES

to **AmpliSens® Escherichioses-FRT** PCR kit

for qualitative detection and differentiation of diarrheagenic *E.coli* DNA in environmental samples and biological 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® Escherichioses-FRT** PCR kit for qualitative detection and differentiation of DNA of different groups of diarrheagenic *E.coli* – enteropathogenic (*EPEC*), enterotoxigenic (*ETEC*), enteroinvasive (*EIEC* (in conjunction with *Shigella* spp.)), enterohemorrhagic (*EHEC*), and enteroaggregative (*EAgEC*), – in environmental samples and 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 GmbH, Germany),
- iCycler iQ5 (Bio-Rad Laboratories, Inc., USA),
- Mx3000P (Stratagene, USA)
- CFX96 (Bio-Rad Laboratories, Inc., USA).

Correspondence of the fluorophores and detection channels

Channel for the fluorophore	Detection channel name for different instrument models[1]
FAM	FAM/Green
JOE	JOE/HEX/R6G/Yellow/Cy3
ROX	ROX/Orange/TxR

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

AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) and Rotor-Gene Q (QIAGEN GmbH, 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 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, or PCR tubes (0.1 ml) with caps from the four-pieces-strips (detection through the bottom of the tube).

Programming the instrument

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.

NOTE: Well 1 must be filled with any test tube except for an empty one. If tubes with different types of reaction mixtures are placed in the thermocycler simultaneously, tube with the **PCR-mix-1-FEP/FRT *EIEC* / *EHEC* / STI** should be placed in the first well. 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. 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".

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

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 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.
4. In the opened window enter the operator name; select the reaction volume – 25 µl.
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:

The amplification program for rotor-type instruments

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Number of cycles*
1	**95**	15 min	–	1
2	**95**	10 s	–	45
	60	25 s	FAM, JOE, ROX	
	72	10 s	–	

Note – The Cy5 and Cy5.5 channels are enabled when required if the “multiprime” format tests are performed.

6. After setting up the temperature profile click the OK button.
7. Set calibration parameters (activate ***Calibrate/Gain Optimization…*** in a new experiment module):
   a) for signal measurement optimisation for the selected channels set calibration from **5Fl** to **10Fl** for the channels FAM/Green, JOE/Yellow and ROX/Orange. It is acceptable to set ***Calibrate/Gain Optimisation…*** value from 3Fl to 7Fl for ***FAM/Green*** and ***JOE/Yellow*** channels.

   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 – AmpliSens. 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 **AmpliSens** 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 opened window check that the reaction volume is 25 µl. Click the ***Next*** button.
4. In the next window the correctness of the amplification program and signal level auto-optimisation 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 amplification as NCA, the Positive control of amplification as C+. Set the type ***Unknown*** opposite all the test samples, the type ***Positive control*** – for the Positive control of amplification, the type ***Negative control*** – for the Negative control of amplification. Set the type ***Standard*** for DNA-calibrators C1 and C2 and enter the values from the *Important product information bulletin* enclosed to the given lot of the PCR kit into the ***Given conc.*** column. 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.

Amplification data analysis in the FAM/Green channel:

1. Activate the ***Analysis*** button in the menu, then 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), set ***Threshold = 0.05***.
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 channels 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	0.05	on	on	10%
JOE/Yellow	0.05	on	on	10%
ROX/Orange	0.05	on	on	10%

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.

AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ5 (Bio-Rad Laboratories, Inc., 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 domed or flat optically transparent caps, or tubes (0.2 ml) with transparent caps from the eight-pieces-strips (detection through the cap of the tube).

Programming the thermocycler

1. Turn on the instrument and the power supply unit of the optical block of the instrument.

NOTE: Warm up the lamp for 15 min before starting the experiment.

2. Start the program iCycler iQ5.
3. Insert the tubes or strips into the reaction module of the thermocycler and program the instrument according to the *Instruction Manual* given by the manufacturer of 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.

Creating the template for the run

1. Set the plate setup (set the order of the tubes in the reaction chamber and the detection of fluorescent signal).
   - click the ***Create New*** button in the ***Selected Plate Setup*** window of the ***Workshop*** module;
   - in the opened window click the ***Whole Plate loading*** button and set the plate setup using the buttons of the upper toolbar. Enter the samples' names in the ***Identifier/Condition*** column in the bar appeared in the screen bottom. Select the fluorescent signal detection in the FAM, JOE/HEX, ROX channels. Click the ***Select/Add Fluorophores*** button, select the fluorophore and tick it in the ***Selected*** column. Click ***OK***. The fluorophore name will appear in the ***Fluorophore*** window. For addition of fluorescence signal measuring for each sample it is necessary to click the fluorophore (activate it) and select the samples on the plate using the ***Fluorophore loading in whole Plate mode***  button under the scheme;
   - 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***;
   - save the set plate setup by clicking the ***Save&Exit Plate Editing*** button. Enter the

file name and click ***Save***.

2. Set all the biological samples as ***Unknown***, positive controls as «+», and negative controls as «–».
3. Set the amplification program. To do this, in the ***Selected Protocol*** window of the ***Workshop*** module click the ***Create New*** button. Set the amplification parameters and save the protocol by activating the ***Save&Exit Protocol Editing*** button. Enter the name of the file and then click ***Save***.

The amplification program for plate-type instruments

Step	***Temperature, °C***	***Time***	***Fluorescence detection***	***Number of cycles***
1	**95**	15 min	–	1
2	**95**	10 s	–	45
	60	25 s	FAM, JOE/HEX, ROX	
	72	10 s	–	

Note – The Cy5 and Cy5.5 channels are enabled when required if the “multiprime” format tests are performed.

4. Before a run it is obligatory to check if the selected protocol (***Selected Protocol***) and the plate scheme (***Selected Plate Setup***) are correct. To begin a run click the ***Run*** button. For the well factors measurement the ***Use Persistent Well Factors*** type is selected by default. Click the ***Begin Run*** button, save the experiment (the results of this experiment will be automatically saved in this file) and click ***OK***.
5. At the end of the run it is necessary to close the software and turn off the instrument (the thermocycler and the optical block).

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:

- select the needed file with the run in the upper left window of the ***Workshop*** module;
- click the ***Edit*** button in the ***Selected Plate Setup*** area of the ***Workshop*** module and edit the plate setup (the files of protocols are saved in the ***SampleFiles*** folder by default);
- click the ***Edit*** button in the ***Selected Protocol*** area of the ***Workshop*** module and check the correctness of the selected protocol (the files of protocols are saved in the ***SampleFiles*** folder by default).

Data analysis:

The obtained results are analyzed by the iCycler iQ5 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 in the corresponding column of the results table.

1. Start the software and open the needed file with data of the analysis in the ***Data File*** window of the ***Workshop*** module. Click the ***Analyze*** button.
2. Select the ***Analysis Mode: PCR Base Line Subtracted Curve Fit*** (is set by default).
3. 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, click the ***Log View*** (logarithmic scale selection) and set (with the left mouse button) the threshold line at the level where the fluorescence curves have a linear character and do not cross with the curves of the negative samples. As a rule, the threshold line is set at the level of 10-20 % of maximum fluorescence obtained for the Positive control in the last amplification cycle. Make sure that the fluorescence curve of the Positive control has the typical exponential growth of fluorescence.
4. In order to analyse the results click the ***Results*** button which is situated under the buttons with the fluorophores' names.

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.

AMPLIFICATION AND DATA ANALYSIS USING Mx3000P (Stratagene, 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 (detection through the cap of the tube).

1. Switch on the instrument. Run the **Stratagene Mx3000P** software.
2. Select ***Quantitative PCR (Multiple Standards)*** and ***Turn lamp on for warm-up*** in the ***New Experiment Options*** window.

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

3. Insert the tubes into the instrument, and close the fixer and the door of the instrument.
4. Select ***Optics Configuration*** in ***Options*** menu and, in the ***Dye Assignment*** tab, set parameter ***FAM*** next to the ***FAM filter set*** item, parameter ***JOE/HEX*** next to the ***HEX/JOE filter set*** and parameter ***ROX***, next to the ***ROX filter set***.
5. Set fluorescence detection parameters in the ***Plate Setup*** menu. To do this:
   - Select all wells with tested tubes or strips;
   - Mark all selected wells as ***Unknown*** in the ***Well type*** window. Select **FAM**, **JOE** and **ROX** 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 for the plate-type instrument (see the *Instruction manual* for the PCR kit). To do this:
   A. Press the ***Import…*** button. Navigate to the folder containing the previous experimental file and open it. The required thermocycling profile will appear in the ***Thermal Profile*** window;
   B. Set the appropriate amplification program in ***Thermal Profile Setup*** menu.

Amplification program for plate-type instruments

Step	***Temperature, °C***	***Time***	***Number of cycles***
1	**95**	15 min	1
2	**95**	10 s	45
	60	25 s *Fluorescent signal detection*	
	72	10 s	

NOTE: If the given PCR kit is used with the kit for setting the parameter of the fluorescence signal detection at the set temperature it is necessary to select the ***All points*** option for the ***Data collection marker for dragging*** parameter and drag it with a mouse from the right part of the box to the field with the necessary temperature.

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

Data analysis

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. Select ***Analysis*** by clicking the corresponding button of the tool bar.
2. The ***Analysis Selection/Setup*** tab will open. Make sure that all the test samples are active (the cells corresponding to the samples should be of a different colour).
3. Select 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 lower ***Dyes shown*** panel. Check the threshold line location and change it, if necessary.

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.

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 (detection through the cap of the tube).

Programming the thermocycler

1. Turn on the instrument and start the Bio-Rad CFX Manager software.
2. Program the instrument according to the *Instruction Manual* provided by the manufacturer

Creating the template for the run

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

Amplification program for plate-type instruments

Step	*Temperature, °C*	*Time*	*Fluorescence detection*	*Number of cycles*
1	**95**	15 min	–	1
2	**95**	10 s	–	45
	60	25 s	FAM, HEX, ROX	
	72	10 s	–	

NOTE: Set ***Ramp Rate* 2,5 °C/s** by clicking the ***Step Options*** button for each step of cycling (see the figure below). Click ***OK***.

Note – The Cy5 and Cy5.5 channels are enabled when required if the “multiprime” format tests are performed.

3. Save the protocol: in the ***Protocol Editor New*** window select ***File***, then ***Save As***, and name the file and click ***Save***.

When the required program is entered or edited, click ***OK*** at the bottom of the window.

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** 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,

confirming the names by the ***Load*** button.

6. Save the plate setup: select ***File*** and then ***Save as*** in the ***Plate Editor New*** window. Enter the file name, click ***Save.***
7. 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.

8. 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 *Ct* (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 *Ct* values are represented in the ***Data Analysis*** window of the ***Quantification*** tab.

Check the correctness of threshold line automatic choice for each channel. The threshold line is to cross only with S-shaped (sigmoid) curves describing the accumulation of the signal detecting positive samples and controls. The threshold line is not to cross the base line. If it happens, it is necessary to set the threshold line level for each channel manually. To do this, tick the ***Log Scale*** item (logarithmic scale selection) and set (with the left mouse button) the threshold line at the level where the fluorescence curves have a linear

character and do not cross with the curves of the negative samples. As a rule, the threshold line is set at the level of 10-20 % of maximum fluorescence obtained for the Positive control in the last amplification cycle. Make sure that the fluorescence curve of the Positive control has the typical exponential growth of fluorescence. To select the curve of C+ sample (or another one appropriate sample), set the cursor to the plate setup or to the results grid.

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.

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
04.09.25 HM	Intended use	Correspondence of the fluorophores and detection channels table was added
	Amplification and data analysis using Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) instruments	The optimization of signal measurement for FAM/Green, JOE/Yellow channels has been changed from «3Fl to 7Fl» to « 5Fl to 10Fl». The note «It is acceptable to set ***Calibrate/Gain Optimisation…*** value from 3Fl to 7Fl for ***FAM/Green*** and ***JOE/Yellow*** channels» has been added
	Amplification and data analysis using iCycler iQ5 (Bio-Rad Laboratories, Inc., USA) instrument	Corrections according to the template
	Amplification and data analysis using Mx3000P (Stratagene, USA)	Corrections according to the template
	Amplification and data analysis using CFX96 (Bio-Rad, USA)	Corrections according to the template