Sacace BIOTECHNOLOGIES

CE 1023

IVD For *in Vitro* Diagnostic Use

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

Handbook

Multiplex Real Time PCR kit for qualitative detection of Chlamydia trachomatis, Neisseria gonorrhoeae and Trichomonas vaginalis

REF B83-100FRT

Σ 100

NAME

T.vaginalis/N.gonorrhoeae/C.trachomatis 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

T.vaginalis/N.gonorrhoeae/C.trachomatis Real-TM PCR kit is an *in vitro* nucleic acid amplification test for multiplex detection of *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis* DNA in clinical materials (urogenital, rectal and oropharyngeal swabs, conjunctival discharge, prostate gland secretion and urine samples) by using real-time hybridization-fluorescence detection.

The results of PCR analysis are taken into account in complex diagnostics of disease.

PRINCIPLE OF PCR DETECTION

C.trachomatis / *Neisseria* / *T.vaginalis* detection by the multiplex polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific *C.trachomatis* / *Neisseria* / *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. **T.vaginalis/N.gonorrhoeae/C.trachomatis 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. **T.vaginalis/N.gonorrhoeae/C.trachomatis 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 *T.vag/N.gon./C.trachomatis*	colorless clear liquid	1.2	1 tube
PCR-mix-2-FRT	colorless clear liquid	0.3	2 tubes
Polymerase (TaqF)	colorless clear liquid	0.03	2 tubes
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

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/100 protocol).*

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA extraction kit.
- Transport medium.
- Disposable powder-free gloves.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers.
- Disposable polypropylene 1,5/2,0 ml tubes.
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes.
- PCR Workstation.
- Real Time Thermal cycler.
- Disposable polypropylene microtubes for PCR.
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.

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.

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.

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 PCR 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

The components of the **T.vaginalis/N.gonorrhoeae/C.trachomatis Real-TM** PCR kit must be stored at 2–8 ºC excepting **Polymerase (TaqF)** and **PCR-mix-2-FRT** that must be stored at -16°C or below.

The kit can be shipped at 2-8°C for no longer than 5 days but should be stored at 2-8°C and -16°C or below immediately on receipt.

STABILITY

T.vaginalis/N.gonorrhoeae/C.trachomatis Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the 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.

The shelf life of reagents before and after the first use is the same, unless otherwise stated.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

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

- *urogenital, rectal, and oropharyngeal swabs:* insert the swab into the nuclease-free 1,5 ml tube and add 0,2 ml of Transport medium. Vigorously agitate swabs 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.
- *prostate gland secretion* stored in “Eppendorf” tube;

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 kit:

⇒ **DNA-Sorb-A** (Sacace, REF K-1-1/A/100).

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

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

REAGENTS PREPARATION (REACTION VOLUME 25 µL):

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

⚠ *Unfreeze PCR-mix-2-FRT before mixing.*

1. Prepare the required number of the tubes for amplification of DNA from clinical and control samples.
2. Prepare in a new sterile tube the **Reaction Mix.** For each sample mix **10*N µl** of **PCR-mix-1-FL *T.vag/N.gon./C.trachomatis***, **5*N µl** of **PCR-mix-2-FRT** and **0,5*N µl** of P**olymerase (TaqF).** Vortex and centrifuge for 2-3 sec.
3. Add **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA** sample to appropriate tube. Mix by pipetting.
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).

5. Insert the tubes in the thermalcycler.

Amplification

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

Step	**Rotor type instruments[1]**			**Plate or modular type instruments[2]**		
	Temperature, °C	***Time***	***Cycles***	***Temperature, °C***	***Time***	***Cycles***
Hold	95	15 min	1	95	15 min	1
Cycling	95	5 sec	5	95	5 sec	5
	60	20 sec		60	20 sec	
	72	15 sec		72	15 sec	
Cycling 2	95	5 sec	40	95	5 sec	40
	60	**20 sec fluorescence detection**		60	**30 sec fluorescence detection**	
	72	15 sec		72	15 sec	

1 For example, Rotor-Gene™ 3000 / Rotor-Gene™ 6000 / Rotor-Gene™ Q (Qiagen), or equivalent.
[2] For example, iCycler iQ5™(BioRad), Mx3000P™/ Mx3000™(Agilent) or equivalent.

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

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

INSTRUMENT SETTINGS

Rotor-type instruments (RotorGene 3000/6000/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	*0.07*	*5-10 %*	*On*

Plate- or modular 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 the level corresponding to 10–20% of maximum fluorescence obtained for the positive control, C+, during the last amplification cycle.

DATA ANALYSIS

- ***Trichomonas vaginalis*** **DNA** amplification product is detected is in the **FAM** fluorescence channel,
- ***Neisseria gonorrhoeae*** **DNA** amplification product is detected in the **JOE** fluorescence channel,
- ***Chlamydia trachomatis*** **DNA** is detected in the **ROX** channel,
- **Internal Control DNA** is detected in the **Cy5** channel.

RESULTS INTERPRETATION

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

The results of the analysis are 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.

Results for controls

Control	Stage for control	Ct channel FAM/Green, JOE/Yellow/HEX, ROX/Orange	Ct channel Cy5/Red	Interpretation
C–	DNA extraction	Neg	Pos	OK
NCA	Amplification	Neg	Neg	OK
C+	Amplification	Pos	Pos	OK

1. The sample is considered to be **positive** for ***Trichomonas vaginalis*** 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 region of exponential fluorescence growth.
2. The sample is considered to be **positive** for ***Neisseria gonorrhoeae*** if its Ct value is detected in the results grid in the JOE channel. Moreover, the fluorescence curve should cross the threshold line in the region of exponential fluorescence growth.
3. The sample is considered to be **positive** for ***Chlamydia trachomatis*** if its Ct value is detected in the results grid in the ROX channel. Moreover, the fluorescence curve should cross the threshold line in the region of exponential fluorescence growth.
4. The sample is considered to be **negative** for *Trichomonas vaginalis, Neisseria gonorrhoeae*, and *Chlamydia trachomatis* if its Ct value is not detected in the results grid in FAM, JOE, and ROX channels and the Ct on the Cy5 channel is less than 36.

Boundary value of the cycle threshold, Ct

Sample	Channel for fluorophore	Ct value	
		Rotor-type instruments	Plate-type instruments
C+	**FAM**	<30	<33
	JOE/HEX	<30	<33
	ROX	<33	<36
	Cy5	<33	<36
Clinical samples, C-	**Cy5**	<33	<36

PERFORMANCE CHARACTERISTICS

Sensitivity

Clinical material	Extraction kit	Microorganism	Sensitivity, GE/ml*
Urogenital swabs	DNA-sorb-A	*C. trachomatis*	$5x10^2$
		N. gonorrhoeae	$5x10^2$
		T.vaginalis	$5x10^2$
Urine (pretreatment is required)	DNA-sorb-A	*C. trachomatis*	$1x10^3$
		N. gonorrhoeae	$1x10^3$
		T.vaginalis	$1x10^3$

Analytical Sensitivity of each microorganism does not change even at high concentrations of two other microorganisms (to 10^9 GE/ml).

** Genome equivalents (GE) of the microorganism per 1 ml of the clinical sample placed in the transport medium.*

Specificity

The analytical specificity of **T.vaginalis/N.gonorrhoeae/C.trachomatis 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.

Nonspecific responses were absent in tests of human DNA samples and DNA of the following microorganisms: *Gardnerella vaginalis, Lactobacillus* spp., *Escherichia coli, Staphylococcus* spp., *Streptococcus* spp., *Candida albicans, Neisseria urealyticum, Neisseria parvum, Mycoplasma hominis, Chlamydia trachomatis, Mycoplasma genitalium, Neisseria* spp., *Ureaplasma urealyticum, Trichomonas vaginalis, Treponema pallidum, Toxoplasma gondii, HSV* types 1 and 2, *CMV,* and *HPV*.

The clinical specificity of **T.vaginalis/N.gonorrhoeae/C.trachomatis Real-TM** PCR kit was confirmed in laboratory clinical trials.

Target regions

Channel for fluorophore	FAM	JOE	ROX	Cy5
DNA-target	T.vaginalis	N.gonorrhoeae	C.trachomatis	Internal Control-FL
Target gene	DNA repeats for PCR identification	16s rRNA gene	cryptic plasmid	genetically engineered construction

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.

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 sample 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 sample 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.

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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

* iCycler iQ5™ is a registered trademark of Bio-Rad Laboratories
* Rotor-Gene™ is a registered trademark of Qiagen
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