



For *in Vitro* Diagnostic Use

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AmpliSens® *Pneumocystis jirovecii (carinii)*-FRT PCR kit

Instruction Manual

AmpliSens®



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1. INTENDED USE.

AmpliSens® *Pneumocystis jirovecii* (carinii)-FRT PCR kit is an in vitro nucleic acid amplification test for qualitative detection of *Pneumocystis jirovecii* (carinii) in the clinical material (bronchoalveolar lavage, sputum, biopsy material, throat washes and swabs) by using real-time hybridization-fluorescence detection.

2. PRINCIPLE OF PCR DETECTION.

Pneumocystis species microorganisms are unicellular fungi that infect respiratory system of mammals including human. *Pneumocystis carinii* was the only species that belonged to *Pneumocystis* genus for a long time. According to modern classification microorganisms dwelling in human organs unite in *Pneumocystis jirovecii* (*Pneumocystis carinii* earlier) species.

Microorganisms of *pneumocystis* genus cause group of diseases called pneumocystosis. Pneumocystosis is associated with lethal outcomes in 65-85% of AIDS suffering patients and considered most significant AIDS markers. Pneumocystosis can be divided into four types: asymptomatic infections, pneumonia of children, pneumonia associated with depressed immunity, and extrapulmonary infections. Due to low virulence of the pathogen, the infection rarely comes out of lungs. However, development of extrapulmonary infections is possible if the infection spreads from lungs to other organs, including lymph nodes, spleen, bone marrow, liver, kidneys, heart, brain, pancreas, etc. Diagnosis of such illnesses happens accidentally, and as a rule, cannot be clinically confirmed. Extrapulmonary pneumocystosis develops in lymph nodes, spleen, bone marrow, gastrointestinal tract, eyes, thyroid gland, adrenals, and kidneys.

Serologic studies results suggest prevalence of pneumocystosis in adults. Initial infection occurs in childhood and 94% of kids have pneumocystis antibodies at four years old. Pneumocystosis cannot be detected by direct method in adults.

Most likely pneumocystis are transmitted with respiratory secretions, however, infective form of microorganism is unknown.

Carriage of pneumocystis in organism was not reliably verified. This issue remains controversial so far. Therefore, people belonging to group of high risk of opportunistic infections development should be tested for pneumocystis. Diagnosis should be based on complex examination data including clinical, X-ray, and laboratory check. Polymerase chain reaction is a very sensitive method that allows detection of single copies of pathogen DNA in bronchoalveolar lavage, sputum, throat washes and swabs, respiratory biopsy material, blood, etc. Consequently, for pneumocystis pneumonia diagnostics this method should be used as supporting, but not main.

Method of detection of *Pneumocystis jirovecii* DNA from clinical material, containing cells, is based on:

1. Extraction of total DNA from cells suspension (whole blood, leucocytes, homogenized biopsy material).
2. Simultaneous real-time amplification of specific region of mitochondrial DNA large subunit and specific region of human β -globin gene, used as endogenous internal control.

Result of *Pneumocystis jirovecii* DNA amplification is registered on JOE/HEX/Yellow fluorescence channel, result of Internal control DNA amplification is registered on FAM/Green channel. DNA-target selected as endogenous internal control is the fragment of human genome and must be present in a sample of DNA from whole blood, leucocytes and biopsy material in sufficient quantity equivalent to that of cells in the sample (no less than 20000 genomes per sample or DNA from 10000 cells). In case of irrelevant storage of clinical sample cells destruction and DNA degradation can occur. Bad DNA extraction from clinical material can lead to significant loss of DNA or presence of inhibitors in purified DNA sample. So, endogenous internal control allows not only to check PCR-analysis steps, but also to assess sampling adequacy and clinical material safety.

AmpliSens® *Pneumocystis jirovecii* (carinii)-FRT PCR kit uses "hot-start", which greatly reduces frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by using of chemically modified polymerase (TaqF). Chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

3. CONTENT.

AmpliSens® *Pneumocystis jirovecii* (carinii)-FRT PCR kit is produced in 1 form:

AmpliSens® *Pneumocystis jirovecii* (carinii)-FRT PCR kit variant FRT-50 F, **REF** R-F2(RG,iQ,Mx)-CE.

<i>Reagent</i>	<i>Description</i>	<i>Volume (ml)</i>	<i>Amount</i>
PCR-mix-1-FRT <i>P.jirovecii</i>/Glob	colorless, clear liquid	0.42	1 tube
PCR-buffer-FRT	colorless, clear liquid	0.3	2 tubes
Polymerase (TaqF)	colorless, clear liquid	0.02	2 tubes
Positive Control DNA <i>P.jirovecii</i> and human DNA	colorless, clear liquid	0.1	1 tube
DNA-buffer	colorless, clear liquid	0.5	1 tube

AmpliSens® *Pneumocystis jirovecii* (carinii)-FRT PCR kit variant FRT-50 F is intended for 55 reactions, including controls.

4. ADDITIONAL REQUIREMENTS.

- DNA isolation kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 2 ml reaction tubes.
- PCR box.
- Personal thermocyclers (for example, Rotor-Gene™ 3000 or Rotor-Gene™ 6000 (Corbett Research, Australia); iQ5 or iQ iCycler (BioRad, USA), Mx3000P (Stratagene, USA) or equivalent).
- “Rotor-Gene”: disposable polypropylene undomed and unsrtipe 0.2 ml microtubes for PCR (for instance, “Axygen”, USA) for 36-well rotor or 0.1 ml microtubes (Corbett Research, Australia) for 72-well rotor.
- “iQ5”, “iQ iCycler”: disposable polypropylene domed 0.2 ml microtubes for PCR (for instance, “Axygen”, USA), stripe domed tubes or 96-wells plate for PCR equipped with heat-proof optical transparent films (Bio-Rad, USA).
- “Mx3000P”: disposable polypropylene domed and stripe/unsrtipe 0.2 ml microtubes for PCR (for instance, “Axygen”, USA) for 36-well rotor or plate for PCR equipped with heat-proof optical transparent films (Bio-Rad, USA).

- Refrigerator for temperature between 2 and 8 °C.
- Deep-freezer with temperature not more than minus16°C.
- Waste bin for used tips.

5. GENERAL PRECAUTIONS.

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store and handle amplicons away from all other reagents.
- Thaw all components thoroughly at room temperature before starting detection.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats, 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 compliance with local authorities requirements.
- Samples 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 contact with the skin, eyes and mucosa. If skin, eyes and mucosa contact immediately flush with water, seek medical attention.
- 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 move to the Amplification and Detection Area. 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.

6. SAMPLING AND HANDLING.



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

AmpliSens® *Pneumocystis jirovecii* (carinii)-FRT PCR kit is intended for analysis of DNA extracted with DNA isolation kits from:

- bronchoalveolar lavage;
- sputum;
- biopsy material;
- throat washes and swabs.

6.1. *Bronchoalveolar lavage* sample should be placed into sterile disposable tube or container. Thoroughly resuspend the sample and transfer 1 ml of material into an Eppendorf tube using the tip with aerosol barrier. Centrifuge the tube for 10 min at 7000 g (8000 - 10000 rpm in 24-well centrifuge and 10000-13000 rpm in 12-well centrifuge). Carefully remove and discard the supernatant using a tip with aerosol barrier and leaving 200 µl of the liquid on the sediment. Resuspend the sample on vortex.

6.2. *Sputum*. Sputum is collected into a sterile disposable container after preliminary mouth rinsing with water. Add «Mucolysin» reagent [REF] 180 to sputum sample in ratio 5:1 (5 volume of Mucolysin per 1 volume of sputum) using graduation on a container. While sputum is liquefied (20-30 min) shake the container occasionally. Transfer 1 ml of the sample into an 1.5 ml Eppendorf tube using tip with aerosol barrier and centrifuge at 7000 g (8000–10000 rpm in 24-well centrifuge or 10000 – 13000 rpm in 12-well centrifuge) for 10 min. Remove and discard the supernatant using vacuum aspirator. Resuspend the pellet in 100 µl of PBS-buffer (or saline solution) and use for DNA extraction.

6.3. Biopsy material samples should be placed in a sterile disposable 1.5 ml tube with tightly sealed caps or container. For further examination place a sample in a sterile porcelain mortar. Add equal volume of saline solution or PBS buffer. Thoroughly homogenize the sample with a pestle. Transfer 100 µl of prepared suspension in a sterile tube for DNA extraction.

6.4. *Throat washes and swabs* are obtained by sterile probe and placed in a tube with transport media.



Only one freeze-thaw cycle of clinical material is allowed.

7. PROTOCOL.

7.1. DNA Isolation.

It's recommended to use the following nucleic acid extraction kits:

- “DNA-sorb-B”, [REF] K1-2-50-CE.

- “RIBO-prep”, [REF] K2-9-Et-50-CE.



Carry the DNA isolation according to the manufacturer's instructions.

7.2. Preparing the PCR.

Total reaction volume - **25 µl**, volume of DNA sample - **10 µl**.

7.2.1 Preparing tubes for PCR. .

1. Preparation of **PCR-buffer-FRT** and **polymerase(TaqF)** mixture:

Add **0.02 ml of polymerase (TaqF)** into the tube that contains **0.3 ml of PCR-buffer-FRT** and vortex carefully. Label the tube indicating the date of preparation. Use only disposable tips with aerosol barrier.



Store prepared mix at the temperature between 2 and 8 °C for 3 month and use as needed.

If smaller amount of prepared mix is intended to be used within 3 month, reaction mix should be prepared in required volume in 15:1 ratio (for example, **150 µl of PCR-buffer-FRT** and **10 µl of polymerase (TaqF)**)

2. Prepare required number of tubes or stripes for amplification of DNA from clinical and control samples, including two controls.
3. Add reagents in the tubes (refer to table 1).

Table 1

Methods of reagents addition in the tubes

First method	Second method
<ol style="list-style-type: none"> 1. Pipette 7 µl of PCR-mix-1-FRT <i>P.jirovecii</i>/Glob in each tube. 2. Add above 8 µl of prepared mix of PCR-buffer-FRT and polymerase (TaqF). 	<ol style="list-style-type: none"> 1. Prepare reaction mix for required number of reactions. In a single tube add PCR-mix-1-FRT <i>P.jirovecii</i>/Glob and prepared mixture of PCR-buffer-FRT and polymerase (TaqF) calculating per 1 reaction: <ul style="list-style-type: none"> – 7 µl of PCR-mix-1-FRT <i>P.jirovecii</i>/Glob; – 8 µl of prepared mixture of PCR-buffer-FRT and polymerase (TaqF). When estimating reaction volume include three additional reactions: negative and positive controls and one extra reaction. 2. Pipette 15 µl of prepared reaction mix in the microtubes.

Table 2

Scheme of reaction mix preparation

The values are calculated including one extra reaction and two controls (positive and negative controls of amplification).

Number of samples to be analyzed	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
PCR-mix-1-FRT <i>P.jirovecii</i> /Glob (μl)	42	49	56	63	70	77	84	91	98	105	112	119	126	133	140
mix of PCR-buffer-FRT and polymerase (TaqF) (μl)	48	56	64	72	80	88	96	104	112	120	128	136	144	152	160
Number of samples to be analyzed	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
PCR-mix-1-FRT <i>P.jirovecii</i> /Glob (μl)	147	154	161	168	175	182	189	196	203	210	217	224	231	238	245
mix of PCR-buffer-FRT and polymerase (TaqF) (μl)	168	176	184	192	200	208	216	224	232	240	248	256	264	272	280

- Using tips with aerosol barrier add **10 μl** of **DNA** obtained from clinical or control samples at the DNA extraction stage into microtubes or part of plate.



When adding DNA sample ensure that sorbent is not transferred into the tube with reaction mix.

- Carry the **control amplification reactions**:

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

C+ - Add **10 μl** of **Positive Control DNA *P.jirovecii* and human DNA** in the tube labeled C+ (Positive Control of Amplification).

7.2.2. Amplification.

7.2.2.1. Rotor-Gene™ 3000 or Rotor-Gene™ 6000.

- Program the Rotor-Gene™ according to manufacturer's manual and Appendix 1.
- Create a temperature profile on your Rotor-Gene™ instrument as follows:

Table 3

Programming of a thermocycler for *Pneumocystis jirovecii* DNA amplification

Step	Temperature, °C	Time	Fluorescence detection	Repeats
Hold	95	15 min	–	1
Cycling	95	15 sec	–	45
	60	45 sec	FAM/Green, JOE/Yellow	

- Fluorescence detection is on FAM/Green and JOE/Yellow fluorometer channels.

- Make the adjustment of the fluorescence channel sensitivity according to Appendix 1.

7.2.2.2. iQ5 or iQ iCycler.

- Program the iQ™ according to manufacturer's manual and Appendix 2.
- Create a temperature profile on your iQ™ instrument as follows:

Table 4

Programming of a thermocycler for *Pneumocystis jirovecii* DNA amplification

Step	Temperature, °C	Time	Fluorescence detection	Repeats
Hold	95	15 min	–	1
Cycling	95	20 sec	–	45
	60	60 sec	FAM, HEX	

- Fluorescence detection is on FAM and HEX fluorometer channels.

- Make the adjustment of the fluorescence channel sensitivity according to Appendix 2.

7.2.2.3. Mx3000P.

- Program the Mx3000P according to manufacturer's manual and Appendix 2.
- Create a temperature profile on your iQ™ instrument as follows:

Table 5

Programming of a thermocycler for *Pneumocystis jirovecii* DNA amplification

Step	Temperature, °C	Time	Fluorescence detection	Repeats
Hold	95	15 min	–	1
Cycling	95	20 sec	–	45
	60	60 sec	FAM, HEX	

- Fluorescence detection is on FAM and HEX fluorometer channels.

- Make the adjustment of the fluorescence channel sensitivity according to Appendix 3.

8. DATA ANALYSIS.

Obtained data (curves of accumulation of fluorescent signals on two channels) are analyzed automatically by software of used devices for amplification and real-time hybridization-fluorescence detection.

Accumulation of **β-globin gene DNA (Internal Control)** amplification product is registered on **FAM/Green** channel; accumulation of ***P.jirovecii* DNA (Positive Control)** amplification product is registered on **JOE/HEX/Yellow** channel.

8.1. Results interpretation.

The results are interpreted by the software of Rotor-Gene™ 3000 or Rotor-Gene™ 6000, iQ5 or iQiCycler or Mx3000P Instrument by the crossing (or not) of the fluorescence curve with the threshold line.

Results for controls

Control	Stage for control	Ct channel FAM/Green	Ct channel JOE/Yellow/HEX	Interpretation
C-	DNA isolation	Neg	Neg	OK
NCA	Amplification	Neg	Neg	OK
C+	Amplification	Pos (< Y*)	Pos (< X*)	OK

- The sample is considered to be positive for *Pneumocystis jirovecii* if its Ct value does not exceed Z* on JOE/Yellow/HEX channel. Fluorescence curve should cross the threshold line in the area of exponential fluorescence growth.
 - The sample is considered to be negative for *Pneumocystis jirovecii* if its Ct value is not defined in the results grid (the fluorescence curve does not cross the threshold line) on JOE/Yellow/HEX channel and in the results grid on FAM/Green channel the Ct value doesn't exceed H*.
 - The sample is considered to be invalid for *Pneumocystis jirovecii* if its Ct value is not defined in the results grid (the fluorescence curve does not cross the threshold line) on JOE/Yellow/HEX channel and in the results grid on FAM/Green channel the Ct value exceed H. PCR is to be repeated for this sample.
 - The sample is considered to be equivocal for *Pneumocystis jirovecii* if its Ct value exceed Z on JOE/Yellow/HEX channel. It is necessary to perform additional analysis in 2 repeats for this sample. If reproducible positive result is received, the sample is considered to be positive. If irreproducible result is received in 2 repeats, the sample is considered to be equivocal.
 - Negative result of analysis is considered to be unreliable if the Ct value exceed H on FAM/Green channel.
- * For X, Y, Z, H values see Appendix 1 in case of using Rotor-Gene™ 3000 or Rotor-Gene™ 6000 Instrument or Appendix 2 in case of using iQ5 or iQiCycler Instrument and Appendix 3 in case of Mx3000P Instrument.

Results are accepted as relevant if both positive and negative controls of amplification along with negative control of extraction are passed.

9. TROUBLESHOOTING.

- If Ct value is present in Negative Control of extraction and Negative Control of Amplification on FAM/Green and JOE/Yellow/HEX channels in results grid, it indicates the contamination of reagent or samples. In such cases results of analysis must be considered as irrelevant. Test analysis must be repeated and measures to detect and eliminate the source of contamination are to be taken.
- If Ct values on FAM/Green channel (Glob) in studying blood or biopsy samples are higher than

Ct values on FAM channel (Glob) in Positive Control of Amplification (Positive control DNA *P. jirovecii* and human DNA), it suggest about failures during extraction stage or insufficient amount of collected sample.

- If Ct value is absent in Positive Control of amplification on FAM/Green and/or JOE/Yellow/HEX channels, results of analysis for all samples are irrelevant. Analysis for all samples should be repeated starting from the PCR stage.

10. STABILITY AND STORAGE.

All components of the **AmpliSens® *Pneumocystis jirovecii* (carinii)-FRT** PCR kit (except for Polymerase(TaqF) and PCR-mix-1-FRT *P.jirovecii*/Glob) are to be stored at the temperature between 2 and 8 °C, when not in use. All components of the **AmpliSens® *Pneumocystis jirovecii* (carinii)-FRT** PCR kit are to be stable until labeled expiration date.



Polymerase(TaqF) and PCR-mix-1-FRT *P.jirovecii*/Glob are to be stored at the temperature not more than minus 16 °C

11. SPECIFICATIONS.

11.1. Sensitivity.

Analytical Sensitivity of **AmpliSens® *Pneumocystis jirovecii* (carinii)-FRT** PCR kit is not less than 500 copies per 1 ml of sample.



The claimed analytical features of **AmpliSens® *Pneumocystis jirovecii* (carinii)-FRT** PCR kit are guaranteed only when additional reagents kits "DNA-sorb-B" or "RIBO-prep" (manufactured by Federal State Institution of Science Central Research Institute of Epidemiology) are used.

11.2. Specificity.

Specificity of **AmpliSens® *Pneumocystis jirovecii* (carinii)-FRT** PCR kit is assured by selection of specific primers and probes, as well as the selection of strict reaction conditions. The primers and probes were checked for possible homologies to all in gene banks published sequences by sequence comparison analysis. Specificity of **AmpliSens® *Pneumocystis jirovecii* (carinii)-FRT** PCR kit was confirmed in laboratory clinical trials.












12. REFERENCES.

- Handbook "Sampling, transportation, storage of clinical material for PCR diagnostics", developed by Federal State Institution of Science Central Research Institute of Epidemiology of Federal Service for Surveillance on Consumers' Rights Protection and Human Well-Being, Moscow, 2008.

13. QUALITY CONTROL.

In compliance with Federal State Institution of Science “Central Research Institute of Epidemiology” ISO 13485 – certified Quality Management System, each lot of **AmpliSens® Pneumocystis jirovecii (carinii)-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

14. EXPLANATION OF SYMBOLS.

	Manufacturer		Temperature limitation
	Use by		Batch code
	For <i>in Vitro</i> Diagnostic Use		Version
	Catalogue number		Contains sufficient for <n> tests
	Authorised representative in the European Community		Consult instructions for use
	Caution, consult accompanying documents	NCA	Negative Control of Amplification
C+	Positive Control of Amplification	C-	Negative control of Extraction