Sacace BIOTECHNOLOGIES

CE 1023

IVD For *in Vitro* Diagnostic Use

For Professional Use Only

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

Handbook

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

REF B61-100FRT

Σ 100

NAME

N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis 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, mycoplasma, gardnerella and trichomoniasis.

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

Kit **N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis Real-TM** is a multiplex Real Time PCR test for the qualitative detection of *Neisseria gonorrhoeae*, *Chlamydia trachomatis, Mycoplasma genitalium and Trichomonas vaginalis* in the urogenital swabs, urine, prostatic liquid and other biological materials.

PRINCIPLE OF ASSAY

N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis detection by the multiplex polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific **N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis** 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 the fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis Real-TM PCR kit is a qualitative test that contains the Internal Control (IC). It must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis Real-TM PCR kit uses “hot-start”, which greatly reduces frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by chemically modified polymerase (TaqF), which is activated by heating at 95 ºC for 15 min.

MATERIALS PROVIDED

Reagent	*Description*	*Volume, ml*	*Quantity*
PCR-mix-1-FL *N.gonorrhoeae* / *C.trachomatis* / *M.genitalium* / *T.vaginalis*	colorless clear liquid	1.1	1 tube
PCR-mix-2-FRT	colorless clear liquid	0.6	1 tube
Polymerase (TaqF)	colorless clear liquid	0.06	1 tube
Positive Control complex (C+)	colorless clear liquid	0.2	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	1 tube
Internal Control-FL (IC)**	colorless clear liquid	1.0	1 tube

**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

- 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 a rotor for 2-ml reaction tubes.
- PCR box.
- Personal thermocyclers (for example, Rotor-Gene 6000 (Corbett Research, Australia); Rotor-Gene Q (Qiagen, Germany), or equivalent).
- Disposable polypropylene microtubes for PCR (0.2- or 0.1-ml; for example, Axygen, USA; Corbett Research, Australia; Qiagen, Germany).
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. 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.

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

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

1. Wear disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterward.
2. Do not pipette by mouth.
3. Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
4. Do not use a kit after its expiration date.
5. Dispose of all specimens and unused reagents in accordance with local regulations.
6. Biosafety Level 2 should be used for materials that contain or are suspected of containing infectious agents.
7. Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant.
8. Avoid contact of specimens and reagents with the skin, eyes and mucous membranes. If these solutions come into contact, rinse immediately with water and seek medical advice immediately.
9. Material Safety Data Sheets (MSDS) are available on request.
10. Use of this product should be limited to personnel trained in the techniques of DNA amplification.
11. PCR reactions are sensitive to contamination. Measures to reduce the risk of contamination in the laboratory include physically separating the activities involved in performing PCR in compliance with good laboratory practice.
12. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where you performed previous step.

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

Sampling of biological materials for PCR-analysis, transportation, and storage are described in details in the handbook of the manufacturer. It is recommended that this handbook is read before beginning of the work.

STORAGE INSTRUCTIONS

All components of the **N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis Real-TM** PCR kit (except for polymerase (TaqF) and PCR-mix-2-FRT) are to be stored at the temperature 2–8 °C, when not in use.

All components of the **N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis Real-TM** PCR kit are to be stable until labeled expiration date. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

PCR-mix-1-FL *N.gonorrhoeae / C.trachomatis / M.genitalium / T.vaginalis* is to be kept away from light.

Polymerase (TaqF) and PCR-mix-2-FRT are to be stored at ≤ –16 °C.

STABILITY

N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis 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.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

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

- *cervical, urethral, 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* (use the first part of the stream);
- *prostatic liquid* stored in “Eppendorf” tube;
- *seminal liquid: t*ransfer about 30 µl of seminal liquid to a polypropylene tube (1,5 ml) and add 70 µl of sterile saline solution;

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

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the "SAMPLE COLLECTION, STORAGE AND TRANSPORT" paragraph, could be used.

Sacace Biotechnologies recommends to use the following kits:

- ⇒ **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)

Extract DNA according to the manufacturer's instructions.

REAGENTS PREPARATION (REACTION VOLUME 25 µL):

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

1. Prepare the required number of the tubes for amplification of DNA from clinical and control samples.
2. For carrying out N reactions (including 2 controls), mix in a new tube: **10*(N+1) µl of PCR-mix-1-FL *N.gonorrhoeae / C.trachomatis / M.genitalium / T.vaginalis*, 5.0*(N+1) µl of PCR-mix-2-FRT** and **0.5*(N+1) µl of polymerase (TaqF)**. Vortex the tube, then centrifuge shortly. Transfer **15 µl** of the prepared mixture to each tube.

3. Using tips with aerosol barrier add **10 µl** of **DNA** obtained from clinical or control samples at the DNA extraction stage into prepared tubes.

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).

Neisseria gonorrhoeae is detected on the FAM/Green channel, *Chlamydia trachomatis* on the JOE/Yellow/HEX/Cy3 channel, *Mycoplasma genitalium* on the ROX/Orange/Texas Red channel, *Trichomonas vaginalis* on Cy5.5/Crimson and *IC DNA* on the Cy5/Red channel.

Amplification

Create a temperature profile on your instrument as follows:

	***Rotor type instrument*[1]**			***Plate type instrument*[2]**		
Step	***Temp. °C***	***Time***	***Cycle repeats***	***Temp. °C***	***Time***	***Cycle repeats***
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***		60	**30 s***	
	72	15 s		72	15 s	

[1] For example Rotor-Gene™ 6000/Q (Qiagen)

[2] For example, SaCycler5-96™ (Sacace), CFX96™ (BioRad).

**fluorescence detection on the channels* FAM/Green, JOE/Yellow/HEX/Cy3, ROX/Orange/Texas Red, Cy5/Red and Cy5.5/Crimson *on the 2-nd step (60°C).*

INSTRUMENT SETTINGS

Rotor-type instruments (Rotor-Gene 6000/Q)

1. Create a template for "Urogenital Assays" by activating in the window *New Run* the programming regime *Advanced.* Choose *Dual Labeled Probe/Hydrolysis probes* and click the button *New.*
2. Select *i*n the new window the carousel type *36-Well Rotor or 72-Well Rotor* and *Reaction Volume (µL)* 25.
3. Set in the window *Edit Profile* program **"STD"** (this program is universal for all Sacace™ Urogenital Assays):
4. Make the adjustment of the fluorescence channel sensitivity: *Channel Setup → Gain Optimisation → Auto Gain Optimisation Setup →Optimise Acquiring* and select *Perform Optimisation Before 1-st Acquisition.*

   For *Green* channel indicate *Min Reading* **5***, Max Reading* **10** and for *Yellow, Orange, Red, Crimson* channels *Min Reading* **4***, Max Reading* **8**.

   In the column *Tube position* program position of the tubes in the carousel of the Rotor-Gene (the 1st position must contains reaction tube with reagents). Close the window *Auto Gain Calibration Setup.*

Results analysis:

1. The results are interpreted with the software of **Rotor-Gene** through the presence of crossing of fluorescence curve with the threshold line. *Neisseria gonorrhoeae* is detected on the Green channel, *Chlamydia trachomatis* on the Yellow channel, *Mycoplasma genitalium* on the Orange channel, *Trichomonas vaginalis* on Crimson and *IC DNA* on the Red channel.

2. Press *Analysis* then select button *Quantitation*. Perform the operation for the channel Green (*Cycling A. Green),* then for the channels Yellow (*Cycling A. Yellow),* Orange *(Cycling A.Orange),* Red *(Cycling A.Red)* and Crimson *(Cycling A.Crimson)*

2.1. Data analysis of *Neisseria gonorrhoeae* DNA

- Click **Green** channel on the curve.
- Select the **Dynamic tube** button in the main window menu.
- In **CT Calculation** menu set **Threshold = 0.1**.
- Select **Outlier Removal** button and type **0** in the text field.
- The Ct (Threshold cycle) values for each sample in channel will be shown in the results grid.

2.2. Data analysis of *Chlamydia trachomatis* DNA

- Click **Yellow** channel on the curve.
- Select the **Dynamic tube** button in the main window menu.
- In **CT Calculation** menu set **Threshold = 0.1**.
- Select **Outlier Removal** button and type **5** in the text field.
- The Ct (Threshold cycle) values for each sample in channel will be shown in the results grid.

2.3. Data analysis of *Mycoplasma genitalium* DNA

- Click **Orange** channel on the curve.
- Select the **Dynamic tube** button in the main window menu.
- In **CT Calculation** menu set **Threshold = 0.1**.
- Select **Outlier Removal** button and type **5** in the text field.
- The Ct (Threshold cycle) values for each sample in channel will be shown in the results grid.

2.4. Data analysis of *Trichomonas vaginalis* DNA

- Click **Crimson** channel on the curve.
- Select the **Dynamic tube** button in the main window menu.
- In **CT Calculation** menu set **Threshold = 0.1**.
- Select **Outlier Removal** button and type **10** in the text field.
- The Ct (Threshold cycle) values for each sample in channel will be shown in the results grid.

2.5. Data analysis of the IC amplification

- Click **Red** channel on the curve.
- Select the **Dynamic tube** button in the main window menu.
- In **CT Calculation** menu set **Threshold = 0.07**.
- Select **Outlier Removal** button and type **5** in the text field.

- The Ct (Threshold cycle) values for each sample in channel will be shown in the results grid.

3. The sample is considered to be positive for *Neisseria gonorrhoeae* if its Ct value is defined in the results grid (the fluorescence curve crosses the threshold line) in the Green channel.
4. The sample is considered to be positive for *Chlamydia trachomatis* if its Ct value is defined in the results grid (the fluorescence curve crosses the threshold line) in the Yellow channel.
5. The sample is considered to be positive for *Mycoplasma genitalium* if its Ct value is defined in the results grid (the fluorescence curve crosses the threshold line) in the Orange channel.
6. The sample is considered to be positive for *Trichomonas vaginalis* if its Ct value is defined in the results grid (the fluorescence curve crosses the threshold line) in the Crimson channel.
7. The sample is considered to be negative for *Chlamydia trachomatis*, *Neisseria gonorrhoeae, Mycoplasma genitalium* and *Trichomonas vaginalis* if its Ct value is not defined in the results grid (the fluorescence curve does not cross the threshold line) in Green, Yellow, Orange and Crimson channels and the Ct value does not exceed the boundary value in the results grid in the Red channel (Ct<33).

Plate-type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

Boundary value of the cycle threshold, Ct

For Ct boundary values of the samples, Negative Control of Extraction and Positive Control of Amplification, see **Table 2**.

Table 2. Results for controls

Control	Stage for control	Ct Green	Ct Yellow	Ct Orange	Ct Crimson	Ct Red	Interpretation
NCE	DNA isolation	Neg	Neg	Neg	Neg	< 33	Valid result
NCA	Amplification	Neg	Neg	Neg	Neg	Neg	Valid result
Pos C+	Amplification	<35	< 35	< 35	< 35	< 33	Valid result

QUALITY CONTROL PROCEDURE

A defined quantity of Internal Control (IC) is introduced into each sample and control at the beginning of sample preparation procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

A negative control of extraction (NCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

PERFORMANCE CHARACTERISTICS

Sensitivity

The analytical sensitivity for *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma genitalium,* and *Trichomonas vaginalis* DNA is not less than $5x10^2$ genome equivalents per 1 ml of sample (GE/ml).

The analytical sensitivity of each microorganism does not change even in the case of high concentration of three other microorganisms.

Specificity

The analytical specificity of **N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis Real-TM** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis. The clinical specificity of **N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis Real-TM** PCR kit was confirmed in laboratory clinical trials.

TROUBLESHOOTING

1. Weak or no signal of the IC (Red 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 with Negative Control of extraction (except for Red channel).
    - 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.
    - 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.

KEY TO SYMBOLS USED

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

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