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For Professional Use Only

AmpliSens® Florocenosis / Aerobes-FRT

PCR kit

Instruction Manual

AmpliSens®

EC REP

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1. INTENDED USE

AmpliSens® Florocenosis / Aerobes-FRT PCR kit is an *in vitro* nucleic acid amplification test for quantitative detection of the DNA of *Enterobacteriaceae* (*E.coli, Klebsiella* spp., *Proteus* spp. etc.), *Staphylococcus* spp. and *Streptococcus* spp. in biological materials (vaginal swabs) by using real-time hybridization-fluorescence detection.

The results of PCR analysis are taken into account in complex diagnostics of disease.

2. PRINCIPLE OF PCR DETECTION

Detection and quantitation of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. DNA by polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection contains two steps: DNA extraction from the biological material and amplification of DNA fragment of microorganism with real-time hybridization-fluorescence detection. The DNA extraction from the clinical material is carried out with the presence of the Internal Control (Internal Control-FL), which allows controlling the procedure of examination of each sample. 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. The results of amplification of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. DNA are detected separately for each type by three different channels.

Channel for fluorophore[1]	FAM	JOE	ROX	Cy5
DNA-target	*Enterobacteriaceae*	*Staphylococcus* spp.	*Streptococcus* spp.	IC

The DNA quantitation by real-time PCR is based on the existence of linear dependence between the logarithm of initial DNA-target concentration in the sample and start of the exponential growth of the fluorescent signal (threshold cycle, *Ct*). For the quantitative analysis simultaneous carried out real-time amplification with real-time detection for the DNA samples obtained from the test samples and DNA-calibrators (the samples with the certain concentration of DNA-target). According to the results of amplification DNA-calibrators built a calibration line on which the determination of the concentration of DNA-target in the test samples.

[1] The name of channels for the corresponding device sees in the guidelines.

AmpliSens® Florocenosis / Aerobes-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® Florocenosis / Aerobes-FRT PCR kit is produced in 1 form:

AmpliSens® Florocenosis / Aerobes-FRT PCR kit variant FRT-100 F **REF** R-B88-100-FT-CE.

AmpliSens® Florocenosis / Aerobes-FRT PCR kit variant FRT-100 F includes:

Reagent		*Description*	*Volume, ml*	*Quantity*
PCR-mix-FL Florocenosis / Aerobes		colorless clear liquid	1.2	1 tube
PCR-buffer-B		colorless clear liquid	0.6	1 tube
Polymerase (TaqF)		colorless clear liquid	0.06	1 tube
TE-buffer		colorless clear liquid	0.2	1 tube
DNA calibrators	**К1 АВ**	colorless clear liquid	0,2	1 tube
	К2 АВ	colorless clear liquid	0.2	1 tube
Negative Control (C–)*		colorless clear liquid	1.2	1 tube
Internal Control-FL (IC)**		colorless clear liquid	1.0	1 tube

* 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 DNA-sorb-AM REF K1-12-100-CE protocol).

AmpliSens® Florocenosis / Aerobes-FRT PCR kit is intended for 110 reactions (including controls and calibrators).

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Transport medium with mucolytic agent or transport medium for swabs.
- Additional materials and equipment for DNA extraction.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with filters (up to 100 µl).
- Tube racks.
- Vortex mixer.

- PCR box.
- Real-time instruments (5 or more detection channels) (for example, Rotor-Gene 6000 (Corbett Research, Australia), Rotor-Gene Q (QIAGEN, Germany), CFX 96 (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 for 2–8 °C.
- Deep-freezer at 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® Florocenosis / Aerobes-FRT PCR kit is intended for the analysis of DNA extracted from biological materials (vaginal swabs).

7. WORKING CONDITIONS

AmpliSens® Florocenosis / Aerobes-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 kits:

- DNA-sorb-AM, **REF** K1-12-100-CE,

Extract DNA according to the manufacturer's instructions.

Extraction of DNA by express methods (for example, EDEM **REF** K2-17-100-CE) is not recommended.

8.2. Preparing PCR

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

8.2.1. Preparing tubes for PCR

Components of the reaction mixture should be mixed immediately before the PCR studies.

1. Vortex the tubes with **PCR-mix-FL Florocenosis / Aerobes, PCR-buffer-B** and **polymerase (TaqF)** and then centrifuge briefly.

   Take the required number of tubes/strips for amplification of the DNA obtained from test and control samples.

2. For 1 reaction, add to a new tube:

   10 µl of PCR-mix-FL Florocenosis / Aerobes,

   5.0 µl of PCR-buffer-B

   0.5 µl of polymerase (TaqF).

In case of a large amount of samples is acceptable preparation of pre-mixed **PCR buffer-B** and **polymerase (TaqF)**. The content of one tube with **polymerase (TaqF) (60 µl)** should be completely transported into the tube with the **PCR buffer-B (600 µl)** and blended precisely not allowing foaming. The tube should be marked and dated.

The prepared mixture is estimated for 110 reactions. The mixture should be stored at 2–8 °C during 3 months and used when it is necessary.

Table 1

Scheme of reaction mixture preparation for variant FRT-100 F

	Reagent volume for specified number of reactions, µl	
Reagent volume per one reaction, µl	**10,0**	**5,0**
Number of biological samples to be tested	**PCR-mix-FL Florocenosis / Aerobes**[2]	**Mix of PCR-buffer-B** and **polymerase (TaqF)**[2]
1	**60**	**30**
2	**70**	**35**
3	**80**	**40**
4	**90**	**45**
5	**100**	**50**
6	**110**	**55**
7	**120**	**60**
8	**130**	**65**
9	**140**	**70**
10	**150**	**75**
11	**160**	**80**
12	**170**	**85**
13	**180**	**90**
14	**190**	**95**
15	**200**	**100**
16	**210**	**105**
17	**220**	**110**
18	**230**	**115**
19	**240**	**120**
20	**250**	**125**
21	**260**	**130**
22	**270**	**135**

[2] Values are calculated taking into account four control reactions (DNA calibrators K1, K2, C– and NCA) and one extra reaction (N+4+1).

23	**280**	**140**
24	**290**	**145**
25	**300**	**150**
30	**350**	**175**

3. Vortex the tube, then centrifuge it briefly. Transfer **15 µl** of the prepared mixture to each tube.
4. Using tips with aerosol filter, add **10 µl** of DNA samples.

In addition samples of DNA to avoid adding sorbent into the PCR reaction mixture

5. Carry out the control reactions:

NCA	- Add **10 µl** of **TE-buffer** to the tube labeled NCA (Negative Control of Amplification).
DNA calibrator K1	- Add **10 µl** of **DNA calibrator K1 AB** first tube labeled K1 and
DNA calibrator K2	**10 µl** of **DNA calibrator K2 AB** to second tube labeled K2
C–	- Add **10 µl** of the sample extracted from **Negative Control (C–)** to the tube labeled C–

8.2.2. Amplification

1. Create a temperature profile on your instrument as follows (see Table 2, 3):

Table 2

AmpliSens unified amplification program for rotor-type[3] and plate type[4] instruments

Step	***Temperature, °C***	***Time***	***Fluorescent signal detection***	***Cycle repeats***
1	50	15 мин	–	1
2	95	15 мин	–	1
3	95	10 с	–	45
	60	20 с	**FAM, JOE, ROX, Cy5, Cy5.5**	

Any combination of the tests can be performed in one instrument simultaneously by the unified amplification program. In case of simultaneously carried out tests only for identify the DNA of the pathogen the first step (50 °C - 15 minutes) can be removed from the program for save time.

Channel for the Cy5.5 fluorophore is activated when it necessary, if tests are conducted in the multiprime format that use the channel.

[3] For example, Rotor-Gene 6000 (Corbett Research, Australia), Rotor-Gene Q (QIAGEN, Germany).
[4] For example, CFX (Bio-Rad, USA).

Table 3

AmpliSens-1 amplification program for rotor-type instruments

	Rotor-type instruments			*Plate-type instruments*		
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 Fluorescence acquiring		**60**	30 s Fluorescence acquiring	
	72	15 s		**72**	15 s	

Fluorescent signal is detected in the channels for the FAM, JOE, ROX and Cy5 fluorophores.

Channel for the Cy5.5 fluorophore is activated when it necessary, if tests are conducted in the multiprime format that use the channel.

2. Insert tubes into the reaction module of the device.

It is recommended to sediment drops from walls of tubes by short centrifugation (1–3 s) before placing them into the instrument.

3. Run the amplification program with fluorescence detection.
4. Analyze results after the amplification program is completed.

9. DATA ANALYSIS

Analysis of the results can be performed by the software of the real-time PCR instrument, and then calculating the concentrations by the Microsoft® Excel software AmpliSens Florocenosis / Aerobes.

Analysis curve of fluorescence signal accumulation in four channels:

- The signal of the *Enterobacteriaceae* DNA amplification product is detected in the channel for the FAM fluorophore.
- The signal of the *Staphylococcus* spp. DNA amplification product is detected in the channel for the JOE fluorophore.
- The signal of the *Streptococcus* spp. DNA amplification product is detected in the channel for the ROX fluorophore.
- The signal of the IC DNA amplification product is detected in the channel for the Cy5 fluorophore.

Results are interpreted by the crossing (or not crossing) of the fluorescence curve with the threshold line that set at the level of exponential growth of fluorescence. That determines

presence (or absence) of *Ct* (cycle threshold) value of a sample in the appropriate cell of the result grid. Obtained *Ct* values are used for plotting a calibration line and determination of concentration of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. DNA in samples.

The values of DNA calibrators concentrations are specified in the *Important Product Information Bulletin* for the PCR kit.

According to the obtained values of *Ct* and the calibration line, the device program automatically calculated values of the number of copies of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. DNA in the reaction tube, and issued in the corresponding column in the result grid (see the description for the device model in the Guidelines [2]). The obtained values are used to calculate the number of genomic equivalents of the microorganisms DNA contained in 1 ml of the sample of biological material according to the formula:

[Number of copies] DNA of microorganisms x K = [Number of genomic equivalents] per 1 ml (GE/ml)

The coefficient K for calculation the result in GE/ml is specified in the *Important Product Information Bulletin* for the PCR kit.

The DNA concentration values of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. reflect the general content of the certain microorganisms in the biological material inserted into transport medium.

If the obtained value is less than $1x10^4$ GE/ml then the output result is “less than $1x10^4$ GE/ml”, if the obtained value is more than $1x10^8$ GE/ml then the output result is “more than $1x10^8$ GE/ml” (according to the linear range of the kit).

The clinical interpretation of the test results should be carried out by the doctor only on the basis of complex examination of the patient according to the anamnesis data, clinical and epidemiological status, keeping into account the existed clinical and methodological recommendations.

The result of the PCR is considered reliable only if the results obtained for Negative Control of amplification as well as for the Negative Control of extraction of DNA and DNA calibrator K2 are according to the Table 2 and the amplification efficiency index E stays in the limits which are specified in the *Important Product Information Bulletin* enclosed in the PCR kit.

Table 4

Results for controls

Control	Stage for control	Calculated concentration or the threshold cycle (*Ct*) value	
		for channels FAM/Green, JOE/Yellow, ROX/Orange	for channel Cy5/Red
K2	PCR	< boundary value	< boundary value
C–	DNA Extraction	Concentration value is absent or < boundary value	< boundary value
NCA	PCR	Concentration value is absent or < boundary value	Ignored

10. TROUBLESHOOTING

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

1. If for the DNA calibrators (K1, K2) the threshold cycle values through the channels for FAM and/or JOE and/or ROX fluorophores are absent or DNA calibrator K2 the *Ct* values exceed the boundary value or the efficiency index E is less than the boundary value which is specified in the *Important Product Information Bulletin.* In this case the amplification should be repeated for all of the samples.
2. If for the Negative Control of extraction (C–) and/or Negative Control of amplification (NCA) the value of the calculated concentration (GE/ml) of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. DNA are more than boundary value PCR study should be repeated for all the samples sample starting from the stage of DNA extraction.
3. If for the test sample the value of the calculated concentration (GE/ml) of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. DNA are absent or less than 10^4 GE/ml and through the channel for Cy5 fluorophore (Internal Control detection) the *Ct* value is absent or exceeds the boundary value. In this case the analysis should be repeated for this sample starting from the stage of DNA extraction.

The detailed information about the interpretation of the results can be found in the Guidelines [2] enclosed to the PCR kit.

11. TRANSPORTATION

AmpliSens® Florocenosis / Aerobes-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® Florocenosis / Aerobes-FRT** PCR kit (except for polymerase (TaqF) and PCR-buffer-B) are to be stored at 2–8 °C when not in use. All

components of the **AmpliSens® Florocenosis / Aerobes-FRT** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

Polymerase (TaqF) and PCR-buffer-B are to be stored at temperature from minus 24 to minus 16 °C

PCR-mix-FL Florocenosis / Aerobes is to be kept away from light

13. SPECIFICATIONS

13.1. Sensitivity

The analytical sensitivity of **AmpliSens® Florocenosis / Aerobes-FRT** PCR kit is the following:

Biological material	Transport medium	Nucleic acid extraction kit	Mcroorganisms	Sensitivity, GE/ml [1)]
Vaginal swabs	Transport Medium with Mucolytic Agent or Transport Medium TM-EDEM or Transport Medium for swabs	DNA-sorb-AM	*Enterobacteriaceae*	$2x10^3$
			Staphylococcus spp.	$2x10^3$
			Streptococcus spp.	$2x10^3$

13.2. Linear range

Linear range for each determining microorganisms group is from $1x10^4$ to $1x10^8$ GE/ml.

13.3. Specificity

The analytical specificity of **AmpliSens® Florocenosis / Aerobes-FRT** PCR kit is ensured by 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.

The nonspecific reactions were absent testing the human DNA samples and the following microorganisms' DNA: *Lactobacillus* spp., *Gardnerella vaginalis, Enterococcus faecium, Neisseria gonorrhoeae, Neisseria* spp., *Chlamydia trachomatis, Mycoplasma hominis, Ureaplasma urealyticum, Trichomonas vaginalis, Candida* spp., *HSV* 1 and 2 types, *CMV.*

During tests with samples DNA of microorganisms belonging to *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp., include *Escherichia coli, Klebsiella*

[1)] Genome equivalents (GE) of the pathogen agent per 1 ml of a sample.

pneumoniae, Proteus mirabilis, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, for each group of positive results were obtained only at channel to detect DNA corresponding microorganisms group and non-specific results is absent for the other channels.

The clinical specificity of **AmpliSens® Florocenosis / Aerobes-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 **AmpliSens® Florocenosis / Aerobes-FRT** PCR kit for detection and quantitation of *Enterobacteriaceae, Staphylococcus* spp. and *Streptococcus* spp. DNA in the biological material (vaginal swabs) 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® Florocenosis / Aerobes-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

16. KEY TO SYMBOLS USED

REF	Catalogue number		Caution
LOT	Batch code	Σ	Sufficient for
RUO	Research use only		Expiration Date
VER	Version		Consult instructions for use
	Temperature limitation	**NCA**	Negative control of amplification
	Manufacturer	**C–**	Negative control of extraction
	Date of manufacture	**C+**	Positive control of Amplification
		IC	Internal control