



For *in Vitro* Diagnostic Use

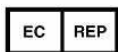
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AmpliSens[®] *Influenza virus A H5N1-FRT* PCR kit

Instruction Manual

AmpliSens[®]



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1. INTENDED USE.

AmpliSens® Influenza virus A H5N1-FRT PCR kit is an in vitro nucleic acid amplification test for qualitative detection of *Influenza virus A* RNA and identifying of H5N1 subtype in the clinical material (nasal and throat swabs or washes; aspirate of trachea; feces; autopsy material) by using real-time hybridization-fluorescence detection.

2. PRINCIPLE OF PCR DETECTION.

Influenza virus A H5N1 detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using special *Influenza virus A* H5N1 primers. In real-time PCR the amplified product is detected using fluorescent dyes. These dyes are usually linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. The real-time PCR monitoring of the 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 H5N1-FRT** PCR kit is a qualitative test, which contain the Internal Control (IC). It must be used in the isolation procedure in order to control the isolation process of each individual sample and to identify possible reaction inhibition. PCR kit uses “hot-start”, which greatly reduces frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase by using wax layer. Wax melting and reaction mix components occur only at 95 °C. In variant FRT-50 F “hot-start” is guaranteed by application of polymerase (TaqF). Chemically modified polymerase (TaqF) activates by heating at 95 °C for 15 min.

3. CONTENT.

AmpliSens® Influenza virus A H5N1-FRT PCR kit is produced in 3 forms:

AmpliSens® *Influenza virus A* H5N1-FRT PCR kit variant FRT (for use with RG) **REF** R-V33(RG)-CE.

AmpliSens® *Influenza virus A* H5N1-FRT PCR kit variant FRT (for use with IQ) **REF** R-V33(IQ)-CE.

AmpliSens® *Influenza virus A* H5N1-FRT PCR kit variant FRT-50 F (for use with SC) **REF** R-V33(SC)-CE.

AmpliSens® Influenza virus A H5N1-FRT PCR kit, variant FRT includes:

Reagent	Description	Volume (ml)	Amount
PCR-mix-1-FEP/FRT Influenza virus A ready-to-use single-dose test tubes (<i>under wax</i>)	colorless, clear liquid	0.008	55 tubes of 0.2 ml
PCR-mix-2-FL	colorless, clear liquid	0.77	1 tube
Positive Control cDNA Influenza virus A (C_A+) 	colorless, clear liquid	0.1	1 tube
TE-buffer	colorless, clear liquid	0.5	1 tube
Negative Control (C-)*	colorless, clear liquid	1.6	3 tubes
Internal Control STI-rec (IC)**	colorless, clear liquid	0.12	5 tubes

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

** add 10 µl of Internal Control during the RNA isolation procedure directly to the sample/lysis mixture (see “RIBO-sorb” **REF** K2-1-Et-50-CE protocol).

Reagents for identifying of Influenza virus A subtype H5N1:

PCR-mix-1-FEP/FRT Influenza virus A H5N1 ready-to-use single-dose test tubes (<i>under wax</i>)	colorless, clear liquid	0.008	55 tubes of 0.2 ml
Positive Control cDNA Influenza virus A H5 (C_{H5}+) 	colorless, clear liquid	0.1	1 tube
Positive Control cDNA Influenza virus A N1 (C_{N1}+) 	colorless, clear liquid	0.1	1 tube

AmpliSens® *Influenza virus A* H5N1-FRT PCR kit variant FRT is intended for 55 reactions, including controls.

AmpliSens® Influenza virus A H5N1-FRT PCR kit, variant FRT-50 F includes:

Reagent	Description	Volume (ml)	Amount
PCR-mix-1-FRT (SC) Influenza virus A	colorless, clear liquid	0.12	5 tubes
PCR-buffer-Flu	colorless, clear liquid	0.28	2 tubes
Polymerase (TaqF)	colorless, clear liquid	0.06	1 tube
Positive Control cDNA Influenza virus A (C_A+) 	colorless, clear liquid	0.1	1 tube
TE-buffer	colorless, clear liquid	0.5	1 tube
Negative Control (C-)*	colorless, clear liquid	1.6	3 tubes
Internal Control STI-rec (IC)**	colorless, clear liquid	0.12	5 tubes

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

** add 10 µl of Internal Control during the RNA isolation procedure directly to the sample/lysis mixture (see “RIBO-sorb” **REF** K2-1-Et-50-CE protocol).

Reagents for identifying of Influenza virus A H5N1 subtype:

PCR-mix-1-FRT (SC) Influenza virus A H5N1	colorless, clear liquid	0.12	5 tubes
Positive Control cDNA Influenza virus A H5 (C_{H5+})	colorless, clear liquid	0.1	1 tube
Positive Control cDNA Influenza virus A N1 (C_{N1+})	colorless, clear liquid	0.1	1 tube

AmpliSens® Influenza virus A H5N1-FRT PCR kit variant FRT-50 F is intended for 55 reactions, including controls.

4. ADDITIONAL REQUIREMENTS.

- RNA isolation kit.
- Reverse transcription kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile RNase-free pipette tips with aerosol barriers (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 2 ml reaction tubes.
- PCR box.
- Rotor-Gene™ 3000 or Rotor-Gene™ 6000 (Corbett Research, Australia) Instrument; iQ5 or iQ iCycler (BioRad, USA) Instrument; SmartCycler II (Cepheid, USA) Instrument.
- Disposable polypropylene microtubes for PCR with 0.5 ml (0.2) capacity (for example, “Axygen”, USA).
- Refrigerator for 2–8 °C.
- Deep-freezer with temperature not more than –16°C.
- Waste bin for used tips.

5. GENERAL PRECAUTIONS.

The user should always pay attention to the following:

- Use sterile RNase-free 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 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 mucose membranes. If any of these solutions 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.
- Workflow in the laboratory must proceed in a one directional manner, beginning in the Extraction

Area and moving 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® Influenza virus A H5N1-FRT PCR kit is intended for analysis of RNA extracted with RNA isolation kits from nasal and throat swabs or washes; aspirate of trachea; feces; autopsy material.

7. PROTOCOL.

7.1. DNA Isolation.

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

- “RIBO-sorb” **REF** K2-1-Et-50-CE.



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

7.2. Reverse transcription.

It’s recommended to use following RT reagents kits for complementary DNA (cDNA) synthesis from RNA.

- “REVERTA-L”, **REF** K3-4-50-CE, which contains RT-G-mix-1.



Carry the reverse transcription procedure according to the manufacturer instruction.

7.3. Preparing the PCR.

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

7.3.1 Preparing tubes for PCR.

7.3.1.1. Detection of Influenza virus A RNA.

Variant FRT

1. Prepare the required number of the tubes with **PCR-mix-1-FEP/FRT Influenza virus A** and wax for amplification of cDNA from clinical and control samples.
2. Add **7 µl** of **PCR-mix-2-FL** to the surface of wax layer of each tube, ensuring that it does not fall under the wax and mix with **PCR-mix-1-FEP/FRT Influenza virus A**.

Variant FRT-50 F

1. Prepare required number of the tubes with **PCR-mix-1-FRT (SC) Influenza virus A** (one tube is intended for 11 reactions). Vortex the tube, then centrifuge shortly.

- For performing N reactions (including 2 controls) mix in a new tube: $10^*(N+1)$ μ l of **PCR-mix-1-FRT (SC) Influenza virus A**, $5.0^*(N+1)$ μ l of **PCR-buffer-Flu** and $0.5^*(N+1)$ μ l of **polymerase (TaqF)**. Vortex the tube, then centrifuge shortly. Transfer 15 μ l of prepared mix into each 0.025 ml tube.

Steps 3 and 4 are applied for both variants.

- Using tips with aerosol barrier add **10 μ l** of **cDNA samples**, obtained at the stage of reverse transcription reaction, into prepared tubes.

- Carry the control amplification reactions:

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

C_{A+} - Add **10 μ l** of **Positive Control cDNA Influenza virus A** to the tube labeled **C_{A+}** (Positive Control of Amplification).

Step 5 is applied only for variant FRT-50 F.

- Centrifuge reaction mix using Smart Cyclor II minicentrifuge.

7.3.1.2. Identifying of *Influenza virus A* H5N1 subtype.



cDNA samples with positive results after detection of Influenza virus A RNA are used for identifying of *Influenza virus A* H5N1 subtype.

Variant FRT

- Prepare the required number of the tubes with **PCR-mix-1-FEP/FRT Influenza virus A H5N1** and wax for amplification of cDNA from clinical and control samples.
- Add **7 μ l** of **PCR-mix-2-FL** to the surface of wax layer of each tube, ensuring that it does not fall under the wax and mix with **PCR-mix-1-FEP/FRT Influenza virus A H5N1**.

Variant FRT-50 F

- Prepare required number of the tubes with **PCR-mix-1-FRT (SC) Influenza virus A H5N1** (one tube is intended for 11 reactions). Vortex the tube, then centrifuge shortly.
- For performing N reactions (including 2 controls) mix in a new tube: $10^*(N+1)$ μ l of **PCR-mix-1-FRT (SC) Influenza virus A H5N1**, $5.0^*(N+1)$ μ l of **PCR-buffer-Flu** and $0.5^*(N+1)$ μ l of **polymerase (TaqF)**. Vortex the tube, then centrifuge shortly. Transfer 15 μ l of prepared mix into each 0.025 ml tube.

Steps 3 and 4 are applied for both variants.

- Using tips with aerosol barrier add **10 μ l** of **cDNA samples**, obtained at the stage of reverse transcription reaction, into prepared tubes.

- Carry the control amplification reactions:

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

C_{H5+} - Add **10 μ l** of **Positive Control cDNA Influenza virus A H5** to the tube labeled **C_{H5+}**.

C_{N1+} - Add **10 μ l** of **Positive Control cDNA Influenza virus A N1** to the tube labeled **C_{N1+}**.

Step 5 is applied only for variant FRT-50 F.

- Centrifuge reaction mix using Smart Cyclor II minicentrifuge.

7.3.2. Amplification.

7.3.2.1. RG

- Program the Rotor-Gene™ according to manufacturer's manual and Appendix 1.

- Create a temperature profile on your Rotor-Gene™ instrument as follows:

- Hold 95 °C – 5 min
- Cycling 95 °C – 10 sec
54 °C – 20 sec
72 °C – 10 sec
Cycle repeats – 10 times.
- Cycling 2 95 °C – 10 sec
54 °C – 20 sec – Detection
72 °C – 10 sec
Cycle repeats – 35 times.

- Fluorescence detection is on the 2-nd pass (**54 °C**) in FAM/Green and JOE/Yellow fluorometer channels.

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

7.3.2.2. iQ

- Program the iQ according to manufacturer's manual and Appendix 2.

- Create a temperature profile on your iQ instrument as follows:

- 95 °C – 5 min
10 cycles: 95 °C – 10 sec / 54 °C – 25 sec / 72 °C – 25 sec
35 cycles: 95 °C – 10 sec / 54 °C – 25 sec (detection) / 72 °C – 25 sec

- Fluorescence detection is on the 2-nd pass (**54 °C**) FAM and JOE in fluorometer channels.

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

7.3.2.3. SC

- Program the Smart Cyclor according to manufacturer's manual and Appendix 3.

- Create a temperature profile on your Smart Cyclor instrument as follows:

- Stage1 Hold 95 °C – 900 sec
- Stage2 2-Temperature Cycle 95 °C – 15 sec
54 °C – 25 sec
72 °C – 25 sec
Repeat – 42

- Fluorescence detection is on the 2-nd pass (**54 °C**) in FAM and Cy3 fluorometer channels.

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

8. DATA ANALYSIS

8.1. Detection of *Influenza virus A RNA*.

Internal Control is detected on the JOE/Yellow/Cy3 fluorescence channel, *Influenza virus A* cDNA is detected on the FAM/Green fluorescence channel.

See **Appendix 1** for data analysis settings for Rotor-Gene™ 3000 or Rotor-Gene™ 6000, **Appendix 2** for data analysis settings iQ iCycler and **Appendix 3** for data analysis settings for SmartCycler II.

8.2. Identifying of *Influenza virus A H5N1* subtype.

Influenza virus A N1 cDNA is detected on the JOE/Yellow/Cy3 fluorescence channel, *Influenza virus A H5* cDNA is detected on the FAM /Green fluorescence channel.

See **Appendix 1** for data analysis settings for Rotor-Gene™ 3000 or Rotor-Gene™ 6000, **Appendix 2** for data analysis settings iQ iCycler and **Appendix 3** for data analysis settings for SmartCycler II.

8.3. Results interpretation.

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

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

8.3.1. Detection of *Influenza virus A RNA*.

Results for controls

Control	Stage for control	Ct channel FAM/Green	Ct channel JOE/Yellow/Cy3	Interpretation
C-	RNA isolation	Neg	Pos (< Y*)	OK
NCA	Amplification	Neg	Neg	OK
CA+	Amplification	Pos (< X*)	Neg	OK

* For X, Y values see Appendix 1 in case of using Rotor-Gene™ 3000 or Rotor-Gene™ 6000 Instrument, Appendix 2 in case of using iQ5 or iQiCycler Instrument or Appendix 3 in case of using SmartCycler II Instrument.

1. The sample is considered to be positive for *Influenza virus A* if Ct value on FAM/Green channel is less than X.
2. The sample is considered to be negative for *Influenza virus A* if its Ct value is not defined in the results grid on FAM/Green channel and in the results grid in the JOE/Yellow/Cy3 channel the Ct value doesn't exceed Y. If Ct value on FAM/Green channel exceed X, PCR should be repeated. If the same result is achieved or Ct value on FAM/Green channel is less than X, the sample is considered to be positive.

8.3.2. Identifying of *Influenza virus A H5N1* subtype.

Results for controls

Control	Stage for control	Ct channel FAM/Green	Ct channel JOE/Yellow/Cy3	Interpretation
NCA	Amplification	Neg	Neg	OK
CH5+	Amplification	Pos (< H*)	Neg	OK
CN1+	Amplification	Neg	Pos (< Z*)	OK

* For Z, H values see Appendix 1 in case of using Rotor-Gene™ 3000 or Rotor-Gene™ 6000 Instrument, Appendix 2 in case of using iQ5 or iQiCycler Instrument or Appendix 3 in case of using SmartCycler II Instrument.

*Influenza virus A N1*cDNA.

1. The sample is considered to be positive for *Influenza virus A N1* if Ct value on JOE/Yellow/Cy3 channel is less than Z.
2. The sample is considered to be negative for *Influenza virus A N1* if its Ct value is not defined in the results grid on JOE/Yellow/Cy3 channel. If Ct value on JOE/Yellow/Cy3 channel exceed Z, PCR should be repeated. If the same result is achieved or Ct value on JOE/Yellow channel is less than Z, the sample is considered to be positive.

Influenza virus A H5 cDNA.

1. The sample is considered to be positive for *Influenza virus A H5* if Ct value on FAM/Green channel is less than H.
2. The sample is considered to be negative for *Influenza virus A H5* if its Ct value is not defined in the results grid on FAM/Green channel. If Ct value on FAM/Green channel exceed H, PCR should be repeated. If the same result is achieved or Ct value on JOE/Yellow channel is less than H, the sample is considered to be positive.

Simultaneous registration of positive signals on the FAM/Green and JOE/Yellow channels indicates that *Influenza virus A H5N1* is present in the sample, or several *Influenza virus* subtypes with hemagglutinin 5 and neuraminidase 1 are present simultaneously.

9. TROUBLESHOOTING.

Results of analysis are not being registered in the following cases:

1. If the signal is registered in Negative Control of extraction on FAM/Green channel (in case of *Influenza virus A RNA* detection) or on any of the channels (in case of *Influenza virus A H5N1* subtype identifying) and in Negative Control of amplification (NCA) in any of the channels, it indicates the contamination of reagents or samples. In this case results of the analysis for all samples are considered invalid. It is necessary to repeat the analysis of all tests, and also to take measures to detect and eliminate the source of contamination.

2. If no signal is detected for Positive Controls of amplification, it can suggest incorrect programming of the temperature profile, incorrect configuration of the PCR reaction or storage conditions for kit components has not complied with manufacturer instruction, or the reagents kit has expired. Programming of the instruments, storage conditions, and the expiration date of the reagents should be checked, and then the PCR should be repeated.
3. If Ct value in results grid for IC (the JOE/Yellow/Cy3 channel for PCR-mix-1-FEP/FRT *Influenza virus A*) exceed Y, analysis should be repeated, beginning with first step.

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

10. STABILITY AND STORAGE.

All components of the **AmpliSens® Influenza virus A H5N1-FRT** PCR kit (except for Polymerase(TaqF), PCR-mix-1-FRT (SC) *Influenza virus A* and PCR-mix-1-FRT (SC) *Influenza virus A H5N1*) are to be stored between 2 and 8 °C than not in use. All components of the **AmpliSens® Influenza virus A H5N1-FRT** PCR kit are to be stable until the labeled expiration date.



Polymerase(TaqF), PCR-mix-1-FRT (SC) *Influenza virus A* and PCR-mix-1-FRT (SC) *Influenza virus A H5N1* are to be stored not more than minus 16 °C.

11. SPECIFICATIONS.

11.1. Sensitivity.

Analytical Sensitivity of **AmpliSens® Influenza virus A H5N1-FRT** PCR kit is no less than 5×10^3 copies/ml.



The claimed analytical features of **AmpliSens® Influenza virus A H5N1-FRT** PCR kit are guaranteed only when additional reagents kit “RIBO-sorb” and “REVERTA-L” (manufactured by Federal State Institution of Science Central Research Institute of Epidemiology) are used.

11.2. Specificity.

Specificity of **AmpliSens® Influenza virus A H5N1-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® Influenza virus A H5N1-FRT** PCR kit was confirmed in laboratory clinical trials.

12. REFERENCES.

1. 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® Influenza virus A H5N1-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		Internal Control complex
	Contains sufficient for <n> tests		Authorized representative in the European Community.
	Consult instructions for use		Caution, consult accompanying documents
	For working with Rotor-Gene™ 3000/6000		For working with iQ5, iQ iCycler
	Positive control		Negative control