Sacace BIOTECHNOLOGIES

IVD For *in Vitro* Diagnostic Use

CE

Gardnerella vaginalis/Lactobacillus species Real-TM Quant

Handbook

Real Time Kit PCR kit for quantitative detection of *Gardnerella vaginalis* and *Lactobacillus spp*.

REF **R-B7-100FRT**

REF **TR-B7-100FRT**

Σ **100**

NAME

Gardnerella vaginalis/Lactobacillus species Real-TM Quant

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 **Gardnerella vaginalis/Lactobacillus species Real-TM Quant** is an *in vitro* Real Time amplification test for quantitative detection of *Gardnerella vaginalis* and *Lactobacillus* spp.

This kit allows to estimate the conditions of vaginal flora and to give with high accuracy the diagnosis of Bacterial vaginosis (BV). Bacterial vaginosis (BV) is considered to be the most common cause of vaginal inflammation among both pregnant and non-pregnant women and prevalences between 4.9% and 36.0% have been reported from European and American studies. It previously was called nonspecific vaginitis or *Gardnerella*-associated vaginitis.

The adult human vagina is a complex ecosystem containing an abundance of microorganisms. In women of childbearing age this system is dominated by *Lactobacillus* spp., a genus of gram-positive, nonmotile rod-like bacteria, a defining characteristic of which is the ability to grow in acid media and tolerate acid conditions (pH < 4.5); lactobacilli also ferment carbohydrates to produce lactic acid and produce H_2O_2 which provides a natural defense against *Gardnerella vaginalis*. In bacterial vaginosis (BV) the balance of flora is changed with reduced numbers of lactobacilli (normal concentration 10^6 – 10^{10} CFU/ml) and an increase in numbers of other facultative and anaerobic species such as anaerobic cocci *Prevotella* spp., *Gardnerella vaginalis*, and *Mobiluncus* spp. (normal concentration < 10^3-10^5 CFU/ml). G. vaginalis is virtually always present at high concentrations in women who have BV but is also detected frequently in normal women and in some cases the concentration of *Gardnerella vaginalis* can reach 10^7-10^8 CFU/ml also in absence of BV, so the most important maker of BV is the ratio of logarithm concentration *Lactobacillus* spp and *G. vaginalis.*

PRINCIPLE OF ASSAY

kit **Gardnerella vaginalis/Lactobacillus species Real-TM Quant** is based on two major processes: isolation of DNA from specimens and multiplex Real Time amplification. Amplification results of *Gardnerella vaginalis* are detected on the Fam/Green channel, amplification results of *Lactobacillus* spp are detected on the Joe/HEX/Yellow channel. For quantification of the DNA of *Lactobacillus* spp and *Gardnerella vaginalis* all calibrators should be used and defined as standards with specific concentrations.

To calculate the concentration of the DNA of *Lactobacillus* spp and *Gardnerella vaginalis* in 1 ml of clinical material use the following formula:

$$C_{DNA\ Gv/ml} = C_{DNA\ Gv} \times \text{coefficient}$$

$$C_{DNA\ L.spp/ml} = C_{DNA\ L.spp} \times \text{coefficient},$$

where

$C_{DNA\ Gv/ml}$ = copies of the DNA *Gardnerella vaginalis*/reaction

$C_{DNA\ L.spp/ml}$= copies of the DNA *Lactobacillus spp*/reaction

Coefficient = 100 – takes into account the volume of DNA in the reaction tube from the volume of the clinical material and the quantity of copies of the amplified gene in the genome of the microorganism

The following equation has to be applied to calculation of the ratio of the concentration DNA *Lactobacillus* spp and DNA *Gardnerella vaginalis:*

$$\mathbf{RC_{Lsp\text{-}Gv} = lg[C_{DNA\ Lspp/ml}] - lg[C_{DNA\ Gv/ml}]}$$

RC < -1,0 – high possibility of BV

RC > 2.0 – low possibility of BV

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (R-B7-100FRT)

Part N° 2 – **"Controls":** Controls

- **Pos DNA *Gardnerella vaginalis/ Lactobacillus* spp-1 BV-**, 0,05 ml
- **Pos DNA *Gardnerella vaginalis/ Lactobacillus* spp-2 BV+**, 0,05 ml

Part N° 3 – **"Gardnerella vaginalis/Lactobacillus species Real-TM Quant":** Real Time amplification

- **PCR-mix-1-FRT *Gardnerella vaginalis/ Lactobacillus* spp**, 0,8 ml
- **PCR- buffer-FRT**, 0,9 ml
- **TaqF Hot Start DNA Polymerase**, 0,06 ml
- **DNA-buffer (C-)**, 0,5 ml
- **Quantitation Standard** (**GL1**, **GL2**, **GL3**), 3 x 0,06 ml;

Contains reagents for 110 samples

Module No.2: Complete Real Time PCR test with DNA purification kit (TR-B7-100FRT)

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

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

Contains reagents for 100 test.

Part N° 2 – **"Controls":** Controls

- **Pos DNA *Gardnerella vaginalis/ Lactobacillus* spp-1 BV-**, 0,05 ml
- **Pos DNA *Gardnerella vaginalis/ Lactobacillus* spp-2 BV+**, 0,05 ml

Part N° 3 – **"Gardnerella vaginalis/Lactobacillus species Real-TM Quant":** Real Time amplification

- **PCR-mix-1-FRT *Gardnerella vaginalis/ Lactobacillus* spp**, 0,8 ml
- **PCR- buffer-FRT**, 0,9 ml
- **TaqF Hot Start DNA Polymerase**, 0,06 ml
- **DNA-buffer (C-)**, 0,5 ml
- **Quantitation Standard** (**GL1**, **GL2**, **GL3**), 3 x 0,06 ml;

Contains reagents for 110 samples

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
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

Store kit at 2-8°C. **PCR-mix-1 and TaqF Polymerase** 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

Gardnerella vaginalis/Lactobacillus species Real-TM Quant 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. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

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

Gardnerella vaginalis/Lactobacillus species Real-TM Quant can analyze DNA extracted with **DNA-Sorb-A** (REF K-1-1/A) from:

- *Posterior fornix vaginal fluid:* insert the swab with clinical material (0,05±0,01 ml) into the nuclease-free sterile 2,0 ml tube and add 0,5 mL of Transport medium (ratio 1:10). Vigorously agitate swabs in medium for 15-20 sec. If the quantity of the taken clinical material is sufficient the transport medium must change the color from rose to yellow and becomes turbid.

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);
- ⇨ **SaMag STD DNA Extraction kit** (Sacace, REF SM007);

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**.
3. Add **100 µl** of **Samples** to the appropriate tube.
4. Prepare Controls as follows:
   - add **100 µl** of **Transport medium** to the tube labeled C*neg*.
   - add **90 µl** of **Transport medium** and **10 µl** of **Pos DNA *Gardnerella vaginalis/ Lactobacillus* spp-1** or **Pos DNA *Gardnerella vaginalis/ Lactobacillus* spp-2**
5. Vortex the tubes and incubate for 5 min at 65°C. Centrifuge for 5-7 sec.
6. Vortex vigorously **Sorbent** and add **20 µl** to each tube.
7. Vortex for 5-7 sec and incubate all tubes for 5 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:

Reaction volume = 25 µl

1. Prepare required quantity of tubes (N + 4 controls (3 standards and 1 negative control).
2. Prepare **Mix** for 120 samples: add into the tube with **PCR- buffer-FRT 60 µl** of **TaqF Hot Start DNA Polymerase** Carefully vortex the tube**.** This mix is stable for 3 months at +4°C.
3. Prepare reaction mix (see table 1). Add for each sample in the new sterile tube **7*(N+1) µl** of **PCR-mix-1-FRT *Gardnerella vaginalis/ Lactobacillus* spp** and **8*(N+1) µl** of **Mix (PCR-buffer-FRT + TaqF Hot Start DNA Polymerase)**
4. Add **15 µl** of **Reaction Mix** into each tube with samples and controls.
5. Add **10 µl** of **extracted DNA** sample to appropriate tube.
6. Prepare for each panel 4 controls:
   - add **10 µl** of **Quantitation Standards** (**GL1, GL2, GL3**) into 3 labeled tubes;
   - add **10 µl** of **DNA-buffer** to the tube labeled Negative Control;

Table 1. Mixes preparation x sample (included calculation of reagents for controls)

Samples	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
µl of PCR-mix-1-FRT	56	63	70	77	84	91	98	105	112	119	126	133	140	147	154
µl of Mix (PCR-buffer-FRT + Hot Start DNA Polymerase)	64	72	80	88	96	104	112	120	128	136	144	152	160	168	176
Samples	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**
µl of PCR-mix-1-FRT	161	168	175	182	189	196	203	210	217	224	231	238	245	252	259
µl of Mix (PCR- buffer-FRT + Hot Start DNA Polymerase)	184	192	200	208	216	224	232	240	248	256	264	272	280	288	296

7. Program position of the tubes and enter the concentrations of the Quantitative Standards (reported on the Quant Data Card) in the Joe/Yellow/Hex (*Lactobacillus* spp), Fam (*Gardnerella vaginalis*) channels in order to generate Standard curves.

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 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™ 6000/Q (Corbett Research, Qiagen)*
[2] *For example, SaCycler-96™ (Sacace), iQ5™ (BioRad); Mx3005P™ (Agilent), ABI® 7500 Real Time PCR (Applied), SmartCycler® (Cepheid)*

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

Lactobacillus spp. is detected on the JOE(Yellow)/HEX/Cy3 channel, *Gardnerella vaginalis* on the FAM (Green) 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*	*005*	*10 %*	*Off*
JOE/Yellow	*from 4 Fl to 8 Fl*	*0.03*	*10 %*	*On*

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.

DATA ANALYSIS:

The fluorescent signal intensity is detected in two channels:

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

– The signal from the *Lactobacillus species* DNA amplification product is detected in the Joe/HEX/Yellow channel.

Interpretation of results

The results are interpreted with the software of instrument through the presence of crossing of fluorescence curve with the threshold line.

Example of calculation with Microsoft® Excel Program:

Name	Calc Conc (copies/PCR)	Calc Conc (copies/PCR)	G.vaginalis (copies/ml)	Lactobacillus sp. (copies/ml)	lg(Lsp)-lg(Gv)
A	B	C	D = B*100	E = C*100	F = LOG(E)-
bv-	268	131336	2.7E+04	1.3E+07	2.7
bv-	271	133356	2.7E+04	1.3E+07	2.7
bv-	*89*	*98324*	*8.9E+03*	*9.8E+06*	*3.0*
bv+	21425	1006	2.1E+06	1.0E+05	-1.3
bv+	26288	1300	2.6E+06	1.3E+05	-1.3
bv+	20756	1026	2.1E+06	1.0E+05	-1.3
bv+	24761	1012	2.5E+06	1.0E+05	-1.4
k-		4			
GL1	204574	1127193			
GL2	2358	13004			
GL3	27	84			

QUALITY CONTROL PROCEDURE

Occurrence of any value Ct in the table of results for the negative control of extraction and for negative control of amplification on Fam/Green channel testifies contamination of reagents or samples. Occurrence of Ct value < 34 (or Calc Conc > 10) in the table of results for the negative control of extraction and for negative control of amplification on Joe/Yellow channel testifies contamination of reagents or samples. In this case results of the analysis for all tests are considered invalid. It is required to repeat the analysis of all tests, and also to take measures to detect and eliminate the source of contamination.

The calculated concentration of the **Pos DNA *Gardnerella vaginalis/ Lactobacillus* spp-1 BV-** and **Pos DNA *Gardnerella vaginalis/ Lactobacillus* spp-2 BV+** is different that reported in Data Card: error during DNA extraction and PCR reagent preparation or incorrect programming of temperature profile. Repeat the test.

SPECIFICATIONS

Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific *Gardnerella vaginalis* and *Lactobacillus* spp. primers and probes. The specificity of the kit **Gardnerella vaginalis/Lactobacillus species Real-TM Real-TM Quant** was 100%. The potential cross-reactivity of the kit **Gardnerella vaginalis/Lactobacillus species Real-TM Real-TM Quant** was tested against the group control. It was not observed any cross-reactivity with other pathogens.

Analytical sensitivity

The kit **Gardnerella vaginalis/Lactobacillus species Real-TM Real-TM Quant** allows to detect *Gardnerella vaginalis* and *Lactobacillus* spp. DNA in 100% of the tests with a sensitivity of not less than 2500 copies/ml. The detection was carried out on the control standard and its dilutions by negative sample.

Target region: 16S rRNA

TROUBLESHOOTING

1. 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.
2. Fam (Green) and Joe/Yellow/Hex 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.
3. 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

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