IVD For *in Vitro* Diagnostic Use

CE

Treponema pallidum Real-TM

Handbook

Real Time PCR kit for qualitative detection of *Treponema pallidum*

REF B20-100FRT

REF TB20-100FRT

100

NAME

Treponema pallidum 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 **Treponema pallidum Real-TM** is a test for the qualitative detection of *Treponema pallidum* in the swabs, plasma, liquor, amniotic liquid, tissue and other biological materials.

PRINCIPLE OF ASSAY

Kit **Treponema pallidum Real-TM** is based on two major processes: isolation of DNA from specimens and Real Time amplification. *Treponema pallidum* DNA is extracted from the specimens, amplified using Real-Time amplification and detected fluorescent reporter dye probes specific for *Treponema pallidum* DNA and Internal Control. Internal Control (IC) serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition. IC is detected in a channel other than the *Treponema pallidum*.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (B20-100FRT)

Part N° 2 – **"Treponema pallidum TM":** Real Time amplification

- **PCR-mix-1-FRT**, 1,2 ml;
- **PCR-mix-2**, 2 x 0,3 ml;
- **TaqF Polymerase**,2 x 0,03 ml;
- **Pos C+**, 0,2 ml;
- **Negative Control C-**, 1,2ml;*
- **Internal Control IC**, 1,0 ml;**
- **DNA-buffer**, 0,5 ml;

Contains reagents for 110 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TB20-100FRT)

Part N° 1 – **"DNA-sorb-B":** Sample preparation

- **Lysis Solution**, 2 x 15 ml;
- **Washing Solution 1**, 2 x 15 ml;
- **Washing Solution 2**, 2 x 50 ml;
- **Sorbent**, 2 x 1,25 ml;
- **DNA-eluent**, 2 x 5 ml.

Contains reagents for 100 tests.

Part N° 2 – **"Treponema pallidum TM":** Real Time amplification

- **PCR-mix-1-FRT**, 1,2 ml;
- **PCR-mix-2**, 2 x 0,3 ml;
- **TaqF Polymerase**,2 x 0,03 ml;
- **Pos C+**, 0,2 ml;
- **Negative Control C-**, 1,2ml;*
- **Internal Control IC**, 1,0 ml;**
- **DNA-buffer**, 0,5 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-B REF K-1-1/B protocol).*

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation:

- DNA extraction kit (Module No. 1)
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with filters
- 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

Treponema pallidum Real-TM must be stored at 2-8°C except for **PCR-mix-2** and **TaqF Polymerase** that must be stored at -16°C. **DNA-sorb-B** 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 -16°C immediately on receipt.

STABILITY

Treponema pallidum 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

IVD **_In Vitro_ Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

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.

**** Only for Module No.2***

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.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

Treponema pallidum Real-TM can analyze DNA extracted with from:

- *cervical, urethral 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.
- *serous exudation from primary syphilis hard chancre and other syphilitic lesions:* insert the swab into the nuclease-free 1,5 ml tube and add 0,2 mL of Transport medium or sterile Saline solution. Agitate swabs in medium for 15-20 sec.
- *plasma* collected blood in ACD or EDTA tubes;
- *liquor* stored in "Eppendorf" tube;
- *amniotic liquid* stored in "Eppendorf" tube;
- *tissue* (≈1,0 gr) homogenized with mechanical homogenizer or scalpel, glass sticks, teflon pestles and dissolved in 1,0 ml of saline water or PBS sterile. Vortex vigorously and incubate 30 min at room temperature. Transfer the supernatant into a new 1,5 ml 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-B** (Sacace, REF K-1-1/B);
- ⇨ **SaMag Bacterial DNA Extraction kit:** for *swabs, serous exudation, plasma, liquor and amniotic liquid* (Sacace, REF SM006).

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

SPECIMEN AND REAGENT PREPARATION (reagents supplied with the module no.2)

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 **300 µl** of **Lysis Solution** and **10 µl** of **Internal Control**.
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 7-10 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 **25 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 8000g and using a micropipette with a plugged aerosol barrier tip, carefully remove and discard supernatant from each tube without disturbing the pellet.
9. Add **300 µl** of **Washing Solution 1** to each tube. Vortex vigorously and centrifuge for 30 sec at 8000g. Remove and discard supernatant from each tube.
10. Add **500 µl** of **Washing Solution 2** to each tube. Vortex vigorously and centrifuge for 30 sec at 8000g. Remove and discard supernatant from each tube.
11. Repeat step 10 and incubate all tubes with open cap for 5 min at 65°C.
12. Resuspend the pellet in **50 µl of DNA-eluent.** Incubate for 5 min at 65°C and vortex periodically.
13. Centrifuge the tubes for 1 min at maximum speed (12000-16000 g). 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 a maximum period of 5 days or frozen at –20°/-80°C.

PROTOCOL:

1. Prepare required quantity of reaction tubes for samples (N) and controls (N+2).
2. Prepare in the new sterile tube for each sample **10*(N+1) µl** of **PCR-mix-1-FRT**, **5,0*(N+1)** of **PCR-mix-2** and **0,5*(N+1)** of **TaqF Polymerase.** Vortex and centrifuge for 2-3 sec.
3. Add to each tube **15 µl** of **Reaction Mix** and **10 µl** of **extracted DNA** sample to appropriate tube. 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.

Amplification

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

	Rotor-type Instruments[1]			Plate- or modular type Instruments[2]		
Step	***Temperature, °C***	***Time***	**Repeats**	***Temperature, °C***	***Time***	**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 *fluorescent signal detection*		**60**	30 s *fluorescent signal detection*	
	72	15 s		**72**	15 s	

[1] For example Rotor-Gene™ 3000/6000/Q (Corbett Research, Qiagen)
[2] For example, SaCycler-96™ (Sacace), CFX96™/iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7300/7500/StepOne Real Time PCR (Applied Biosystems), SmartCycler® (Cepheid), LineGeneK® (Bioer)

Fluorescence is detected at the 2nd step of Cycling 2 stage (60 °C) in FAM/Green and JOE/Yellow/Hex/Cy3 fluorescence channels.

Treponema pallidum is detected on the FAM (Green) channel, *IC DNA* on the JOE(Yellow)/HEX/Cy3 channel

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	*0.1*	*5 %*	*On*
JOE/Yellow	*from 4 Fl to 8 Fl*	*0.1*	*5 %*	*Off*

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

Sample	Channel for fluorophore	Ct boundary value	
		Rotor-type instruments	Plate-type instruments
C+	FAM/Green	33	36
	JOE/Yellow/Hex/Cy3	30	33
Clinical samples, C-	JOE/Yellow/Hex/Cy3	30	33

DATA ANALYSIS

The fluorescent signal intensity is detected in two channels:

– The signal from the *Treponema pallidum* DNA amplification product is detected in the FAM/Green channel;

– The signal from the Internal Control amplification product is detected in the JOE/Yellow/HEX/Cy3 channel.

Interpretation of results

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

Principle of interpretation:

— *Treponema pallidum* DNA is **detected** in a sample if its Ct value is present in the FAM channel. The fluorescence curve should cross the threshold line in the area of exponential fluorescence growth.

— *Treponema pallidum* DNA is **not detected** in a sample if its Ct value is absent in the FAM channel (fluorescence curve does not cross the threshold line) while the Ct value in the JOE channel is less than 33.

— The result is **invalid** if the Ct value of a sample in the FAM channel is absent while the Ct value in the JOE channel is either absent or greater than the specified boundary value (Ct > 33). It is necessary to repeat the PCR analysis of such samples.

The result of analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (Table 1).

Table 1. Results for controls

Control	Stage for control	Ct channel Fam	Ct channel Joe	Interpretation
NCE	DNA isolation	NEG	POS	Valid result
NCA	Amplification	NEG	NEG	Valid result
C+	Amplification	POS	POS	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.

SPECIFICATIONS

Sensitivity

The analytical sensitivity of **Treponema pallidum Real-TM** PCR kit is specified in the table below.

Clinical material	DNA extraction kit	Analytical sensitivity, GE/ml*
Urogenital swabs	DNA-sorb-A	5×10^2

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

Specificity

The analytical specificity of **Treponema pallidum Real-TM** PCR kit is ensured by selection of specific primers and probes as well as by selection of stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis. There were no nonspecific responses during examination of human DNA as well as DNA panel of the following microorganisms: *Mycoplasma hominis, Lactobacillus spp., Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus agalactiae, Candida albicans, Neisseria gonorrhoeae, Ureaplasma urealyticum, Ureaplasma parvum, Mycoplasma genitalium, Neisseria flava, Neisseria subflava, Neisseria sicca, Neisseria mucosa, Chlamydia trachomatis, Trichomonas vaginalis, Gardnerella vaginalis, Toxoplasma gondii, HSV* type 1 and 2, *CMV,* and *HPV.*

TROUBLESHOOTING

1. Weak or no signal of the IC (Joe/Hex/Cy3 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
    - Improper DNA extraction.
        - ⇒ Repeat analysis starting from the DNA extraction stage
    - 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. Fam (Green) signal 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.

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	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
	Expiration Date	**IC**	Internal Control

* SaCycler™ is a registered trademark of Sacace Biotechnologies
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* Rotor-Gene™ is a registered trademark of Qiagen
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