N.gonorrhoeae/C.trachomatis/ M.genitalium Real-TM

Handbook

Multiplex Real Time PCR kit for the detection of *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Mycoplasma genitalium*

REF TB67-100FRT

100

NAME

N.gonorrhoeae/C.trachomatis/M.genitalium Real-TM

INTRODUCTION

STDs (sexually transmitted diseases) refer to a variety of bacterial, viral and parasitic infections that are acquired through sexual activity. Some STDs, such as syphilis and gonorrhea, have been known for centuries — while others, such as HIV, have been identified only in the past few decades. STDs are caused by more than 25 infectious organisms. As more organisms are identified, the number of STDs continues to expand. Common STDs include: chlamydia, gonorrhea, herpes, HIV, HPV, syphilis, gardnerella and trichomoniasis.

The Chlamydia trachomatis is nonmotile, gram-negative bacterial pathogen and is the most common sexually transmitted bacterial agent. The prevalence of *C. trachomatis* infection in sexually active adolescent women, the population considered most at risk, generally exceeds 10%, and in some adolescent and STD clinic populations of women, the prevalence can reach 40%. The prevalence of *C. trachomatis* infection ranges from 4 to 10% in asymptomatic men and from 15 to 20% in men attending STD clinics. Chlamydial infections in newborns occur as a result of perinatal exposure; approximately 65% of babies born from infected mothers become infected during vaginal delivery.

The development of tests based on nucleic acid amplification technology has been the most important advance in the field of STD diagnosis. Because nucleic acid amplification is exquisitely sensitive and highly specific, it offers the opportunity to use noninvasive sampling techniques to screen for infections in asymptomatic individuals who would not ordinarily seek clinical care.

INTENDED USE

The kit **N.gonorrhoeae/C.trachomatis/M.genitalium Real-TM** is a multiplex Real Time PCR test for the qualitative detection of *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Mycoplasma genitalium* DNA in clinical materials (urogenital, rectal, and pharyngeal swabs; conjunctival discharge; prostate gland secretion; and urine samples) by using real-time hybridization-fluorescence detection.

PRINCIPLE OF ASSAY

The kit **N.gonorrhoeae/C.trachomatis/M.genitalium Real-TM** is based on two major processes: isolation of DNA from specimens and Real Time amplification. DNA is extracted from the specimens, amplified using Real-Time amplification and detected fluorescent reporter dye probes specific for *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Mycoplasma genitalium* DNA and Internal Control. Internal Control (IC) serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition.

MATERIALS PROVIDED

Part N° 1 – **"DNA-Sorb-A":** sample preparation

- **Lysis Solution**, 2 x 15 ml;
- **Sorbent**, 2 x 1,0 ml;
- **Washing Solution**, 2 x 50 ml;
- **DNA-eluent**, 2 x 5 ml;
- **Transport medium** , 2 x 15 ml.

Contains reagents for 100 tests.

Part N° 2 - **N.gonorrhoeae/C.trachomatis/M.genitalium Real-TM"**: Real Time amplification

- **PCR-mix-1-FL C.trachomatis/Neisseria/M.genitalium**, 1,2 ml;
- **PCR-mix-2-FRT**, 2 x 0,3 ml;
- **Polymerase (TaqF)**, 2 x 0,03 ml;
- **Positive Control complex (C+)**, 0,2 ml;
- **DNA-buffer**, 0,5 ml;
- **Negative Control (C-)***, 1,2 ml;
- **Internal Control-FL (IC)****, 1,0 ml;

Contains reagents for 110 tests.

* *must be used in the isolation procedure as Negative Control of Extraction.*
** *add 10 µl of Internal Control during the DNA isolation directly to the sample/lysis mixture (see DNA-Sorb-A REF K-1-1/A protocol).*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- Biological cabinet
- Desktop microcentrifuge for "eppendorf" type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- 1,5 ml polypropylene sterile tubes
- Biohazard waste container
- Refrigerator
- Freezer

Zone 2: Real Time amplification:

- Real Time Thermal cycler
- Reaction tubes
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

N.gonorrhoeae/C.trachomatis/M.genitalium Real-TM must be stored at -20°C. **DNA-sorb-A** must be stored at 2-8°C. The kits can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

N.gonorrhoeae/C.trachomatis/M.genitalium Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

The user should always pay attention to the following:

- ⚠ Lysis Solution contains guanidine thiocyanate*. Guanidine thiocyanate is harmful if inhaled, or comes into contact with skin or if swallowed. Contact with acid releases toxic gas. (Xn; R: 20/21/22-36/37/38; S: 36/37/39).
- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens 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 other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- 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.

PRODUCT USE LIMITATIONS

Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

N.gonorrhoeae/C.trachomatis/M.genitalium Real-TM can analyze DNA extracted from:

- *cervical, urethral, rectal, conjunctival swabs:* insert the swab into the nuclease-free 1,5 ml tube and add 0,2 ml of Transport medium. Vigorously agitate swabs in medium for 15-20 sec.
- *urine sediment:* collect 10-20 ml of first-catch urine in a sterile container. Centrifuge for 30 min at 3000 x g, carefully discard the supernatant and leave about 200 µl of solution. Resuspend the sediment. Use the suspension for the DNA extraction.
- *prostatic liquid* stored in "Eppendorf" tube;
- *seminal liquid:* maintain semen for 40 min in darkness until liquefaction. Use 100 µl for the DNA extraction.

It is recommended to process samples immediately after collection. Store samples at 2–8 °C for no longer than 24 hours, or freeze at –20/80°C. Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

The following isolation kit is recommended:

⇒ **DNA-Sorb-A** (Sacace, REF K-1-1/A);

⇒ **SaMag STD DNA Extraction kit** (Sacace, REF SM007).

Please carry out the DNA extraction according to the manufacturer's instructions. Add 10 µl of Internal Control during the DNA isolation procedure directly to the sample/lysis mixture.

(Note: the Sacace Internal Control is the same for all urogenital infectious Real Time kits)

SPECIMEN AND REAGENT PREPARATION

1. Lysis Solution **and** Washing Solution **(in case of their storage at +2-8°C) should be warmed up to 60–65°C until disappearance of ice crystals. Prepare required quantity of 1.5 ml polypropylene tubes including one tube for** Negative Control of Extraction**.**
2. Add to each tube **10 µl** of **Internal Control** and **300 µl** of **Lysis Solution**.
3. Add **100 µl** of **Samples** to the appropriate tube.
4. Prepare Controls as follows:
   - add **100 µl** of **C–** (**Neg Control** provided with the amplification kit) to the tube labeled C*neg*.
5. Vortex the tubes and incubate for 5 min at 65°C. Centrifuge for 5-7 sec. If the sample is not completely dissolved it is recommended to re-centrifuge the tube for 5 min at a maximum speed (12000-16000 g.) and transfer the supernatant into a new tube for DNA extraction.
6. Vortex vigorously **Sorbent** and add **20 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 3 min at room temperature. Repeat this step.
8. Centrifuge all tubes for 30 sec at 5000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet. Change tips between the tubes.
9. Add **500 µl** of **Washing Solution** to each tube. Vortex very vigorously and centrifuge for 30 sec at 10000g. Remove and discard supernatant from each tube.
10. Repeat step 9 and incubate all tubes with open cap for 5-10 min at 65°C.
11. Resuspend the pellet in **100 µl of DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
12. Centrifuge the tubes for 1 min at 12000g.
13. The supernatant contains DNA ready for amplification. If amplification is not performed in the same day of extraction, the processed samples can be stored at 2-8°C for at maximum period of 5 days or frozen at –20°/-80°C.

PROTOCOL:

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

1. Prepare required quantity of reaction tubes for samples (N) and controls (N+2).
2. Prepare in the new sterile tube **10*N µl** of **PCR-mix-1-FRT**, **5*N µl** of **PCR-mix-2-FRT** and **0,5*N µl** of **TaqF DNA Polymerase.** Vortex and centrifuge briefly.
3. Add to each tube **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA**. Mix by pipetting.
4. Prepare for each panel 2 controls:
   - add **10 µl** of **DNA-buffer** to the tube labeled Amplification Negative Control;
   - add **10 µl** of **Positive Control C+** to the tube labeled Amplification Positive Control;
5. Insert the tubes in the thermalcycler.

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line. *Neisseria gonorrhoeae* is detected on the FAM (Green) channel, *Chlamydia trachomatis* on the JOE (Yellow)/Cy3/HEX channel, *Mycoplasma genitalium* on the ROX (Orange)/TexasRed channel and *IC DNA* on the Cy5 (Red) channel

Table.1 Temperature profile*

Step	**Rotor-type instruments[1]**			**Plate-type instruments[2]**		
	Temperature, °C	***Time***	***Cycle repeats***	***Temperature, °C***	***Time***	***Cycle repeats***
Hold	95	15 min	1	95	15 min	1
Cycling	95	5 s	5	95	5 s	5
	60	20 s		60	20 s	
	72	15 s		72	15 s	
Cycling 2	95	5 s	40	95	5 s	40
	60	**20 s (fluorescence detection)**		60	**30 s (fluorescence detection)**	
	72	15 s		72	15 s	

[1] For example Rotor-Gene™ 3000/6000 (Corbett Research, Australia)
[2] For example, *SaCycler-96™ (Sacace),* iQ5™/iQ iCycler™ (BioRad, USA); Mx3000P/Mx3005P™ (Stratagene, USA), Applied Biosystems® 7500 Real Time PCR (Applera), SmartCycler® (Cepheid), LineGeneK® (Bioer)

Fluorescence is detected at the 2nd step of Cycling 2 stage (60 °C) in FAM/Green, JOE/Green/Cy3/Hex, ROX/Orange/TexasRed, and Cy5/Red fluorescence channels.

* the **"STD 65-60-45 RG-TaqF"** program reported in Sacace™ Urogenital kits can be also used

INSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 3000/6000, RotorGene Q)

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*
Cy5/Red	*0.1*	*5 %*	*On*

Test settings for data analysis for plate type instruments

The threshold line is to be installed at the level corresponding to 10-20 % of maximum level of fluorescence, registered for the Positive Control of Amplification (C+) in the last amplification cycle.

RESULTS INTERPRETATION

Neisseria gonorrhoeae is detected on the FAM (Green) channel, *Chlamydia trachomatis* on the JOE (Yellow)/Cy3/HEX channel, *Mycoplasma genitalium* on the ROX (Orange)/TexasRed channel and *IC DNA* on the Cy5 (Red) channel.

The results are interpreted by the software of the PCR instrument by the crossing (or not crossing) of the fluorescence curve with the threshold line.

The result of the analysis is considered reliable only if the results obtained for both Positive and Negative Controls of Amplification as well as for the Negative Control of Extraction are correct.

Table. **Results for controls**

Control	Stage for control	Ct in channels		Interpretation
		FAM, JOE, ROX	Ct channel Cy5	
C–	DNA extraction	Neg	Pos (< 33)	OK
NCA	Amplification	Neg	Neg	OK
C+	Amplification	Pos (< 33)	Pos (< 33)	OK

1. The sample is considered to be **positive** for *Neisseria gonorrhoeae* if its Ct value is detected in the results grid (the fluorescence curve crosses the threshold line) in the FAM (Green) channel.
2. The sample is considered to be **positive** for *Chlamydia trachomatis* if its Ct value is detected in the results grid (the fluorescence curve crosses the threshold line) in the JOE (Yellow)/Cy3/HEX channel.
3. The sample is considered to be **positive** for *Mycoplasma genitalium* if its Ct value is detected in the results grid (the fluorescence curve crosses the threshold line) in the ROX (Orange)/TexasRed channel.
4. The sample is considered to be **negative** for *Neisseria gonorrhoeae*, *Chlamydia trachomatis,* and *Mycoplasma genitalium* if its Ct value is not detected in the results grid (the fluorescence curve does not cross the threshold line) in FAM (Green), JOE (Yellow)/Cy3/HEX and ROX (Orange)/TexasRed channels and the Ct value does not exceed the boundary Ct value (< 33) in the results grid in the Cy5 (Red) channel.

PERFORMANCE CHARACTERISTICS

Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific *Neisseria gonorrhoeae*, *Chlamydia trachomatis and Mycoplasma genitalium* primers and probes. The specificity of the kit **N.gonorrhoeae/C.trachomatis/M.genitalium Real-TM** was 100%.

The potential cross-reactivity of the kit **N.gonorrhoeae/C.trachomatis/M.genitalium Real-TM** was tested against the group control. It was not observed any cross-reactivity with other pathogens (*Gardnerella vaginalis, Lactobacillus* spp., *Escherichia coli, Staphylococcus* spp., *Streptococcus* spp., *Candida albicans, Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma hominis, Trichomonas vaginalis, Treponema pallidum, Toxoplasma gondii, HSV* type 1 and 2, *CMV,* and *HPV)*

Analytical sensitivity

The kit **N.gonorrhoeae/C.trachomatis/M.genitalium Real-TM** allows to detect *Neisseria gonorrhoeae*, *Chlamydia trachomatis and Mycoplasma genitalium* DNA in 100% of the tests with a sensitivity of not less than 500 copies/ml. The detection was carried out on the control standard and its dilutions by negative sample.

TROUBLESHOOTING

1. Weak or no signal of the IC (Cy5 channel) for the Negative Control of extraction.
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow to the manufacturer's instructions.
        - ⇒ Re-centrifuge all the tubes before pipetting of the extracted DNA for 2 min at maximum speed (12000-16000 g) and take carefully supernatant. Don't disturb the pellet, sorbent inhibit reaction.
    - The reagents storage conditions didn't comply with the instructions.
        - ⇒ Check the storage conditions
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the PCR conditions and select for the IC detection the fluorescence channel reported in the protocol.
    - The IC was not added to the sample during the pipetting of reagents.
        - ⇒ Make attention during the DNA extraction procedure.
2. Weak or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
3. Any signal on Fam(Green), Joe (Yellow)/Hex/Cy3, Rox (Orange)/TexasRed channels with Negative Control of extraction.
    - Contamination during DNA extraction procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips between tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
4. Any signal with Negative Control of PCR (DNA-buffer).
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive control at last.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

EXPLANATION OF SYMBOLS

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
	Expiration Date	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
		IC	Internal Control

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