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GUIDELINES

to AmpliSens® *Streptococcus agalactiae*-screen-titre-FRT PCR kit

for detection and quantitative analysis of *Streptococcus agalactiae* DNA in the biological material by the polymerase chain reaction (PCR) with real-time fluorescence hybridization detection

AmpliSens®

Federal Budget Institute of Science "Central Research Institute for Epidemiology"
3A Novogireevskaya Street
Moscow 111123 Russia

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INTENDED USE

The guidelines describe the procedure of using **AmpliSens® *Streptococcus agalactiae*-screen-titre-FRT** PCR kit for detection and quantitative analysis of *Streptococcus agalactiae* DNA in the biological material (blood plasma, cerebrospinal fluid (CSF), oropharyngeal swabs, epithelial swabs of lateral vaginal walls, anorectal swabs) by the polymerase chain reaction (PCR) with real-time fluorescence-hybridization detection using the following instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- Rotor-Gene Q (QIAGEN GmbH, Germany);
- iCycler iQ, iCycler iQ5 (Bio-Rad Laboratories, Inc., USA);
- CFX96 (Bio-Rad Laboratories, Inc., USA);

and also in combination with the automatic station for the nucleic acids extraction NucliSENS easyMAG (bioMérieux, France).

Correspondence of names of fluorophores and detection channels

Channel for the fluorophore	The detection channels' names for different instruments[1)]
FAM	FAM/Green
JOE	JOE/HEX/R6G/Yellow/Cy3
ROX	ROX/Orange/TxR

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

WORK WITH THE NucliSENS easyMAG (bioMérieux, France) AUTOMATED NUCLEIC ACID EXTRACTION SYSTEM

Variant 1

DNA extraction with the sample lysis outside of the instrument (off-board mode)

This extraction method allows the reduction of the NucliSENS lysis buffer consumption and is more preferred for the analysis of biological material samples containing conglomerate.

1. Switch on the NucliSENS easyMAG instrument and prepare it for the DNA extraction according to the *Instruction manual*.
2. In the window for input of test samples enter the following parameters:
   - ***Name***,
   - ***Matrix*** – select ***Plasma***,
   - ***Volume*** – ***0.1 ml***,
   - ***Eluate*** – ***55 µl***,
   - ***Type*** – ***Lysed***,
   - ***Priority*** – ***normal***.
3. Create a new protocol of DNA extraction and save it. In the protocol outline that lysis and incubation of the samples are taking place outside of the instrument by selecting ***On-board Lysis Buffer Dispensing – No***, ***On-board Lysis Incubation – No***.
4. Relocate the samples table into the created protocol.
5. Prepare the required number of specialized disposable tubes for extraction of DNA in the NucliSENS easyMAG instrument, (including positive and negative controls of extraction). To the inner walls of each tube add **10 µl** of **Internal Control STI-87 (IC)**. Add **550 µl** of **NucliSens lysis buffer**.

NOTE: If the material containing conglomerate is used for lysis, it is advised to use the tubes with volume of 1.5 ml. After the end of incubation (**step 8**), the tubes must be centrifuged at 10,000 rpm for 1 min on a microcentrifuge and then transfer the supernatant to the specialized tubes for DNA extraction in NucliSENS easyMAG.

6. Add **100 µl** of prepared probes into each tube containing the **NucliSens lysis buffer** and the **Internal Control STI-87 (IC)** using disposable tips with filters and carefully, but thoroughly mix by pipetting (it is advised to avoid the transfer of conglomerates and large particles to the test tubes).
7. To the tube labelled Negative Control of Extraction (C–) add **100 µl** of the **Negative Control (C–)**. To the tube labelled **Positive Control of Extraction (PCE)** add **90 µl** of

Negative Control (C–) and **10 µl** of **Positive Control DNA *Streptococcus agalactiae* and human DNA**.

8. Incubate the tubes for 10 min at room temperature.
9. Intensively vortex the tube with **NucliSens magnetic silica** (bioMérieux, France).To each tube, using disposable filter tips, add **10 µl** of the **NucliSens magnetic silica** and thoroughly mix by pipetting. The magnetic silica must be evenly distributed throughout the tube volume.
10. Load the sample tubes into the instrument, position the caps and start the DNA extraction with sample lysis program outside the instrument (***off* board mode**).
11. After the extraction procedure is completed, remove the sample tubes from the instrument and proceed with them to the DNA amplification area.

Variant 2

DNA extraction with automated lysis of sample inside the instrument (on-board mode)

1. Switch on the NucliSENS easyMAG instrument and prepare it for the DNA extraction according to the *Instruction manual*.
2. In the window for input of test samples enter the following parameters:
   - ***Name***,
   - ***Matrix*** – select ***Plasma***,
   - ***Volume*** – ***0.1-1.0 ml***,
   - ***Eluate*** – ***55 µl***,
   - ***Type*** – ***Primary***,
   - ***Priority*** – ***normal***.
3. Create new protocol of DNA extraction and save it. In the protocol outline that lysis and incubation of the samples are taking place automatically inside the instrument by selecting ***On-board Lysis Buffer Dispensing – Yes*****,** ***On-board Lysis Incubation – Yes*****.**
4. Relocate the samples table into the created protocol.
5. Add **100 µl** of prepared probes into each tube using disposable filter tips and carefully, but thoroughly mix by pipetting.
6. To the tube labelled Negative Control of Extraction (C–) add **100 µl** of the **Negative Control (C–)**. To the tube labelled **Positive Control of Extraction (PCE)** add **90 µl** of **Negative Control (C–)** and **10 µl** of **Positive Control DNA *Streptococcus agalactiae* and human DNA**.

7. Using sterile filter tips in a separate 2 ml tube mix the **NucliSens magnetic silica** and **Internal Control STI-87 (IC)** in the following ratio:

Table 1

Number of samples for DNA extraction	Volume of NucliSens magnetic silica, µl	Volume of Internal Control (C–), µl
1	**10**	**10**
24 (complete instrument load)	**250** (with 25 probes in reserve)	**250** (from two tubes)

8. Mix the tube contents thoroughly. The **NucliSens magnetic silica** and **Internal Control STI-87 (IC)** mix can be stored for no longer than **30 min**.
9. Load the sample tubes into the instrument, position the caps and start the DNA extraction with sample lysis program inside the instrument (***on* board mode**).
10. Wait for the NucliSENS easyMAG instrument to pause in the ***Instrument State-Idle*** (approx. 15 min).
11. Thoroughly vortex the tube containing the **NucliSens magnetic silica** and **Internal Control STI-87 (IC)** mix until homogeneous state is reached.
12. Open the lid of the instrument and add **20 µl** of **prepared mixture of NucliSens magnetic silica and Internal Control STI-87 (IC)** to each tube using disposable filter tips. Thoroughly mix the contents of each tube using adjustable pipette with 200 µl disposable filter tips.
13. Set the instrument to continue the DNA extraction program.
14. After the DNA extraction procedure is completed, remove the sample tubes from the instrument and proceed with them to the DNA amplification area.

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.

NOTE: 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. 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* given by the manufacturer of the 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 ***New run*** window.
2. Select the ***TwoStep/Hidrolysis Probes*** template in the tab for edition and click the ***New*** button.
3. In the opened window select ***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 and choose the reaction mix volume: ***Reaction volume*** – **25 µl**. Tick the box next to ***15 µl oil layer volume***. 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 parameters:

Table 2

"AmpliSens-1" 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	–	
	72	15 s	–	
Cycling 2	**95**	5 s	–	40
	60	20 s	FAM/Green, JOE/Yellow, ROX/Orange	
	72	15 s	–	

6. After setting up the temperature profile click the ***OK*** button.
7. In the ***New Run Wizard*** window press the ***Calibrate/Gain Optimisation***. In the newly opened window
   a) For signal measurement optimisation for the selected channels set calibration from **5Fl** to **10Fl** for all the channels FAM/Green, JOE/Yellow, ROX/Orange.

      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-1**. 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.
9. 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.

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 and the ***15 µl oil layer volume*** option is activated. 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. The calibration curve plotting and calculation of the *Streptococcus agalactiae* DNA concentration are performed automatically in accordance with the *Ct* values.

Amplification data analysis in the JOE/Yellow channel:

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

Results analysis in the FAM/Green, ROX/Orange channels is carried out similarly to results analysis in the JOE/Yellow 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	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 iQ and 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 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. Switch on the instrument and the power supply unit of the optical part of the instrument.

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

2. Start the program iCycler iQ/iQ5.
3. Insert the microtubes 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 «–». Set the calibrators C1 and C2 in the test channels as ***Std (Standard***). Enter the values specified in the *Important Product Information Bulletin* enclosed to the given lot of the PCR kit in the ***Quantity*** column. If different calibrators' values in the detection channels are set then the ***Whole Plate Loading*** **button** should be **inactive.**
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***.

"AmpliSens-1" amplification program for plate-type instruments

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

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. The calibration curve plotting and calculation of the *Streptococcus agalactiae* DNA concentration are performed automatically in accordance with the *Ct* values.

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. For each channel check that automatic threshold line setting is correct. Normally, the threshold line should intercept only sigmoid curves of fluorescence accumulation for positive samples and controls and it should not intercept the basal line. If otherwise, increase the threshold level by pressing ***Log View*** button and set the threshold to the level where fluorescence curves have linear shape and do not intercept negative samples curves.
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 CFX96 (Bio-Rad Laboratories, Inc., 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.

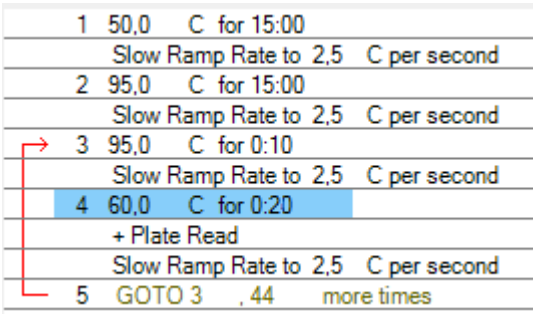
Creating the template for the run

1. In the ***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*** 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-1 amplification program for rotor-type instruments

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	
	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***.



Note – The Cy5 and Quasar 705 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***, 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** fluorophores and click ***OK***. In the ***Sample type*** menu select ***Unknown*** for all the samples, except for the DNA-calibrators. 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.

   Set the calibrators C1 and C2 for all the channels as ***Sample type*** – ***Standard***. Enter a concentration in the ***Concentration*** box according to the *Important Product Information Bulletin* enclosed to the PCR kit, 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 *Ct* (threshold cycle) value in the corresponding column of the results table. The calibration curve plotting and calculation of the *Streptococcus agalactiae,* IC Glob, IC STI-87 DNA concentrations are performed automatically in accordance with the *Ct* values.

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.
3. Click the ***View/Edit Plate…*** button on the toolbar and set the samples names and calibrators concentrations in the opened window.

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 CFX96 for research 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 (herewith, curves are S-shaped in a linear scale)	Set the threshold line at the level of 10-20 % of maximum fluorescence obtained for the Positive control in the last amplification cycle (in logarithmic scale)
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

CALCULATION OF *Streptococcus agalactiae* DNA CONCENTRATION

The concentration calculation for *Streptococcus agalactiae* DNA in 1 ml of the sample upon extraction from 100 µl should use this formula:

***Calculated concentration of Streptococcus agalactiae DNA* x 100 = copies/ml**

Each PCR kit contains the *Important Product Information Bulletin*, in which the DNA calibrators' concentrations, necessary for the concentration calculations of the analysed probes and for estimation of obtained results reliability.

An example of calculation of *Streptococcus agalactiae* DNA copies in 1 ml of sample using the Excel table, see Table 4.

1. Copy the samples' names to the first column of the Excel table (Column A).
2. Copy the concentration values of *Streptococcus agalactiae* (in the channel for JOE/HEX fluorophore) to the second column of the Excel table (Column B).
3. In the next table enter the formula (=B*100), as shown in Table 4 here. The concentration values of *Streptococcus agalactiae* in 1 ml of the sample will appear in cells of the latter column.

Table 4

Example of concentration calculation of *Streptococcus agalactiae* DNA copies in 1 ml of sample using the Excel table

Name	Calc Conc (copies/reaction)Yellow (JOE/HEX)	*Streptococcus agalactiae* DNA, copies/ml of sample
A	B	= B*100
1	42	4200
2		
3	12	1200
4	7	700
5		
K1 *SAG*	10457	
K1 *SAG*	9563	
K2 *SAG*	96	
K2 *SAG*	104	
NCA		

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
13.07.23 EM	Footer	**REF** R-B77-100-FT(RG,iQ)-CE was added
23.07.25 HM	Through the text	Corrections according to the template
	Amplification and data analysis using CFX96 (Bio-Rad Laboratories, Inc., USA)	The section was added