



For Professional Use Only

eSens Streptococcus pyogenes QT PCR kit

REF ES3810A

Instructions for Use

1 INTENDED USE

eSens Streptococcus pyogenes QT PCR kit is an *in vitro* nucleic acid amplification test for quantitative detection of *Streptococcus pyogenes* DNA in the biological material (oropharyngeal swabs, whole blood, tissue (biopsy, surgical) material, synovial fluid, discharge of erosive and ulcerative lesions of the skin, cerebrospinal fluid (CSF), urine) using real-time hybridization-fluorescence detection of amplified products.

Indications and contra-indications for use of the reagent kit

The reagent kit is used for the analysis of biological material, taken from the persons suspected of bacterial infection (infection caused by *Streptococcus pyogenes*).

There are no contra-indications with the exception of cases when the material cannot be taken for medical reasons.

NOTE: The results of PCR analysis are taken into account in complex diagnostics of disease.

2 PRINCIPLE OF PCR DETECTION

The principle of testing is based on the DNA extraction from test samples together with the exogenous internal control (Internal Control-FL (IC)) and simultaneous amplification of DNA fragments of the detected microorganism and DNA of the internal control with hybridization-fluorescence detection. Exogenous internal control (Internal Control-FL (IC)) allows to control all PCR-analysis stages of each individual sample and to identify possible reaction inhibition.

Amplification of DNA fragments with the use of specific primers and Taq-polymerase enzyme are performed with the DNA samples obtained at the extraction stage. In the real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The real-time monitoring of

fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

The quantitative analysis of *Streptococcus pyogenes* DNA is based on the linear dependence between the initial concentration of DNA target in a test sample and the cycle threshold (*Ct*) (the cycle of beginning of fluorescence signal exponential growth). For the quantitative analysis amplification of DNA from the test samples is carried out simultaneously with DNA-calibrators (samples with the known concentration of the DNA target). Values for DNA calibrators are assigned according to the manufacturer's measurement procedure according to paragraph 5.6 of ISO 17511-2003. Based on the amplification results of DNA-calibrators a calibration line is plotted and it is used for the estimation of concentration of the DNA target in the test samples.

eSens Streptococcus pyogenes QT PCR kit contains the system for prevention of contamination by amplicons using the enzyme uracil-DNA-glycosylase (UDG) and deoxyuridine triphosphate (dUTP). The enzyme UDG recognizes and catalyzes the destruction of the DNA containing deoxyuridine, but has no effect on DNA containing deoxythymidine. Deoxyuridine is absent in the authentic DNA, but is always present in amplicons, because dUTP is a part of dNTP mixture in the reagents for the amplification. Due to the deoxyuridine containing contaminating amplicons are sensitive to the destruction by UDG before the DNA-target amplification. So the amplicons cannot be amplified.

The enzyme UDG is thermolabile. It is inactivated by heating at temperature above 50 °C. Therefore, UDG does not destroy the target amplicons which are accumulated during PCR.

The results of amplification are registered in the following fluorescence channels:

Table 1

Channel for fluorophore	FAM	JOE
DNA-target	Internal Control-FL (IC) DNA	<i>Streptococcus pyogenes</i> DNA
Target gene	Artificially synthesized sequence	erythrogenic toxin B (<i>speB</i>) gene

3 CONTENT

eSens Streptococcus pyogenes QT PCR kit (ES3810A) includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL <i>Streptococcus pyogenes</i>	clear liquid from colorless to light lilac colour	1.2	1 tube
PCR-bufer-H	colorless clear liquid	0.6	1 tube
C1 SP	colorless clear liquid	0.2	1 tube
C2 SP	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.2	1 tube
Internal Control-FL (IC)*	colorless clear liquid	1.0	1 tube
Negative Control (C-)**	colorless clear liquid	1.2	2 tubes
Positive Control <i>Streptococcus pyogenes</i>***	colorless clear liquid	0.1	1 tube

* add **10 µl** of **Internal Control-FL (IC)** during the DNA extraction procedure directly to the sample/lysis mixture.

** must be used in the extraction procedure as Negative Control of Extraction.

*** must be used in the extraction procedure as Positive Control of Extraction.

eSens Streptococcus pyogenes QT PCR kit is intended for 110 reactions (including controls).

4 ADDITIONAL REQUIREMENTS

- Transport medium with mucolytic agent
- Transport medium.
- Plastic container (50-60 ml) for sampling, storage and transportation of biological samples.
- Vacuette blood collection system.
- Urine analysis preservative tube.
- Urine transfer straw.
- Swab for collection, transportation and storage of biological samples
- Reagent for pretreatment of whole peripheral and umbilical blood.
- Sterile tools (individual for each sample) for homogenization (porcelain mortar and mallet) or homogenizer for pretreatment of viscera material.
- Microcentrifuge for Eppendorf tubes (RCF max. 12,000 x g).
- Vortex mixer.
- Vacuum aspirator with flask for removing supernatant.
- DNA extraction kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free and pipette tips with filters (up to 100 µl, 200 µl).
- Tube racks.
- PCR box.
- Real-time instruments with 3 (or more) independent detection channels (for example, Rotor-Gene Q (QIAGEN, Germany), CFX 96 Touch, CFX 96 Opus (Bio-Rad, USA), QuantStudio 5 (Thermo Fisher Scientific), or equivalent).
- Disposable polypropylene PCR tubes:
 - a) tightly closed 2.0-ml tubes for sampling;
 - b) screwed or tightly closed 1.5-ml tubes for pretreatment and preparation of reaction mixture for PCR;
 - c) thin-walled 0.2-ml PCR tubes with optical transparent domed or flat caps or strips of eight 0.2-ml tubes with optical transparent caps if a plate-type instrument is used for PCR;
 - d) thin-walled 0.2-ml PCR tubes with flat caps or strips of four 0.1-ml Rotor-Gene PCR tubes if a rotor-type instrument is used for PCR kit.
- Refrigerator with the range from 2 to 8 °C.
- Deep-freezer with the range from minus 24 to minus 16 °C.
- Reservoir for used tips.

5 GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol filters and use a new tip for every procedure.
- Store all extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a distantly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable protective gloves and laboratory cloths, and protect eyes while samples and reagents handling. Thoroughly wash hands afterward.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.

- Do not use the PCR kit if the internal packaging was damaged or its appearance was changed.
- Do not use the PCR kit if the transportation and storage conditions according to the Instruction Manual were not observed.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in a biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid inhalation of vapors, samples and reagents contact with the skin, eyes and mucous membranes. Harmful if swallowed. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice if necessary.
- While observing the conditions of transportation, operation and storage, there are no risks of explosion and ignition.
- Safety Data Sheets (SDS) are available on request.
- The PCR kit is intended for single use for PCR analysis of specified number of samples (see the section "Content").
- The PCR kit is ready for use in accordance with the Instruction Manual. Use the PCR kit strictly for intended purpose.
- Use of this product should be limited to personnel trained in the DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment, and reagents to the area where the previous step was performed.



Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

6 SAMPLING AND HANDLING

eSens Streptococcus pyogenes QT PCR kit is intended for the analysis of DNA extracted with DNA extraction kits from the biological material (oropharyngeal swabs, whole blood, tissue (biopsy, surgical) material, synovial fluid, discharge of erosive and ulcerative lesions of the skin, cerebrospinal fluid (CSF), urine).

Sampling

6.1 Oropharyngeal swabs.

The material is taken with a sterile dry probe. Rotate the probe over the tonsillar area, palatine arches, and posterior area of the oropharynx. When the material is obtained, place the working part of the probe into the sterile disposable tube with transport medium. Break off the plastic stick at the distance no more than 0.5 cm from the working part. Close the tube with the working part of the probe in it. Mark the tube.

6.2 Whole blood.

Blood should be taken after overnight fasting or in 3 hour after eating by a disposable 0.8-1.1 mm diameter needle into the tube with EDTA (special vacuum system Vacuette (lavender caps – 6 % EDTA)). After blood sampling the tube should be gently inverted several times for the thoroughly mixing with the anticoagulant. (Otherwise, blood will coagulate and DNA extraction will be impossible!) Place the tube in the tube rack.

6.3 Tissue (biopsy, surgical) material.

The material should be taken from the proposed pathogen location, from the lesional tissue or the area surrounding the lesional tissue.

The tissue pieces (no more than 5 mm in a diameter) should be placed into the disposable 2.0-ml tubes with 500 µl of **Transport Medium with Mucolytic Agent** (952-CE).

The tissue pieces (more than 5 mm in a diameter) should be placed into the disposable 50-ml containers with wide mouth.

6.4 Synovial fluid.

Synovial fluid should be collected in an amount no less than 1 ml using disposable needles into disposable 2.0-ml tubes.

6.5 Discharge of erosive and ulcerative lesions of the skin.

Samples are collected rotating dry sterile probe onto the erosive and ulcerative lesions of the skin. When the material is obtained, place the working part of the probe into the sterile disposable tube with 500 µl of **Transport Medium with Mucolytic Agent** (952-CE). Break off the plastic stick at the distance no more than 0.5 cm from the working part. Close the tube with the working part of the probe in it. Mark the tube.

6.6 Cerebrospinal fluid (CSF).

Cerebrospinal fluid is collected in an amount no less than 1 ml by puncturing the lumbar, suboccipital area, or cerebral ventricles using sterile puncture needle into disposable 2.0-ml tubes.

6.7 Urine.

The first portion of first void urine is taken for PCR-analysis in an amount no more than 15 ml into the dry sterile container (60 ml).

The above-mentioned samples can be stored before pretreatment/PCR analysis:

- at the temperature from 18 to 25 ° – no more than 8 hours;
- at the temperature from 2 to 8 °C – no more than 2 days;
- at the temperature from minus 24 to minus 16 °C – for 1 year (except for whole blood and urine samples).

Only one freeze-thawing cycle is acceptable.

NOTE: Freezing of whole blood and urine samples is unacceptable!

It is allowed to transport the above-mentioned material at the temperature from 2 to 8 °C for 1 day.

Pretreatment

Pretreatment of oropharyngeal swabs and discharge of erosive and ulcerative lesions of the skin is not required.

6.8 Whole blood.

The whole blood samples are to be pretreated. Transfer 1.0 ml of whole blood to the disposable 1.5-ml tube. Centrifuge at 8,000-9,000 g (for example, 12,000-13,000 rpm for the MiniSpin Eppendorf microcentrifuge) for 5 minutes. Remove plasma. Add 1.0 ml of **Hemolytic** (137-CE) to the pellet. Gently vortex the tubes and leave them for 15 minutes at room temperature (from 18 to 25°C), stirring occasionally. Centrifuge at 4,000 g (for example, 8,000 rpm for the MiniSpin Eppendorf microcentrifuge) for 5 minutes. Remove the supernatant using vacuum aspirator leaving 100 µl of the pellet. After washing the cell pellet should be white, only a small pinkish bloom on the pellet is allowed (the remains of the destroyed erythrocytes). The washing using **Hemolytic** (137-CE) may be repeated if

necessary. The obtained pellet must be immediately lysed (in case of extraction using **RIBO-prep** (K2-9-Et-100-CE) add **300 µl of Solution for Lysis** and then extract DNA in accordance with the *Instruction Manual* enclosed to the **RIBO-prep** reagent kit without adding **Solution for Lysis** once again).

The pretreated samples of whole blood can be stored before the PCR-analysis:

- at the temperature from 18 to 25 °C – for no more than 6 hours;
- at the temperature from minus 24 to minus 16 °C – for 1 year;
- at the temperature ≤ -68 °C – for a long time.

Only one freeze-thawing cycle is acceptable.

6.9 Tissue (biopsy, surgical) material.

Place the tissue (biopsy, surgical) material (5-10 mm in a diameter) to a sterile porcelain mortar and grind it up using a mallet. Add 1 ml of **Transport Medium with Mucolytic Agent** (952-CE) to the obtained homogenate and thoroughly mix using a mallet. Use 100 µl of suspension for DNA extraction. Place the tissue (biopsy, surgical) material (less than 5 mm in a diameter) to a sterile porcelain mortar and grind it up using a mallet. Add 0.5 ml of **Transport Medium with Mucolytic Agent** to the obtained homogenate and thoroughly mix using a mallet. Use 100 µl of suspension for DNA extraction.

6.10 Synovial fluid.

Transfer 1.0 ml of synovial fluid to the disposable 1.5-ml tube. Centrifuge at 8,000-9,000 g (for example, 12,000-13,000 rpm for the MiniSpin Eppendorf microcentrifuge) for 5 minutes. Remove the supernatant leaving 100 µl of the pellet for subsequent DNA extraction.

6.11 Cerebrospinal fluid (CSF).

Transfer 1.0 ml of cerebrospinal fluid (CSF) to the disposable 1.5-ml tube. Centrifuge at 8,000-9,000 g (for example, 12,000-13,000 rpm for the MiniSpin Eppendorf microcentrifuge) for 5 minutes. Remove the supernatant leaving 100 µl of the pellet for subsequent DNA extraction.

6.12 Urine.

The urine samples are to be pretreated.

Transfer 1 ml of urine into the sterile disposable 1.5-ml tube. Centrifuge at 8,000-9,000 g (for example, 12,000-13,000 rpm for the MiniSpin Eppendorf microcentrifuge) for 5 min. Carefully remove the supernatant using the vacuum aspirator and leaving the pellet and 100 µl of supernatant. Add 100 µl of **Transport Medium with Mucolytic Agent** (952-CE) and vortex thoroughly. Extraction should be carried out from 100 µl of the sample.

The pretreated samples of above-mentioned material can be stored before the PCR-analysis:

- at the temperature from 18 to 25 °C – for no more than 6 hours;
- at the temperature from 2 to 8 °C – for no more than 1 day;
- at the temperature from minus 24 to minus 16 °C – for a year;
- at the temperature ≤ -68 °C – for a long time.

Only one freeze-thawing cycle is acceptable.

Interfering substances and limitations of using test material samples

The next samples are inapplicable for analysis:

- the samples of biological material collected more than 48 hours before the delivery to the laboratory;
- the whole blood samples, collected in the tubes with heparin as anticoagulant,
- the whole blood samples, containing blood clot or which has been exposed to freezing.

In order to control the DNA extraction efficiency and possible reaction inhibition the Internal Control (Internal Control-FL (IC)) is used in the PCR kit. The Internal Control is added in each biological sample

at the extraction stage. The presence of internal control signal after the amplification testifies the effectiveness of nucleic acid extraction and the absence of PCR inhibitors.

Potential interfering substances

Endogenous and exogenous substances that may be present in the biological material (oropharyngeal swabs, whole blood, tissue (biopsy, surgical) material, synovial fluid, discharge of erosive and ulcerative lesions of the skin, cerebrospinal fluid (CSF), urine) used for the study were selected to assess potential interference.

6.13 Whole blood.

Samples of whole venous blood taken in tubes with EDTA as an anticoagulant without adding and with the addition of potentially interfering substances in concentrations exceeding the upper limit of the normal concentration of these substances in whole venous blood were tested to assess the effect of endogenous substances (Table 2).

Samples of whole venous blood taken in tubes with heparin and in tubes with EDTA as an anticoagulant were tested to assess the effect of exogenous substances (anticoagulants) (Table 2).

Sample of whole blood with *Streptococcus pyogenes* ATCC 19615™ strain were tested at concentrations 1×10^5 and 4×10^2 GE/ml. Each sample was tested in two repeats.

6.13 Oropharyngeal swabs.

Oropharyngeal swabs without adding and with addition of exogenous potential interfering substances, such as Chlorhexidine (5% solution for local and external use), Stomatophyt (15% solution for local use) and Miramistin (0,01% solution for local use) were tested. Chlorhexidine concentration in sample was 0,5 %, Stomatophyt – 1,5 %, Miramistin – 0,001 % (Table 2).

Oropharyngeal swabs with *Streptococcus pyogenes* ATCC 19615™ strain were tested at concentrations 1×10^5 and 4×10^2 GE/ml. Each sample was tested in two repeats.

6.14 Urine.

Urine samples with high Albumine content (500 mg/l) were tested to assess the effect of high concentrations of endogenous substances. Also the urine samples with acidity above and below normal were tested (pH 4,0; pH 9,0) (Table 2).

Urine samples with adding Azithromycin were tested to assess the effect of exogenous potentially interfering substance. Substance's concentration in sample was 0,8 mg/ml.

Urine samples with *Streptococcus pyogenes* ATCC 19615™ strain were tested at concentrations in 1×10^5 and 4×10^2 GE/ml. Each sample was tested in two repeats.

6.15 Discharge of erosive and ulcerative lesions of the skin.

Discharge of erosive and ulcerative lesions of the skin without adding and with addition of exogenous potential interfering substances, such as iodine (5 % solution for external use). Iodine concentration in sample was 0,5% (Table 2).

Discharge of erosive and ulcerative lesions of the skin with *Streptococcus pyogenes* ATCC 19615™ strain were tested at concentrations 1×10^5 and 4×10^2 GE/ml. Each sample was tested in two repeats.

6.16 Cerebrospinal fluid (CSF).

Cerebrospinal fluid (liquor) samples with high Glucose content (10 mmol/l) and with high Leucocytes content ($500/\text{mm}^3$) were tested to assess the effect of high concentrations of endogenous substances/ingredients (Table 2).

Liquor samples with *Streptococcus pyogenes* ATCC 19615™ strain were tested at concentrations 1×10^5 and 4×10^2 GE/ml. Each sample was tested in two repeats.

6.17 Synovial fluid.

Synovial fluid samples without adding and with adding of exogenous potential interfering substances, such as Cefazolin sodium were tested. Cefazolin sodium concentration sample was 64 µg/ml (Table 2).

Synovial fluid samples with *Streptococcus pyogenes* ATCC 19615™ strain were tested at concentrations 1x10⁵ and 4x10² GE/ml. Each sample was tested in two repeats.

6.18 Tissue (biopsy, surgical) material.

Tissue material samples without adding and with addition of exogenous potential interfering substances, such as Ceftriaxone were tested. Ceftriaxone concentration in sample was 257 µg/ml (Table 2).

Tissue material samples with *Streptococcus pyogenes* ATCC 19615™ strain were tested at concentrations 1x10⁵ and 4x10² GE/ml. Each sample was tested in two repeats.

Table 2

Test material	Potential interferent aspect	Potential interferent	Tested concentration	Interference presence
Whole blood	Endogenous substances	Total bilirubin	210 µmol/l (upper limit of normal – 21 µmol/l)	Not detected
		Total cholesterol	78 mmol/l (upper limit of normal – 7,8 mmol/l)	Not detected
		Triglycerides	37,0 mmol/l (upper limit of normal – 3,7 mmol/l)	Not detected
		Hemoglobin	250 g/l (upper limit of normal – 170 g/l)	Not detected
	Exogenous substances	Potassium EDTA	to 2,0 mg/ml	Not detected
		Lithium heparin	to 12 ME/ml	<u>Detected</u>
Oropharyngeal swabs	Exogenous substances	Chlorhexidine	0,5 %	Not detected
		Stomatophyt	1,5 %	Not detected
		Miramistin	0,001 %	Not detected

Urine	Endogenous substances	Urine overacidification	pH 4,0 (pH value is in the normal of 5,0 – 7,0)	Not detected
		Urinary alkalinizing	pH 9,0 (pH value is in the normal 5,0 – 7,0)	Not detected
		Albumine	500 mg/l (upper limit of normal – 20 mg/l)	Not detected
	Exogenous substances	Azithromycin	0,8 mg/ml	Not detected
Discharge of erosive and ulcerative lesions of the skin	Exogenous substances	Iodine (potassium iodide)	0,5%	Not detected
Cerebrospinal fluid (CSF)	Endogenous substances	Glucose	10 mmol/l (upper limit of normal – 3,89 mmol/l)	Not detected
		Leucocytes	500 /mm ³ (upper limit of normal – 20 / mm ³)	Not detected
Synovial fluid	Exogenous substances	Cefazolin sodium	64 µg/ml	Not detected
Tissue (biopsy, surgical) material	Exogenous substances	Ceftriaxone	257 µg/ml	Not detected

7 WORKING CONDITIONS

eSens Streptococcus pyogenes QT PCR kit should be used at 18–25 °C.

8 PROTOCOL

8.1 DNA extraction

Any commercial nucleic acid extraction kit, if IVD-CE validated for the indicated specimen types, could be used.

Ecoli Dx, s.r.o. recommends:

- For the manual extraction

- **RIBO-prep** (K2-9-Et-100-CE).

- For the automatic extraction

- **ePure Bacterial DNA Extraction Kit** (E2006)

NOTE: Extract the DNA according to the manufacturer's protocol.

The DNA extraction for each sample is carried out in the presence of **Internal Control-FL (IC)**.

8.2 Preparing PCR

8.2.1 Preparing tubes for PCR

The type of tubes depends on the PCR instrument used for analysis. Use disposable filter tips for adding reagents, DNA and control samples into tubes.

The total reaction volume is **25 µl**, the volume of the DNA sample is **10 µl**.

1. Calculate the required quantity of each reagent for reaction mixture preparation. For one reaction:

**10 µl of PCR-mix-FL *Streptococcus pyogenes*,
5 µl of PCR-buffer-H.**

Prepare the reaction mixture for the total number of test and control samples plus several extra reactions. See numbers of control samples in item 7.

NOTE: Prepare the reaction mixture just before use.

2. Thaw the tubes with **PCR-mix-FL *Streptococcus pyogenes* and PCR-buffer-H**. Thoroughly vortex the tubes with **PCR-mix-FL *Streptococcus pyogenes* and PCR-buffer-H** and sediment the drops by vortex.
3. In a new tube prepare the reaction mixture. Mix the required quantities of **PCR-mix-FL *Streptococcus pyogenes* and PCR-buffer-H**. Sediment the drops by vortex.
4. Take the required number of the tubes or strips taking into account the number of test samples and control samples.
5. Transfer **15 µl** of the prepared reaction mixture to each tube. Discard the unused reaction mixture.
6. Add **10 µl of DNA samples** obtained at the DNA extraction stage from test samples to the prepared tubes.
7. Carry out the control amplification reactions:

C1	–	Add 10 µl of C1 SP to two tubes labeled C1
C2	–	Add 10 µl of C2 SP to two tubes labeled C2
C-	–	Add 10 µl of the sample extracted from the Negative Control (C-) reagent to the tube labeled C-
PCE	–	Add 10 µl of the sample extracted from Positive Control <i>Streptococcus pyogenes</i> to the tube labeled PCE
NOTE:		It is also necessary to carry out Negative Control of Amplification (NCA) at suspicion on possible contamination
NCA	–	Add 10 µl of TE-buffer to the tube labeled NCA

8.2.2 Amplification

1. Create a temperature profile on your instrument as follows:

Table 4

eSens unified amplification and fluorescence detection program

	Rotor-type Instruments (e.g Rotor-Gene Q or equivalent)		Plate-type Instruments (e.g CFX 96 Touch, CFX 96 Opus, QuantStudio 5 or equivalent)	
Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	50	15 min	–	1
2	95	15 min	–	1
3	95	10 s	–	45
	60	20 s	FAM, JOE	

NOTE: Any combination of the tests including test with reverse transcription and amplification can be performed in one instrument simultaneously with the use of the unified amplification program. If several tests in “multiprime” format are carried out simultaneously, the detection is enabled in other used channels except for the specified ones. If only the tests for pathogen agent DNA detection are performed in one instrument then the first step of reverse transcription (50 °C – 15 minutes) can be omitted for time saving

2. Adjust the fluorescence channel sensitivity according to the *Technical Sheet*.
3. Insert tubes into the reaction module of the instrument.

NOTE: It is recommended to sediment drops from walls of tubes by short centrifugation before placing them into the instrument.
Insert empty tubes at the edges of reaction module in case of incomplete filling of plate-type instrument.

4. Run the amplification program with fluorescence detection.
5. Analyze results after the amplification program is completed.

9 DATA ANALYSIS

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in two channels:

Table 5

Channel for the fluorophore	FAM	JOE
Signal registration, indicating the amplification product accumulation	Internal Control-FL (IC) DNA	<i>Streptococcus pyogenes</i> DNA

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a Ct value of the DNA sample in the corresponding column of the results grid.

Principle of interpretation is the following:

Based on the obtained *Ct* values and specified concentration values of DNA calibrators (C1 and C2) a calibration line is automatically plotted and the values of *Streptococcus pyogenes* DNA GE in 1 ml of tested and control samples are calculated.

NOTE: Concentration values of calibrators are specified in the *Technical Sheet* enclosed to the PCR kit.

Table 6

Results interpretation for the test samples

Result	Interpretation
Invalid	The <i>Ct</i> value in the channel for the FAM fluorophore is absent or determined greater than the boundary value. The PCR analysis (beginning with the DNA extraction stage) should be repeated for this sample.
<i>Streptococcus pyogenes</i> DNA is not detected	The <i>Ct</i> value for <i>Streptococcus pyogenes</i> DNA is absent or determined greater than the boundary value and the <i>Ct</i> value determined in the channel for the FAM fluorophore is less than the boundary value. The result is <i>Streptococcus pyogenes</i> is not detected.
less than 1×10^3 GE/ml	<i>Streptococcus pyogenes</i> DNA was detected in concentration less than the linear measurement range of the PCR kit. The result is less than 1×10^3 <i>Streptococcus pyogenes</i> GE/ml
$X \times 10^y$ GE/ml	Calculated concentration value (copies/ml) is in the lower limit of measurement range of the PCR kit. The result is <i>Streptococcus pyogenes</i> DNA is detected in concentration $X \times 10^y$ copies/ml
greater than 1×10^7 GE/ml	<i>Streptococcus pyogenes</i> DNA was detected in concentration greater than the upper limit of measurement range of the PCR kit. The result is greater than 1×10^7 <i>Streptococcus pyogenes</i> GE/ml. If the accurate quantification is required, the extracted sample is to be diluted by TE-buffer reagent (for example, 100-fold dilution) and the PCR-analysis is to be repeated from the amplification stage. The result obtained after repeated analysis should be multiplied by the coefficient of the sample dilution.

NOTE: Boundary *Ct* values are specified in the *Technical Sheet* enclosed to the PCR kit.

The results of the analysis is considered reliable only if the results obtained for controls of amplification and extraction stages are correct (according to Table 7 and the *Technical Sheet* enclosed to the PCR kit).

Table 7

Results for controls

Control	Stage for control	Amplification results in the channel for fluorophore	
		FAM	JOE
C-	DNA extraction	Ct value < boundary value	Ct value is absent
PCE	DNA extraction	Ct value < boundary value	Ct value < boundary value, concentration value is within the range
NCA	PCR	Ct value is absent	Ct value is absent
C1	PCR	Ct value and calculated concentration are defined	Ct value and calculated concentration are defined
C2	PCR	Ct value and calculated concentration are defined	Ct value and calculated concentration are defined

NOTE: Boundary Ct values and the range of Positive Control *Streptococcus pyogenes* concentration are specified in the *Technical Sheet* enclosed to the PCR kit.

10 TROUBLESHOOTING

Results of analysis are not taken into account in the following cases:

1. The Ct value determined for the Positive Control of Extraction (PCE) in the channels for the FAM and/or JOE fluorophores is greater than the boundary Ct value or absent. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples.
2. The calculated concentration of the Positive Control *Streptococcus pyogenes* does not fit in the range specified in the *Technical Sheet*. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples.
3. The Ct value is determined for the Negative Control of Extraction (C-) in the channel for the JOE fluorophore. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples in which specific DNA was detected.
4. The Ct value is determined for the Negative Control of amplification (NCA) in the channels for the FAM and/or JOE fluorophores. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The amplification and detection should be repeated for all samples in which specific DNA was detected.
5. The Ct values and calculated concentration are absent for the DNA-calibrators C1 and C2 in either of the specified channels for fluorophores. The amplification and detection should be repeated for all the samples.
6. The correlation coefficient R^2 is less than 0.98 when plotting the calibration curve. Check the correctness of set concentrations of calibrators in accordance with the *Technical Sheet*. If the

improper result has been obtained again the amplification and detection for all the samples should be repeated.

- The Ct value is determined for the test sample, whereas the area of typical exponential growth of fluorescence is absent (the graphic looks like approximate straight line). It is necessary to check the correctness of selected threshold line level or parameters of base line calculation. If the result has been obtained with the correct level of threshold line (base line), the amplification and detection should be repeated for this sample.

11 TRANSPORTATION

eSens Streptococcus pyogenes QT PCR kit should be transported at 2–8 °C for no longer than 5 days.

12 STABILITY AND STORAGE

All components of the **eSens Streptococcus pyogenes QT PCR kit** are to be stored at 2–8 °C when not in use (except for PCR-buffer-H and PCR-mix-FL *Streptococcus pyogenes*). All components of the **eSens Streptococcus pyogenes QT PCR kit** are stable until labeled expiration date. The shelf life of opened reagents is the same as that of unopened reagents, unless otherwise stated.

NOTE: PCR-buffer-H and PCR-mix-FL *Streptococcus pyogenes* are to be stored at the temperature from minus 24 to minus 16 °C

NOTE: PCR-mix-FL *Streptococcus pyogenes* is to be kept away from light

13 SPECIFICATIONS

13.1 Measurement range and limit of detection

Test material	Transport medium	Nucleic acid extraction kit	PCR kit	Limit of detection, GE/ml	Measurement range, GE/ml
Oropharyngeal swabs	transport medium	RIBO-prep	ES3810A	4x10 ²	1x10 ³ – 1x10 ⁷
Whole blood	—				
Tissue (biopsy, surgical) material	Transport Medium with Mucolytic Agent				
Cerebrospinal fluid (CSF)	—				
Synovial fluid	—				
Discharge of erosive and ulcerative lesions of the skin	Transport Medium with Mucolytic Agent				
Urine	—				

13.2 Analytical specificity

The analytical specificity of **eSens Streptococcus pyogenes QT PCR kit** is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes have been checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

The PCR kit detects the DNA fragments of *Streptococcus pyogenes*: *Streptococcus pyogenes* ATCC 19615™ from ATCC collection (American Type Culture Collection, CША) with concentration $\geq 1 \times 10^7$ GE/ml. The analytical specificity of the PCR kit was proved on testing DNA/RNA of the following microorganisms/strains as well as human DNA:

- Strains: *Acinetobacter baumannii* ATCC 19606™, *Candida albicans* ATCC 14053™, *Enterococcus faecalis* ATCC 29212™, *Escherichia coli* ATCC 25922™, *Haemophilus influenzae* ATCC 33930™, *Klebsiella oxytoca* ATCC 49131™, *Klebsiella pneumoniae* ATCC 27736™, *Listeria grayi* ATCC 25401™, *Listeria innocua* ATCC 33090™, *Listeria monocytogenes* ATCC 7644™, *Moraxella catarrhalis* ATCC 25240™, *Neisseria gonorrhoeae* ATCC 19424™, *Neisseria meningitidis* ATCC 13102™, *Neisseria meningitidis* ATCC 13090™, *Proteus mirabilis* ATCC 12453™, *Pseudomonas aeruginosa* ATCC 15442™, *Staphylococcus aureus* ATCC 6538P™, *Staphylococcus epidermidis* ATCC 12228™, *Staphylococcus haemolyticus* ATCC 29970™, *Staphylococcus saprophyticus* ATCC 49907™, *Streptococcus agalactiae* ATCC 12386™ from ATCC collection (American Type Culture Collection, USA) with concentration $\geq 1 \times 10^7$ GE/ml;
- Strains: *Human gammaherpesvirus 4* NIBSC N°09/260 with concentration = 5×10^6 UI/ml, *Human polyomavirus 1* NIBSC N°14/212 with concentration = 2×10^7 UI/ml, *Human polyomavirus 2* NIBSC N°14/114 with concentration = 1×10^7 UI/ml, *Primate erythrovirus* NIBSC N° 99/802 with concentration = 5×10^5 UI/ml, *Human betaherpesvirus 5* NIBSC N° 09/162 with concentration = 5×10^6 UI/ml from NIBSC collection (National Institute for Biological Standards and Control, UK);
- Clinical isolates of strains and isolates panel which is at FBIS CRIE disposal: *Candida glabrata*, *Candida parapsilosis*, *Enterovirus* spp., *Human alphaherpesvirus 1*, *Human alphaherpesvirus 2*, *Human alphaherpesvirus 3*, *Human betaherpesvirus 6A*, *Human betaherpesvirus 6B*, *Human betaherpesvirus 7*, *Pneumocystis jirovecii*, *Streptococcus mitis*, *Streptococcus sanguis*, *Toxoplasma gondii* with concentration $\geq 1 \times 10^4$ GE/ml;
- Human DNA (Sigma Aldrich, USA) with concentration $\geq 1 \times 10^6$ GE/ml.

The nonspecific responses were not observed while testing the DNA samples of the above mentioned microorganisms, as well as human DNA.

The clinical specificity of **eSens Streptococcus pyogenes QT PCR kit** was confirmed in laboratory clinical trials.

The information about known interfering substances is specified in the *Interfering substances and limitations of using test material samples*.

13.3 Reproducibility, repeatability and trueness

Repeatability and reproducibility were determined by testing of quality control sample (QCS Positive Control *Streptococcus pyogenes* DNA) with concentrations 1×10^6 , 1×10^5 and 1×10^4 GE/ml which lay within the measurement range.

Table 8

Reproducibility

PCR kit	Initial concentration value, GE/ml	Number of repeats	Average concentration value, lg	Standard deviation (SD)	Coefficient of variation (CV), %
ES3810A	1×10^6	80	6,07	0,05	0,83
	1×10^5	80	5,12	0,05	1,01
	1×10^4	80	4,12	0,06	1,45

Table 9

Repeatability

PCR kit	Initial concentration value, GE/ml	Number of repeats	Average concentration value, lg	Standard deviation (SD)	Coefficient of variation (CV), %
ES3810A	1×10^6	40	6,05	0,03	0,48
	1×10^5	40	5,03	0,04	0,84
	1×10^4	40	4,05	0,05	1,14

Trueness was determined by testing of quality control sample (QCS Positive Control *Streptococcus pyogenes* DNA) with known concentration.

Table 10

Trueness

PCR kit	Number of repeats	Average concentration value, lg	Specified value, lg	Bias (B), %
ES3810A	100	6,78	6,78	0,00

13.4 Diagnostic characteristics

The samples of biological material (oropharyngeal swabs, whole blood, tissue material, synovial fluid, discharge of erosive and ulcerative lesions of the skin, cerebrospinal fluid (CSF), urine) taken from the 550 persons with streptococcal infection and 210 persons without streptococcal infection were used to confirm the diagnostic specificity of **eSens Streptococcus pyogenes QT PCR kit**.

Table 11

The results of testing eSens Streptococcus pyogenes QT PCR kit

Sample type	The results of application of eSens Streptococcus pyogenes QT PCR kit		Results of using the reference assay *	
			Positive	Negative
Oropharyngeal swabs	230 samples were tested	Positive	134	0
		Negative	0	96
Whole blood	80 samples were tested	Positive	31	0
		Negative	0	49
Tissue (biopsy, surgical) material	80 samples were tested	Positive	29	0
		Negative	0	51
Synovial fluid	80 samples were tested	Positive	33	0
		Negative	0	47
Discharge of erosive and ulcerative lesions of the skin	80 samples were tested	Positive	28	0
		Negative	0	52
Cerebrospinal fluid (CSF)	80 samples were tested	Positive	26	0
		Negative	0	54
Urine	130 samples were tested	Positive	63	0
		Negative	0	67

* Bacterial inoculation on the PYR Agar culture medium. "PYR Agar for isolation and identification of *S.pyogenes*".

Diagnostic characteristics of eSens *Streptococcus pyogenes* QT PCR kit

Sample type	Diagnostic sensitivity, (with a confidence level of 95 %) in the interval, %	Diagnostic specificity, (with a confidence level of 95 %) in the interval, %
Oropharyngeal swabs	97,2-100	96,2-100
Whole blood	88,7-100	92,7-100
Tissue (biopsy, surgical) material	88,0-100	93,0-100
Synovial fluid	89,4-100	92,4-100
Discharge of erosive and ulcerative lesions of the skin	87,6-100	93,1-100
Cerebrospinal fluid (CSF)	86,7-100	93,4-100
Urine	94,3-100	94,6-100

Correlation of *Streptococcus pyogenes* DNA concentration values with values obtained by reference method*.

*** QX100 system is for providing droplet digital PCR (QX100 droplet digital PCR)**

344 samples of different biological material contained *Streptococcus pyogenes* DNA in concentration from 400 to $9,9 \times 10^6$ GE/ml were analyzed.

Approximation accuracy value (R^2) for each type of biological material amounted over 80%.

14 QUALITY CONTROL

The production process, including batch release, is carried out in accordance with an established quality management system certified according to ISO 13485.

15 KEY TO SYMBOLS USED

 REF	Catalogue number		Caution
 LOT	Batch code		Contains sufficient for <n> tests
 IVD	<i>In vitro</i> diagnostic medical device		Use-by Date
 VER	Version		Consult instructions for use
	Temperature limit		Keep away from sunlight
	Manufacturer	C-	Negative control of extraction
	Date of manufacture	C+	Positive control of amplification
	Authorized representative in the European Community	C1, C2	DNA-calibrators
PCE	Positive control of extraction	IC	Internal control

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
01_04/2022		

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