IVD For *in Vitro* Diagnostic Use

For Professional Use Only

GUIDELINES

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

Federal State Institution of
Science Central Research
Institute of Epidemiology
3A Novogireevskaya Street
Moscow 111123 Russia

TABLE OF CONTENTS

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

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

1. INTENDED USE

Guidelines describe the procedure of detection of STIs and other reproductive tract infections in clinical material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection using real-time PCR instruments: Rotor-Gene 3000 and 6000 (Corbett Research), iCycler iQ and iQ5 (Bio-Rad), Mx3000P, and Mx3005 (Stratagene).

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***	***Amount***
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 during the DNA extraction directly to the sample/lysis mixture (see "DNA-sorb-AM" **REF** K1-12-100-CE, K1-12-50-CE protocol).

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

PCR kit variant FRT-100 F includes:

Reagent	*Description*	*Volume, ml*	*Amount*
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 during the DNA extraction directly to the sample/lysis mixture (see the "DNA-sorb-AM" **REF** K1-12-100-CE, K1-12-50-CE protocol).

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 barriers (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 2-ml reaction tubes.
- PCR box.
- Personal thermocyclers (for example, Rotor-Gene 3000 or Rotor-Gene 6000 (Corbett Research, Australia); iCycler iQ or iQ5 (Bio-Rad, USA) or equivalent).
- Disposable polypropylene tubes for PCR (0.1- or 0.2-ml; for example, Axygen, USA).
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin 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 and handle amplicons away from all other reagents.
- Thaw all components thoroughly at room temperature before starting detection.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats, protect eyes while samples and reagents handling. Thoroughly wash hands afterward.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all samples and unused reagents in compliance with local authorities' requirements.
- Samples should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid contact with the skin, eyes, and mucosa. If skin, eyes, or mucosa contact,

immediately flush with water and seek medical attention.

- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment, and reagents to the area in which 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

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 clinical material is used:

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

Clinical material should be placed into tubes with a transport medium recommended by 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.

Only one freeze–thaw cycle of clinical 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 clinical samples is performed in special container with cooling elements.

The following types of clinical 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.kruzei*** DNA;
- vaginal swabs are used for detection of ***Gardnerella vaginalis*** DNA;

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

- Transport Medium with Mucolytic Agent, **REF** 952-CE.
- Transport Medium for Swabs, **REF** 956-CE, 987-CE.

If DNA is extracted with EDEM Reagent Kit (manufactured by CRIE), Transport Medium TM-EDEM, **REF** 1533-CE is recommended for pretreatment of urine samples

7. WORKING CONDITIONS

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

8. PROTOCOL

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

- DNA-sorb-AM, **REF** K1-12-100-CE, K1-12-50-CE.
- DNA-sorb-B, **REF** K1-2-100-CE, K1-2-50-CE (for prostate secretion, whole blood, and

spinal fluid).

Extract DNA according to the manufacturer's instructions.

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 barrier 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 barrier 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 TM-EDEM transport medium using a new tip with the aerosol barrier for each sample.
3. Centrifuge the tubes containing 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 barrier for each sample.
5. Add 0.5 ml of 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 the TM-EDEM should be used for DNA extraction procedure.

   Thus obtained urine pellet in the 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.

8.1 DNA EXTRACTION

A. 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 clinical materials. 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 clinical samples can be used for PCR diagnostic tests.

DNA-sorb-AM nucleic acid extraction kit variant 50 or 100 includes:

Reagent	*Description*	*Variant 50*		*Variant 100*	
		Volume (ml)	*Quantity*	*Volume (ml)*	*Quantity*
Lysis Solution	colorless clear liquid	15	1 vial	30	1 vial
Washing Buffer	colorless clear liquid	50	1 vial	100	1 vial
Universal Sorbent	white suspension	1.0	1 tube	1.0	2 tubes
TE-buffer for DNA elution	colorless clear liquid	5.0	1 tube	5.0	2 tubes
Internal Control complex (ICc)*	colorless clear liquid	1.0	1 tube	1.0	1 tube
Internal Control-FL**	colorless clear liquid	1.0	1 tube	1.0	1 tube
Negative Control	colorless clear liquid	1.2	1 tube	1.2	1 tube

***** should be used during DNA extraction procedure if followed by PCR-analysis with electrophoretic detection.

****** should be used during DNA extraction procedure if followed by PCR-analysis with hybridization-fluorescent detection.

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 clinical 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 specified in item 8.1 A.

DNA extraction procedure

1. Add **10 µl** of **Internal Control complex** (in case of electrophoretic detection) or **10 µl** of **Internal Control-FL** (in case of hybridization-fluorescence detection) to each sterile disposable tube.

If different detection methods are used within one test run, both internal controls can be added (10 µl of each).

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 barrier.

If the number of clinical 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 barrier. 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 barrier) 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 barrier. 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.

B. 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 50 or 100 includes:

Reagent	*Description*	*Variant 50*		*Variant 100*	
		Volume (ml)	*Amount*	*Volume (ml)*	*Amount*
Lysis Solution	colorless clear liquid	15	1 vial	30	1 vial
Washing Solution 1	colorless clear liquid	15	1 vial	30	1 vial
Washing Solution 2	colorless clear liquid	50	1 vial	100	1 vial
Universal Sorbent	white suspension	1.25	1 tube	1.25	2 tubes
TE-buffer for DNA elution	colorless clear liquid	5.0	1 tube	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 clinical 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 complex** (in case of electrophoretic detection) or **10 µl** of **Internal Control-FL** (in case of hybridization-fluorescence detection) to each sterile disposable tube.

If different detection methods are used within one test run, both internal controls can be added (10 µl of each).

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. 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.

C. 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 clinical materials (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 CRIE (including the MULTIPRIME series kits).

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

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).

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

[1] Urine samples should be preliminary treated.

Clinical material obtained from a patient is transferred into TM-EDEM transport medium, in which it is stored and transported to a laboratory. For DNA extraction, an aliquot of a clinical 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 clinical material and, thereby, is a quality marker of laboratory analysis of clinical samples.

EDEM reagent kit includes:

Reagent	*Description*	*Volume (ml)*	*Quantity*
Transport mediumTM-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. Spin down 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 clinical material in TM-EDEM transport medium 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 item 8.1 B to obtain the urine pellet in the TM-EDEM transport medium. To do this, the additional reagent **TM-EDEM transport medium** (50 ml) is to be used.

DNA extraction procedure

1. Transfer **100 µl** of clinical material in the TM-EDEM transport medium into the prepared tubes with **IC-diluent** using a new tip with aerosol barrier for each sample. Add **100 µl** of the **TM-EDEM transport medium** 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.**

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).

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 clinical material in TM-EDEM transport medium 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 the tubes with **PCR-mix-1-FL** and wax for amplification of DNA from clinical 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 clinical 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 clinical or control samples at the DNA extraction stage into the prepared tubes using tips with aerosol barrier.
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 a sample extracted from the **Negative Control** to the tube labeled C- (Negative Control of Extraction).

B. Amplification

1. Create a temperature profile in your PCR instrument as follows:

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 *fluorescent signal detection*		**60**	30 s *fluorescent signal detection*	
	72	15 s		**72**	15 s	

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

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.

[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.

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 from 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.

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 (Table 2).

Table 2

Results for controls

Control	Stage for control	Ct value in channel		Interpretation
		FAM fluorophore	JOE fluorophore	
C–	DNA extraction	Neg	Pos (< boundary Ct value)	OK
NCA	Amplification	Neg	Neg	OK
C+	Amplification	Pos (< boundary Ct value)	Pos (< boundary Ct value)	OK

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 (Crimson) *
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	–
N.gonorrhoeae/ C.trachomatis/ T. vaginalis	*Neisseria gonorrhoeae*	*Chlamydia trachomatis*	*Trichomonas vaginalis*	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 channel) is available in Rotor-Gene 6000 and Rotor-Gene Q instruments.

Principle of interpretation

- The microorganism DNA is **found** 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 found** 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 found** 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 fragments 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.

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 (Table 4).

Table 4

Results for controls

Control	Stage for control	Ct channels FAM / JOE	Ct channel ROX	Interpretation
C–	DNA extraction	Neg	Pos (< boundary Ct value)	OK
NCA	Amplification	Neg	Neg	OK
C+	Amplification	Pos (< boundary Ct value)	Pos (< boundary Ct value)	OK

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.

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.

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

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.

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 the Rotor-Gene 3000 instrument, use the Rotor-Gene 6 program version 6.1 or higher. When working with the Rotor-Gene 6000 or Rotor-Gene Q instruments, use the Rotor-Gene 6000 program version 1.7 (build 67) or higher.

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** amplification program. Click ***OK*** when finished.

AmpliSens-1 program

Step	*Temperature, °C*	*Time*	Cycles
Hold	**95**	15 min	1
Cycling	**95**	5 s	5
	60	20 s	
	72	15 s	
Cycling 2	**95**	5 s	40
	60	20 s Acquiring*	
	72	15 s	

*Acquiring—fluorescent signal is detected at 60 ºC of stage Cycling 2 (***Acquiring to Cycling A***) in the ***FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red,*** and ***Crimson*** channels.

AmpliSens-1 is a general program for conducting tests for detection STIs with AmpliSens PCR kits. Therefore, any combination of tests including tests for identifying *human papillomaviruses (HPV HCR)* can be carried out simultaneously in the same instrument.

Another program, **60-45 RG**, can be exceptionally applied for PCR kits **variant FRT** (wax layer is used). It allows the run time to be to reduced by 10 min. To do this, create a new template and enter the **60-45 RG** program in the ***Edit Profile*** window:

60-45 RG program

Step	*Temperature, °C*	*Time*	Cycles
Hold	**95**	5 min	1
Cycling	**95**	5 s	5
	60	20 s	
	72	15 s	
Cycling 2	**95**	5 s	40
	60	20 s Acquiring*	
	72	15 s	

Acquiring fluorescent signal is enabled as described for AmpliSens-1 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. For JOE/Yellow, ROX/Orange, Cy5/Red, and 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** or **60-45 RG**. 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 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** 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** 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.

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 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 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.

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.

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

2.4 Ct values of 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 5.

Activate the ***More Settings***/***Outlier Removal*** button and in the text field enter the value specified in Table 5.

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 5

Parameters of analysis of results in the FAM/Green channel

PCR kit	Threshold	More Settings/ Outlier Removal	Slope Correct
Chlamydia trachomatis	**0.1**	**0**	off
Neisseria gonorrhoeae-screen	**0.1**	**0**	off
Neisseria gonorrhoeae-test	**0.1**	**0**	off
Neisseria gonorrhoeae	**0.1**	**0**	off
Mycoplasma genitalium	**0.1**	**0**	off
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 **found** 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 found** 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 (Table 6, 7).

Table 6

Results for controls

Control	Stage for control	Ct in FAM/Green channel	Ct in JOE/Yellow channel	Interpretation
C–	DNA extraction	Neg	Detected value <30	OK
NCA	Amplification	Neg	Neg	OK
C+	Amplification	Pos (< boundary Ct value)	Detected value < 30	OK

Table 7

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

PCR kit variant FRT	Boundary Ct value for C+ in the *Green* channel
Chlamydia trachomatis	30
HSV I, II	
CMV	
Candida albicans	
Neisseria gonorrhoeae-screen	33
Neisseria gonorrhoeae-test	
Neisseria gonorrhoeae (1^{st} and 2^{nd} reactions)	
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 20 (**FAM/Green, JOE/Yellow, ROX/Orange**, or **Crimson** channel). The product of amplification 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** (5%) 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). Activate the ***Slope Correct*** button. 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 8 (on – for Crimson channel, off – for other channels).

Activate the ***More Settings*/*Outlier Removal*** button and in the text field enter value specified in Table 8.

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 8

Parameters of result analysis for MULTIPRIME PCR kit

Detection channel	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	**0.1**	**0**	off
JOE/Yellow	**0.1**	**5**	off
ROX/Orange	**0.1**	**5**	off
Crimson (if used)	**0.1**	**5**	off
Cy5/Red	**0.07**	**5**	on

4. Principle of interpretation

– The microorganism DNA is **found** 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 found** 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 (Tables 9, 10).

Table 9

Results for controls

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

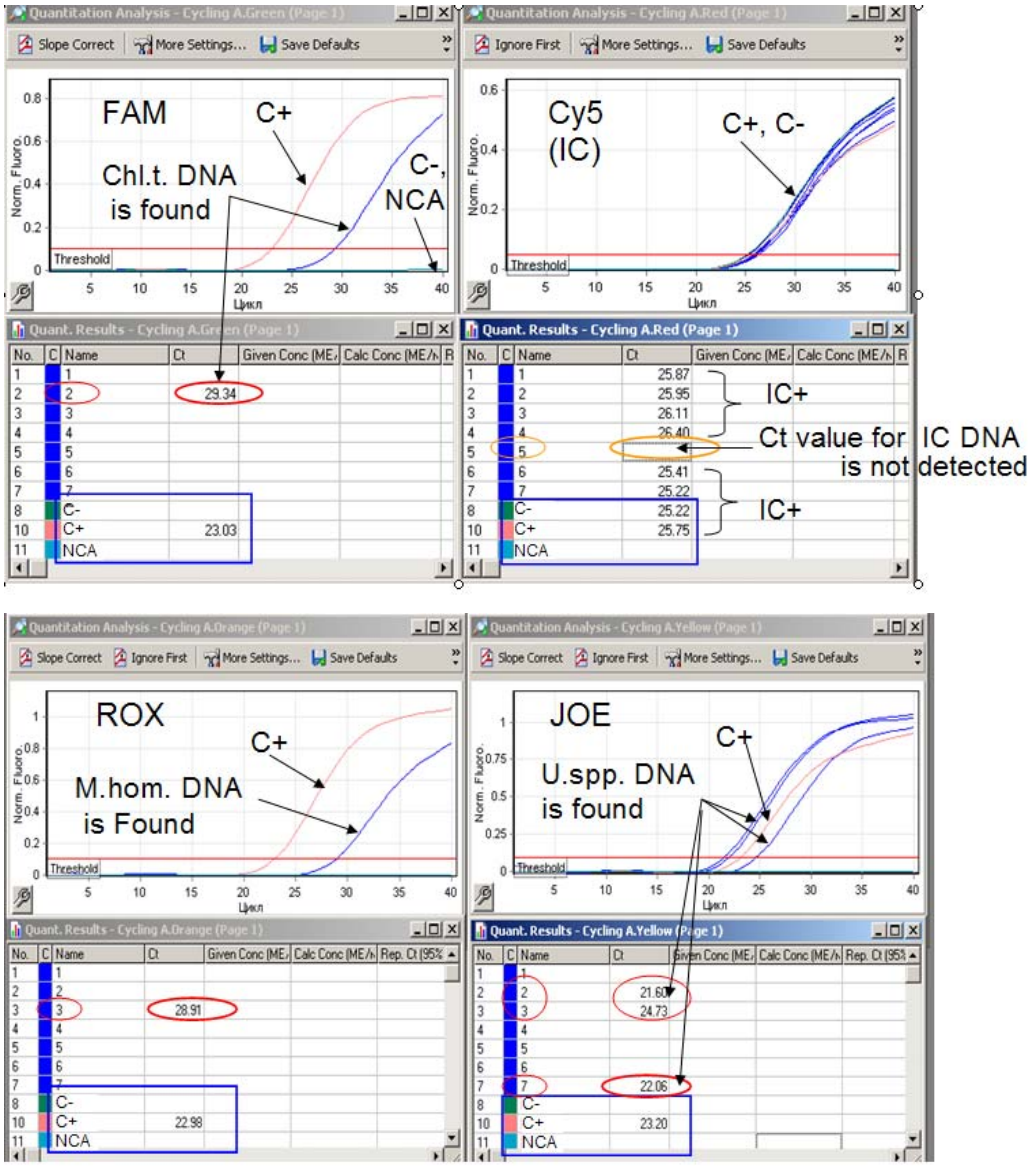
Table 10

Boundary Ct value for positive control (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	33
C.trachomatis / Ureaplasma / M.genitalium / M.hominis	35	35	35	33	33
C.trachomatis / Ureaplasma / M.genitalium	30	33	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 Nos. 1, 4, and 6.

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

1. Set the **AmpliSens-1** (Table 11) general amplification and detection program.

Table 11

AmpliSens-1 program for iCycler iQ or iQ5 instruments

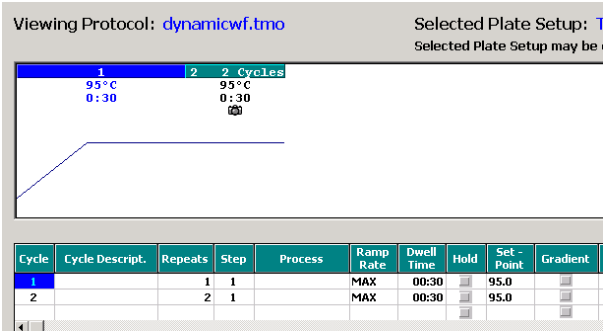
Step	*Temperature, °C*	*Time*	*Cycle repeats*
1	**95**	15 min	1
2	**95**	5 s	5
	60	20 s	
	72	15 s	
3	**95**	5 s	40
	60	30 s *fluorescent signal detection	
	72	15 s	

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.

AmpliSens-1 general program allows simultaneously conducting any combination of tests for detection of DNA of sexually transmitted infections with PCR kits manufactured by CRIE, including the tests for identifying and genotyping *Human Papillomaviruses* (HPV HCR).

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 HEX detection channels. If a MULTIPRIME PCR kit is used activate FAM, HEX, ROX, and Cy5 channels. Save the plate setup. Click the ***Run with selected protocol*** button.

   – **iCycler iQ 5 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 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 paragraph 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 **HEX** channel.

1. Data analysis of the specific microorganism amplification

1.1. Select data in the **FAM** channel (iCycler iQ) 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 **HEX** channel (iCycler iQ) 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 **found** 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 found** 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 12 and 13)

Table 12

Results for controls

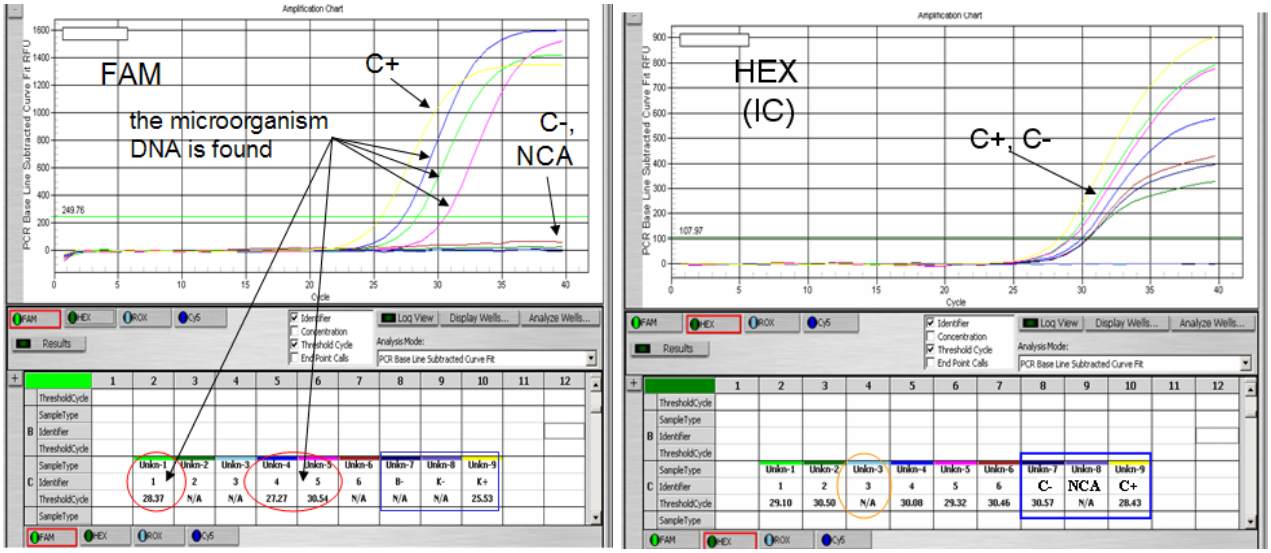
Control	**Stage for control**	**Ct**	
		FAM channel	**HEX channel**
C–	DNA extraction	N/A (absent)	Detected Ct value < 33
NCA	PCR	N/A (absent)	N/A (absent)
C+	PCR	Detected Ct value is less than the specified boundary Ct value	Detected Ct value < 33

Table 13

Boundary Ct values for positive control in the FAM channel

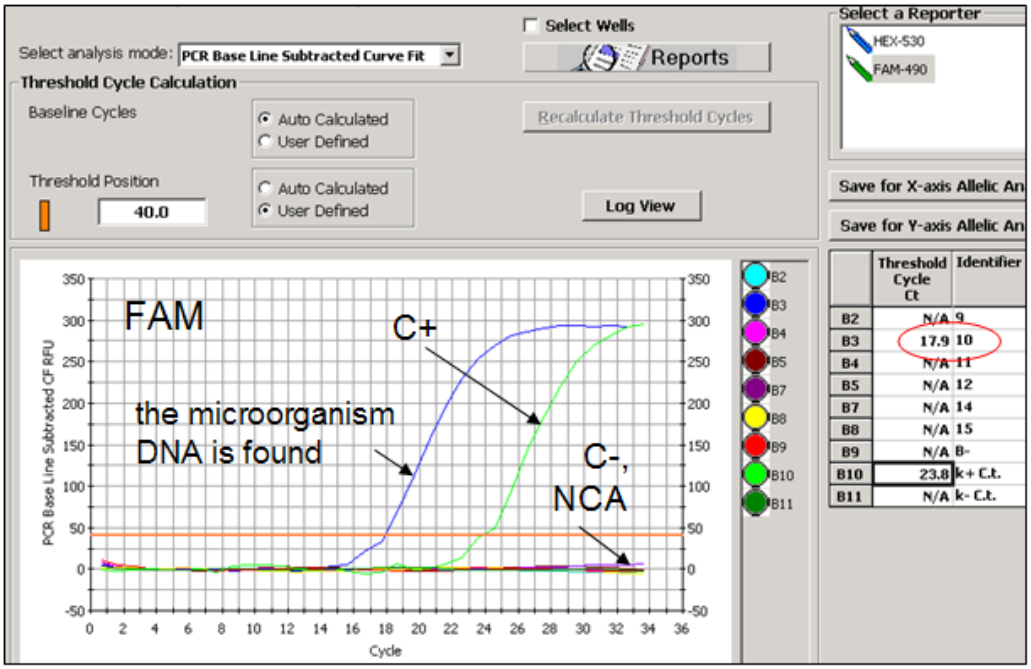
PCR kit	Ct value in FAM channel for C+
Chlamydia trachomatis	33
HSV I, II	
CMV	
Candida albicans	
Neisseria gonorrhoeae-screen	36
Neisseria gonorrhoeae-test	
Neisseria gonorrhoeae (1st and 2nd reaction)	
Mycoplasma genitalium	
Trichomonas vaginalis	
Treponema pallidum	
Ureaplasma species	
Mycoplasma hominis	
Gardnerella vaginalis	

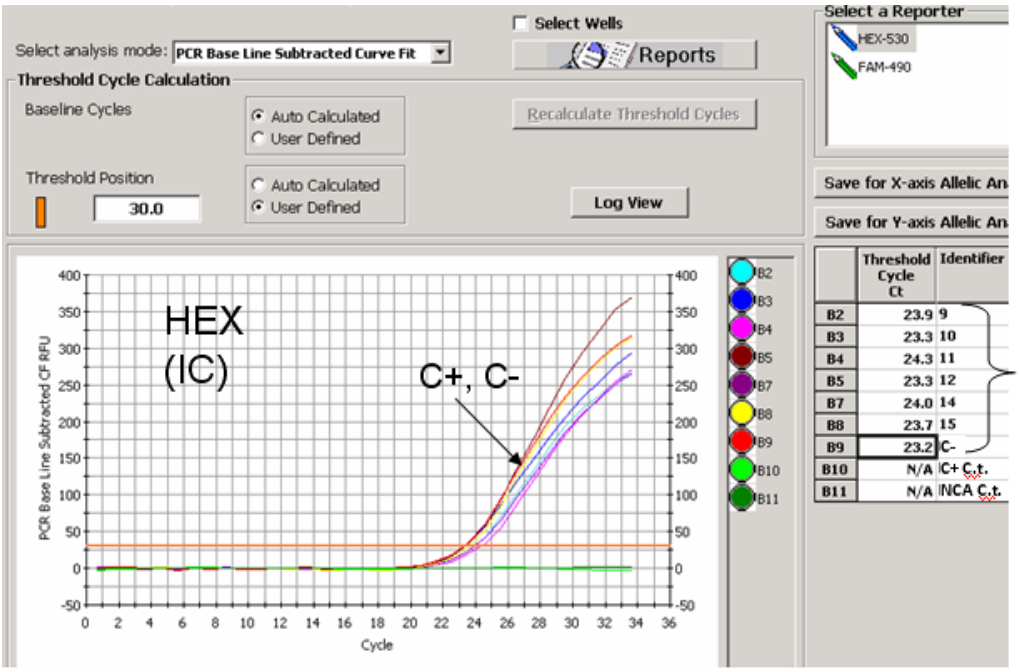
Example of results obtained using the iCycler iQ instrument:



- The results for negative controls, C– and NCA, are negative; the Ct value detected for C– in the 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 Nos. 1, 4, and 5.
- Ct value not exceeding the boundary Ct value (33) is detected in the HEX channel for all samples except for sample No. 3.
- DNA of the specific microorganism is not found in samples Nos. 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 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 Nos. 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, 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 iQ 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 **found** 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 found** 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, 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 Table 14 and 15).

Table 14

Results for controls

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

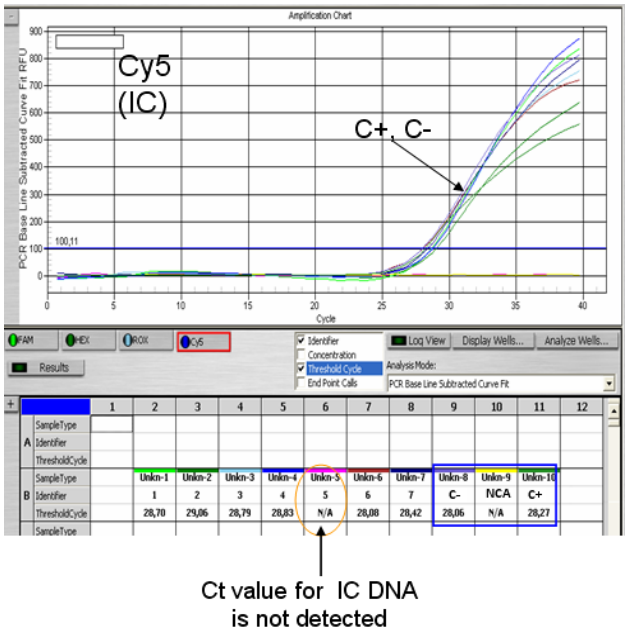
Table 15

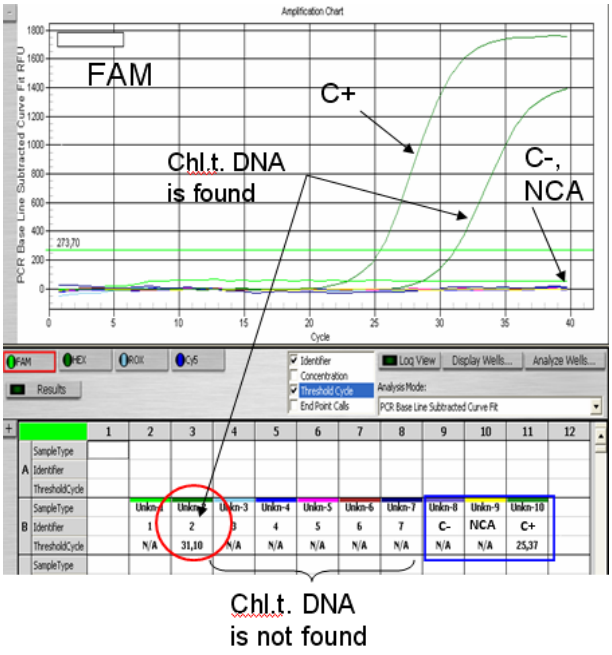
Boundary Ct values for positive control C+

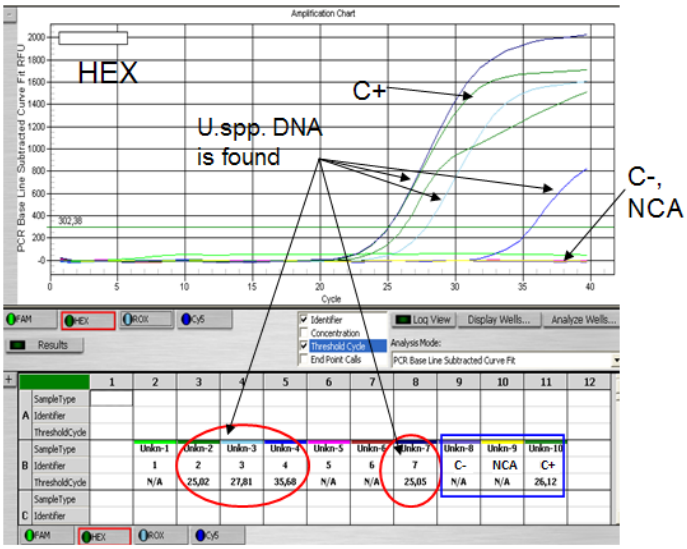
PCR kit	Ct value in channel		
	FAM	HEX	ROX
C.trachomatis / Ureaplasma / M.genitalium	33	36	36
C.trachomatis / Ureaplasma / M.hominis	33	36	36
N.gonorrhoeae / C.trachomatis / M.genitalium	36	33	36
C.albicans / C.glabrata / C. krusei	36	36	36
U.parvum / U.urealyticum	36	36	36
HSV-typing	36	33	36
T.vaginalis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

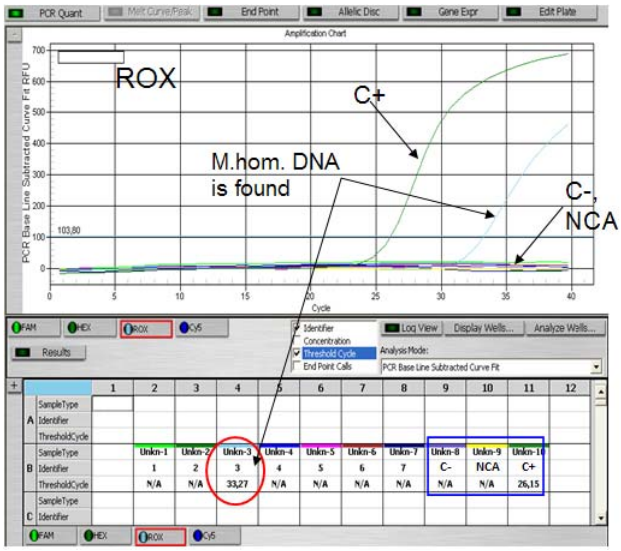
Example of results obtained using the iCycler iQ instrument for a MULTIPRIME PCR kit

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 HEX channel (*Ureaplasma* spp. in here).
- Sample No. 3 shows the presence of DNA of microorganisms detected in 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 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 Nos. 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** channels. If a MULTIPRIME PCR kit is use, enable detection in **FAM, JOE, ROX,** and **Cy5** channels.
2. Assign execution of the **Amplisens-1 Mx** 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 16

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 Acquiring*	
	72	15 s	

Acquiring fluorescent signal is enabled at 60 ºC of Segment 3 in the **FAM, JOE ROX,** and **Cy5** channels.

AmpliSens-1 is a general program for conducting tests for detection of STIs with AmpliSens PCR kits. Therefore, any combination of tests including the tests for identifying *human papillomaviruses (HPV HCR)* can be carried out simultaneously in the same PCR instrument.

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** 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** 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 CRIE. The same threshold level set for the JOE 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 **found** 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 found** 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 17 and 18)

Table 17

Result for controls

Control	**Stage for control**	**Ct value**	
		FAM	**JOE**
C–	DNA extraction	N/A (absent)	Detected Ct value < 33
NCA	PCR	N/A (absent)	N/A (absent)
C+	PCR	Detected Ct value is less than the specified boundary Ct value	Detected Ct value < 33

Table 18

Boundary Ct values for positive control C+ in FAM channel

PCR kit	Ct
Chlamydia trachomatis	33
HSV I, II	
CMV	
Candida albicans	
Neisseria gonorrhoeae-screen	36
Neisseria gonorrhoeae-test	
Neisseria gonorrhoeae (1^{st} and 2^{nd} reaction)	
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 20** (**FAM**, or **JOE**, 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 **found** 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 found** 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 and 2, 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 Table 19 and 20).

Table 19

Results for controls

Control	Stage for control	Ct value	
		Channel for detection of specific microorganism DNA amplification	**Channel for detection of IC DNA amplification (Cy5 or ROX)**
C–	DNA extraction	Absent	Detected Ct value < 36
NCA	PCR	Absent	Absent
C+	PCR	Detected Ct value is less than the specified boundary Ct value	Detected Ct value < 36

Table 20

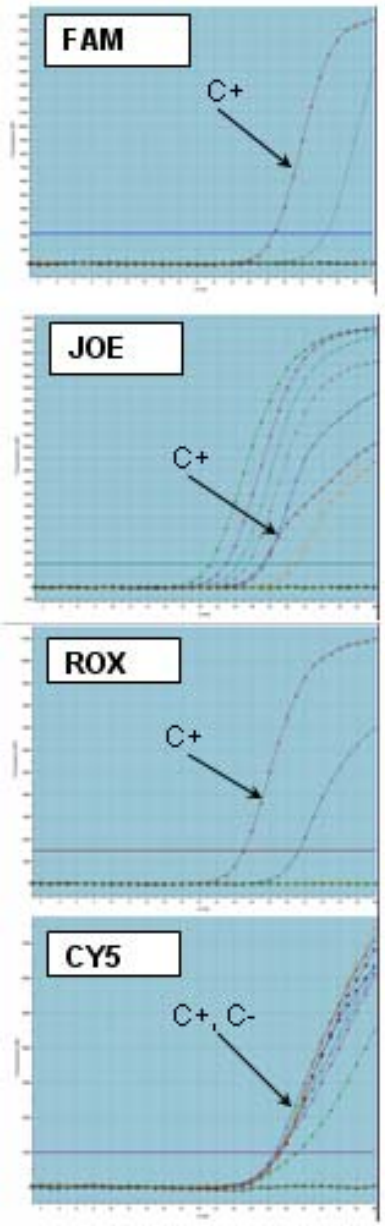
Boundary Ct values for Positive Control of Amplification (C+)

PCR kit (test)	Ct value in channel		
	FAM	HEX	ROX
C.trachomatis / Ureaplasma / M.genitalium	33	36	36
C.trachomatis / Ureaplasma / M.hominis	33	36	36
N.gonorrhoeae / C.trachomatis / M.genitalium	36	33	36
C.albicans / C.glabrata / C. krusei	36	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 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 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.