# AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT PCR kit

For Professional Use Only

# Instruction Manual

## KEY TO SYMBOLS USED

| Symbol | Meaning | Symbol | Meaning |
|---|---|---|---|
| REF | Catalogue number | | Caution |
| LOT | Batch code | Σ | Contains sufficient for <n> tests |
| IVD | *In vitro* 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 |
| EC REP | Authorized representative in the European Community | C1, C2 | DNA-calibrators |
| PCE | Positive control of extraction | IC | Internal control |

## 1. INTENDED USE

**AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT** 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.

The PCR kit variant FRT-100 FN 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 | *Streptococcus pyogenes* DNA |
| Target gene | Artificially synthesized sequence | erythrogenic toxin B (*speB*) gene |

## 3. CONTENT

**AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT** PCR kit is produced in 2 forms:

**variant FRT-100 FN** REF H-2171-1-1-CE.

**variant FRT-L** REF H-2172-1-14-CE.

**Variant FRT-100 FN** includes:

| Reagent | Description | Volume, ml | Quantity |
|---|---|---|---|
| **PCR-mix-FL *Streptococcus pyogenes*** | 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 *Streptococcus pyogenes******* | 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 (see **RIBO-prep**, REF K2-9-Et-100-CE protocol).

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

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

**Variant FRT-100 FN** is intended for 110 reactions (including controls).

**Variant FRT-L** includes:

| Reagent | Description | Volume, ml | Quantity |
|---|---|---|---|
| **PCR-mix *Streptococcus pyogenes*-Lyo** | white powder | - | 96 tubes of 0.2 ml |
| **C1 SP** | colorless clear liquid | 0.5 | 1 tube |
| **C2 SP** | colorless clear liquid | 0.5 | 1 tube |
| **TE-buffer** | colorless clear liquid | 0.5 | 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 *Streptococcus pyogenes******* | 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 (see **RIBO-prep**, REF K2-9-Et-100-CE protocol).

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

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

**Variant FRT-L** is intended for 96 reactions (including controls).

## 4. ADDITIONAL REQUIREMENTS

- Transport medium with mucolytic agent
- Transport medium for storage and transportation of respiratory swabs.
- 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), CFX96 (Bio-Rad, USA)).
- 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 reaction mixture preparation for PCR kit variant FRT-100 FN;
  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 kit variant FRT-100 FN;
  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 variant FRT-100 FN.
- 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.




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## 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

**AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT** 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***

*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 500 µl of **Transport Medium for Storage and Transportation of Respiratory Swabs** (REF 959-CE, REF 957-CE, REF 958-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.

*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.

*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** (REF 952-CE; REF 953-CE).

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

*Synovial fluid.*

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

*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** (REF 952-CE; REF 953-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.

*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.

*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.

*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** (REF 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** (REF 137-CE) may be repeated if necessary. The obtained pellet must be immediately lysed (in case of extraction using **RIBO-prep** 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.

*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** (REF 952-CE; REF 953-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** (REF 952-CE; REF 953-CE) to the obtained homogenate and thoroughly mix using a mallet. Use 100 µl of suspension for DNA extraction.

*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.

*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.

*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** (REF 952-CE; REF 953-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.

*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 $1x10^5$ and $4x10^2$ GE/ml. Each sample was tested in two repeats.

*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 $1x10^5$ and $4x10^2$ GE/ml. Each sample was tested in two repeats.

*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 $1x10^5$ and $4x10^2$ GE/ml. Each sample was tested in two repeats.

*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 $1x10^5$ and $4x10^2$ GE/ml. Each sample was tested in two repeats.

*Cerebrospinal fluid (CSF)*

Cerebrospinal fluid (liquor) samples with high Glucose content (10 mmol/l) and with high Leucocytes content ($500/mm^3$) 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 $1x10^5$ and $4x10^2$ GE/ml. Each sample was tested in two repeats.

*Synovial fluid*
Synovial fluid samples without additing and with additing of exogenous potential interfering substances, such as Cefazolin sodium were tested. Cefazolin sodium concentration sample was 64 µg/ml (Table 2).
Sinovvial fluid samples with *Streptococcus pyogenes* ATCC® 19615TM strain were tested at concentrations $1x10^5$ and $4x10^2$ GE/ml. Each sample was tested in two repeats.

*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® 19615TM strain were tested at concentrations $1x10^5$ and $4x10^2$ 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 | Detected |
| 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

**AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT** PCR kit should be used at 18–25 °C.

## 8. PROTOCOL

### 8.1. DNA Extraction

It is recommended to use the following nucleic acid extraction kits:

- **RIBO-prep**, REF K2-9-Et-100-CE.

**If using the RIBO-prep kit** extract the DNA according to the manufacturer's protocol.

The volumes of reagents and samples when the DNA is extracted by the RIBO-prep reagent kit:

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

**NOTE:** Add **10 µl** of **Internal Control-FL (IC)** to each tube.

The volume of the test sample is **100 µl.**

Add **100 µl of Negative Control (C–)** into the tube labeled C– (Negative Control of Extraction).

Add **10 µl of Positive Control *Streptococcus pyogenes*** and **90 µl of Negative Control (C–)** into the tube labeled PCE (Positive Control of Extraction).

The volume of elution is **50 µl.**

### 8.2. Preparing PCR

### 8.2.1 Preparing tubes for PCR

**Variant FRT-100 FN**

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

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 *Streptococcus pyogenes*** 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** |

**Variant FRT-L**

The total reaction volume is **25 µl**, the volume of the **DNA** sample is **25 µl**.
Use disposable filter tips for adding reagents, DNA and control samples into tubes.

1. Take the required number of the tubes with ready-to-use lyophilized reaction mixture **PCR-mix *Streptococcus pyogenes*-Lyo** for amplification of DNA from test and control samples (see numbers of control samples in point 3).
2. Add **25 µl** of **DNA samples** extracted from test samples into the prepared tubes.
3. Carry out the control reactions:

| | |
|---|---|
| **C1** | – Add **25 µl** of **C1 SP** to two tubes labeled **C1** |
| **C2** | – Add **25 µl** of **C2 SP** to two tubes labeled **C2** |
| **C-** | – Add **25 µl** of **the sample extracted from the Negative Control (C-) reagent** to the tube labeled **C-** |
| **PCE** | – Add **25 µl** of **the sample extracted from Positive Control *Streptococcus pyogenes*** 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 **25 µl** of **TE-buffer** to the tube labeled **NCA** |

**NOTE:** Mix the tubes thoroughly by pipetting avoiding foaming.

### 8.2.2. Amplification

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

Table 4

**AmpliSens unified amplification and fluorescence detection program for rotor-[1] and plate-type instruments[2]**

| 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 *Important Product Information Bulletin.*
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.

[1] For example, Rotor-Gene Q (QIAGEN, Germany).
[2] For example, CFX 96 (Bio-Rad).


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## 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 | *Streptococcus pyogenes* 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 *Important Product Information Bulletin* enclosed to the PCR kit.

Table 6

**Results interpretation for the test samples**

| Result | Interpretation |
|---|---|
| Invalid | The *Ct* 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. |
| *Streptococcus pyogenes* DNA is not detected | The *Ct* value for *Streptococcus pyogenes* DNA is absent or determined greater than the boundary value and the *Ct* value determined in the channel for the FAM fluorophore is less than the boundary value. The result is ***Streptococcus pyogenes* is not detected**. |
| less than $1x10^3$ GE/ml | ***Streptococcus pyogenes*** DNA was detected in concentration less than the linear measurement range of the PCR kit. The result is **less than $1x10^3$ *Streptococcus pyogenes* GE/ml** |
| X x $10^y$ GE/ml | Calculated concentration value (copies/ml) is in the lower limit of measurement range of the PCR kit. The result is ***Streptococcus pyogenes* DNA is detected in concentration X x $10^y$ copies/ml** |
| greater than $1x10^7$ GE/ml | *Streptococcus pyogenes* DNA was detected in concentration greater than the upper limit of measurement range of the PCR kit. The result is **greater than $1x10^7$ *Streptococcus pyogenes* 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 *Important Product Information Bulletin* 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 *Important Product Information Bulletin* 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 *Important Product Information Bulletin* 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 *Important Product Information Bulletin.* 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 *Important Product Information Bulletin.* If the improper result has been obtained again the amplification and detection for all the samples should be repeated.
7. 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.

If you have any further questions or if encounter problems, please contact our Authorized representative in the European Community.

## 11. TRANSPORTATION

**AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT** PCR kit should be transported at 2–8 ºC for no longer than 5 days.

## 12. STABILITY AND STORAGE

All components of the **AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT** 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 **AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT** 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

**NOTE:** PCR-mix *Streptococcus pyogenes*-Lyo is to be kept in packages with a desiccant 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 for Storage and Transportation of Respiratory Swabs | RIBO-prep | variant FRT-100 FN, variant FRT-L | $4x10^2$ | $1x10^3$ – $1x10^7$ |
| 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 | — | | | | |

The claimed features are achieved while respecting the rules specified in the section *Sampling and Handling*

### 13.2. Analytical specificity

The analytical specificity of **AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT** 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, США) with concentration ≥ $1x10^7$ 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 ≥ $1x10^7$ GE/ml;
- Strains: *Human gammaherpesvirus* 4 NIBSC №09/260 with concentration = $5x10^6$ UI/ml, *Human polyomavirus* 1 NIBSC №14/212 with concentration = $2x10^7$ UI/ml, *Human polyomavirus* 2 NIBSC №14/114 with concentration = $1x10^7$ UI/ml, *Primate erythroparvovirus* NIBSC № 99/802 with concentration = $5x10^5$ UI/ml, *Human betaherpesvirus* 5 NIBSC № 09/162 with concentration = $5x10^6$ 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 ≥ $1x10^4$ GE/ml;
- Human DNA (Sigma Aldrich, USA) with concentration ≥ $1x10^6$ 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 **AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT** 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 $1x10^6$; $1x10^5$ and $1x10^4$ 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), % |
|---|---|---|---|---|---|
| variant FRT-100 FN | $1x10^6$ | 80 | 6,07 | 0,05 | 0,83 |
| | $1x10^5$ | 80 | 5,12 | 0,05 | 1,01 |
| | $1x10^4$ | 80 | 4,12 | 0,06 | 1,45 |
| variant FRT-L | $1x10^6$ | 80 | 6,11 | 0,08 | 1,29 |
| | $1x10^5$ | 80 | 5,16 | 0,09 | 1,71 |
| | $1x10^4$ | 80 | 4,13 | 0,09 | 2,21 |

Table 9

**Repeatability**

| PCR kit | Initial concentration value, GE/ml | Number of repeats | Average concentration value, lg | Standard deviation (SD) | Coefficient of variation (CV), % |
|---|---|---|---|---|---|
| variant FRT-100 FN | $1x10^6$ | 40 | 6,05 | 0,03 | 0,48 |
| | $1x10^5$ | 40 | 5,03 | 0,04 | 0,84 |
| | $1x10^4$ | 40 | 4,05 | 0,05 | 1,14 |
| variant FRT-L | $1x10^6$ | 40 | 6,07 | 0,03 | 0,54 |
| | $1x10^5$ | 40 | 5,07 | 0,05 | 0,89 |
| | $1x10^4$ | 40 | 4,09 | 0,06 | 1,39 |

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), % |
|---|---|---|---|---|
| variant FRT-100 FN | 100 | 6,78 | 6,78 | 0,00 |
| variant FRT-L | 100 | 6,72 | 6,78 | 0,91 |

## 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 **AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT** PCR kit.

Table 11

**The results of testing AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT PCR kit**

| Sample type | The results of application of AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT PCR kit | | Results of using the reference assay[3] | |
|---|---|---|---|---|
| | | | 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 |

Table 12

**Diagnostic characteristics of AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT 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[4].**

344 samples of different biological material contained *Streptococcus pyogenes* DNA in concentration from 400 to $9,9x10^6$ GE/ml were analyzed.

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

## 14. REFERENCES


1. Spellerberg B., Brandt C. Laboratory Diagnosis of Streptococcus pyogenes (group A streptococci) // Basic Biology to Clinical Manifestations. – 2016. – https://www.ncbi.nlm.nih.gov/books/NBK343617/
2. Reglinski M., Sriskandan S. Streptococcus pyogenes // Molecular Medical Microbiology (Second Edition). – 2015. – Vol.2. – P. 675-716


## 15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science "Central Research Institute for Epidemiology" ISO 13485-Certified Quality Management System, each lot of the **AmpliSens® *Streptococcus pyogenes*-screen/monitor-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

**List of Changes Made in the Instruction Manual**

| VER | Location of changes | Essence of changes |
|---|---|---|
| 12.09.19 PM | Through the text | Corrections according to the template. Autopsy material was deleted. The text formatting was changed |
| | 1. Intended use | Information about bacterial infection (infection caused by *Streptococcus pyogenes*) was added. Phrase «-without distinction of form and presence of manifestation» was deleted<br>The subsection Indications and contra-indications for use of the reagent kit was added |
| | 2. Principle of RCR Detection | Information about assigning values to calibrators was added |
| | 6. Sampling and handling | Information about Transport medium with Mucolitic Agent was added.<br>The information about freezed urine samples in Interfering substances and limitations of using test material samples subsection was deleted<br>The Potential interfering substances subsection was added |
| | 9. Data analysis | In table 4 Results interpretation for the test samples in results: less than $1x10^3$ GE/ml and greater than $1x10^7$ GE/ml term "DNA" was deleted |
| | 13. Specifications | The section was actualized |
| | 14. References | The section was actualized |
| 03.06.20 KK | Footer | The phrase "Not for use in the Russian Federation" was added |
| 11.03.21 MM | — | The name, address and contact information for Authorized representative in the European Community was changed |

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

[4] QX100 system is for providing droplet digital PCR (QX100 droplet digital PCR)