IVD For *in Vitro* Diagnostic Use

CE

Candidosis Real-TM Quant

Handbook

Multiplex Real Time PCR test for the quantitative detection of *Candida albicans, Candida glabrata, Candida krusei, Candida parapsilosis* and *Candida tropicalis.*

REF F5-100FRT

REF TF5-100FRT

100

NAME

Candidosis Real-TM Quant

INTRODUCTION

Candida albicans is a diploid fungus that grows both as yeast and filamentous cells and a causal agent of opportunistic oral and genital infections in humans. Systemic fungal infections (fungemias) including those by *C. albicans* have emerged as important causes of morbidity and mortality in immunocompromised patients (e.g., AIDS, cancer chemotherapy, organ or bone marrow transplantation). In addition, hospital-acquired infections by C. albicans have become a cause of major health concerns.

Candida glabrata is a haploid yeast of the genus Candida, previously known as Torulopsis glabrata. Until recently, *C. glabrata* was thought to be a primarily non-pathogenic organism. However, with the ever increasing population of immunocompromised individuals, trends have shown *C. glabrata* to be a highly opportunistic pathogen of the urogenital tract, and of the bloodstream (Candidemia). It is especially prevalent in HIV positive people.

Candida krusei is a budding yeast (a species of fungus) involved in chocolate production. *Candida krusei* is an emerging fungal nosocomial pathogen primarily found in the immunocompromised and those with hematological malignancies. It has natural resistance to fluconazole, a standard antifungal agent. It is most often found in patients who have had prior fluconazole exposure, sparking debate and conflicting evidence as to whether fluconazole should be used prophylactically. Mortality due to *C. krusei* fungemia is much higher than the more common *C. albicans*.

Candida parapsilosis is a fungal species of the yeast family that has become a significant cause of sepsis and of wound and tissue infections in immuno-compromised patients. The immune system is a major player in *Candida parapsilosis* infections.

C. tropicalis is taxonomically close to C. albicans and shares many pathogenic traits. C. tropicalis is particularly virulent in neutropenic hosts commonly with hematogenous seeding to peripheral organs

INTENDED USE

Kit **Candidosis Real-TM Quant** is a multiplex Real Time PCR test for the quantitative detection of *Candida albicans, Candida glabrata, Candida krusei, Candida parapsilosis and Candida tropicalis* DNA in the human clinical materials.

PRINCIPLE OF ASSAY

Kit **Candidosis Real-TM Quant** is based on two major processes: isolation of DNA from specimens and Real Time amplification.

MATERIALS PROVIDED

Module No.1: Real Time PCR kit (F5-100FRT)

Part N° 2 – **"Candidosis Real-TM Quant":** 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;
- **Negative Control C-**, 1,2 ml;*
- **Internal Control IC**, 1,0 ml;**
- **DNA-buffer**, 0,5 ml;
- **Standard**
  - **QSG1**, 0,2 ml
  - **QSG2**, 0,2 ml

Contains reagents for 110 tests.

Module No.2: Complete Real Time PCR test with DNA purification kit (TF5-100FRT)

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 – **"C.albicans/C.glabrata/C.krusei/C.parapsilosis/C.tropicalis Real-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;
- **Negative Control C-**, 1,2 ml;*
- **Internal Control IC**, 1,0 ml;**
- **DNA-buffer**, 0,5 ml;
- **Standard**
  - **QSG1**, 0,2 ml
  - **QSG2**, 0,2 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:

- 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

Candidosis Real-TM Quant must be stored at 2-8°C except for **PCR-mix-2** and **TaqF Polymerase** that must be stored at -16°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 -16°C immediately on receipt.

STABILITY

Candidosis 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.

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

Candidosis Real-TM Quant can analyze DNA extracted from:

- *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.

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.

The following kit is recommended:

- ⇨ **DNA-Sorb-A** (Sacace, REF K-1-1/A);
- ⇨ **SaMag STD 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 **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-** (**Negative Control C-** provided with the amplification kit) to the tube labeled NCE.
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:

1. Prepare required quantity of tubes or PCR plate.
2. Prepare for each sample in the new sterile tube **10*N µl** of **PCR-mix-1**, **5*N µl** of **PCR-mix-2** and **0,5*N µl** of **TaqF Polymerase.**
3. Add **15 µl** of **Reaction Mix** into each tube.
4. Add **10 µl** of **extracted DNA** sample to appropriate tube with Reaction Mix.
5. Prepare for qualitative run 1 positive control and 1 negative control:
   - add **10 µl** of **QSG2** to the tube labeled C*pos*;
   - add **10 µl** of **DNA-buffer** to the tube labeled C*neg*;
6. For quantitative analysis prepare 4 tubes and perform QSG1 and QSG2* standards **twice**.
   **QSG1 and QSG2 values are specific for each lot and are reported in the Quant Data Card provided in the kit.*

Close tubes and transfer them into the instrument in this order: samples, negative controls, Standards.

Amplification

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

	Rotor-type Instruments[1]			Plate-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™ 6000/Q (Corbett Research, Qiagen)*
[2] *For example, SaCycler-96-5x™ (Sacace), CFX-96 (Bio-rad)*

Fluorescence is detected at the 2nd step of Cycling 2 stage (60 °C) in FAM/Green, JOE/Yellow/HEX/Cy3, ROX/Orange/Texas Red, Cy5/Red and Cy5.5 fluorescence channels.
The results are interpreted with the software of the instrument through the presence of crossing of fluorescence curve with the threshold line.

Candida albicans is detected on the FAM (Green) channel, *Candida glabrata* on the JOE (Yellow) channel, *Candida krusei* on the ROX (Orange) channel, *Candida parapsilosis* and *Candida tropicalis* on the Cy5 (Red) and the IC DNA on the Cy5.5 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*	*10-20 %*	*On*
JOE/Yellow	*from 4 Fl to 8 Fl*	*0.1*	*10-20 %*	*On*
ROX/Orange	*from 4 Fl to 8 Fl*	*0.1*	*10-20 %*	*On*
Cy5/Red	*from 4 Fl to 8 Fl*	*0.1*	*10-20 %*	*On*
Cy5.5	*from 4 Fl to 8 Fl*	*0.1*	*10-20 %*	*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 corresponding to 10-20% of maximum fluorescence obtained in QSG2 (for all channels except Cy5.5/Crimson). In Cy5.5/Crimson Channel set the threshold at a level corresponding to 10-20% of maximum fluorescence obtained in negative control of extraction (NCE).

Boundary values of the cycle threshold, Ct

Sample	Channel	Ct for rotor type instrument	Ct for plate type instrument
QSG1	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	*23*	*26*
QSG2	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	*33*	*36*
Clinical Samples, NCE	*Cy5.5/Crimson*	*35*	*37*
NCE, NCA	*FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red*	*absent*	*absent*

DATA ANALYSIS

The fluorescent signal intensity is detected in five channels:

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

– The signal from the *Candida glabrata* DNA amplification product is detected in the JOE (Yellow) channel;

– The signal from the *Candida krusei* DNA amplification product is detected in the ROX (Orange) channel;

– The signal from the *Candida parapsilosis* and *Candida tropicalis* amplification product is detected in the Cy5 (Red) channel;

– The signal from the IC amplification product is detected in the Cy5.5 channel.

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 Fam (Green)	Ct Joe (Yellow)	Ct Rox (Orange)	Ct Cy5 (Red)	Ct Cy5.5	Interpretation
NCE	DNA isolation	Neg	Neg	Neg	Neg	POS	Valid result
NCA	Amplification	Neg	Neg	Neg	Neg	Neg	Valid result
QSG2	Amplification	POS	POS	POS	POS	-	Valid result

QUANTITATIVE ANALYSIS

Candida DNA quantity is determined using the following formula:

Number of Genome Equivalents per ml (GE/ml*) = calculated copies/reaction x coefficient K **

** GE/ml = Genome Equivalents per 1 ml of sample*

*** Coefficient K is provided in the DataCard enclosed in the kit*

If calculated values are greater than $2x10^5$ GE/ml, the results should be reported as “greater than $2x10^5$ GE/ml”, if they are less than 200 GE/ml, the results should be reported as “less than 200 GE/ml”.

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

Analytical specificity

The analytical specificity of the primers and probes was validated with negative samples. They did not generate any signal with the specific *Candida albicans, Candida glabrata, Candida krusei, Candida parapsilosis and Candida tropicalis* primers and probes.

The potential cross-reactivity of the kit **Candidosis Real-TM Quant** was tested against the group control. It was not observed any cross-reactivity with other pathogens like *Gardnerella vaginalis, Lactobacillus spp., Escherichia coli, Staphylococcus spp., Streptococcus spp., Chlamydia trachomatis, Neisseria gonorrhoeae, Neisseria spp., Mycoplasma genitalium, Trichomonas vaginalis, Treponema pallidum, Toxoplasma gondii, HSV 1 and 2 types, CMV, HPV.*

The specificity of the kit **Candidosis Real-TM Quant** was 100%.

Linear Range

The linear range for quantitative detection of kit **Candidosis Real-TM Quant** is from 200 to $2x10^5$ GE/ml. If calculated values are greater than $2x10^5$ GE/ml, the results should be reported as “greater than $2x10^5$ GE/ml”, if they are less than 200 GE/ml, the results should be reported as “less than 200 GE/ml”.

Analytical sensitivity

The kit **Candidosis Real-TM Quant** allows to detect *Candida albicans, Candida glabrata, Candida krusei, Candida parapsilosis and Candida tropicalis* DNA in 100% of the tests with a sensitivity of not less than **100 GE/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 (Cy5.5 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, Cy5 (Red) 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.

KEY TO SYMBOLS USED

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	IVD	For *in Vitro* Diagnostic Use

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* Rotor-Gene™ is a registered trademark of Qiagen
* CFX-96™ is a registered trademark of BioRad

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