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For Professional Use Only

AmpliSens® *Influenza virus* A-type-H5, H7, H9-FRT

PCR kit

Instruction Manual

AmpliSens®

EC REP

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1. INTENDED USE

AmpliSens® *Influenza virus* A-type-H5, H7, H9-FRT PCR kit is an *in vitro* nucleic acid amplification test for typing (identification) of *Influenza virus* A subtypes H5, H7, H9 in *Influenza virus* cultures and biological material containing *Influenza virus* A RNA using real-time hybridization-fluorescence detection of amplified products.

AmpliSens® *Influenza virus* A-type-H5, H7, H9-FRT PCR kit can be used with suspected influenza without distinction of form and presence of manifestation.

The material for PCR analysis is the cDNA samples obtained from human biological material: nasal swabs (inferior nasal meatus), oropharyngeal swabs (posterior pharyngeal wall), sputum (or tracheal aspirates), bronchoalveolar lavage, autopsy material in which the *Influenza virus* A RNA was detected. In case of lower respiratory tract diseases (bronchitis, bronchiolitis, pneumonia) the most informative material is sputum (or tracheal aspirates) and bronchoalveolar lavage.

PCR kit should be used for analysis of cDNA samples in which the *Influenza virus* A RNA was detected within the analysis of biological material and viruses cultures with the use of **AmpliSens® *Influenza virus* A/B-FRT** PCR kit manufactured by Federal Budget Institute of Science "Central Research Institute for Epidemiology". The reagents kits recommended by Federal Budget Institute of Science "Central Research Institute for Epidemiology" should be used for the RNA extraction and cDNA synthesis.

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

2. PRINCIPLE OF PCR DETECTION

Typing (identification) of subtypes H5, H7, H9 by the polymerase chain reaction (PCR) is based on the amplification of the haemagglutinin gene fragments of given subtypes of *Influenza virus* A using specific primers. In the real-time PCR, the amplified product is detected with the use of three 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® *Influenza virus* A-type-H5, H7, H9-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® *Influenza virus* A-type-H5, H7, H9-FRT PCR kit is produced in 1 form:

AmpliSens® *Influenza virus* A-type-H5, H7, H9-FRT PCR kit variant FRT-50 F, **REF** R-V66-F-CE

AmpliSens® *Influenza virus* A-type-H5, H7, H9-FRT PCR kit variant FRT-50 F includes:

Reagent	*Description*	*Volume, ml*	*Quantity*
PCR-mix-FL *Influenza virus* A H5, H7, H9	colorless clear liquid	0.6	1 tube
PCR-buffer-B	colorless clear liquid	0.3	1 tube
Polymerase (TaqF)	colorless clear liquid	0.03	1 tube
Positive Control *Influenza virus* A H5, H7, H9 (C+*Influenza virus* A H5, H7, H9)	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.2	1 tube

AmpliSens® *Influenza virus* A-type-H5, H7, H9-FRT PCR kit is intended for 55 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- 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.
- PCR box.
- Real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia); Rotor-Gene Q (QIAGEN, Germany); iCycler iQ5 (Bio-Rad, USA); 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 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® *Influenza virus* A-type-H5, H7, H9-FRT PCR kit is intended for analysis of

the RNA extracted with RNA extraction kits from *Influenza virus* cultures and biological material containing *Influenza virus* A RNA (nasal swabs (inferior nasal meatus), oropharyngeal swabs (posterior pharyngeal wall), sputum (or tracheal aspirates), bronchoalveolar lavage and autopsy material).

Pretreatment

For all manipulations consult **AmpliSens® *Influenza virus* A/B-FRT** PCR kit *Instruction manual*. *Influenza virus* cultures testing is recommended to carry out after the prior dilution to the concentration not more than 10^5 GE/ml (ie the *Ct* value in the channel for the ROX fluorophore detected with the use of **AmpliSens® *Influenza virus* A/B-FRT** PCR kit must be not less than the *Ct* value for Positive Control *Influenza virus* A / *Influenza virus* B / STI in the same channel).

7. WORKING CONDITIONS

AmpliSens® *Influenza virus* A-type-H5, H7, H9-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. RNA extraction

RNA extraction should be caried out in accordance with **AmpliSens® *Influenza virus* A/B-FRT** PCR kit *Instruction manual* and *Guidelines*.

Extract the RNA according to the manufacturer's protocol.

8.2. Reverse transcription

Complementary DNA (cDNA) synthesis from the RNA should be caried out in accordance with **AmpliSens® *Influenza virus* A/B-FRT** PCR kit *Instruction manual* and *Guidelines*.

8.3. Preparing PCR

8.3.1 Preparing tubes for PCR

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

1. Thaw the tubes with **PCR-mix-FL *Influenza virus* A H5, H7, H9**. Vortex the tubes with **PCR-mix-FL *Influenza virus* A H5, H7, H9, PCR-buffer-B** and **polymerase (TaqF)** and then centrifuge briefly.
2. Take the required number of tubes/strips for amplification of the cDNA obtained from test and control samples.
3. For N reactions, add to a new tube:

10*(N+1) µl of PCR-mix-FL *Influenza virus* A H5, H7, H9,

5*(N+1) µl of PCR-buffer-B

0.5*(N+1) µl of polymerase (TaqF). (see the scheme of reaction mixture preparation in table 1).

4. Vortex the tube with prepared mixture, then centrifuge it briefly to sediment the drops.

Table 1

Scheme of reaction mixture preparation

	Reagent volume for specified number of reactions		
Reagent volume per one reaction, µl	**10.0**	**5.0**	**0.5**
Number of reactions[1]	**PCR-mix-FL *Influenza virus* A H5, H7, H9**	**PCR-buffer-B**	**Polymerase (TaqF)**
6	**60**	**30**	**3.0**
8	**80**	**40**	**4.0**
10	**100**	**50**	**5.0**
12	**120**	**60**	**6.0**
14	**140**	**70**	**7.0**
16	**160**	**80**	**8.0**
18	**180**	**90**	**9.0**
20	**200**	**100**	**10.0**
22	**220**	**110**	**11.0**
24	**240**	**120**	**12.0**
26	**260**	**130**	**13.0**
28	**280**	**140**	**14.0**
30	**300**	**150**	**15.0**
32	**320**	**160**	**16.0**

5. Transfer **15 µl** of the prepared mixture to each tube.
6. Add **10 µl** of **cDNA samples** obtained at the RNA reverse transcription stage.
7. Carry out the control amplification reactions:

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

C+ - Add **10 µl** of **Positive Control *Influenza virus* A H5, H7, H9 (C+*Influenza virus* A H5, H7, H9)** to the tube labeled C+.

[1] Number of test samples including the controls of amplification, and one extra reaction (N+2+1).

8.3.2. Amplification

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

Table 2

Amplification program rotor-type and plate-type instruments

	Rotor-type instruments[2]			***Plate-type instruments***[3]		
Step	***Temperature, °C***	***Time***	***Step***	***Temperature, °C***	***Time***	***Step***
1	95	15 min	1	95	15 min	1
2	95	10 s	10	95	10 s	10
	54	20 s		54	25 s	
	72	10 s		72	25 s	
3	95	10 s	35	95	10 s	35
	54	20 s Fluorescence acquiring		54	25 s Fluorescence acquiring	
	72	10 s		72	25 s	

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

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.

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

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

9. DATA ANALYSIS

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

– The signal of amplification product of the haemagglutinin gene fragment cDNA of *Influenza virus* A subtype H5 is detected in the channel for the FAM fluorophore.
– The signal of amplification product of the haemagglutinin gene fragment cDNA of *Influenza virus* A subtype H7 is detected in the channel for the JOE fluorophore.
– The signal of amplification product of the haemagglutinin gene fragment cDNA of *Influenza virus* A subtype H9 is detected in the channel for the ROX 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.

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

Principle of interpretation is the following:

– ***Influenza virus* A subtype H5 is identified** if the *Ct* value determined in the results grid for this sample in the channel for the FAM fluorophore is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*.
– ***Influenza virus* A subtype H7 is identified** if the *Ct* value determined in the results grid for this sample in the channel for the JOE fluorophore is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*.
– ***Influenza virus* A subtype H9 is identified** if the *Ct* value determined in the results grid for this sample in the channel for the ROX fluorophore is less than the boundary *Ct* value specified in the *Important Product Information Bulletin*.
– The given *Influenza virus* A subtype is not identified (not detected) if the *Ct* values in the specified detection channel are absent.
– The result is **equivocal** if the *Ct* value determined in the respective channel is greater than the boundary *Ct* value specified in the *Important Product Information Bulletin*. In this case, the PCR analysis of respective sample should be repeated. If the same result is obtained or the *Ct* value is determined less than threshold cycle, the sample is considered positive.

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 are correct (seeTable 3).

Table 3

Results for controls

Control	**Stage for control**	***Ct* value in the channel for fluorophore**		
		FAM	**JOE**	**ROX**
NCA	PCR	Absent	Absent	Absent
C+	PCR	**<**boundary value	**<**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 Control of Amplification (C+) in any channel is greater than the boundary *Ct* value or absent, the amplification should be repeated for all samples in which negative results was obtained in the respective channel.

2. If the *Ct* value is determined for the Negative Control of Amplification (NCA) in any channel, the amplification should be repeated for all samples in which positive result was obtained in the respective channel.

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

11. TRANSPORTATION

AmpliSens® *Influenza virus* A-type-H5, H7, H9-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® *Influenza virus* A-type-H5, H7, H9-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-mix-FL *Influenza virus* A H5, H7, H9, PCR-buffer-B, and polymerase (TaqF)). All components of the **AmpliSens® *Influenza virus* A-type-H5, H7, H9-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-FL *Influenza virus* A H5, H7, H9, PCR-buffer-B, and polymerase (TaqF) are to be stored at the temperature from minus 24 to minus 16 °C.

13. SPECIFICATIONS

13.1. Sensitivity

Clinical material	PCR kit	Sensitivity, GE/ml[4]
Nasal and oropharyngeal swabs, sputum (or tracheal aspirates), bronchoalveolar lavage, autopsy material in which the *Influenza virus* A RNA was detected	PCR kit variant FRT-50 F	1×10^3

13.2. Specificity

The analytical specificity of **AmpliSens® *Influenza virus* A-type-H5, H7, H9-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.

The PCR kit detects the haemagglutinin genes fragments of the claimed *Influenza virus* A subtypes (H5, H7 and H9). The PCR kit specific activity is confirmed by analysis of strains of *Avian Influenza virus* A/Anhui/1/2013 (H7N9), A/Hong Kong/1073/99 (H9N2),

[4] Genome equivalents (GE) of the pathogen agent per 1 ml of a sample transferred into the specified transport medium.

A/chiken/Moscow/2/07 (H5N1), and also by analysis of experimental samples of human material with addition of *Avian Influenza virus* strains.

The activity of PCR kit components was absent in respect of haemagglutinin genes fragments of *Influenza virus* A subtypes H1, H2, H3, H4, H13, H8, H6, H10, H11, H12, *Influenza virus* B, and also cDNA/DNA of strains and isolates of the main pathogens causing acute respiratory diseases as well as normal microflora of human nasal cavity and oropharynx and human DNA.

The clinical specificity of **AmpliSens® *Influenza virus* A-type-H5, H7, H9-FRT** PCR kit was confirmed in laboratory clinical trials.

13.3. Diagnostic characteristics

Results of PCR kit characteristics testing:

Samples description	Samples type	Number of samples	Results of using AmpliSens® *Influenza virus* A-type-H5, H7, H9-FRT PCR kit
Biological material containing *Influenza virus* A/H5 RNA[5]	Nasal and oropharyngeal swabs	100	Positive 100%
	Sputum	100	Positive 100%
	Autopsy material	100	Positive 100%
Biological material containing *Influenza virus* A/H7 RNA[5]	Nasal and oropharyngeal swabs	100	Positive 100%
	Sputum	100	Positive 100%
	Autopsy material	100	Positive 100%
Biological material containing *Influenza virus* A/H9 RNA[5]	Nasal and oropharyngeal swabs	100	Positive 100%
	Sputum	100	Positive 100%
	Autopsy material	100	Positive 100%
Biological material that does not contain *Influenza viruses* A/H5, A/H7, A/H9 RNA[6]	Nasal and oropharyngeal swabs	100	Negative 100%

In accordance with the submitted data the **diagnostic sensitivity** of the **AmpliSens® *Influenza virus* A-type-H5, H7, H9-FRT** PCR kit is 98-100 % with a confidence coefficient of 90 % for all type of the biological material.

[5)] Model samples of biological material containing recombinant quality control samples was used as samples containing *Influenza viruses* A/H5, A/H7 and A/H9.

[6)] Biological material samples from patients with suspected influenza containing *Influenza virus* A/H1N1pdm2009, *Parainfluenza viruses, Rhinoviruses* (that was proved by testing with AmpliSens® *Influenza virus* A/B-FRT, AmpliSens® *Influenza virus* A/H1-swine-FRT and AmpliSens® ARVI-screen-FRT PCR kits) was used as samples that do not contain *Influenza viruses* A/H5, A/H7 and A/H9.

The **diagnostic specificity** of the **AmpliSens® *Influenza virus* A-type-H5, H7, H9-FRT** PCR kit is 98-100 % with a confidence coefficient of 90 %.

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® *Influenza virus* A-type-H5, H7, H9-FRT** PCR kit for typing (identification) of *Influenza virus* A subtypes H5, H7, H9 in *Influenza virus* cultures and biological material containing *Influenza virus* A RNA by real-time hybridization-fluorescence detection of amplified products 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® *Influenza virus* A-type-H5, H7, H9-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
IVD	*In vitro* diagnostic medical device		Expiration Date
VER	Version		Consult instructions for use
	Temperature limitation		Keep away from sunlight
	Manufacturer	**NCA**	Negative control of amplification
	Date of manufacture	**C+**	Positive control of amplification
EC REP	Authorised representative in the European Community		