IVD For *in Vitro* Diagnostic Use

CE

# Cryptococcus neoformans Real-TM

# Handbook

## Real Time PCR Kit for detection of Cryptococcus neoformans

**REF F4-100FRT**

**Σ 100**

## NAME

**Cryptococcus neoformans Real-TM**

## INTRODUCTION

Cryptococcosis, caused by Cryptococcus neoformans, is the most common fungal disease in HIV infected persons and it is the AIDS-defining illness in 60-70% of HIV infected patients.

## INTENDED USE

**Cryptococcus neoformans Real-TM** kit is a Real-Time test for the qualitative detection of *Cryptococcus neoformans*.

## PRINCIPLE OF ASSAY

**Cryptococcus neoformans Real-TM** kit is a Real-Time test for the qualitative detection *Cryptococcus neoformans* DNA in the biological material (cerebrospinal fluid, bronchoalveolar lavage, sputum, blood, skin lesions aspirate, viscera biopsy and autopsy material) by using real-time hybridization-fluorescence detection of amplified products. The DNA extraction is carried out with the internal control sample (IC) which helps control the test procedure for each sample.

*Cryptococcus neoformans* detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific Cryptococcus neoformans primers. In the real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

*Cryptococcus neoformans* DNA amplification is detected on JOE(Yellow)/HEX/Cy3 channel and the IC DNA amplification is detected on FAM (Green) channel.

## MATERIALS PROVIDED

- **PCR-mix-1 Cryptococcus**, 1,2 ml
- **PCR-mix-2- FRT**, 0,6 ml
- **TaqF DNA Polymerase**, 0,06 ml
- **TE-buffer**, 0,5 ml
- **Negative Control C-***, 1,2 ml
- **Pos C+1** *(Low)*, 0,2 ml
- **Pos C+2** *(Low)*, 0,2 ml
- **Internal Control IC****, 2 x 0,6 ml

Contains reagents for 110 tests.

** must be used during the sample preparation procedure: add 100 µl of C– (Negative Control) to labeled Cneg;*

***add 10 µl of Internal Control to all samples during the DNA isolation procedure directly to the sample/lysis mixture*

## MATERIALS REQUIRED BUT NOT PROVIDED

- DNA isolation kit
- Desktop microcentrifuge for “eppendorf” type tubes
- Vortex mixer
- Disposable gloves, powderless
- Biohazard waste container
- Refrigerator, Freezer
- Real Time Thermal cycler
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters

## STORAGE INSTRUCTIONS

**Cryptococcus neoformans Real-TM** must be stored -20°C. The **Cryptococcus neoformans Real-TM** kit can be shipped at 2-8°C.

## STABILITY

**Cryptococcus neoformans Real-TM** Test 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:

- 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.

## 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. 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

**Cryptococcus neoformans Real-TM** PCR kit is intended for analysis of DNA extracted from the biological material (cerebrospinal fluid, bronchoalveolar lavage, sputum, blood, skin lesions aspirate, viscera biopsy and autopsy material)

## 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/RNA-Prep** (Sacace, REF K-2-9);

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.

## 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-FRT** and **0,5*N µl** of **Hot Start DNA 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 each run 2 positive controls and 1 negative control:
   - add **10 µl** of **Positive control DNA *Crypt.*-1 (C+$_1$)** to the tube labeled C+$_1$;
   - add **10 µl** of **Positive control DNA *Crypt.*-2 (C+$_2$)** to another one tube labeled C+$_2$
   - add **10 µl** of **TE-buffer** to the tube labeled NCA;

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

## INSTRUMENT SETTINGS

**Settings for rotor-type instruments (Rotor-Gene 3000, Rotor-Gene 6000, Rotor-Gene Q)**

| Channel | Calibrate / Gain Optimisation | Threshold | More Settings/ Outlier Removal | Slope Correct |
|---|---|---|---|---|
| FAM/Green | from 5Fl to 10Fl | 0,03 | 10 % | on |
| JOE/Yellow | from 5Fl to 10Fl | 0,03 | 10 % | on |

**Settings for 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.

## RESULTS INTERPRETATION

The results are interpreted through the presence of crossing of fluorescence curve with the threshold line. To set threshold put the line at such level where curves of fluorescence are linear.

- *Cryptococcus* DNA amplification is detected on JOE(Yellow)/HEX/Cy3 channel;
- Exogenous Internal Control IC is detected on FAM (Green) channel.

Results are accepted as relevant if positive and negative controls of amplification and extraction are passed.

**Results for controls**

| Sample | FAM channel | JOE/HEX channel |
|---|---|---|
| C– | *< 30* | Absent |
| $C+_1$ | *< 25* | *< 25* |
| $C+_2$ | *< 32* | *< 32* |
| Test samples | *< 30* | *< 37* |
| NCA | Absent | Absent |

- The sample is considered to be positive for *Cryptococcus* if in the channel JOE (Yellow)/HEX/ Cy3 the value of **Ct** is different from zero (Ct<37);
- The sample is considered to be uncertain for *Cryptococcus* if its Ct value is more than 37 on JOE(Yellow)/HEX/Cy3 channel. Additional double study of this sample should be conducted;
- Specimens with Ct <30 in the channel FAM (Green) and absent fluorescence signal in the channel JOE(Yellow)/HEX/Cy3 are interpreted as negative.
- Specimens with absent signal in the FAM (Green) and JOE(Yellow)/HEX/Cy3 are interpreted as invalid.

## PERFORMANCE CHARACTERISTICS

### Analytical sensitivity

| Biological material | Nucleic acid extraction kit | Sensitivity, copies/ml |
|---|---|---|
| - cerebrospinal fluid,<br>- bronchoalveolar lavage,<br>- sputum,<br>- blood,<br>- skin lesions aspirate,<br>- viscera biopsy and autopsy material | DNA/RNA-prep | 400 |

### Specificity

The analytical specificity of ***Cryptococcus neoformans*** **Real-TM** PCR kit is ensured by the selection of specific primers and probes as well as stringent reaction conditions. The primers and probes have been checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

Specificity of PCR kit for qualitative detection of *Cryptococcus neoformans* was studied on strains of fungi: *Penicillium brevicompactum, Penicillium chrysogenum, Trichoderma harzianum, Trichothecium roseum, Trichoderma viride, Trichoderma koningii, Fusarium solani, Fusarium poae, Fusarium oxysporum, Fusarium sambucinum, Fusarium verticillioides, Mucor plumbeus, Mucor hiemalis, Mucor racemosus, Mucor pusillus, Aspergillus versicolor, Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Rhizopus stolonifer, Rhizopus oryzae, Rhizopus microsporus, Scedosporium apiospermum, Trichosporon beigelii, Neurospora sitophila, Stachybotrys chartarum, Paecilomyces fulvus, Cladosporium cladosporioides, Wallemia sebi, Geotrichum candium, Candida albicans, Candida glabrata, Candida krusei; and* human DNA. Nonspecific reactions (falce-positive results) were absent.

## 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 controls (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 (Fam/channel).
    - The PCR was inhibited.
        - ⇒ Make sure that you use a recommended DNA extraction method and follow to the manufacturer's instructions.
    - 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.
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. JOE(Yellow)/HEX/Cy3 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.
        - ⇒ 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 |




***Sacace Biotechnologies Srl***
*via Scalabrini, 44 – 22100 – Como – Italy Tel +390314892927 Fax +390314892926*
*mail:* ***info@sacace.com*** *web:* ***www.sacace.com***