

IVD

For Professional Use Only

**GUIDELINES**  
**to AmpliSens<sup>®</sup> *Streptococcus agalactiae*-**  
**screen-titre-FRT PCR kit**

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

**AmpliSens<sup>®</sup>**



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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® *Streptococcus agalactiae*-screen-titre-FRT** PCR kit for detection and quantitative analysis of *Streptococcus agalactiae* DNA in the clinical 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:

- NucliSENS easyMAG automated system (bioMérieux, France);
- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- Rotor-Gene Q (QIAGEN, Germany);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA).

## 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 clinical 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 <b>NucliSens magnetic silica</b> , µl	Volume of <b>Internal Control (C–)</b> , µl
<b>1</b>	<b>10</b>	<b>10</b>
<b>24</b> (complete instrument load)	<b>250</b> (with 25 probes in reserve)	<b>250</b> (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

Load the microtubes into the rotor of the Rotor-Gene 3000/6000/Q thermocycler so that the first tube is located in the well №1; place the rotor into the instrument, close the lid (the rotor wells are numbered, those numbers are used further on for programming the probes position in the thermocycler).

**NOTE:** Balance the rotor of the instrument if it is not loaded entirely. Fill the spare wells with empty tubes (don't use the tubes left after previous experiments). Well 1 must be filled with any tested tube except for an empty one.

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

### Programming the thermocycler

1. Press the **New** button in the main menu of the instrument.
2. In the newly opened window choose the **Advanced** template for the start of experiment and select **Dual Labelled Probe/Hydrolysis probes**. Press the **New** button.
3. In the newly opened window select **36-Well Rotor** (or **72-Well Rotor**) and outline that you are not using tubes with domed lids (**No Domed Tubes** (Rotor-Gene 3000))/that the locking ring is attached (**Locking Ring Attached** (Rotor-Gene 6000 and Rotor-Gene Q)). Press the **Next** button.
4. In the newly opened window set the name of the operator and choose the reaction mix volume: **Reaction volume – 25 µl**. For Rotor-Gene 6000 or Rotor-Gene Q tick the box next to **15 µl oil layer volume**. Press the **Next** button.
5. In the newly opened window set the temperature profile for the experiment. To do this, press the **Edit profile** button and set the following 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. Press the **OK** button.

7. In the **New Run Wizard** window press the **Calibrate/Gain Optimisation**. In the newly opened window, **Auto Gain Calibrator Setup**, press **Calibrate Acquiring/Optimise** button
  - a) To calibrate the FAM/Green, JOE/Yellow and ROX/Orange channels press the **Calibrate Acquiring/Optimise Acquiring**
  - b) To set calibration for all channels set the **Min reading** to **5 FI** and the **Max Reading** at **10 FI**. Tick the box next to **Perform Calibration Before 1<sup>st</sup> Acquisition/Perform Optimisation Before 1<sup>st</sup> Acquisition** Press the **Close** button.
8. Press the **Next** button, and start amplification by pressing **Start run**.
9. Name the experiment and save it on the disc (this file will automatically save the results of the experiment).
10. Insert the data into the samples table (it will open automatically after amplification starts). In the **Name** column insert the names/numbers of the tested clinical samples. The negative control of amplification must be labelled as **NCA** and the positive control as **C+**. Next to all tested clinical samples select the **Unknown** type, next to the positive control of amplification – **Positive control** type, for the negative control of amplification – **NTC** type. For **DNA calibrators** – **Standard** type and enter their concentrations in **Given Conc.** column. The concentration values for calibrators are found in the *Important Product Information Bulletin*. For wells corresponding to the empty tubes select the **None** type.
11. Start the amplification

**NOTE:** Samples indicated as **None** will not be analysed.

#### **Data analysis:**

*Results analysis of Streptococcus agalactiae DNA amplification reaction (JOE/Yellow channel):*

1. Check that the calibrators are defined and their concentrations are set in the samples table (in case of quantitative analysis).
2. 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.
3. Cancel the automatic choice of the threshold line level by activating the **Threshold** button.
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 table (the **Quant. Results** window) the **Ct** values will appear.
8. For the negative control of extraction (C-) – **Negative Control (C-)** – the **Ct** values must be absent.
9. For the negative control of amplification (NCA) – **TE-buffer** – the **Ct** values must be absent.
10. For the positive control of extraction (PCE) – **Positive Control DNA Streptococcus agalactiae and human DNA** – the calculated concentration value must be within the range of boundary values given in the Important Product Information Bulletin (for quantitative analysis).
11. For the DNA calibrators, in case of quantitative analysis, – **K1 SAG** and **K2 SAG** – the **Ct** values and the concentration values (**Calc Conc (copies/reaction)**) should appear.

Results analysis of IC Glob amplification reaction (FAM/Green channel):

1. Check that the calibrators are defined and their concentrations are set in the samples table (in case of quantitative analysis).
2. Activate the **Analysis** button in the menu, select the **Quantitation** mode of the analysis, and activate the **Cycling A. FAM/Cycling A. Green, Show** button.
3. Cancel the automatic choice of the threshold line level by activating the **Threshold** button.
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 table (the **Quant. Results** window) the **Ct** values will appear for **Glob DNA** for each tested sample, whilst for quantitative analysis – the concentration values (**Calc Conc (copies/reaction)**) will appear.
8. For the negative control of extraction (C-) – **Negative Control (C-)** – the **Ct** value **>30**.
9. For the negative control of amplification (NCA) – **TE-buffer** – the **Ct** value **>30**.
10. For the positive control of extraction (PCE) – **Positive Control DNA Streptococcus agalactiae and human DNA** – the **Ct** value and the concentration value will appear (for quantitative analysis).



11. For the DNA calibrators, in case of quantitative analysis, – **K1 SAG** and **K2 SAG** – the *Ct* values and the concentration values (**Calc Conc (copies/reaction)**) will appear.

Results analysis of Internal Control STI-87 (IC) amplification reaction (ROX/Orange channel):

1. Check that the calibrators are defined and their concentrations are set in the samples table (in case of quantitative analysis).
2. Activate the **Analysis** button in the menu, select the **Quantitation** mode of the analysis, and activate the **Cycling A. ROX/Cycling A. Orange, Show** button.
3. Cancel the automatic choice of the threshold line level by activating the **Threshold** button.
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 table (the **Quant. Results** window) the *Ct* values will appear for **Internal Control STI-87 (IC)** for each tested sample, whilst for quantitative analysis – the concentration values (**Calc Conc (copies/reaction)**) will appear. Wherein the *Ct* value must not exceed the boundary value given in the *Important Product Information Bulletin*.
8. For the negative control of amplification (NCA) – **TE-buffer** – the *Ct* values must be absent.
9. For the DNA calibrators, in case of quantitative analysis, – **K1 SAG** and **K2 SAG** – the *Ct* values and the concentration values (**Calc Conc (copies/reaction)**) will appear.

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

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.

**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. In the ***Selected Protocol*** window of the ***Workshop*** module press the ***Create new*** button to create a new amplification protocol.
2. In the newly opened window set the corresponding parameters, see Table 3:

Table 3

**“AmpliSens-1” 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	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	–	

3. Name the protocol and save it.
4. Create new ***Plate Setup*** (set the order of the tubes in the reaction chamber and the detection of fluorescent signal). Set the sample loading scheme.
5. In the newly opened window select the ***Unknown*** type for all clinical samples, for positive controls select “+”, for negative controls select “-“. For calibrators in ***JOE/HEX, FAM*** and ***ROX*** channels select ***Standard*** and set the concentration as shown in the ***Important Product Information Bulletin***. Upon setting the calibrators, the ***Whole Plate Loading*** button must be inactive. For all samples and calibrators set the fluorescence estimation in three channels – ***JOE/HEX, FAM, ROX***.
6. Name the sample loading scheme and save it.
7. Press the ***Run*** button. In the newly opened window tick the box next to ***Use Persistent Well Factors***, activate the ***Begin Run*** button and save the experiment.

**Data analysis:**

1. Start the program and open the saved file. To do this in the ***Workshop*** module press ***Data file*** and choose your data file. Change mode to ***Data Analysis***.
2. Analyse data separately for each channel.
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 (with the aid of the left mouse button). In the results table (**Quant. Results**) the *Ct* values will appear for the analysed channel.

4. For results analysis press **PCR Quant** button (iCycler iQ) or activate the **Results** button (directly under the fluorophore name buttons) (iCycler iQ5).

#### Results analysis for *Streptococcus agalactiae* amplification reaction (JOE/HEX channel)

Check that in the samples table DNA calibrators are defined and their concentrations are set (for quantitative analysis).

The *Ct* values for ***Streptococcus agalactiae* DNA** will appear in the results table.

1. For the negative control of extraction (C-) – **Negative Control (C-)** – the *Ct* values must be absent.
2. For the negative control of amplification (NCA) – **TE-buffer** – the *Ct* values must be absent.
3. For the positive control of extraction (PCE) – **Positive Control DNA *Streptococcus agalactiae* and human DNA** – the calculated concentration value must be within the range of boundary values given in the *Important Product Information Bulletin* (for quantitative analysis).
4. For the DNA calibrators – **K1 SAG** and **K2 SAG** – the *Ct* values and the concentration values should appear.

#### Results analysis for IC Glob amplification reaction (FAM channel)

Check that in the samples table DNA calibrators are defined and their concentrations are set (for quantitative analysis).

1. The *Ct* values for each analysed sample or the concentration values (**copies/reaction**) for qualitative analysis will appear in the results table.
2. For the negative control of extraction (C-) – **Negative Control (C-)** – the *Ct* value **>35**.
3. For the negative control of amplification (NCA) – **TE-buffer** – the *Ct* value **>35**.
4. For the positive control of extraction (PCE) – **Positive Control DNA *Streptococcus agalactiae* and human DNA** – the *Ct* values and the concentration values will appear.
5. For the DNA calibrators – **K1 SAG** and **K2 SAG** – the *Ct* values and the concentration values (**copies/reaction**) should appear for the FAM fluorophore channel.

#### Results analysis for Internal Control STI-87 (IC) amplification reaction (ROX channel)

Check that in the samples table DNA calibrators are defined and their concentrations are set (for quantitative analysis).

1. In the results table the *Ct* values should appear for STI-87 DNA for each analysed

sample and for the negative control of extraction (C–), for quantitative analysis – concentration values will appear (***copies/reaction***). Wherein the *Ct* value must not exceed the boundary values given in the *Important Product Information Bulletin*.

2. For the negative control of amplification (NCA) – **TE-buffer** – the *Ct* values must be absent.
3. For the DNA calibrators – **K1 SAG** and **K2 SAG** – the *Ct* values and the concentration values (***copies/reaction***) should appear (for quantitative analysis).

### **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**

<b>Name</b>	<b>Calc Conc (copies/reaction) Yellow (JOE/HEX)</b>	<b><i>Streptococcus agalactiae</i> DNA, copies/ml of sample</b>
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 Guidelines

VER	Location of changes	Essence of changes
12.11.14 ME	AMPLIFICATION AND DATA ANALYSIS USING Rotor-Gene 3000/6000 (Corbett Research, Australia) AND Rotor-Gene Q (QIAGEN, Germany) INSTRUMENTS	The results analysis is corrected for the C– and NCA samples in the FAM/Green channel Corrections according the template
	AMPLIFICATION AND DATA ANALYSIS USING iCycler iQ and iCycler iQ5 (Bio-Rad, USA) INSTRUMENTS	The results analysis is corrected for the C– and NCA samples in the FAM channel
08.05.15 PM	Intended use	The clinical material was clarified
25.12.20 EM	Through the text	The symbol  was changed to NOTE:
	Cover page	The phrase “Not for use in the Russian Federation” was added
11.03.21 MM	Front page	The name, address and contact information for Authorized representative in the European Community was changed