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For Professional Use Only

AmpliSens® *Cryptococcus neoformans*-FRT

PCR kit

Instruction Manual

AmpliSens®

EC REP

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1. INTENDED USE

AmpliSens® *Cryptococcus neoformans*-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *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 results of PCR analysis are taken into account in complex diagnostics of disease.

2. PRINCIPLE OF PCR DETECTION

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.

AmpliSens® *Cryptococcus neoformans*-FRT PCR kit is a qualitative test that contains the Internal Control (Internal Control STI-87 (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.

AmpliSens® *Cryptococcus neoformans*-FRT PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by the separation of nucleotides and Taq-polymerase by using chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

3. CONTENT

AmpliSens® *Cryptococcus neoformans*-FRT PCR kit is produced in 1 form:

AmpliSens® *Cryptococcus neoformans*-FRT PCR kit variant FRT-100 F, **REF** R-F4-F(RG,iQ)-CE

AmpliSens® *Cryptococcus neoformans*-FRT PCR kit variant FRT-100 F includes:

Reagent	*Description*	*Volume, ml*	*Quantity*
PCR-mix-1-FRT *Cryptococcus*	colorless clear liquid	1.2	1 tube
PCR-mix-2-FRT	colorless clear liquid	0.6	1 tube
Polymerase (TaqF)	colorless clear liquid	0.06	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Positive control DNA *Crypt.*-1 ($C+_1$)	colorless clear liquid	0.2	1 tube
Positive control DNA *Crypt.*-2 ($C+_2$)	colorless clear liquid	0.2	1 tube
Negative control (C–)*	colorless clear liquid	1.2	2 tubes
Internal control STI-87 (IC)**	colorless clear liquid	0.6	2 tubes

* must be used in the extraction procedure as Negative Control of Extraction.

** add 10 µl of Internal Control during the DNA extraction procedure directly to the sample/lysis mixture (see RIBO-prep, **REF** K2-9-Et-100-CE protocol).

AmpliSens® *Cryptococcus neoformans*-FRT PCR kit is intended for 110 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free pipette tips with aerosol filters (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- PCR box.
- Real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia); Rotor-Gene Q (QIAGEN, Germany); iCycler iQ5 (Bio-Rad, USA)).
- Disposable polypropylene PCR tubes (0.1- or 0.2-ml):

a) 0.2-ml PCR tubes with optical transparent domed or flat caps if a plate-type instrument is used;
b) 0.2-ml PCR tubes with flat caps or strips of four 0.1-ml Rotor-Gene PCR tubes if a rotor-type instrument is used.

- Refrigerator with the range from 2 to 8 °C.
- Deep-freezer with the range from minus 24 to minus 16 °C.
- Reservoir for used tips.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol filters and use a new tip for every procedure.
- Store all extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a distantly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable protective gloves and laboratory cloths, and protect eyes while samples and reagents handling. Thoroughly wash hands afterward.
- 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 samples and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid samples and reagents contact with the skin, eyes and mucous membranes. If these solutions come into contact, rinse the injured area 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 DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment, and reagents to the area where the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

6. SAMPLING AND HANDLING

Obtaining samples of biological materials for PCR-analysis, transportation, and storage are described in the manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

AmpliSens® *Cryptococcus neoformans*-FRT PCR kit is intended for analysis of the DNA extracted with DNA extraction kits from the biological material (cerebrospinal fluid, bronchoalveolar lavage, sputum, blood, skin lesions aspirate, viscera biopsy and autopsy material).

7. WORKING CONDITIONS

AmpliSens® *Cryptococcus neoformans*-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. DNA extraction

It is recommended to use the following nucleic acid extraction kit:

– RIBO-prep, **REF** K2-9-Et-100-CE.

Extract the DNA according to the manufacturer's protocol.

8.2. Preparing PCR

8.2.1 Preparing tubes for PCR

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

1. Prepare the mixture of **PCR-mix-2-FRT** and **polymerase (TaqF).** For this, add the whole volume of **polymerase (TaqF) (60 µl)** into the tube with **PCR-mix-2-FRT (600 µl).** Carefully vortex the tube, avoiding foaming. Centrifuge briefly (1-2 s) to sediment the drops. Mark the preparation date on the tube.

Prepared mixture is intended for analysis of 120 samples. Store the mixture at the temperature from 2 to 8 °C for 3 months and use as it is necessary.

If the prepared mixture cannot be used within 3 months prepare the mixture for less number of reactions. For example, mix **150 µl of PCR-mix-2-FRT** and **15 µl of polymerase (TaqF)** (prepared mixture is intended for 30 reactions).

2. Prepare the reaction mixture. Take into account that it is necessary to carry out 3 control reactions (positive controls of amplification - Positive control DNA *Crypt.*-1 ($C+_1$), Positive control DNA *Crypt.*-2 ($C+_2$), negative control of amplification - DNA-

buffer) even for 1 test sample. Moreover take the reagents with a reserve: prepare the reagents for (N+1) reactions for analysis of N samples.

3. Mix in a new tube **PCR-mix-1-FRT *Cryptococcus*** and prepared mixture of **PCR-mix-2-FRT** and **polymerase (TaqF).** Calculate the reagents volumes on the basis that for 1 reaction it is needed:

   10 µl of PCR-mix-1-FRT *Cryptococcus*,

   5 µl of mixture of PCR-mix-2-FRT and polymerase (TaqF).

   One can calculate the reagents volumes for needed number of reactions including test and control samples analysis in accordance with the scheme of reaction mixture preparation (see table 1).

Table 1

Scheme of reaction mixture preparation

	Reagent volumes for specified number of reactions	
Reagent volume per one reaction, µl	**10.0**	**5.0**
Number of clinical samples	**PCR-mix-1-FRT *Cryptococcus*** [1]	**Mixture of PCR-mix-2-FRT и polymerase (TaqF)**[1]
1	**50**	**25**
2	**60**	**30**
3	**70**	**35**
4	**80**	**40**
5	**90**	**45**
6	**100**	**50**
7	**110**	**55**
8	**120**	**60**
9	**130**	**65**
10	**140**	**70**
11	**150**	**75**
12	**160**	**80**
13	**170**	**85**
14	**180**	**90**
15	**190**	**95**
16	**200**	**100**
17	**210**	**105**
18	**220**	**110**
19	**230**	**115**
20	**240**	**120**
21	**250**	**125**
22	**260**	**130**
23	**270**	**135**
24	**280**	**140**
25	**290**	**145**
30	**340**	**170**

[1] The volumes are specified with account of reserve (one extra reaction) and necessity of carrying out 3 controls of amplification (positive controls - Positive control DNA *Crypt.*-1 ($C+_1$), Positive control DNA *Crypt.*-2 ($C+_2$) and negative control - DNA-buffer).

4. Take the required number of tubes for amplification of the DNA obtained from clinical and control samples.
5. Add **15 µl** of prepared reaction mixture to each tube.
6. Using tips with aerosol filter, add **10 µl** of **DNA samples** obtained at the DNA extraction stage from test and control samples to the tubes with reaction mixture.
7. Carry out the control amplification reactions:

NCA - Add **10 µl** of **DNA-buffer** to the tube labeled NCA (Negative Control of Amplification)

$C+_1$, - Add **10 µl** of **Positive control DNA *Crypt.*-1 ($C+_1$)** to the tube labeled $C+_1$,

$C+_2$ add **10 µl** of **Positive control DNA *Crypt.*-2 ($C+_2$)** to another one tube labeled $C+_2$

C– - Add **10 µl** of **the sample extracted from the Negative Control (C–) reagent** to the tube labeled C–.

8.2.2. Amplification

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

Table 2

AmpliSens-1 amplification program

	***Rotor-type instruments*[2]**			***Plate-type instruments*[3]**		
Step	***Temperature, °C***	***Time***	***Cycles***	***Temperature, °C***	***Time***	***Cycles***
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		**60**	30 s	
	72	15 s		**72**	15 s	

Fluorescent signal is detected in the channels for the FAM and JOE fluorophores (when another tests are performed simultaneously the detection in another channels is enabled).

2. Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin* and Guidelines [2].
3. Insert tubes into the reaction module of the device.
4. Run the amplification program with fluorescence detection.
5. Analyze results after the amplification program is completed.

[2] For example, Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia), Rotor-Gene Q (QIAGEN, Germany).
[3] For example, iCycler iQ5 (Bio-Rad, USA).

9. DATA ANALYSIS

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in two channels:

– The signal of the IC DNA (Internal control STI-87 (IC)) amplification product is detected in the channel for the FAM fluorophore.
– The signal of the *Cryptococcus neoformans* DNA amplification product is detected in the channel for the JOE fluorophore.

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a *Ct* value of the cDNA sample in the corresponding column of the results grid.

Principle of interpretation is the following:

– ***Cryptococcus neoformans*** DNA is **detected** if the *Ct* value determined in the results grid in the channel for the JOE fluorophore is not more than the boundary *Ct* value specified in the *Important Product Information Bulletin*. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.
– ***Cryptococcus neoformans*** DNA is **not detected** if the *Ct* value is not determined (absent) or greater than the boundary *Ct* value specified in the *Important Product Information Bulletin* in the channel for JOE fluorophore, whereas the *Ct* value determined in the channel for the FAM fluorophore is not more than the boundary *Ct* value specified in the *Important Product Information Bulletin*.
– The result is **invalid** if the *Ct* value is not determined (absent) in the channel for FAM fluorophores, whereas the *Ct* value in the channel for the JOE fluorophore is not determined (absent) or greater than the specified boundary *Ct* value. In such cases, the PCR analysis of this clinical sample should be repeated.

Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit. See also Guidelines [2]

The result of the 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 (seeTable 3).

Table 3

Results for controls

Control	Stage for control	*Ct* value in the channel for fluorophore	
		FAM	**JOE**
C–	DNA extraction	**<**boundary value	Absent
NCA	PCR	Absent	Absent
$C+_1$	PCR	**<**boundary value	**<**boundary value
$C+_2$	PCR	**<**boundary value	**<**boundary value

10. TROUBLESHOOTING

Results of analysis are not taken into account in the following cases:

1. If the *Ct* value determined for the Positive Controls of Amplification ($C+_1$ and $C+_2$) in the channel for the **JOE** fluorophore is greater than the boundary *Ct* value or absent, the amplification should be repeated for all samples in which *Cryptococcus neoformans* DNA was not detected.
2. If the *Ct* value is determined for the Negative Control of Amplification (NCA) and/or Negative Control of Extraction (C–) in the channel for the **JOE** fluorophore, the PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples in which *Cryptococcus neoformans* DNA was detected. Take measures to detect the source of contamination.

If you have any further questions or if you encounter problems, please contact our Authorized representative in the European Community.

11. TRANSPORTATION

AmpliSens® *Cryptococcus neoformans*-FRT PCR kit should be transported at 2–8 °C for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *Cryptococcus neoformans*-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-mix-1-FRT *Cryptococcus*, PCR-mix-2-FRT, and polymerase (TaqF)). All components of the **AmpliSens® *Cryptococcus neoformans*-FRT** PCR kit are stable until the expiry date stated on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

PCR-mix-1-FRT *Cryptococcus*, PCR-mix-2-FRT, and polymerase (TaqF) are to be stored at the temperature from minus 24 to minus 16 °C.

PCR-mix-1-FRT *Cryptococcus* is to be kept away from light.

13. SPECIFICATIONS

13.1. Sensitivity

Biological material	Nucleic acid extraction kit	PCR kit	Sensitivity, copies/ml
- cerebrospinal fluid, - bronchoalveolar lavage, - sputum, - blood, - skin lesions aspirate, - viscera biopsy and autopsy material	RIBO-prep	PCR kit variant FRT-100 F	400

13.2. Specificity

The analytical specificity of **AmpliSens® *Cryptococcus neoformans*-FRT** 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.

The clinical specificity of **AmpliSens® *Cryptococcus neoformans*-FRT** PCR kit was confirmed in laboratory clinical trials.

14. REFERENCES

1. Handbook “Sampling, Transportation, and Storage of Clinical Material for PCR diagnostics”, developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology” of Federal Service for Surveillance on Consumers’ Rights

Protection and Human Well-Being, Moscow, 2010.

2. Guidelines to the **AmpliSens® *Cryptococcus neoformans*-FRT** PCR kit for qualitative detection of *Cryptococcus neoformans* DNA in the biological material (cerebrospinal fluid, bronchoalveolar lavage, sputum, blood, skin lesions aspirate, viscera biopsy and autopsy material) by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology”.

15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science “Central Research Institute for Epidemiology” ISO 13485-Certified Quality Management System, each lot of the **AmpliSens® *Cryptococcus neoformans*-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

16. KEY TO SYMBOLS USED

REF	Catalogue number	Σ	Sufficient for
LOT	Batch code		Expiration Date
IVD	*In vitro* diagnostic medical device		Consult instructions for use
VER	Version		Keep away from sunlight
	Temperature limitation	**NCA**	Negative control of amplification
	Manufacturer	**C–**	Negative control of extraction
	Date of manufacture	**C+**	Positive control of amplification
	Caution	**IC**	Internal control
EC REP	Authorised representative in the European Community		