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GUIDELINES

Real-Time PCR Detection of STIs and Other Reproductive Tract Infections

AmpliSens®

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The list of reagent kits AmpliSens® manufactured by FBIS CRIE for detection of STIs and other reproductive tract infections by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection

Detectable microorganisms (infectious agents)	Reagents kits
Bacterial infections	
Chlamydia trachomatis	AmpliSens® *Chlamydia trachomatis*-FRT MULTIPRIME series kits
Neisseria gonorrhoeae	AmpliSens® *Neisseria gonorrhoeae*-screen-FRT MULTIPRIME series kits
Treponema pallidum	AmpliSens® *Treponema pallidum*-FRT MULTIPRIME series kits
Mycoplasma genitalium	AmpliSens® *Mycoplasma genitalium*-FRT MULTIPRIME series kits
Ureaplasma parvum, ***Ureaplasma urealyticum***	AmpliSens® *Ureaplasma* spp.-FRT MULTIPRIME series kits
Mycoplasma hominis	AmpliSens® *Mycoplasma hominis*-FRT MULTIPRIME series kits
Gardnerella vaginalis	AmpliSens® *Gardnerella vaginalis*-FRT MULTIPRIME series kits
Virus infections – herpesviruses	
***HSV* I, II**	AmpliSens® *HSV* I, II-FRT AmpliSens® *HSV*-typing-FRT MULTIPRIME series kits
CMV	AmpliSens® *CMV*-FRT MULTIPRIME series kits
Protozoal infections	
Trichomonas vaginalis	AmpliSens® *Trichomonas vaginalis*-FRT MULTIPRIME series kits
Mycotic infections	
Candida albicans	AmpliSens® *Candida albicans*-FRT
Candida albicans, ***Candida glabrata,*** ***Candida krusei***	MULTIPRIME series kits

Simultaneously detectable microorganisms	Reagents kits of MULTIPRIME series
Chlamydia trachomatis* / *Ureaplasma* spp. / *Mycoplasma genitalium	AmpliSens® *C. trachomatis / Ureaplasma / M. genitalium*-MULTIPRIME-FRT
Chlamydia trachomatis* / *Ureaplasma* spp. / *Mycoplasma hominis	AmpliSens® *C. trachomatis / Ureaplasma / M. hominis*-MULTIPRIME-FRT
Neisseria gonorrhoeae* / *Chlamydia trachomatis* / *Mycoplasma genitalium* / *Trichomonas vaginalis	AmpliSens® *N.gonorrhoeae / C.trachomatis / M.genitalium / T.vaginalis*-MULTIPRIME-FRT
Chlamydia trachomatis* / *Ureaplasma* spp. / *Mycoplasma genitalium* / *Mycoplasma hominis	AmpliSens® *C.trachomatis / Ureaplasma / M.genitalium / M.hominis*-MULTIPRIME-FRT
Trichomonas vaginalis* / *Neisseria gonorrhoeae* / *Chlamydia trachomatis	AmpliSens® *T.vaginalis / N.gonorrhoeae / C.trachomatis*-MULTIPRIME-FRT
Neisseria gonorrhoeae* / *Chlamydia trachomatis* / *Mycoplasma genitalium	AmpliSens® *N.gonorrhoeae / C.trachomatis / M.genitalium*-MULTIPRIME-FRT
***HSV* I / *HSV* II**	AmpliSens® *HSV*-typing-FRT
Ureaplasma parvum* / *Ureaplasma urealyticum	AmpliSens® *U.parvum / U.urealyticum*-FRT (detection and differentiation)
Trichomonas vaginalis* / *Neisseria gonorrhoeae	AmpliSens® *T.vaginalis / N.gonorrhoeae*-MULTIPRIME-FRT
Candida albicans* / *Candida glabrata* / *Candida krusei	AmpliSens® *C.albicans / C.glabrata / C.krusei*-MULTIPRIME-FRT
HSV / CMV	AmpliSens® *HSV / CMV*-MULTIPRIME-FRT

1. INTENDED USE

The guidelines describe the procedure of detection of STIs and other reproductive tract infections in biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using real-time PCR instruments:

- Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia);
- iCycler iQ, iCycler iQ5 (Bio-Rad, USA);
- Mx3000P, Mx3005P (Stratagene, USA);
- CFX96 (Bio-Rad, USA).

2. PRINCIPLE OF PCR DETECTION

Detection of microorganisms by the polymerase chain reaction (PCR) is based on the amplification of a pathogen genome specific region using specific primers. In real-time PCR, the amplified product is detected using 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 Internal Control (IC) is used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition. It is possible to automate data analysis and reduce the subjectivity in the interpretation of results. Simultaneous amplification and detection of several DNA targets in a single reaction is possible. Optimization ensures a high sensitivity to each DNA target. The use of multiplex PCR allows a 3-4-fold increase in the efficiency of analysis without extending the instrument base. Reagent kits of the MULTIPRIME series are intended for multiplex PCR analysis.

3. CONTENT

PCR kit variant FRT includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-1-FL ready-to-use single-dose test tubes (*under wax)*	Solution containing primers, dNTP, and oligonucleotide probes	0.01	110 tubes of 0.2 ml
PCR-mix-2-FL-red	Buffer solution containing Taq-polymerase and Mg^{2+}	1.1	1 tube
Positive Control complex (C+)	Solution containing specific fragments of DNA of analyzed microorganisms	0.2	1 tube
DNA-buffer	Buffer solution	0.5	1 tube
Negative Control (C–)*	Buffer solution	1.2	1 tube
Internal Control-FL (IC)**	Phage (λgt67) particle solution containing a cloned genetically engineered construct with an artificial nucleotide sequence nonhomologous to known microorganisms and viruses and complementary to the fluorescent probe which is included in the PCR kit	1.0	1 tube

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

** add **10 µl** of Internal Control-FL (IC) during the DNA extraction directly to the sample/lysis mixture (see **DNA-sorb-AM**, **DNA-sorb-B** protocols).

PCR kit variant FRT is intended for 110 reactions (including controls).

PCR kit variant FRT-100 F includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-1-FL	Solution containing primers, dNTP, and oligonucleotide probes	1.2	1 tube
PCR-mix-2-FRT	Buffer solution containing Taq-polymerase and Mg^{2+}	0.3	2 tubes
Polymerase (TaqF)	Solution containing modified Taq-polymerase	0.03	2 tubes
Positive Control complex (C+)	Solution containing specific fragments of DNA of analyzed microorganisms	0.2	1 tube
DNA-buffer	Buffer solution	0.5	1 tube
Negative Control (C-)*	Buffer solution	1.2	1 tube
Internal Control-FL (IC)**	Phage (λgt67) particle solution containing a cloned genetically engineered construct with an artificial nucleotide sequence nonhomologous to known microorganisms and viruses and complementary to the fluorescent probe which is included in the PCR kit	1.0	1 tube

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

** add **10 µl** of Internal Control-FL (IC) during the DNA extraction directly to the sample/lysis mixture (see the **DNA-sorb-AM**, **DNA-sorb-B** protocols).

PCR kit variant FRT-100 F is intended for 110 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Transport medium.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol filters (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 2-ml reaction tubes.
- PCR box.
- Real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia); iCycler iQ or iCycler iQ5 (Bio-Rad, USA), Mx3000P (Stratagene, USA), CFX 96 (Bio-Rad, USA)).
- Disposable polypropylene PCR tubes (0.1- or 0.2-ml) when working with PCR kit variant FRT-100 F:
  a) 0.2-ml PCR tubes with optical transparent domed or flat caps if a plate-type instrument is used;
  b) 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.
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature 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 barriers and use a new tip for every procedure.
- Store all extracted positive material (specimens, 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 afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- 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 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 samples and reagents contact with the skin, eyes, and mucous membranes. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice immediately.
- Safety Data Sheets (SDS) are available on request.
- Use of this product should be limited to personnel trained in 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 in 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

NOTE: Obtaining samples of biological materials for PCR-analysis, transportation and storage are described in the manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

The following material is used for analysis: urogenital, rectal, and oropharyngeal swabs; conjunctival secretion; exudate of blisters and erosive-ulcerative lesions of skin and mucous membranes; urine sediment (use the first portion of the morning specimen); and prostate secretion.

The following biological material is used:

1. Women: cervical, vaginal, and urethral swabs and urine.
2. Men: urethral swabs, urine, and prostate secretion.
3. Children: urine, conjunctival secretion.

Biological material should be placed into tubes with a transport medium recommended by FBIS CRIE.

The obtained samples (except for urine) can be transported and stored under the following conditions:

- At the room temperature for 48 h;
- At 2–8 °C for two weeks;
- At ≤ –20 °C for the month;

- At ≤ –68 °C for a long time;

Samples placed to the Transport Medium with Mucolytic agent can be transported and stored under the following conditions:

- At the room temperature (18–25 °C) for 28 days;
- At 2–8 °C for 3 months;
- At ≤ –20 °C for a long time.

NOTE: Only one freeze–thaw cycle of biological material is allowed.

The obtained urine samples can be transported and stored under the following conditions:

- At the room temperature for 6 h;
- At 2–8 °C for 24 h;

Transportation of test samples is performed in special container with cooling elements.

The following types of biological material are used for microorganism DNA detection:

- urogenital swabs, urine sediment, and prostate secretion are used for detection of ***Chlamydia trachomatis***; ***Neisseria gonorrhoeae***; ***Mycoplasma genitalium***, ***M.hominis***; ***Trichomonas vaginalis***; ***Ureaplasma* spp.**, ***U. parvum***, ***U.urealyticum*** DNA;
- conjunctival secretion as well as rectal and oropharyngeal swabs are used for detection of ***Chlamydia trachomatis*** and ***Neisseria gonorrhoeae*** DNA;
- urogenital, rectal, and oropharyngeal swabs as well as exudate of blisters and erosive-ulcerative lesions of skin and mucous membranes are used for detection of ***HSV* I, II** and ***Treponema pallidum*** DNA.
- whole blood, cerebrospinal fluid and conjunctival secretion are used for detection of ***HSV* I, II** DNA as well;
- urogenital swabs, urine, saliva, and whole blood are used for detection of ***CMV*** DNA;
- urogenital and oropharyngeal swabs and urine are used for detection of ***Candida albicans***, ***C.glabrata***, and ***C.krusei*** DNA;
- vaginal swabs are used for detection of ***Gardnerella vaginalis*** DNA;

The following transport media (manufactured by FBIS CRIE) are recommended for transportation and storage of biological material:

- **Transport Medium with Mucolytic Agent**.
- **Transport Medium for Swabs**.

Pretreatment (only for urine samples)

A. Pretreatment of urine samples with the DNA-sorb-AM reagent kit for subsequent DNA extraction

1. Shake the vial with the urine.
2. Transfer 1 ml of urine to a 1.5-ml sterile disposable tube using a new tip with aerosol filter for each sample.
3. Centrifuge the tube at 10,000 g (12,000 rpm at MiniSpin centrifuge, Eppendorf) for 5 min. If urine contains excess salts, resuspend only the upper layer of salt pellet in 1 ml and centrifuge again.
4. Discard the supernatant using a vacuum aspirator with a trap flask without disturbing the pellet; use a new tip without aerosol filter for each sample.
5. Add the transport medium to the pellet (final volume, 0.2 ml). Mix thoroughly the content of the tubes using a vortex mixer. Thus pretreated urine samples (urine pellet in the transport medium) can be stored:
   - at 2–8 °C for 24 h;
   - at ≤ –20 °C for one week;
   - at ≤ –68 °C for a long time.

B. Pretreatment of urine samples with the EDEM reagent kit for subsequent DNA extraction

1. Shake the vial with urine.
2. Transfer 1 ml of urine to a tube with 0.5 ml of Transport medium TM-EDEM using a new tip with the aerosol filter for each sample.
3. Centrifuge the tubes containing Transport medium TM-EDEM and urine at 12,000 rpm for 5 min in a MiniSpin centrifuge (Eppendorf).
4. Discard the supernatant using a vacuum aspirator with a trap flask without disturbing the pellet; use a new tip without aerosol filter for each sample.
5. Add 0.5 ml of Transport medium TM-EDEM to each tube with urine pellet using a new tip for each tube. Close tubes tightly. Mix thoroughly the content of the tubes on a vortex mixer to resuspend the pellet. Centrifuge at 1500–3000 rpm for 2-3 s to spin down the drops from the walls of the tube and the cap.
6. Obtained samples of urine pellet in Transport medium TM-EDEM should be used for DNA extraction procedure.

   Thus obtained urine pellet in Transport medium TM-EDEM can be stored:
   - at the room temperature (18–25 °C) for 48 h;
   - at 2–8 °C for 14 days;

– at ≤ –20 °C for a long time.

7. WORKING CONDITIONS

Reagents kits should be used at 18–25 °C.

8. PROTOCOL

8.1 DNA EXTRACTION

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

- **DNA-sorb-AM**;
- **DNA-sorb-B** (for prostate secretion, whole blood, and cerebrospinal fluid);
- **EDEM**.

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

8.1.1 DNA extraction with DNA-sorb-AM reagent kit

DNA-sorb-AM nucleic acid extraction kit is a reagent kit for rapid and efficient manual extraction and purification of DNA from various biological material. Lysis solution contains a chaotropic agent (guanidine chloride) that lyses cells and denatures cell proteins. The nucleic acids are then adsorbed on silica particles. DNA extracted from test samples can be used for PCR diagnostic tests.

DNA-sorb-AM nucleic acid extraction kit variant 100 includes:

Reagent	Description	Volume, ml	Quantity
Lysis Solution	solution containing chaotropic agent	30	1 vial
Washing Buffer	solution containing isopropanol	100	1 vial
Universal Sorbent	suspension of silica particles	1.0	2 tubes
TE-buffer for DNA elution	buffer solution for DNA elution	5.0	2 tubes

Preparation to DNA extraction

1. Turn on the thermostat and set the temperature at 65 °C.
2. **Lysis Solution** (if stored at 2–8 °C) should be heated to 65 °C until the ice crystals disappear (ice crystals can appear at the bottom of the vial).
3. Take the required number of 1.5-ml disposable sterile tubes, label them, and place in a tube rack.
4. Centrifuge the tubes with test samples at 1500–3000 rpm for 5 s, then carefully mix using a vortex mixer, and place in a tube rack.
5. When using the first portion of the morning specimen for analysis, pretreat it to obtain the urine pellet in the transport medium as described above.

DNA extraction procedure

1. Add **10 µl** of **Internal Control-FL** to each sterile disposable tube.

NOTE: **Internal Control-FL** and **Negative Control (C–)** are included in the PCR kit.

2. Thoroughly resuspend **Universal Sorbent** on a vortex mixer. Add **20 µl** of **Universal Sorbent** and **300 µl** of **Lysis Solution** to each test tube using tips with aerosol filter.

NOTE: If the number of test samples exceeds 50, it is recommended that the whole volume of sorbent and IC are transferred to the tube with Lysis Solution (2 ml of Universal Sorbent and 1 ml of IC per 30 ml of Lysis Solution). Thoroughly stir this suspension and transfer 330 µl of it to the tubes. The prepared mixture can be stored at room temperature for 2 days. Stir well before use.

3. Add **100 µl** of a sample to the tube using a tip with aerosol filter. Add **100 µl** of **Negative Control** to the tube with Negative Control of Extraction (**C–)**.
4. Tightly close the caps, thoroughly mix the tubes on a vortex mixer, and incubate them at 65 °C for 5 min in a thermostat. After incubation, mix the contents of the tubes on a vortex once again and incubate at room temperature for another 2 min.
5. Centrifuge all tubes at 10,000 rpm for 30 s and carefully remove the supernatant from each tube with a vacuum aspirator without disturbing the pellet. Use a new tip (without aerosol filter) for every tube.
6. Add **1 ml** of **Washing Buffer** into each tube. Vortex until the sorbent is completely resuspended.
7. Repeat step 5.
8. Incubate all tubes with open caps at 65 °C for 5–10 min (for sorbent predrying).
9. Add **100 µl** of **TE-buffer** for DNA elution using tip with aerosol filter. Vortex until the sorbent is completely resuspended. Incubate tubes at 65 °C for 5 min. The elution volume can be adjusted to 150 µl.
10. Centrifuge tubes at 12,000 rpm for 1 min. The supernatant contains purified DNA and is ready for PCR amplification. The purified DNA can be stored:
    - at 2–8 °C for 1 week;
    - at –16 °C for 1 year.

If samples are analyzed once again, mix the content of the tubes on a vortex mixer and repeat centrifugation in accordance with item 10.

8.1.2 DNA extraction with DNA-sorb-B reagent kit

The principle of extraction with the DNA-sorb-B reagent kit corresponds to the principle specified above for the DNA-sorb-AM reagent kit.

DNA-sorb-B nucleic acid extraction kit variant 100 includes:

Reagent	Description	Volume, ml	Quantity
Lysis Solution	solution containing chaotropic agent	30	1 vial
Washing Solution 1	solution containing chaotropic agent and isopropanol	30	1 vial
Washing Solution 2	solution containing isopropanol	100	1 vial
Universal Sorbent	suspension of silica particles	1.25	2 tubes
TE-buffer for DNA elution	buffer solution for DNA elution	5.0	2 tubes

Preparation to DNA extraction

1. Turn on the thermostat and set temperature at 65 °C.
2. **Lysis Solution** and **Washing Solution 1** (if stored at 2–8 °C) should be heated to 65 °C until ice crystals disappear.
3. Take the required number of 1.5-ml sterile disposable tubes, label them, and place in a tube rack.
4. Centrifuge the tubes with test samples at 1500–3000 rpm for 5 s, then carefully mix using a vortex mixer, and place in a tube rack.

DNA extraction procedure

1. Add **10 µl** of **Internal Control-FL** to each sterile disposable tube.

NOTE: **Internal Control-FL** and **Negative Control (C–)** are included in the PCR kit.

2. Add **300 µl** of **Lysis Solution** to each prepared tube.
3. Add **100 µl** of **a sample** to the tubes with Internal Control and Lysis Solution. Add **100 µl** of **Negative Control** to the tube labeled **C–.**
4. Vortex the tubes and then incubate at 65 °C for 5 min. Centrifuge all tubes at 5,000 rpm for 5 s. If a sample hasn't dissolved completely, centrifuge the tube at 12000 rpm for 5 min, transfer the supernatant to a new tube, and use for DNA extraction.
5. Thoroughly resuspend **Universal Sorbent** on vortex mixer. Add **25 µl** of **Universal Sorbent** into each test tube using a new tip**.** Vortex the tubes, then place them in a tube rack for 2 min. Vortex once again and place the tubes for 5 min in a tube rack.
6. Centrifuge all tubes at 5000 rpm for 30 s. Discard the supernatant using a vacuum aspirator. Use a new tip for every tube.
7. Add **300 µl** of **Washing Solution 1** to each tube. Vortex until the sorbent is completely resuspended.
8. Repeat step 6.
9. Add **500 µl** of **Washing Solution 2** to each tube. Vortex until sorbent is completely

resuspended.

10. Centrifuge at 10,000 rpm for 30 s. Discard the supernatant using a vacuum aspirator. Use a new tip for every tube.
11. Repeat steps 9-10. Remove the supernatant entirely.
12. Incubate all tubes with open caps at 65 °C for 5-10 min.
13. Add **50 µl** of **TE-buffer for DNA elution**. Mix the contents of the tubes on a vortex mixer. Incubate the tubes at 65 °C for 5 min, vortex occasionally while incubating.
14. Centrifuge tubes at 12,000 rpm for 1 min. The supernatant contains purified DNA and is ready for PCR amplification. The purified DNA can be stored:
    - at 2–8 °C for 1 week;
    - at ≤ –16 °C for 1 year.

8.1.3 DNA extraction by express method with the EDEM reagent kit

Reagent kit for Extraction of DNA by Express Method (EDEM) is intended for the treatment of different types of biological material (urogenital, oropharyngeal, and conjunctival swabs; erosive-ulcerative lesions of skin and mucous membranes; and first portions of human urine samples[1]) with subsequent tests for the presence of STIs and other reproductive tract infections by using hybridization-fluorescence detection and PCR kits manufactured by FBIS CRIE (including the MULTIPRIME series kits).

The reagent kit EDEM is intended for qualitative PCR-analysis and primary screening of patients.

NOTE: This reagents kit is not intended for quantitative PCR analysis or monitoring after treatment (for these purposes, DNA-sorb-AM reagents kit is used for DNA extraction).

NOTE: Samples must be placed into tubes with Transport medium TM-EDEM only (the EDEM reagent kit contains Transport medium TM-EDEM).

Biological material obtained from a patient is transferred into Transport medium TM-EDEM, in which it is stored and transported to a laboratory. For DNA extraction, an aliquot of a test sample is transferred into a tube with “IC-diluent”, after which it is thermally processed to destroy cell membranes, viral coats, and other biopolymer complexes, and to ensure DNA release. Insoluble components are pelleted on the tube bottom by centrifuging; the supernatant containing DNA is used for PCR. The internal control sample (IC) contained in “IC-diluent” is isolated simultaneously with DNA from biological material and, thereby, is a quality marker of laboratory analysis of test samples.

[1] Urine samples should be preliminary treated.

EDEM reagent kit includes:

Reagent	Description	Volume, ml	Quantity
Transport medium TM-EDEM	colorless clear liquid	0.5	100 tubes
IC-diluent	colorless clear liquid	0.3	100 tubes
PCR-buffer-Background	colorless clear liquid	0.5	2 tubes

Preparation to DNA extraction

1. Switch on the thermostat and set the temperature at 95 °C.
2. Prepare and place the required number of tubes with **IC-diluent** into the tube rack and label them. Sediment the drops of solution from tube walls and caps by short centrifuging at 1500–3000 rpm for 2–3 s.
3. Before starting DNA extraction, mix the content of tubes with biological material in Transport medium TM-EDEM by vortexing and spin down the drops of material from tube walls and caps by short centrifuging at 1500–000 rpm for 2–3 s. Place the prepared tubes into tube rack.
4. Urine samples should be preliminary treated in accordance with chapter “Pretreatment of urine samples with the EDEM reagent kit for subsequent DNA extraction to obtain the urine pellet in the Transport medium TM-EDEM”. To do this, the additional reagent **Transport medium TM-EDEM** is to be used.

DNA extraction procedure

1. Transfer **100 µl** of biological material in the Transport medium TM-EDEM into the prepared tubes with **IC-diluent** using a new tip with aerosol filter for each sample. Add **100 µl** of the **Transport medium TM-EDEM** into the tube for Negative Control of Extraction (C–).
2. Tightly close all tubes, carefully mix the contents by vortexing (prevent spraying), and incubate in a thermostat at **95 °C** for **5 min.**

NOTE: Close tightly the tubes so that they would not open during heating.

3. After the end of incubation, place the tubes into a desktop centrifuge and centrifuge **at 14,000 rpm for 1 min.** Thus obtained DNA samples are ready for PCR analysis with hybridization-fluorescence detection.

DNA samples can be stored for one week at 2–8 °C or for one year at ≤ –16 °C (it is necessary to vortex and recentrifuge the tube contents according to item 3 if PCR analysis of DNA samples is performed once again).

NOTE: In case of invalid or equivocal result of PCR analysis obtained with the use of EDEM reagent kit, repeat DNA extraction procedure. To do this, 100 µl of biological material in Transport medium TM-EDEM should be treated with the DNA-sorb-AM reagent kit according to its instruction manual.

8.2. REAL-TIME PCR

A. Preparing tubes for PCR

Variant FRT

Total reaction volume is **30 µl**, the volume of DNA sample is **10 µl**.

1. Prepare the required number of tubes with **PCR-mix-1-FL** and wax for amplification of DNA from test and control samples.
2. Add **10 µl** of **PCR-mix-2-FL-red** to the surface of wax layer of each tube, so that it does not fall under the wax and mix with **PCR-mix-1-FL**.

Variant FRT-100 F

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

1. Thaw the **PCR-mix-2-FRT** tube. Vortex the tubes with **PCR-mix-1-FL, PCR-mix-2-FRT,** and **polymerase (TaqF)** then centrifuge briefly.
   Collect the required number of the tubes/strips for amplification of DNA obtained from test and control samples.
2. For N reactions (including 2 controls) mix in a new tube:
   10*(N+1) µl of **PCR-mix-1-FL;**
   5.0*(N+1) µl of **PCR-mix-2-FRT;**
   0.5*(N+1) µl of **polymerase (TaqF).**
   Vortex the tube, then centrifuge briefly. Transfer **15 µl** of the prepared mixture to each tube.

Steps 3 and 4 are carried out in both variants.

3. Add **10 µl** of **DNA** obtained from test or control samples at the DNA extraction stage into the prepared tubes using tips with aerosol filter.
4. Carry out the control amplification reactions:

NCA – Add **10 µl** of **DNA-buffer** to the tube labeled NCA (Negative Control of Amplification).

C+ – Add **10 µl** of **Positive Control complex** to the tube labeled C+ (Positive Control of Amplification).

C– – Add **10 µl** of the sample extracted from the **Negative Control (C–) reagent** to the tube labeled C– (Negative Control of Extraction).

B. Amplification

1. Create a temperature profile in your PCR instrument (table 1 or table 1a):

Table 1

AmpliSens-1 program

	Rotor-type Instruments[2]			Plate-type Instruments[3]		
Step	***Temperature, °C***	***Time***	***Cycles***	***Temperature, °C***	***Time***	***Cycles***
1	**95**	15 min	1	**95**	15 min	1
2	**95**	5 s	5	**95**	5 s	5
	60	20 s		**60**	20 s	
	72	15 s		**72**	15 s	
3	**95**	5 s	40	**95**	5 s	40
	60	20 s Fluorescence detection		**60**	30 s Fluorescence detection	
	72	15 s		**72**	15 s	

Table 1a

AmpliSens-1M program

	Rotor-type instruments[2]			Plate-type instruments[3]		
Step	***Temperature, °C***	***Time***	***Cycles***	***Temperature, °C***	***Time***	***Cycles***
1	**95**	15 min	1	**95**	15 min	1
2	**95**	20 s	5	**95**	20 s	5
	60	20 s		**60**	20 s	
	72	15 s		**72**	15 s	
3	**95**	20 s	40	**95**	20 s	40
	60	20 s Fluorescence detection		**60**	30 s Fluorescence detection	
	72	15 s		**72**	15 s	

If **AmpliSens® *HSV* I, II-FRT**, **AmpliSens® *HSV* / *CMV*-MULTIPRIME-FRT**, **AmpliSens® *HSV*-typing-FRT** PCR kits are used for analysis (separately or in combination with any other kits), the **AmpliSens-1M** program is recommended for running amplification and real-time detection (see Table 1a).

The instrument programming is described in detail below in chapter “Conducting Real-Time PCR with the Use of Different Instruments” of this Guidelines manual.

2. 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

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

[2] For example, Rotor-Gene 3000, Rotor-Gene 6000, Rotor-Gene Q or equivalent.
[3] For example, iCycler iQ, iQ5, Mx3000P, Mx3000, DT-96 or equivalent.

9. DATA ANALYSIS

The analysis of results was performed by the software of the instrument used. The fluorescent signal intensity is detected in the channels assigned for detection of amplification products of DNA fragments of specific microorganisms and in the channel assigned for detection of amplification product of IC DNA.

The results are interpreted by the crossing (or not-crossing) of the fluorescence curve with the threshold line set at a specific level and are shown as the presence (or absence) of ***Ct*** (cycle threshold) in the results grid.

To analyze results in each channel, set the threshold line at the required level and activate the required options in accordance with the instrument user manual and the chapter "Conducting Real-Time PCR with the Use of Different Instruments" of this Guidelines manual.

A. If PCR kits for detection of a single microorganism are used

The fluorescent signal intensity is detected in two channels:

- The signal from the amplification product of DNA of the analyzed microorganism is detected in the FAM channel;
- The signal of the Internal Control amplification product is detected in the JOE channel.

Interpretation of results

Principle of interpretation:

- The microorganism DNA is **detected** in a sample if its *Ct* value is detected in the results grid in the FAM channel. Moreover, the fluorescence curve should cross the threshold line in the area of exponential fluorescence growth.
- The microorganism DNA is **not detected** in a sample if its *Ct* value is not detected in the results grid in the FAM channel (the fluorescence curve does not cross the threshold line), whereas the *Ct* value in the JOE channel is less than the boundary *Ct* value specified.
- The result is **invalid** if the *Ct* value of a sample in the FAM channel is not detected (absent), whereas the *Ct* value in the JOE channel is either absent or greater than the boundary *Ct* value specified. Repeat the PCR test for such a sample.

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed in the PCR kit and in the chapter "Conducting Real-Time PCR with the Use of Different Instruments" of this Guidelines manual.

The result of the analysis is considered reliable only if the results of both Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (see Table 2).

Table 2

Results for controls

Control	Stage for control	*Ct* value in the channel for fluorophore	
		FAM	JOE
C–	DNA extraction	Absent	< boundary *Ct* value
NCA	PCR	Absent	Absent
C+	PCR	< boundary *Ct* value	< boundary *Ct* value

B. If MULTIPRIME PCR kits are used

The fluorescent signal intensity is detected in each channels assigned for detection of amplification products of DNA fragments of specific microorganisms and in the channel assigned for detection of amplification product of the IC DNA. Designations of channels are indicated in Table 3 and in the Instruction Manual to the PCR kit used.

MULTIPRIME PCR kits can be divided in two groups: PCR kits for detection of three or four microorganisms – group 1, and PCR kits for detection of two microorganisms (duplex) – group 2.

The signal of amplification product of IC DNA is detected in the Cy5 channel if PCR kits belonging to group 1 are used. The signal of the amplification product of IC DNA is detected in the ROX channel if PCR kits belonging to group 2 (duplex) are used.

Table 3

Channels for detection of signal indicating amplification of microorganism DNA and internal control DNA fragments

PCR kit (test), group 1	Channel for fluorophore				
	FAM	JOE	ROX	Cy5	Cy5.5*
N.gonorrhoeae/ C.trachomatis/ M.genitalium/ T.vaginalis	*Neisseria gonorrhoeae*	*Chlamydia trachomatis*	*Mycoplasma genitalium*	IC	*Trichomonas vaginalis*
C.trachomatis/ Ureaplasma/ M.genitalium/ M.hominis	*Chlamydia trachomatis*	*Ureaplasma* spp.	*Mycoplasma genitalium*	IC	*Mycoplasma hominis*
C.trachomatis/ Ureaplasma/ M.genitalium	*Chlamydia trachomatis*	*Ureaplasma* spp.	*Mycoplasma genitalium*	IC	—
C.trachomatis/ Ureaplasma / M.hominis	*Chlamydia trachomatis*	*Ureaplasma* spp.	*Mycoplasma hominis*	IC	—
T. vaginalis/ N.gonorrhoeae/ C.trachomatis	*Trichomonas vaginalis*	*Neisseria gonorrhoeae*	*Chlamydia trachomatis*	IC	—
N.gonorrhoeae/ C.trachomatis/ M.genitalium	*Neisseria gonorrhoeae*	*Chlamydia trachomatis*	*Mycoplasma genitalium*	IC	—
C.albicans/ C.glabrata/ C. krusei	*Candida albicans*	*Candida glabrata*	*Candida krusei*	IC	—

PCR kit (test), group 2 (duplex)					
U. parvum / U. urealyticum	*Ureaplasma parvum*	*Ureaplasma urealyticum*	IC	—	—
HSV-typing	*HSV* II	*HSV* I	IC	—	—
T.vaginalis / N.gonorrhoeae	*Trichomonas vaginalis*	*Neisseria gonorrhoeae*	IC	—	—
HSV / CMV	*HSV*	*CMV*	IC	—	—

* The channel for the Cy5.5 fluorophore (Crimson or Quasar 705 channel) is available in Rotor-Gene 6000, Rotor-Gene Q, CFX96 instruments.

Principle of interpretation

- The microorganism DNA is **detected** in a sample if its *Ct* value is detected in the results grid in the channel assigned for detection of this microorganism (in accordance with instruction manual to PCR kit). Moreover, the fluorescence curve of this sample should cross the threshold line in the typical exponential growth phase.
- The microorganism DNA is **not detected** in a sample if its *Ct* value is not detected (absent) in the results grid in the required channel (the fluorescence curve does not cross the threshold line).
- DNA of any analyzed microorganisms is **not detected** in a sample if its *Ct* values are not detected (absent) in the results grid in the required channel assigned for detection of amplification products of DNA of specific microorganisms (the fluorescence curve does not cross the threshold line), whereas the *Ct* value for the Internal Control is detected in the appropriate channel and it is less than the boundary *Ct* value specified.
- The result is **invalid** if none *Ct* value is detected in the channels assigned for detection of amplification products of DNA fragments of specific microorganisms, whereas the *Ct* value in the channel for detection of the Internal Control amplification product is either absent or greater than the specified boundary *Ct* value. Repeat the PCR assay for such samples.

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed in the PCR kit and in the chapter “Conducting Real-Time PCR with the Use of Different Instruments” of this Guidelines manual.

The results of analysis are considered reliable only if the results of both Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (see Table 4).

Table 4

Results for controls

Control	Stage for control	*Ct* value in channel	
		for detection of amplification products of DNA of specific microorganisms	for detection of amplification products of Internal Control DNA
C–	DNA extraction	Absent	< boundary value
NCA	PCR	Absent	Absent
C+	PCR	< boundary value	< boundary value

10. TROUBLESHOOTING

A. If PCR kits for detection of a single microorganism are used

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

1. If the *Ct* value of Positive Control of amplification (C+) in the FAM channel is absent or greater than the boundary *Ct* value, amplification should be repeated for all samples in which the microorganism DNA was not detected.
2. If the *Ct* value is detected for C– and/or for NCA in the FAM channel, PCR assay should be repeated starting from the DNA extraction stage for all samples in which the microorganism DNA was detected.

NOTE: If a *Ct* value is repeatedly detected for C– and/or for NCA in the FAM channel, it indicates contamination of reagents or samples. In such cases, the results of analysis must be considered as invalid. Test analysis must be repeated and measures to detect and eliminate the source of contamination must be taken.

3. If a *Ct* value of a sample is detected in the results grid in the FAM channel but the fluorescence curve does not have a typical exponential growth phase (the curve is linear), the result should not be considered as positive. This may suggest incorrect setting of the threshold line or other analysis parameters. If threshold levels (as well as other analysis settings) are correct, amplification of such samples should be repeated.

B. If MULTIPRIME PCR kits are used

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

1. If no signal is detected for Positive Control of Amplification (C+) or the signal is greater than the specified boundary *Ct* value in more than one channel assigned for detection of amplification products of DNA fragments of specific microorganisms, PCR should be repeated for all samples for which *Ct* values in these channels were not detected.
2. If a *Ct* value is present for the Negative Control of Extraction (C–) and/or for the Negative Control of Amplification (NCA) in the channels assigned for detection of amplification products of DNA fragments of specific microorganisms, PCR analysis

should be repeated for all samples for which a *Ct* value in these channels was detected.

NOTE: If a *Ct* value is detected for C– and/or for NCA in the channels assigned for detection of amplification product of microorganism DNA in the second run, this indicates contamination of reagents or samples. In such cases, the results of analysis must be considered as invalid. Test analysis must be repeated and measures to detect and eliminate the source of contamination must be taken.

3. If a *Ct* value of a sample is detected in the results grid in the FAM channel but the fluorescence curve does not have a typical exponential growth phase (the curve is linear), the result should not be considered as positive. This may suggest incorrect setting of the threshold line or other analysis parameters. If threshold level is correct (as well as other analysis settings), amplification should be repeated of such a sample to get correct result.

11. CONDUCTING REAL-TIME PCR WITH THE USE OF DIFFERENT INSTRUMENTS

CONDUCTING REAL-TIME PCR WITH THE USE OF Rotor-Gene 3000, Rotor-Gene 6000, and Rotor-Gene Q INSTRUMENTS

When working with Rotor-Gene 3000 one should use the Rotor-Gene version 6.1 and higher software and the Rotor-Gene 6000 versions 1.7 (build 67) software or higher for Rotor-Gene 6000 and Rotor-Gene Q instruments.

A. Creating a template

Hereinafter, the terms specific for different instruments are listed in the following order: for the Rotor-Gene 3000 instrument / for the Rotor-Gene 6000 (or Rotor-Gene Q). If terms for different instruments coincide, only one term is shown.

1. In the ***New Run*** window, select the ***Advanced*** mode. Select any template (for example, ***Dual Labeled Probe/Hydrolysis probes***) for editing and click the ***New*** button. Select ***36-Well Rotor*** in the next window. Tick the ***No Domed Tubes/ Locking ring attached*** line.
2. Set the reaction mixture volume: ***Reaction Volume (µL)***
   - **30** for Rotor-Gene 3000;
   - **25** for Rotor-Gene 6000. Tick the ***15 µL oil layer volume*** box to activate this option.
3. In the ***Edit profile*** window, set the **AmpliSens-1** (table 5) or **Amplisens-1M** (table 6) amplification program. Click ***OK*** when finished.

Table 5

AmpliSens-1 program

Step	*Temperature, °C*	*Time*	*Cycles*
1	**95**	15 min	1
2	**95**	5 s	5
	60	20 s	
	72	15 s	
3	**95**	5 s	40
	60	20 s Fluorescence detection*	
	72	15 s	

Table 6

AmpliSens-1M program

Step	*Temperature, °C*	*Time*	*Cycles*
1	**95**	15 min	1
2	**95**	20 s	5
	60	20 s	
	72	15 s	
3	**95**	20 s	40
	60	20 s Fluorescence detection*	
	72	15 s	

* Fluorescence detection is assigned at the second step (60 ºC) of the second cycling block (Step 3) (***Acquiring to Cycling A***) in the ***FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red,*** and ***Crimson*** channels.

NOTE: **AmpliSens-1** and **AmpliSens-1M** are universal programs for conducting tests for detection of STIs and other infections of reproductive system with AmpliSens PCR kits. Therefore, all this tests or any combination of tests including tests for *human papillomaviruses (HPV HCR)* detection and genotyping can be carried out simultaneously in the same instrument. If **AmpliSens® *HSV* I,II-FRT, AmpliSens® *HSV* / *CMV*-MULTIPRIME-FRT**, **AmpliSens® *HSV*-typing-FRT** PCR kits are used for analysis (separately or in combination with any other kits), the **AmpliSens-1M** program is recommended for running amplification and real-time detection.

Other programs, **60-45 RG** and **60-45 RG-M**, can be exceptionally used for PCR kits **variant FRT** (with a wax layer used) (they substitute the **AmpliSens-1** and **AmpliSens-1M** programs, respectively). This allows the run time to be reduced by 10 min. To do this, create a new template and enter the **60-45 RG or 60-45 RG-M** program in the ***Edit Profile*** window:

Table 7

60-45 RG program

Step	*Temperature, °C*	*Time*	*Cycles*
1	**95**	5 min	1
2	**95**	5 s	5
	60	20 s	
	72	15 s	
3	**95**	5 s	40
	60	20 s Fluorescence detection*	
	72	15 s	

Table 8

60-45 RG-M program

Step	*Temperature, °C*	*Time*	*Cycles*
1	**95**	5 min	1
2	**95**	20 s	5
	60	20 s	
	72	15 s	
3	**95**	20 s	40
	60	20 s Fluorescence detection *	
	72	15 s	

* Fluorescence detection is assigned as described for **AmpliSens-1** and **AmpliSens-1M** program.

4. Adjust the fluorescence channel sensitivity. In the ***Channel Setup*** window, select the ***Calibrate/Gain Optimisation*** button. In the opened ***Auto Gain Calibration Setup*** window, click the ***Calibrate Acquiring/Optimise Acquiring*** button. For the FAM/Green channel, enter **5** in the ***Min Reading*** line and **10** in the ***Max Reading*** line (it is acceptable to set ***Calibrate/Gain Optimisation…*** value from 5 FI to 10 FI for the *JOE/Yellow* channel). For JOE/Yellow, ROX/Orange, Cy5/Red, and Cy5.5/Crimson channels, enter **4** in the ***Min Reading*** line and **8** in the ***Max Reading*** line.

   In the ***Tube position*** column, specify the number of the test tube as **1**, which means automatic selection of the ***gain*** parameter. Tick the ***Perform Calibration Before 1st Acquisition/ Perform Optimisation Before 1st Acquisition*** box. Close the ***Auto Gain Calibration Setup*** window.
5. Proceed to the next window. Click the ***Save Template*** button. Enter the template name corresponding to the name of amplification program: **AmpliSens-1, AmpliSens-1M** or **60-45 RG, 60-45 RG M**. Save the template in the offered ***Templates*** folder (in the ***Quick Start Templates*** subfolder) and close the ***New Run Wizard*** window. The created template will appear in the template list in the ***New Run*** window.

The AmpliSens-1 template can be used for conducting any amplification tests for detection

of STIs with use of PCR kits manufactured by FBIS CRIE.

B. Use of the created template

1. Place the tubes into the rotor so that the first well is loaded with a tube filled with the reaction mixture prepared for the run (see Notes 1 and 2). Fix the locking ring, secure the rotor, and close the lid.
2. To start run with the prepared template, select the ***Advanced*** tab in the ***New Run Wizard*** window of the ***New Run*** menu. Select the template with **AmpliSens-1** or **AmpliSens-1M** amplification program from the drop-down list box (set as described in section A. Creating Template). If a PCR kit variant FRT (with a wax layer) is used, the template with the **60-45 RG** or **60-45 RG-M** amplification program can be selected.
3. Select ***36-Well Rotor*** or ***72-Well Rotor*** and tick the ***No Domed 0.2 ml Tubes***/***Locking ring attached*** line. Proceed to the next window.
4. Make sure that the reaction volume is correct. Make sure that ***15 µL oil layer volume*** is selected for Rotor-Gene 6000 or Rotor-Gene Q. Proceed to the next window.
5. Check the correctness of the amplification program and automatic optimization gain parameters.

NOTE: If MULTIPRIME PCR kits are not used for the run, unable fluorescence detection in the ROX/Orange, Cy5/Red, and Crimson channels in the ***Edit Profile*** window (FAM/Green and JOE/Yellow channels are activated).

6. Start the program by clicking the ***Start*** button. Make sure that rotor is secured and the lid is closed. Enter the file name for result data and click ***Save***.
7. In the table of samples, define the order of samples by entering the name and type (***Unknown***) of each sample. Click ***Finish/OK***.

NOTE: Rotor-Gene 6000 and Rotor-Gene Q instruments allow editing the table of samples before the run starts. To do this, select the ***Edit Samples Before Run Started*** button in the ***User Preferences*** submenu of the ***File*** menu. See Note 3.

8. Proceed to interpretation of results when the run is completed.

NOTE: When PCR run is completed, the tubes should be removed from the rotor and discarded.

Note 1. The first tube in the rotor is used for automatic optimization of the level of signal; therefore, the first tube should contain the reaction mixture. If several tests for detection of STIs with PCR kits manufactured by FBIS CRIE are conducted within the same run, any tube containing reaction mixture can be placed in the first well of the rotor. If several MULTIPRIME tests are simultaneously carried out, the first well should be loaded with the tube analyzed in the maximum number of channels.

Note 2. Do not place tubes that already passed amplification run in the rotor iteratively. It

is acceptable to leave some rotor wells unloaded.

Note 3. If tests for detection of *human papillomavirus* (*HPV*) DNA and different tests for detection of STI with PCR kits manufactured by FBIS CRIE are simultaneously conducted, it is necessary to create the second page in the table of samples. In this page all samples tested for HPV should be defined while the other samples should have ***None*** type. This is important for data analysis.

Note 4. If PCR kits **AmpliSens® *HSV* I, II-FRT**, **AmpliSens® *HSV* / *CMV*-MULTIPRIME-FRT**, **AmpliSens® *HSV*-typing-FRT** are used for analysis (separately or in combination with any other kits), the **AmpliSens-1M** program is recommended for running amplification and real-time detection.

Analysis of result obtained with Rotor-Gene 3000, Rotor-Gene 6000, or Rotor-Gen Q instruments

Hereinafter, the terms specific for different instruments are listed in the following order: for the Rotor-Gene 3000 instrument / for the Rotor-Gene 6000 (or Rotor-Gene Q). If terms for different instruments coincide, only one term is shown.

If PCR kits for detection of a single microorganism are used, the fluorescence signal is detected in two channels: the amplification product of the DNA fragment of the specific microorganism is detected in the **FAM/Green** channel; the amplification product of the Internal Control DNA is detected in the **JOE/Yellow** channel.

1. Select the ***Analysis*** sign in the main menu, select the ***Quantitation*** tab in the drop-down menu, and then select the required channel. Perform operation for FAM/Green channel by selecting ***Cycling A FAM/Cycling A Green***; perform operation for JOE/Yellow channel by selecting ***Cycling A JOE/Cycling A Yellow***.

2. Data analysis of **IC DNA** amplification in the **JOE/Yellow** channel.
   2.1 Select normalized curves in the **JOE/Yellow** channel.
   2.2 Make sure that the ***Dynamic tube*** button is activated (set by default). Activate the ***More Settings*/*Outlier Removal*** button and enter **5** (5 %) in the text field. For **AmpliSens® *Chlamydia trachomatis*-FRT** and **AmpliSens® *Mycoplasma genitalium*-FRT** PCR kits activate ***Slope Correct*** button, then activate ***Outlier Removal*** button and enter **0-10** (0-10 %) in the text field.
   2.3 In the ***CT Calculation*** menu, set ***Threshold* = 0.1.**
   2.4 *Ct* values for each sample in the **JOE/Yellow** channel will appear in the results grid (***Quant. Results – Cycling A JOE/Quant. Results/Quant. Results – Cycling A Yellow***).

3. Data analysis of the microorganism DNA amplification in the FAM/Green channel.

3.1 Select normalized curves in the **FAM/Green** channel.

3.2 Make sure that the ***Dynamic tube*** button is activated (set by default).

The ***Slope Correct*** button should be turned off or on as specified in Table 9.

Activate the ***More Settings*/*Outlier Removal*** button and in the text field enter the value specified in Table 9.

3.3 In the ***CT Calculation*** menu, set ***Threshold* = 0.1.**

3.4 *Ct* values of each sample in **FAM/Green** channel will appear in the results grid (***Quant. Results – Cycling A FAM/Quant. Results/Quant. Results – Cycling A Green***).

Table 9

Parameters of analysis of results in the FAM/Green channel

PCR kit	Threshold	More Settings/ Outlier Removal	Slope Correct
Chlamydia trachomatis	**0.1**	**0-10**	on
***Neisseria gonorrhoeae*-screen**	**0.1**	**0**	off
Mycoplasma genitalium	**0.1**	**0-10**	on
Ureaplasma species	**0.1**	**0**	off
Mycoplasma hominis	**0.1**	**0**	off
***HSV* I, II**	**0.1**	**0**	off
CMV	**0.1**	**0**	off
Gardnerella vaginalis	**0.1**	**0**	off
Treponema pallidum	**0.1**	**5**	on
Trichomonas vaginalis	**0.1**	**5**	on
Candida albicans	**0.1**	**0**	off

4. Principle of interpretation

- The microorganism DNA is **detected** in a sample if its *Ct* value is detected in the results grid in the **FAM/Green** channel. The fluorescence curve should cross the threshold line at the typical exponential growth phase.
- The microorganism DNA is **not detected** in a sample if its *Ct* value is not detected in the results grid in the **FAM/Green** channel (the fluorescence curve does not cross the threshold line), whereas the *Ct* value detected in the JOE/Yellow channel is less than **30**.
- The result is **invalid** if the *Ct* value of a sample is not detected in the **FAM/Green** channel whereas the *Ct* value in the **JOE/Yellow** channel is either absent or greater than **30**. Repeat the PCR test for such samples.

The result of analysis is considered reliable only if the results of both Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (see Tables 10, 11).

Table 10

Results for controls

Control	Stage for control	*Ct* value in the channel	
		FAM/Green	JOE/Yellow
C–	DNA extraction	Absent	Detected value <30
NCA	PCR	Absent	Absent
C+	PCR	< boundary *Ct* value	Detected value < 30

Table 11

Boundary *Ct* value for C+ in the FAM/Green channel

PCR kit variant FRT	Boundary *Ct* value for C+ in the FAM/Green channel
Chlamydia trachomatis	30
HSV I, II	
CMV	
Candida albicans	
Neisseria gonorrhoeae-screen	33
Mycoplasma genitalium	
Trichomonas vaginalis	
Treponema pallidum	
Ureaplasma species	
Mycoplasma hominis	
Gardnerella vaginalis	

If MULTIPRIME PCR kits are used, the fluorescent signal is detected in all channels enabled for detection. The products of amplification of DNA of the analyzed microorganisms are detected in the channels listed in Table 3 (**FAM/Green, JOE/Yellow, ROX/Orange**, or **Crimson** channel). The amplification product of IC DNA is detected in the **Cy5/Red** channel for group 1 PCR kits or in the **ROX/Orange** channel for group 2 PCR kit (duplex).

Interpretation of results is based on the data obtained for each channel assigned for detection of the analyzed microorganisms as well as for detection of Internal Control in accordance with Table 3.

1. Select the ***Analysis*** sign in the main menu and the ***Quantitation*** tab in the drop-down menu, after which select the required channel (for example, select ***Cycling A FAM/Cycling A Green*** for the FAM/Green channel, ***Cycling A JOE/Cycling A Yellow*** for the JOE/Yellow channel, etc.)
2. Data analysis of **IC DNA** amplification
   2.1 Group 1 PCR kits. Select normalized curves in the **Cy5/Red** channel. Make sure that ***Dynamic tube*** button is activated (set by default). Activate the ***Slope Correct*** button. Turn on the ***More Settings***/***Outlier Removal*** button and enter **5-10** (5-10

%) in the text field. In the ***CT Calculation*** menu, set ***Threshold* = 0.07.** *Ct* values of each sample in **Cy5/Red** channel will appear in the results grid (***Quant. Results – Cycling A Cy5/Quant. Results/Quant. Results – Cycling A Red***).

2.2 Group 2 PCR kits (duplex). Select normalized curves in the **ROX/Orange** channel. Make sure that the ***Dynamic tube*** button is activated (set by default). Turn on the ***More Settings*/*Outlier Removal*** button and enter **5** (5%) in the text field. In the ***CT Calculation*** menu, set ***Threshold* = 0.1.** *Ct* values of each sample in **ROX/Orange** channel will appear in the results grid (***Quant. Results – Cycling A ROX/Quant. Results/Quant. Results – Cycling A Orange***).

3. Data analysis of the **microorganism DNA** amplification

Results should be consecutively analyzed as described below in each channel used.

3.1 Select the ***Analysis*** sign in the main menu, select the ***Quantitation*** tab in the drop-down menu, and then select the required channel.

3.2 Select window of normalized curves in the required channel.

3.3 Make sure that the ***Dynamic tube*** button is activated (set by default).

The ***Slope Correct*** button should be turned off or on as specified in Table 12.

Activate the ***More Settings*/*Outlier Removal*** button and in the text field enter value specified in Table 12.

3.4 In the ***CT Calculation*** menu set ***Threshold* = 0.1.**

3.5 *Ct* values of each sample in the required channel will appear in the results grid (***Quant. Results*** window).

For convenient interpretation of results, we recommend that the *Ct* value column is copied and entered into the corresponding column in Excel.

Table 12

Parameters of result analysis for MULTIPRIME PCR kit

Detection channel	Threshold	More Settings/ Outlier Removal	Slope Correct
AmpliSens® *C.trachomatis / Ureaplasma / M.genitalium*-MULTIPRIME-FRT PCR kit, AmpliSens® *C.trachomatis / Ureaplasma / M.hominis*-MULTIPRIME-FRT PCR kit, AmpliSens® *T.vaginalis / N.gonorrhoeae / C.trachomatis*-MULTIPRIME-FRT PCR kit, AmpliSens® *C.albicans / C.glabrata / C.krusei*-MULTIPRIME-FRT PCR kit			
FAM/Green	0.1	0 %	Off
JOE/Yellow	0.1	5 %	Off
ROX/Orange	0.1	5 %	Off
Cy5/Red	0.07	5-10 %	On
AmpliSens® *HSV*-typing-FRT PCR kit, AmpliSens® *U.parvum / U.urealyticum*-FRT PCR kit			
FAM/Green	0.1	0 %	Off
JOE/Yellow	0.1	5 %	Off
ROX/Orange	0.1	5 %	Off

Detection channel	Threshold	More Settings/ Outlier Removal	Slope Correct
AmpliSens® *N.gonorrhoeae / C.trachomatis / M.genitalium*-MULTIPRIME-FRT PCR kit			
FAM/Green	0.1	5-10 %	On
JOE/Yellow	0.1	5-10 %	On
ROX/Orange	0.1	5-10 %	On
Cy5/Red	0.07	5-10 %	On
AmpliSens® *N.gonorrhoeae / C.trachomatis / M.genitalium / T.vaginalis*-MULTIPRIME-FRT PCR kit			
FAM/Green	0.1	0 %	Off
JOE/Yellow	0.1	5 %	Off
ROX/Orange	0.1	5 %	Off
Cy5/Red	0.07	5 %	On
Cy5.5/Crimson	0.1	10 %	On
AmpliSens® *C.trachomatis / Ureaplasma / M.genitalium / M.hominis*-MULTIPRIME-FRT PCR kit			
FAM/Green	0.1	0 %	Off
JOE/Yellow	0.1	5 %	Off
ROX/Orange	0.1	5 %	Off
Cy5/Red	0.07	5 %	On
Cy5.5/Crimson	0.1	20 %	On
AmpliSens® *T.vaginalis / N.gonorrhoeae*-MULTIPRIME-FRT PCR kit			
FAM/Green	0.1	5 %	Off
JOE/Yellow	0.1	5 %	Off
ROX/Orange	0.1	5 %	Off
AmpliSens® *HSV / CMV*-MULTIPRIME-FRT PCR kit			
FAM/Green	0.1	0 %	Off
JOE/Yellow	0.1	10 %	Off
ROX/Orange	0.1	5 %	Off

4. Principle of interpretation
   - The microorganism DNA is **detected** in a sample if its ***Ct*** value is detected in the results grid in the channel assigned for detection of the given microorganism. The fluorescence curve should cross the threshold line in the typical exponential growth phase.
   - The microorganism DNA is **not detected** in a sample if its ***Ct*** value is not detected in the results grid in the channel assigned for detection of this microorganism (the fluorescence curve does not cross the threshold line), whereas the *Ct* value in the channel assigned for detection of the internal control amplification product (**Cy5/Red** channel for group 1 PCR kits or **ROX/Orange** channel for group 2 PCR kits) is detected and less than **33**.
   - The result is **invalid** if the *Ct* value of a sample is absent in all channels assigned for detection of specific microorganisms, whereas the *Ct* value detected in the channel assigned for the internal control amplification product is either absent or greater than **33**. Repeat the PCR test for such samples.
5. For automatic analysis of results, the **AmpliSens<*PCR kit*>Results Matrix** program supplied by the manufacturer can be used. The obtained data should be analyzed as

described in items 1 and 2. *Ct* values should be copied from the results grid to the clipboard and entered in the corresponding column of the program for automatic analysis of results.

The result of the analysis is considered reliable only if the results of both Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (see Tables 13, 14).

Table 13

Results for controls

Control	Stage for control	*Ct* values in channels	
		FAM/Green, JOE/Yellow, ROX/Orange, and Cy5.5/Crimson (if required)	ROX/Orange or Cy5/Red
If PCR kit for detection of 3 or 4 microorganisms is used (group 1)			
C–	DNA extraction	Absent	Detected value < 33
NCA	PCR	Absent	Absent
C+	PCR	Pos (< boundary *Ct* value)	< boundary *Ct* value
If PCR kit for detection of 2 microorganisms is used (group 2)			
C–	DNA extraction	Absent	Detected value < 33
NCA	PCR	Absent	Absent
C+	PCR	< boundary *Ct* value	< boundary *Ct* value

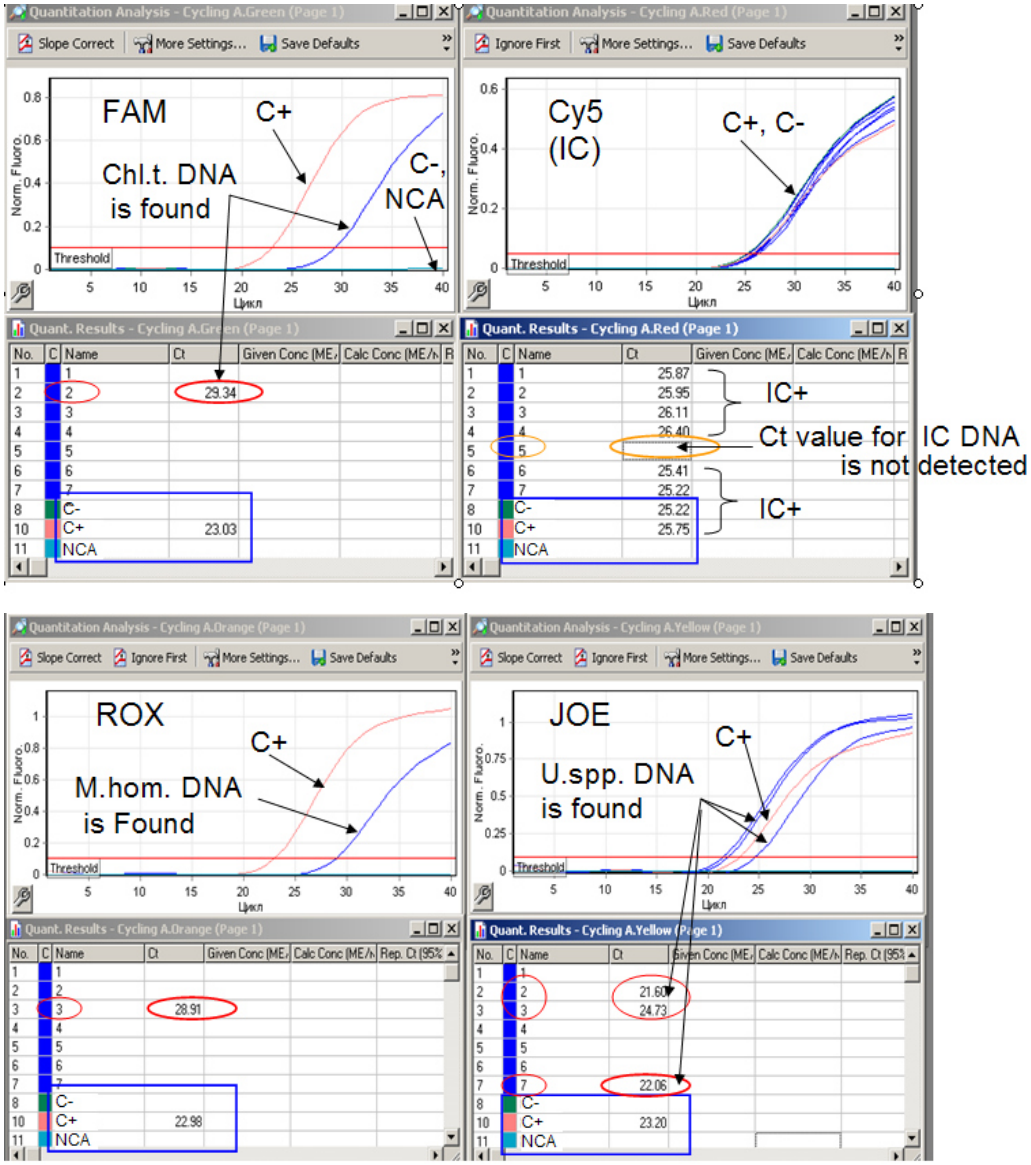
Table 14

Boundary *Ct* value for positive control of amplification (C+)

PCR kit, group 1	Boundary *Ct* value in channel				
	FAM/Green	JOE/Yellow	ROX/Orange	Cy5/Red	Crimson
N.gonorrhoeae / C.trachomatis / M.genitalium / T.vaginalis	35	35	35	33	35
C.trachomatis / Ureaplasma / M.genitalium / M.hominis	35	35	35	33	35
C.trachomatis / Ureaplasma / M.genitalium	30	33	33	33	–
T.vaginalis/ N.gonorrhoeae / C.trachomatis	30	30	33	33	–
C.trachomatis / Ureaplasma / M.hominis	30	33	33	33	–
N.gonorrhoeae / C.trachomatis / M.genitalium	33	30	33	33	–
C.albicans / C.glabrata / C. krusei	33	33	33	33	–
PCR kit, group 2 – duplex					
U. parvum / U. urealyticum	33	33	33	–	–
HSV-typing	33	30	33	–	–
T.vaginalis / N.gonorrhoeae	33	33	33	–	–
HSV / CMV	30	30	33	–	–

Examples of obtained results:

Results obtained with **AmpliSens®** ***C.trachomatis / Ureaplasma / M.genitalium*-MULTIPRIME-FRT** PCR kit



- The result for negative controls, C– and NCA, is negative; The *Ct* value detected for C– in the Cy5/Red channel (detection of IC) is less than 33. The result for positive control, C+, is positive, *Ct* values do not exceed the boundary *Ct* values in all channels. The results for controls correspond with specified values. The results for test samples are valid.
- Sample No.2 shows the presence of DNA of the microorganisms that are detected in the FAM/Green channel (*Chlamydia trachomatis* in here) as well as DNA of the microorganisms detected in the JOE/Yellow channel (*Ureaplasma* spp. in here).
- Sample No.3 shows the presence of DNA of the microorganisms detected in the JOE/Yellow and ROX/Orange channels (*Ureaplasma* spp. and *Mycoplasma hominis*, respectively, in here).
- Sample No.7 shows the presence of DNA of the microorganism detected in the JOE/Yellow channel (*Ureaplasma* spp. in here).
- *Ct* values less than 33 are detected for all samples except for sample No. 5 in the Cy5/Red channel.
- Sample No.5 shows an invalid result, that is, *Ct* values are absent in all channels.
- None of the microorganisms of interest was found in samples No. 1, 4, and 6.

CONDUCTING REAL-TIME PCR WITH THE USE OF iCycler iQ or iQ5 INSTRUMENTS

1. Set the **AmpliSens-1** (Table 15) or **AmpliSens-1M** (Table 16) universal amplification and detection program.

Table 15

AmpliSens-1 program for iCycler iQ or iQ5 instruments

Step	*Temperature, °C*	*Time*	*Cycle repeats*
1	**95**	15 min	1
2	**95**	5 s	5
	60	20 s	
	72	15 s	
3	**95**	5 s	40
	60	30 s Fluorescence detection*	
	72	15 s	

Table 16

AmpliSens-1M for iCycler iQ or iQ5 instruments

Step	*Temperature, °C*	*Time*	*Cycle repeats*
1	**95**	15 мин	1
2	**95**	20 с	5
	60	20 с	
	72	15 с	
3	**95**	20 с	40
	60	30 с Fluorescence detection*	
	72	15 с	

* Fluorescence detection is assigned in the second step (60 ºC) of the second cycling block (Step 3) in the **FAM, JOE/HEX, ROX, Cy5** channels.

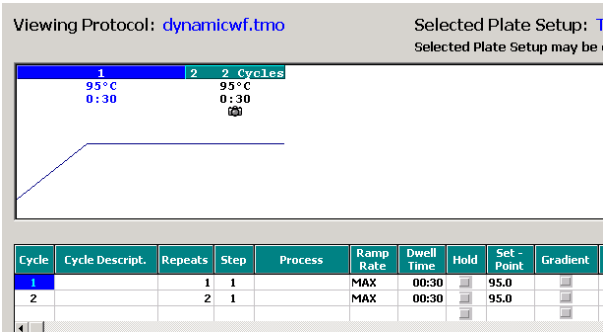
To do this, select or create this program in the ***Protocol*** module (***View Protocols*** for iCycler iQ). For iCycler iQ5, click the ***Run with selected Plate Setup*** button to start the program.

NOTE: **AmpliSens-1** and **AmpliSens-1M** are universal programs for conducting tests for detection of STIs and other infections of reproductive system with AmpliSens PCR kits. Therefore, all this tests or any combination of tests including tests for *human papillomaviruses (HPV HCR)* detection and genotyping can be carried out simultaneously in the same instrument. If **AmpliSens® *HSV* I,II-FRT, AmpliSens® *HSV* / *CMV*-MULTIPRIME-FRT**, **AmpliSens® *HSV*-typing-FRT** PCR kits are used for analysis (separately or in combination with any other kits), the **AmpliSens-1M** program is recommended for running amplification and real-time detection.

It is not recommended running the MULTIPRIME-format test and single pathogen detection tests (tests with different combinations of detection channels) simultaneously in the iCycler iQ Instrument. If these tests are to be conducted within the same run, then the ***External Well Factors Plate*** option should be selected for the well factor determination

and the tube kit with a special External Well Factor Solution (Bio-Rad) should be used for start up.

Before programming the iCycler iQ instrument, make sure that the dinamicwf.tmo protocol is set as follows (standard):



2. Set the plate setup, that is, tubes order in the reaction chamber and the detection of fluorescent signal for all tubes in the required channels, in the ***Edit Plate Setup*** window of the ***Workshop*** module. If a PCR kit for detection of a single microorganism is used, activate the FAM and JOE/HEX detection channels. If a MULTIPRIME PCR kit is used activate FAM, JOE/HEX, ROX, and Cy5 channels. Save the plate setup. Click the ***Run with selected protocol*** button.
   - **iCycler iQ5 instrument**. In the ***Selected Plate Setup*** window of the ***Workshop*** module press the ***Create New*** or ***Edit*** button. Edit the plate setup in the ***Whole Plate loading*** mode. To turn on the second fluorophore use  sign. Set ***Sample Volume*** as ***30 µl***, ***Seal Type*** as ***Domed Cap***, and ***Vessel Type*** as ***Tubes.*** Press ***Save &Exit Plate Editing.***
   - **iCycler iQ instrument.** Edit the plate setup in the ***Edit Plate Setup*** window of the ***Workshop*** module. Press the ***Run with selected protocol*** button to save and activate the created plate setup.
3. Proceed to item 4 if a PCR kit variant FRT (“hot start” is ensured by using a wax layer) is used. If a PCR kit variant FRT-100 F (TaqF polymerase is applied) is used, insert the tubes into the reaction chamber in accordance with the created plate setup. Secure the instrument.
4. Start the **AmpliSens-1** or **AmpliSens-1M** program along with the created plate setup.
   - **iCycler iQ5 instrument.** Ensure that the ***Selected Protocol*** and ***Selected Plate Setup*** are set correctly before starting the program. To start the program, click the ***Run*** button. Select the ***Use Persistent Well Factor*** option (set by default) for detection of a well factor.
   - **iCycler iQ instrument.** Ensure that the selected protocol and plate setup are set

correctly in the ***Run Prep*** window. For determination of the well factor, select the ***Experimental Plate*** option (set by default) below the ***Select well factor source*** line (see point 1). Set the reaction mix volume as **30 µ**l. Press ***Run*** to start.

5. Proceed to item 6 if PCR kit variant FRT-100 F is used.

   If PCR kit variant FRT is used, press the ***Pause*** button when the temperature in the reaction chamber reaches 95 ºC, open the instrument, and insert the tubes into the wells in accordance with the created plate setup. Close the lid and press the ***Resume Run*** button (for iCycler iQ5) or ***Continue Running Protocol*** button (for iCycler iQ).
6. Proceed to data analysis when the program is done.
7. At the end of the work, close the program and shut down the instrument.

Data analysis. iCycler iQ and iQ5 instruments

The obtained data are interpreted with the software of iCycler iQ or iQ5 instruments. The results are interpreted by the crossing (or not-crossing) of the fluorescence curve with the threshold line set at a certain level and it is shown as the presence (or absence) of a *Ct* (cycle threshold) value in the results grid.

If PCR kits for detection of a single microorganism DNA are used, fluorescence signal is detected in two channels: amplification product of a DNA fragment of a specific microorganism is detected in the **FAM** channel and the amplification product of the Internal Control DNA is detected in the **JOE/HEX** channel.

1. <u>Data analysis of the specific microorganism amplification</u>

1.1. Select data in the **FAM** channel (iCycler iQ5) or activate the **FAM-490** sign in the ***Select a Reporter*** window (iCycler iQ). Make sure that the ***PCR Base Line Subtracted Curve*** **Fit** mode is activated (set by default).

1.2. Set the threshold line at the level of **10-20 %** of maximum level of fluorescence, obtained for the Positive Control (**C+**) in the last amplification cycle (the fluorescence level for the Positive Control is considered as equal to the nearest digital scale mark). The fluorescence curve for Positive Control should represent typical exponential growth of fluorescence. The threshold line can also be set at default if it fits in this range.

In order to select Positive Control graph (or any other object), use the ***Display Wells (Select Wells)*** button or point tab cursor on a desired graph and double click.

- **iCycler iQ5 instrument**

  To set the threshold line level, move it using the left mouse button or select the ***Baseline Threshold*** menu (in the drop-down menu, which appears after clicking the right mouse button in the fluorescence graph window), then select the ***User***

Define option and insert required value in the ***Threshold Position*** text field. Results grid will be displayed after clicking the ***Results*** button.

- **iCycler iQ instrument**

  To set the threshold line level, either move it using the left mouse button or select the ***User Defined*** option, insert the required value in the ***Threshold Position*** text field, and press the ***Recalculate Threshold Cycles*** button.

NOTE: Selected threshold level can be used for data analysis in further runs performed with the same PCR kit and conducted on the same Instrument, in case the new calibration was not performed.

2. Data analysis of the IC amplification

Select data in **JOE/HEX** channel (iCycler iQ5) or activate **HEX-530** sign in the ***Select a Reporter*** window (iCycler iQ). Select the ***PCR Base Line Subtracted Curve Fit*** mode (set by default). Set the threshold line at the level of 10–20 % of the maximum fluorescence intensity recorded for the Positive Control (C+) in the last amplification cycle. The fluorescence curve for Positive Control (C+) should contain a section of typical exponential fluorescence growth. The threshold line can also be set at default if it fits in this range.

NOTE: The selected threshold level can be used for IC data analysis of the other tests carried out with AmpliSens PCR kits for detection of pathogens of sexually transmitted diseases. The same threshold level in the HEX channel can be used in further runs conducted on the same Instrument in case a new calibration has not been performed.

3. Principle of interpretation

- The microorganism DNA is **detected** in a sample if its *Ct* value is detected in the results grid in the **FAM** channel. The fluorescence curve should cross the threshold line in the typical exponential growth phase.
- The microorganism DNA is **not detected** in a sample if **N/A** appears in the results grid in the **FAM** channel (the fluorescence curve does not cross the threshold line), whereas the ***Ct*** value detected in the **HEX** channel is less than **33**.
- The result is **invalid** if the ***Ct*** value of the sample is not detected in the **FAM** channel (**N/A** appears), whereas the *Ct* value in the **HEX** channel is either absent (**N/A**) or greater than **33**. Repeat the PCR test for such samples.

The result of the analysis is considered reliable only if the results of both Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (see Tables 17 and 18)

Table 17

Results for controls

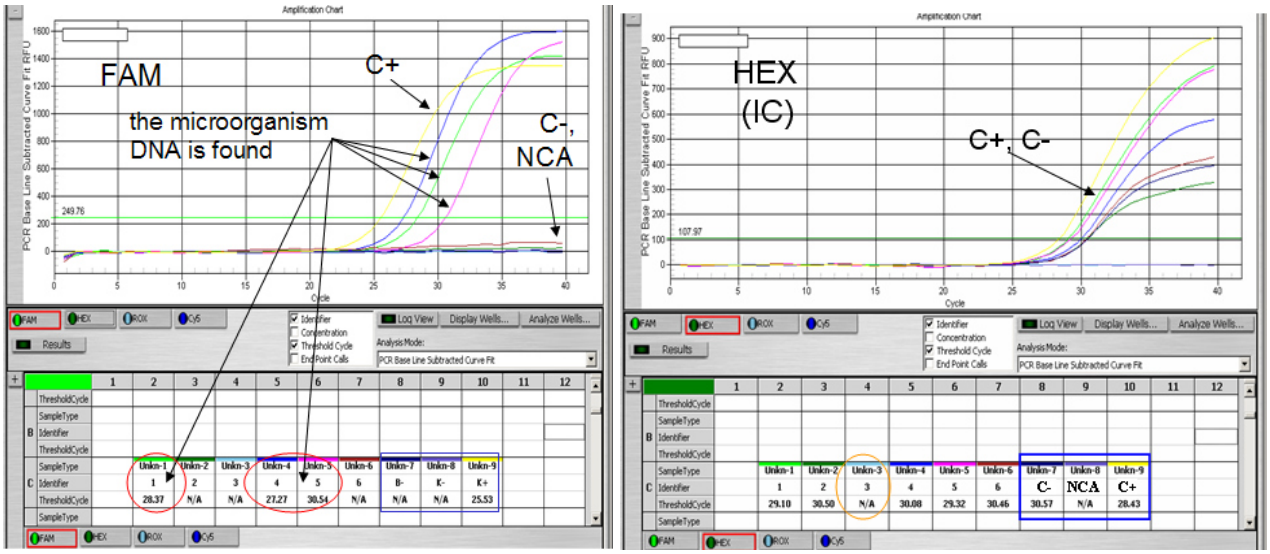
Control	Stage for control	*Ct* value in channel	
		FAM	JOE/HEX
C–	DNA extraction	N/A (absent)	Detected value < 33
NCA	PCR	N/A (absent)	N/A (absent)
C+	PCR	< boundary *Ct* value	Detected value < 33

Table 18

Boundary *Ct* values for positive control in the FAM channel

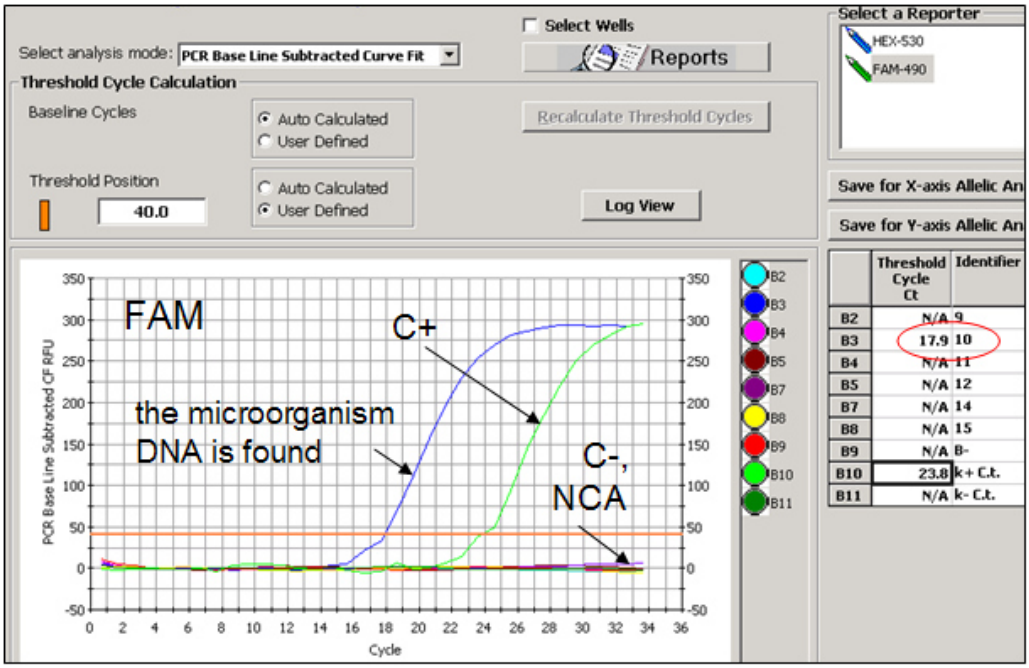
PCR kit	*Ct* value in FAM channel for C+
Chlamydia trachomatis	33
HSV I, II	
CMV	
Candida albicans	
Neisseria gonorrhoeae-screen	36
Mycoplasma genitalium	
Trichomonas vaginalis	
Treponema pallidum	
Ureaplasma species	
Mycoplasma hominis	
Gardnerella vaginalis	

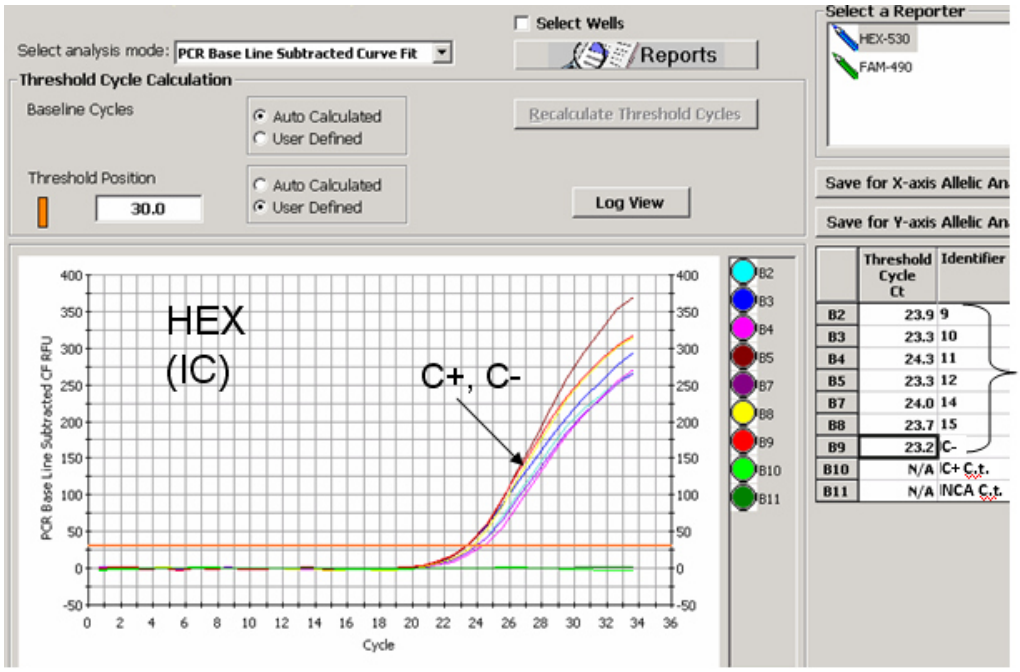
Example of results obtained using the iCycler iQ instrument:



- The results for negative controls, C– and NCA, are negative; the *Ct* value detected for C– in the JOE/HEX channel (detection of IC) is less than 33. The result for positive control, C+, is positive. *Ct* values in the FAM channel do not exceed the boundary *Ct* values. The results for controls correspond to the boundary *Ct* values specified. The results for test samples are valid.
- DNA of the specific microorganism was found in samples No. 1, 4, and 5.
- *Ct* value not exceeding the boundary *Ct* value (33) is detected in the JOE/HEX channel for all samples except for sample No. 3.
- DNA of the specific microorganism is not found in samples No. 2 and 6.
- Sample No. 3 shows an invalid result, that is, *Ct* values are absent in both channels. Analysis of this sample should be repeated.

Example of results obtained using the iCycler iQ instrument:





- The results for negative controls, C– and NCA, are negative; The *Ct* value detected for C– in the JOE/HEX channel (detection of IC) is less than 33. The result for positive control, C+, is positive. *Ct* values in the FAM channel do not exceed the boundary *Ct* values specified. The results for controls correspond to the boundary *Ct* values specified. The results for test samples are valid.
- Sample No. 10 (B3) shows the presence of a specific microorganism.
- DNA of the specific microorganism is not found in samples No. 9 and 11–15.

If MULTIPRIME PCR kits are used

Fluorescence signal is detected in all channels enabled for detection. The product of amplification of the analyzed microorganism DNA is detected in the channel specified in Table 3 (**FAM, JOE/HEX (JOE fluorophore), or ROX** channels). The product of amplification of the IC DNA is detected in the **Cy5** channel if a PCR kits for detection of three microorganisms is used or in the **ROX** channel if a PCR kit for detection of two microorganisms (duplex) is used.

The interpretation of results is based on data obtained from each channel assigned for detection of analyzed microorganisms as well as for detection of Internal Control in accordance with Table 3.

1. Data analysis of the IC DNA amplification

   1.1 Select data in the channel assigned for detection of IC: **Cy5** channel if PCR kit for detection of three microorganisms is used (for iCycler iQ instrument select the **Cy5-635** sign in the ***Select a Reporter*** window) or **ROX** channel if two microorganisms (duplex) are tested (for the iCycler iQ instrument, select the **ROX-575** sign in the ***Select a Reporter*** window). Make sure that the ***PCR Base Line Subtracted Curve*** **Fit** mode is activated (set by default).

   1.2 Set the threshold line at a level of **10–20 %** of the maximum fluorescence level obtained for the Positive Control (**C+**) in the last amplification cycle (the fluorescence level for the Positive Control is considered as equal to the nearest digital scale mark). The fluorescence curve for the Positive Control should contain a segment of a typical exponential growth of fluorescence. The threshold line can also be set by default if it fits in this range.

   In order to select Positive Control curve (or any other object), use the ***Display Wells (Select Wells)*** button or the point tab cursor at a desired curve and double click.

   - **iCycler iQ5 instrument**

     To set the threshold line level, move it using the left mouse button or select the ***Baseline Threshold*** menu (in the drop-down menu, which appears after clicking the right mouse button at window with fluorescence curves), then select the ***User Define*** option and insert the required value in the ***Threshold Position*** text field. The results grid will be displayed after clicking the ***Results*** button.

   - **iCycler iQ instrument**

     To set the threshold line level, either move it using the left mouse button or select ***User Defined*** option, enter the required value in the ***Threshold Position*** text field, and click the ***Recalculate Threshold Cycles*** button.

NOTE: The selected threshold level can be used for data analysis in further runs performed with the same PCR kit and conducted on the same Instrument if a new calibration was not performed.

2. Data analysis of the specific microorganism amplification

Obtained results should be consistently analyzed as described below for each channel used.

2.1 Select the required channel (for the iCycler iQ instrument, select the sign in the ***Select a Reporter*** window) in the analysis window. Make sure that the ***PCR Base Line Subtracted Curve*** **Fit** mode is activated (set by default).

2.2 Set the threshold line at the level of **10–20 %** of the maximum fluorescence level recorded for the Positive Control (**C+**) in the last amplification cycle (the fluorescence level for the Positive Control is considered as equal to the nearest digital scale mark). The fluorescence curve for the Positive Control should represent typical exponential growth of fluorescence. The threshold line can also be set by default if it fits in this range.

2.3 For convenient interpretation of results we recommend that the *Ct* value column is copied and entered into the corresponding column in Excel.

3. Interpretation of results

Principle of interpretation

- The microorganism DNA is **detected** in a sample if its *Ct* value is detected in the results grid in the channel assigned for detection of the amplified DNA fragment of this microorganism. The fluorescence curve should cross the threshold line in the typical exponential growth phase.
- The microorganism DNA is **not detected** in a sample if *Ct* value is not detected in the results grid in the channel assigned for detection of amplified DNA fragment of this microorganism (the fluorescence curve does not cross the threshold line), whereas the *Ct* value detected in the channel assigned for IC DNA is less than **36** (**Cy5** or **ROX** channel for tests of the first or second group, respectively).
- The result is **invalid** if the *Ct* of the sample is not detected in all channels assigned for detection of the amplified DNA fragment of specific microorganisms, whereas the *Ct* value in the channel assigned for the IC DNA is either absent or greater than **36**. Repeat the PCR test for such samples.

4. For automatic analysis of results, the **AmpliSens<*PCR kit*>Results Matrix** program supplied by the manufacturer can be used. Obtained data should be analyzed as described in items 1 and 2, The *Ct* values should be copied from the results grid in the

clipboard and entered in the corresponding column of the program for automatic analysis of results.

The result of the analysis is considered reliable only if the results of Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (see Tables 19 and 20).

Table 19

Results for controls

Control	Stage for control	*Ct* value in the channel	
		for detection of specific microorganism DNA amplification	for detection of IC DNA amplification (Cy5 or ROX)
C–	DNA extraction	N/A (absent)	Detected value < 36
NCA	PCR	N/A (absent)	N/A (absent)
C+	PCR	< boundary *Ct* value	< boundary *Ct* value

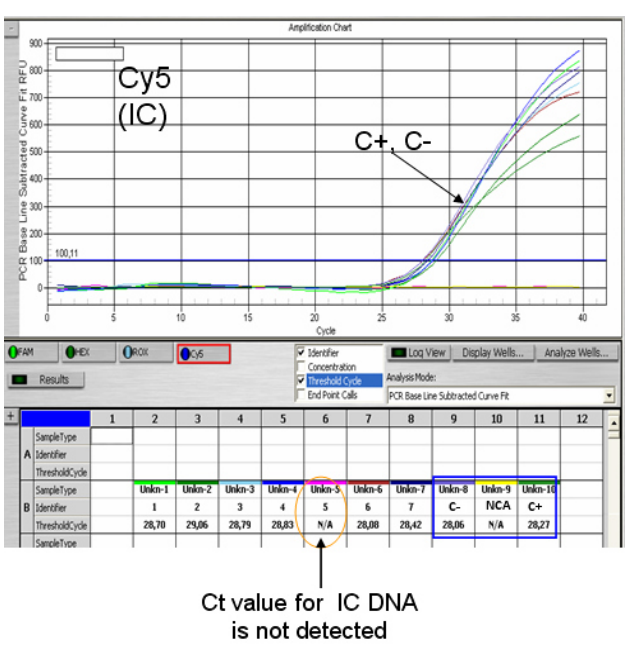
Table 20

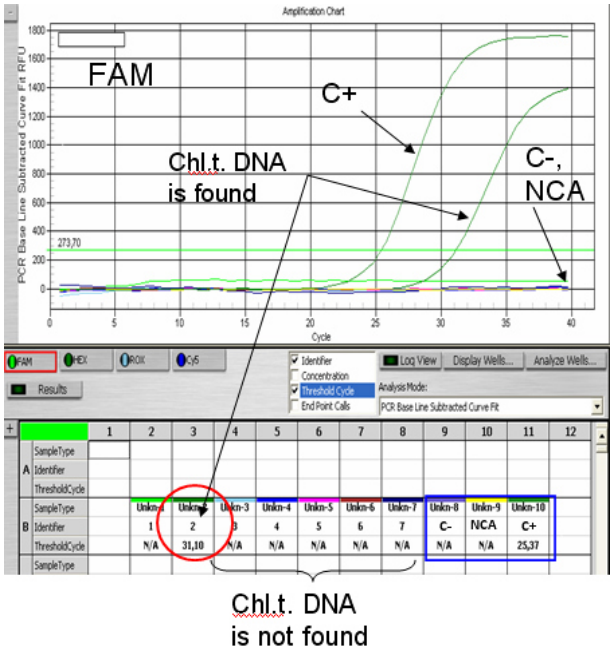
Boundary *Ct* values for positive control of amplification (C+)

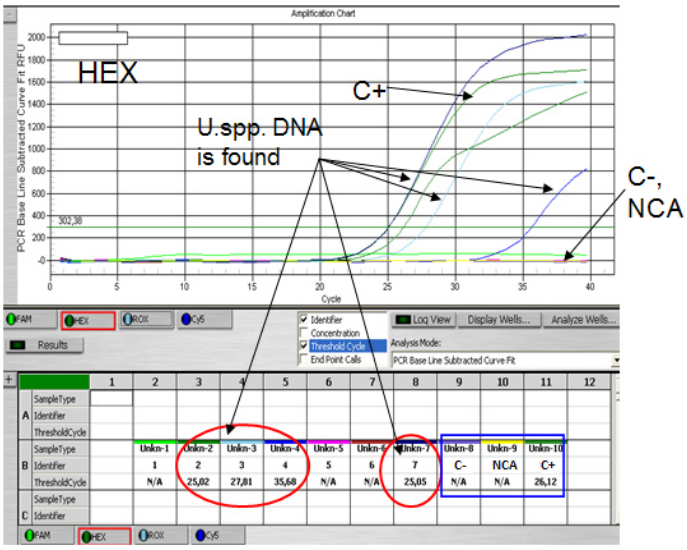
PCR kit	*Ct* value in channel				
	FAM	HEX	ROX	Cy5	Cy5.5
C.trachomatis / Ureaplasma / M.genitalium	33	36	36	36	—
C.trachomatis / Ureaplasma / M.hominis	33	36	36	36	—
N.gonorrhoeae / C.trachomatis / M.genitalium	36	33	36	36	—
C.albicans / C.glabrata / C. krusei	36	36	36	36	—
T.vaginalis / N.gonorrhoeae/ C.trachomatis	33	33	36	36	—
U.parvum / U.urealyticum	36	36	36	—	—
HSV-typing	36	33	36	—	—
T.vaginalis / N.gonorrhoeae	36	36	36	—	—
HSV / CMV	33	33	36	—	—

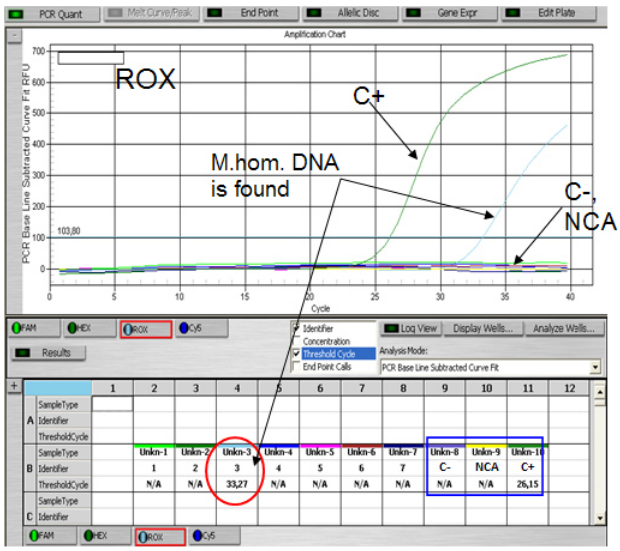
Example of results obtained using the iCycler iQ instrument for a MULTIPRIME PCR kit

Results obtained with **AmpliSens®** ***C.trachomatis* / *Ureaplasma* / *M.genitalium*-MULTIPRIME-FRT** PCR kit:









- The results for negative controls, C– and NCA are negative; the *Ct* value detected for C– in the Cy5 channel (detection of IC) is less than 36. The result for positive control, C+, is positive. *Ct* values do not exceed the boundary *Ct* values in all channels. The results for controls correspond to the specified *Ct* values. The results for test samples are valid.
- *Ct* values less than 36 are detected for all samples except for No. 5 in the Cy5 channel.
- Sample No. 2 shows the presence of DNA of microorganisms detected in the FAM channel (*Chlamydia trachomatis* in here) as well as DNA of microorganisms detected in the JOE/HEX channel (*Ureaplasma* spp. in here).
- Sample No. 3 shows the presence of DNA of microorganisms detected in JOE/HEX and ROX channels (*Ureaplasma* spp. and *Mycoplasma hominis*, respectively, in here).
- Sample No. 4 shows the presence of DNA of the microorganism detected in the JOE/HEX channel (*Ureaplasma* spp. in here).
- Sample No. 5 shows an invalid result, that is, *Ct* values are absent in all channels.
- None of the specific microorganisms was found in samples No. 1, 5, and 6.

CONDUCTING REAL-TIME PCR WITH THE USE OF Mx3000P OR Mx3005P INSTRUMENTS

1. Create a plate setup, which shows the order of tubes in the module and the settings of fluorescence detection in the tubes in the required channel, in the ***Plate Setup*** window. Indicate all samples as ***Unknown***, tick the names of fluorophores to be detected, click the ***Show Well Names*** button, and enter names of samples. If a PCR kit for detection of a single microorganism is used, enable detection in **FAM** and **JOE/HEX** channels. If a MULTIPRIME PCR kit is use, enable detection in **FAM, JOE/HEX, ROX,** and **Cy5** channels.
2. Assign execution of the **Amplisens-1** (table 21) or **AmpliSens-1M** (table 22) program. To do this, select or create the program in the ***Thermal Profile Setup*** module. Save the file with the specified program and the required plate setup and click the ***Run*** button for running.

Table 21

AmpliSens-1 program

Step	***Temperature, °C***	***Time***	***Cycles***
Segment 1	**95**	15 min	1
Segment 2	**95**	5 s	5
	60	20 s	
	72	15 s	
Segment 3	**95**	5 s	40
	60	30 s Fluorescence detection*	
	72	15 s	

Table 22

AmpliSens-1M program

Step	***Temperature, °C***	***Time***	***Cycles***
Segment 1	**95**	15 min	1
Segment 2	**95**	20 s	5
	60	20 s	
	72	15 s	
Segment 3	**95**	20 s	40
	60	30 s Fluorescence detection*	
	72	15 s	

***** Fluorescence detection is assigned in the second step (60 ºC) of the second cycling block (*Segment 3*) in the **FAM, JOE/HEX, ROX, Cy5** channels.

NOTE: **AmpliSens-1** and **AmpliSens-1M** are universal programs for conducting tests for detection of STIs and other infections of reproductive system with AmpliSens PCR kits. Therefore, all this tests or any combination of tests including tests for *human papillomaviruses (HPV HCR)* detection and genotyping can be carried out simultaneously in the same instrument. If **AmpliSens® *HSV* I,II-FRT, AmpliSens® *HSV* / *CMV*-MULTIPRIME-FRT**, **AmpliSens® *HSV*-typing-FRT** PCR kits are used for analysis (separately or in combination with any other kits), the **AmpliSens-1M** program is recommended for running amplification and real-time detection.

3. Transfer the reaction tubes into the wells of the instrument in accordance with the specified plate setup. Secure the lid.
4. It is recommended that the option of lamp shutdown after the run completion is activated (the box is ticked).
5. When the run is completed, proceed to analysis of results.
6. Close the program and switch the instrument off when the work with the instrument is finished.

Data analysis. Mx3000P and Mx3005P instruments

The obtained data are interpreted with the software of Mx3000P and Mx3005P PCR instruments. The result are interpreted by the crossing (or not crossing) of the fluorescence curve with the threshold line and is shown as the presence (or absence) of the *Ct* (cycle threshold) value in the results grid.

If PCR kits for detection of a single microorganism are used

Fluorescence signal is detected in two channels: the amplification product of a DNA fragment of a specific microorganism is detected in the **FAM** channel and the amplification product of the Internal Control DNA is detected in the **JOE/HEX** channel.

1. Data analysis of the specific microorganism DNA amplification

   Select data in the **FAM** channel in the ***Result/Amplification Plots*** window of the ***Analysis*** module. Set the threshold line at the level of **10–20 %** of the maximum fluorescence level recorded for the Positive Control (C+) in the last amplification cycle (the fluorescence level for the Positive Control is considered as equal to the nearest digital scale mark). The fluorescence curve for the Positive Control should contain a typical segment of the exponential growth of fluorescence. The threshold line can also be set by default if it fits in this range.

   To select the curve of the C+ sample (or another sample) in the ***Analysis Selection/Setup*** window, select the required well and shift to the ***Results*** window.

NOTE: The selected threshold level can be used for interpretation of results obtained for the same pathogen with the given PCR kit.

2. Data analysis of the Internal Control DNA amplification

   Select data in the **JOE/HEX** channel in the ***Result/Amplification Plots*** window of the ***Analysis*** module. Set the threshold line at the level of **10–20 %** of maximum fluorescence level recorded for the Positive Control (**C+**) in the last amplification cycle (the fluorescence level for the Positive Control is considered as equal to the nearest digital scale mark). The fluorescence curve for the Positive Control should contain a segment of the typical exponential growth of fluorescence. The threshold line can also be set by default if it fits in this range.

NOTE: The selected threshold level can be used for interpretation of results of the amplified Internal Control DNA obtained with other STI tests for detection of a single microorganism performed with PCR kits manufactured by FBIS CRIE. The same threshold level set for the JOE/HEX channel can be used for further runs conducted with the same PCR instrument.

3. To obtain the overall results grid, activate data in both channels in the ***Results/Amplification Plots*** window and select the ***Text Report*** option in the ***Area to analyze*** menu list. The results grid can be exported to Excel (to do this, press the right mouse button and select the ***Export Text Report to Excel*** option from the menu displayed).
4. Interpretation of results

Principle of interpretation:

- The microorganism DNA is **detected** in a sample if its *Ct* value is detected in the results grid in the **FAM** channel (make sure that the FAM-490 sigh is selected in the ***Select a Reporter*** window). The fluorescence curve should cross the threshold line in the typical exponential growth phase.
- The microorganism DNA is **not detected** in a sample if **N/A** appears in the results grid in the **FAM** channel (the fluorescence curve does not cross the threshold line), whereas the *Ct* value detected in the **JOE** channel is less than **33**.
- The result is **invalid** if the *Ct* value of the sample is not detected in the **FAM** channel (**N/A** appears), whereas the *Ct* value in the **JOE** channel is either absent or greater than **33**. Repeat the PCR test for such samples.

The result of analysis is considered reliable only if the results of Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (see Tables 23 and 24)

Table 23

Result for controls

Control	Stage for control	*Ct* value in channel	
		FAM	**JOE/HEX**
C–	DNA extraction	N/A (absent)	Detected value < 33
NCA	PCR	N/A (absent)	N/A (absent)
C+	PCR	< boundary *Ct* value	Detected value < 33

Table 24

Boundary *Ct* values for positive control C+ in FAM channel

PCR kit	*Ct* value
Chlamydia trachomatis	33
HSV I, II	
CMV	
Candida albicans	
Neisseria gonorrhoeae-screen	36
Mycoplasma genitalium	
Trichomonas vaginalis	
Treponema pallidum	
Ureaplasma species	
Mycoplasma hominis	
Gardnerella vaginalis	

If MULTIPRIME PCR kits are used

Fluorescence curves are analyzed in all channels used for detection. For each specific microorganism, the result of its DNA fragment amplification is detected in the specific channel defined in the PCR kit Instruction Manual and in **Table 3** (**FAM**, or **JOE/HEX**, or **ROX** channels). The result of IC DNA amplification is obtained in the **Cy5** channel if a PCR kit for detection of three microorganisms is used or in the **ROX** channel if a PCR kit for detection of two microorganisms (duplex) is used.

Interpretation of results is based on the presence or absence of ***Ct*** values in the channels in accordance with the channel assignment (detection of the specific microorganism DNA or IC DNA) specified in **Table 3**.

1. Data analysis of IC DNA amplification

   1.1 Select data in the required channel in the **Analysis** module of the ***Results/Amplification Plots*** window: the Cy5 channel if a PCR kit for detection of three microorganisms is used or the ROX channel if a PCR kit for detection of two microorganisms (duplex) is used.

   1.2 Set the threshold line at the level of **10–20 %** of the maximum fluorescence level recorded for the Positive Control (C+) in the last amplification cycle (the fluorescence level for the Positive Control is considered as equal to the nearest digital scale mark). The fluorescence curve for the Positive Control should contain

a segment of the typical exponential growth of fluorescence. The threshold line can also be set by default if it fits in this range.

To select positive control curve (C+) or another sample, select this sample in the result list left to the ***Amplification Plots*** window and shift to the ***Result*** window. To activate all analyzed samples, click the ***Select all*** button in the right bottom corner (below the result list).

2. Data analysis of the specific microorganism amplification

   The obtained results should be consistently analyzed in each channel used as described below:

   2.1 Select the required channel in the ***Results/Amplification Plots*** of the ***Analysis*** module.

   2.2 Set the threshold line at the level of **10–20 %** of the maximum fluorescence level recorded for the Positive Control (C+) in the last amplification cycle (the fluorescence level for the Positive Control is considered as equal to the nearest digital scale mark). The fluorescence curve for the Positive Control should contain a segment of the typical exponential growth of fluorescence. The threshold line can also be set by default if it fits in this range.

3. To obtain the overall results grid, activate data in all channels in the ***Results/Amplification Plots*** window and select the ***Text Report*** option in the ***Area to analyze*** menu list. The results grid can be exported to Excel (to do this, press the right mouse button and select the ***Export Text Report to Excel*** option from the menu displayed).

4. Principle of interpretation

   - The microorganism DNA is **detected** in a sample if its *Ct* value is detected in the results grid in the channel assigned for detection of amplified DNA fragment of this microorganism. Moreover, the fluorescence curve should cross the threshold line at the typical exponential growth phase.
   - The microorganism DNA is **not detected** in a sample if the *Ct* value is not detected in the results grid in the channel assigned for detection of an amplified DNA fragment of this microorganism (the fluorescence curve does not cross the threshold line), whereas the *Ct* value detected in the channel assigned for the IC DNA is less than **36** (**Cy5** or **ROX** channel for tests of the first or second group, respectively).
   - The result is **invalid** if the *Ct* value of the sample is not detected in all channels assigned for detection of amplified DNA fragment of specific microorganisms; whereas, *Ct* in the channel assigned for IC DNA is either absent or greater than **36**.

It is necessary to repeat the PCR test for such a sample.

5. For automatic analysis of results, the **AmpliSens<*PCR kit*>Results Matrix** program supplied by the manufacturer can be used. The obtained data should be analyzed as described in items 1-3, *Ct* values should be copied from the results grid in the clipboard and entered in the corresponding column of the program for the automatic result analysis.

The result of the analysis is considered reliable only if the results of Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (see Tables 25 and 26).

Table 25

Results for controls

Control	Stage for control	*Ct* value in channel	
		for detection of specific microorganism DNA amplification	for detection of IC DNA amplification (Cy5 or ROX)
C–	DNA extraction	Absent	Detected value < 36
NCA	PCR	Absent	Absent
C+	PCR	< boundary *Ct* value	< boundary *Ct* value

Table 26

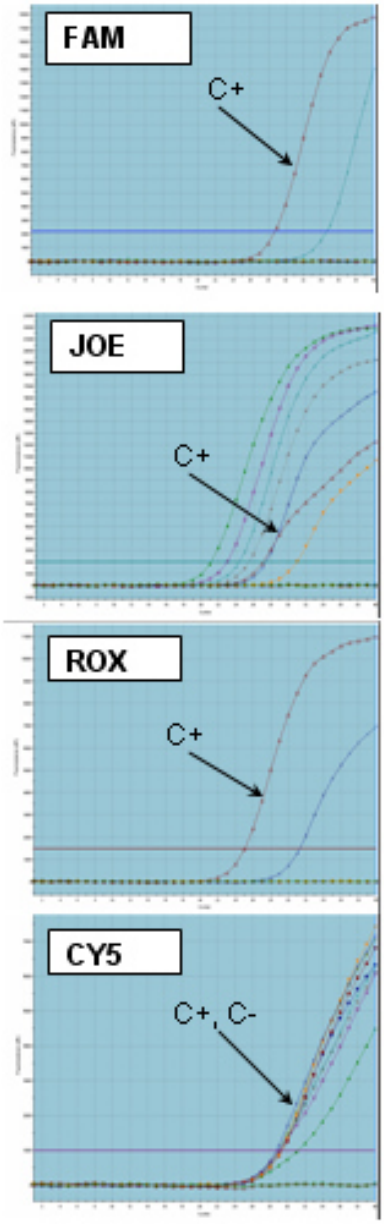
Boundary *Ct* values for Positive Control of Amplification (C+)

PCR kit (test)	*Ct* value in channel				
	FAM	HEX	ROX	Cy5	Cy5.5
C.trachomatis / Ureaplasma / M.genitalium	33	36	36	36	—
C.trachomatis / Ureaplasma / M.hominis	33	36	36	36	—
N.gonorrhoeae / C.trachomatis / M.genitalium	36	33	36	36	—
C.albicans / C.glabrata / C.krusei	36	36	36	36	—
T.vaginalis / N.gonorrhoeae/ C.trachomatis	33	33	36	36	—
U. parvum / U. Urealyticum	36	36	36	—	—
HSV-typing	36	33	36	—	—
T.vaginalis / N.gonorrhoeae	36	36	36	—	—
HSV / CMV	36	36	36	—	—

Example of results obtained with the Mx3000P instrument for a MULTIPRIME PCR kit

Results obtained with **AmpliSens®** ***C.trachomatis / Ureaplasma / M.genitalium*-MULTIPRIME-FRT** PCR kit:

Well Name	Dye	Well	Ct (dR)
C-	CY5	B3	29.3
C-	ROX	B3	No Ct
C-	JOE	B3	No Ct
C-	FAM	B3	No Ct
1	CY5	B4	29.39
1	ROX	B4	No Ct
1	JOE	B4	No Ct
1	FAM	B4	No Ct
2	CY5	B5	31.19
2	ROX	B5	No Ct
2	JOE	B5	21.16
2	FAM	B5	No Ct
3	CY5	B6	29.16
3	ROX	B6	31.1
3	JOE	B6	25.54
3	FAM	B6	No Ct
4	CY5	B7	29.01
4	ROX	B7	No Ct
4	JOE	B7	No Ct
4	FAM	B7	No Ct
5	CY5	B8	28.96
5	ROX	B8	No Ct
5	JOE	B8	24.38
5	FAM	B8	34.67
6	CY5	B9	29.06
6	ROX	B9	No Ct
6	JOE	B9	22.97
6	FAM	B9	No Ct
C+	CY5	F2	28.74
C+	ROX	F2	25.03
C+	JOE	F2	27.11
C+	FAM	F2	28.8
NCA	CY5	F4	No Ct
NCA	ROX	F4	No Ct
NCA	JOE	F4	No Ct
NCA	FAM	F4	No Ct



- The results for negative controls, C– and NCA, are negative; the *Ct* value detected for C– in the Cy5 channel (detection of IC) is less than 36. The result for positive control, C+, is positive. *Ct* values do not exceed the boundary *Ct* values in all channels. The results for controls correspond to the specified *Ct* values. The results for test samples are valid.
- Samples Nos. 2, 3, and 6 show the presence of DNA of the microorganisms detected in the JOE/HEX channel (*Ureaplasma* spp. in here). Sample No.3 shows the presence of DNA of microorganisms detected in the ROX channel (*Mycoplasma hominis* in here).
- Sample No. 5 shows the presence of DNA of the microorganisms detected in the FAM channel (*Chlamydia trachomatis* in here) and DNA of the microorganisms detected in the JOE/HEX channel (*Ureaplasma spp.* in here).
- Samples Nos. 1 and 4 shows the absence of DNA of analyzed microorganisms. The *Ct* value in the Cy5 channel (IC detection) does not exceed 36.

CONDUCTING REAL-TIME PCR WITH THE USE OF CFX96 INSTRUMENT

1. Turn on the instrument and start the **Bio-Rad CFX Manager** program.
2. Select ***Create a new Run*** (or select ***New*** and then ***Run…*** in the ***File*** menu).
3. In the ***Run Setup*** window, select ***Protocol*** and click the ***Create new…*** button. Set amplification parameters (time, temperature, cycles, and fluorescence acquiring cycle) in the opened ***Protocol Editor – New*** window (see table 27 or table 28). Set ***Sample Volume*** **– 30 µl.**

Table 27

AmpliSens-1 program for CFX96

Step	*Temperature, °C*	*Time*	*Cycles*
1	**95**	15 min	1
2	**95**	5 s	5
	60	20 s	
	72	15 s	
3	**95**	5 s	40
	60	30 s Fluorescence detection	
	72	15 s	

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

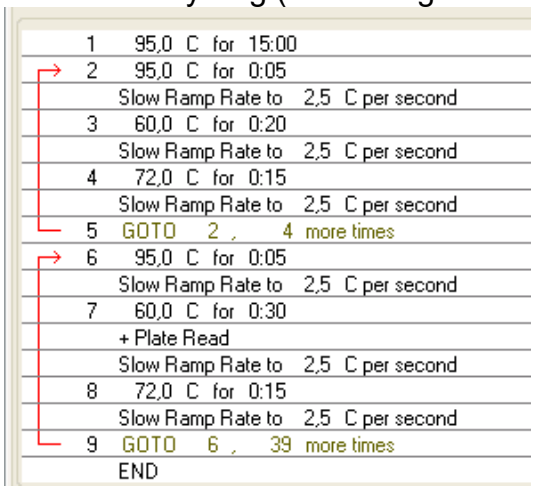


Table 28

AmpliSens-1M program for CFX96

Step	*Temperature, °C*	*Time*	*Cycles*
1	**95**	15 min	1
2	**95**	20 s	5
	60	20 s	
	72	15 s	
3	**95**	20 s	40
	60	30 s Fluorescence detection	
	72	15 s	

* Fluorescence detection is assigned in the second step (60 ºC) of the second cycling block (Step 3) in the **FAM**, **HEX, ROX, Cy5, Quasar 705** channels.

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

NOTE: **AmpliSens-1** and **AmpliSens-1M** are universal programs for conducting tests for detection of STIs and other infections of reproductive system with AmpliSens PCR kits. Therefore, all this tests or any combination of tests including tests for *human papillomaviruses (HPV HCR)* detection and genotyping can be carried out simultaneously in the same instrument. If **AmpliSens® *HSV* I,II-FRT, AmpliSens® *HSV* / *CMV*-MULTIPRIME-FRT**, **AmpliSens® *HSV*-typing-FRT** PCR kits are used for analysis (separately or in combination with any other kits), the **AmpliSens-1M** program is recommended for running amplification and real-time detection.

4. Set the tube order and fluorescence acquiring for all tubes in the appropriate channels in the ***Plate*** window of the ***Experiment Setup*** module. If a single microorganism is detected: select ***Unknown*** in the ***Sample type*** menu; check off ***FAM*** and ***HEX*** while pressing the ***Select Fluorophores…*** button; click ***OK***; then check off fluorescence detection for the specified tubes in the required channels. If MULTIPRIME PCR kits are used: check off **FAM**, **HEX**, **ROX**, **Cy5** and **Quasar 705**. Fill out the ***Sample name*** window. Click ***Save***.
5. Click the ***Open Lid*** button. Place the reaction tubes in the cells of the instrument in accordance with the plate setup. Click ***Close Lid***.
6. Click the ***Start Run*** button to run the selected AmpliSens-1 program with the specified plate setup.
7. Proceed to the analysis of results after the end of the run.

Analysis of results obtained with the CFX96 instrument

The obtained results are analyzed by the CFX96 software. Results are analyzed based on the presence (or absence) of fluorescence curve crossing the threshold line set at the specific level that corresponds with the presence (or absence) of ***Ct*** (cycle threshold) value in an appropriate cell of the results grid.

If PCR kits for detection of a single microorganism are used, the fluorescence signal is detected in two channels:

- the amplification product of the **DNA fragment of the specific microorganism** is detected in the **FAM** channel,
- the amplification product of the **Internal Control DNA** is detected in the **HEX** channel.

1. Results of analysis of amplification of a DNA fragment of a **target microorganism**.
   1.1 Check off the box (below the fluorescence graph) corresponding to the data obtained on the ***FAM*** channel in the ***Quantitation*** tab of the ***Data Analysis*** window.

1.2 Set the threshold line at a level corresponding to 10-20 % of the maximum fluorescence level obtained for C+ sample during the last amplification cycle (fluorescence level is equal to the nearest digital scale mark). Moreover, the fluorescence graph of C+ sample should have typical exponential fluorescence growth. The threshold line can be set automatically (by default) if it falls in the specified range.

To select the graph of C+ sample (or any other object), set the cursor in the plate setup or in the results grid.

To set the threshold line, either drag it with the left mouse button or select the ***Baseline Threshold*** menu (from the dropdown menu which appears when the fluorescence graph is clicked with the right mouse button), then the ***User Defined*** option, and enter the required value in the text field.

NOTE: The set threshold level can be used for analysis of results of further runs with the same PCR kit in case a new calibration of the instrument was not performed.

2. Data analysis of the Internal Control DNA in the HEX channel.

   Perform similar procedures for data obtained in the **HEX** channel.

NOTE: The selected threshold level can be used for result analysis of Internal Control amplification obtained in other tests performed with the use of PCR kits manufactured by FBIS CRIE for detection of STIs. The same threshold level in the **HEX** channel can be used for further runs with the use of the same PCR kit in case a new calibration of the instrument was not performed.

3. Interpretation of results:
   a) DNA of a microorganism is **detected** if a *Ct* value is defined for the sample in the FAM channel in the results grid. Moreover, the fluorescence curve of this sample should cross the threshold line in the area of typical exponential growth of fluorescence.
   b) DNA of a microorganism is **not detected** if N/A is indicated for the *Ct* value in the FAM channel in the results grid (fluorescence graph doesn't cross the threshold line), whereas the *Ct* value detected and indicated in the results grid for the HEX channel does not exceed the boundary *Ct* value specified in the Table 29 or in the *Important Product Information Bulletin* enclosed in PCR kit.
   c) The result of analysis is **invalid** if *Ct* value is absent (not detected) in the FAM channel (N/A is indicated in the results grid), whereas the *Ct* value detected in the HEX channel is absent (N/A is indicated) or exceed the value indicated in the *Important Product Information Bulletin* enclosed in the PCR kit or indicated in

Table 29. In such cases, either repeat amplification and real-time detection of the required test sample or repeat the analysis beginning with the DNA extraction stage.

The result of the analysis is considered reliable only if the results of both Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (see Tables 29, 30).

Table 29

Results for controls

Control	Stage for control	*Ct* value in channel	
		FAM	HEX
C–	DNA extraction	N/A (absent)	Detected value < 33
NCA	PCR	N/A (absent)	N/A (absent)
C+	PCR	< boundary *Ct* value	Detected value < 33

Table 30

Boundary *Ct* value for positive control (C+) in FAM channel

PCR kit	Boundary *Ct* value for C+ in FAM channel
Chlamydia trachomatis	33
HSV I, II	
CMV	
Candida albicans	
Neisseria gonorrhoeae-screen	36
Mycoplasma genitalium	
Trichomonas vaginalis	
Treponema pallidum	
Ureaplasma species	
Mycoplasma hominis	
Gardnerella vaginalis	

If MULTIPRIME PCR kits are used

Fluorescence signal is detected in all channels enabled for detection. Results of amplification of **DNA fragment of the target microorganisms** are detected in the specific channels indicated in the PCR kit Instruction Manual and in **Table 3** (**FAM, HEX, ROX, or QUASAR 705** channels). Results of amplification of **Internal Control DNA** are detected in the **Cy5** channel if a PCR kit for detection of three microorganisms is used or in the **ROX** channel if a PCR kit for detection of two microorganisms (duplex) is used.

The interpretation of results is based on data obtained from each channel assigned for detection of analyzed microorganisms as well as for detection of Internal Control in accordance with **Table 3.**

1. Analysis of results of **Internal Control DNA** amplification

1.1 Select data obtained in the channel assigned for detection of Internal Control: the **Cy5** channel if PCR kits for detection of three microorganisms are used or the **ROX** channel if PCR kits for detection of two microorganisms (duplex) are used.

1.2 Set the threshold line at a level corresponding to 10-20 % of the maximum fluorescence level obtained for the C+ sample during the last amplification cycle (fluorescence level is equal to the nearest digital scale mark). Moreover, the fluorescence graph of the C+ sample should have typical exponential fluorescence growth. The threshold line can be set automatically (by default) if it falls in the specified range.

To select the graph of C+ sample (or any other object) set the cursor in the plate setup or in the results grid.

NOTE: This threshold level can be used for analysis of results obtained in further runs of the same PCR kit in case a new calibration of the instrument was not performed.

2. Analysis of results of amplification of a **DNA fragment of microorganism**

Analyze results for each channel to be used as follows:

2.1 Select the required channel in the window of analysis module.

2.2 Set the threshold line at a level corresponding to 10-20 % of the maximum fluorescence level obtained for C+ sample during the last amplification cycle (fluorescence level is equal to the nearest digital scale mark). Moreover, the fluorescence graph of C+ sample should have a typical exponential fluorescence growth. The threshold line can be set automatically (by default) if it falls in the specified range.

To select the graph of C+ sample (or any other object), set the cursor in the plate setup or in the results grid.

NOTE: This threshold level can be used for analysis of results obtained in further runs of the same PCR kit in case a new calibration of the instrument was not performed

2.3 For more convenient interpretation of result import the table containing *Ct* values in an Excel spreadsheet.

3. Interpretation of results:

a) DNA of a microorganism is **detected** if *Ct* value is defined in the channel assigned for obtaining signals of microorganism DNA amplification. Moreover, fluorescence curve of this sample should cross the threshold line at the area of typical exponential growth of fluorescence.

b) Microorganism DNA is **not detected** if *Ct* value is absent (fluorescence curve

doesn't cross the threshold line) in the channel assigned for obtaining signal of microorganism DNA amplification; whereas, *Ct* value detected in the channel assigned for signal of IC DNA amplification (**Cy5** channel for the first group of tests or **ROX** channel for second group of tests) is less than the boundary value specified in the *Important Product Information Bulletin* enclosed in the PCR kit or specified in **Table 31.**

c) Result of analysis is **invalid** if *Ct* value is not detected in the channels assigned for recording the signal of the amplified DNA fragments of the target microorganisms; whereas the *Ct* value detected in the channel assigned for recording the signal of amplified Internal Control DNA is absent or exceed the boundary *Ct* value specified in the *Important Product Information Bulletin* enclosed in the PCR kit or specified in **Table 31.** In such cases either repeat amplification and real-time detection of the required test sample or repeat the analysis beginning with DNA extraction stage.

The result of the analysis is considered reliable only if the results of both Positive and Negative Controls of amplification as well as Negative Control of extraction are correct (see Tables 31, 32).

Table 31

Results for controls

Control	Stage for control	*Ct* value in the channel	
		for detection of amplification of microorganism DNA	for detection of amplification of Internal Control DNA (Cy5 or ROX)
C–	DNA extraction	Absent	Detected value < 36
NCA	PCR	Absent	Absent
C+	PCR	< boundary *Ct* value	< boundary *Ct* value

Table 32

Boundary *Ct* values for the Positive Control of amplification (C+)

PCR kit (test)	Boundary *Ct* value in channel				
	FAM	HEX	ROX	Cy5	Quasar 705
N.gonorrhoeae / C.trachomatis / M.genitalium / T.vaginalis	38	38	38	36	38
C.trachomatis / Ureaplasma / M.genitalium / M.hominis	38	38	38	36	38
C.trachomatis / Ureaplasma / M.genitalium	33	36	36	36	—
C.trachomatis / Ureaplasma / M.hominis	33	36	36	36	—
N.gonorrhoeae / C.trachomatis/ M.genitalium	36	33	36	36	—
C.albicans / C.glabrata / C. krusei	36	36	36	36	—
T.vaginalis / N.gonorrhoeae / C.trachomatis	33	33	36	36	—
U.parvum / U.Urealyticum	36	36	36	—	—
HSV-typing	36	33	36	—	—
T.vaginalis / N.gonorrhoeae	36	36	36	—	—
HSV / CMV	33	33	36	—	—

12. KEY TO SYMBOLS USED

	Catalogue number		Caution
	Batch code		Contains sufficient for <n> tests
	Research use only		Use-by-date
	Version		Consult instructions for use
	Manufacturer		Keep away from sunlight
	Date of manufacture		Temperature limit

List of Changes Made in the Guidelines

VER	Location of changes	Essence of changes
12.02.16 ME	8.1.1 DNA extraction with DNA-sorb-AM reagent kit 8.1.2 DNA extraction with DNA-sorb-B reagent kit	The description of the reagents was corrected.
	8.2. Real-time PCR	The phrase “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” was added
	Conducting real-time PCR with the use of Rotor-Gene 3000, Rotor-Gene 6000, and Rotor-Gene Q instruments	In the Results analysis (Table 8 and Data analysis of IC DNA amplification) the value of Outlier Removal parameter was changed from “5” to “5-10”.
	Conducting real-time PCR with the use OF iCycler iQ or iQ5 instruments Conducting real-time PCR with the use of CFX96 instrument	The phrase about the fluorescence detection at second stage (60 ºC) of the second cycling block (step 3) in the corresponding channels was added after the amplification program for iCycler IQ or iCycler IQ5, CFX96 instruments.
	16. Key to symbols used	The chapter was added
09.11.18 PM	Text	Specifications on the use of the "AmpliSens-1M" amplification program
	Conducting real-time PCR with the use of Rotor-Gene 3000, Rotor-Gene 6000, and Rotor-Gene Q instruments	In the Parameters of result analysis for MULTIPRIME PCR kit (Table 12) the value of outliner removal for JOE/Yellow was specified and notes were added
09.12.19 EM	Throughout the text	The information about AmpliSens® *Neisseria gonorrhoeae*-test-FRT PCR kit, Transport medium TM-EDEM reagent, DNA-sorb-AM **REF** K1-12-50-CE was deleted
04.06.21 KK	Front page	The phrase “For research use only. Not for diagnostic procedures” was added
	Through the text	The symbol  was changed to **NOTE:**
22.06.23 EM	Through the text	The reference numbers were deleted; clinical material was changed to biological material
28.06.24 PM	Conducting real-time PCR with the use of Rotor-Gene 3000, Rotor-Gene 6000, and Rotor-Gene Q instruments	The information about parameters for results analysis in Table 12 was expanded for all MULTIPRIME PCR kits
23.07.24 PM	Conducting real-time PCR with the use of Rotor-Gene 3000, Rotor-Gene 6000, and Rotor-Gene Q instruments	The information about parameters for results analysis was corrected for AmpliSens® *Chlamydia trachomatis*-FRT and AmpliSens® *Mycoplasma genitalium*-FRT PCR kits