



For *in Vitro* Diagnostic Use

AmpliSens® *Influenza virus A H5N1-FEP* PCR kit Instruction Manual



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Ecoli s.r.o., Studenohorská 12
841 03 Bratislava 47
Slovak Republic
Tel.: +421 2 6478 9336
Fax: +421 2 6478 9040
ecoli@ecoli.sk
www.ecoli.sk www.pcrdiagnostics.eu



Federal State Institution of Science
Central Research Institute of Epidemiology
3A Novogireevskaya Street
Moscow 111123 Russia

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 end-point hybridization-fluorescence detection of amplified products.

2. PRINCIPLE OF PCR DETECTION.

Influenza virus A and H5N1 subtype 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 Fluorescent End-Point 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. A multi channel rotor-type fluorometer is specially designed to detect fluorescent excitation from the fluorophores in a reaction mix after PCR. It allows the accumulating product detection without re-opening the reaction tubes after the PCR run. AmpliSens® *Influenza virus A H5N1-FRT* PCR kit is qualitative test, which contains 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. The wax melting and reaction mix component occurs only at 95°C.

3. CONTENT.

AmpliSens® *Influenza virus A H5N1-FEP* PCR kit is produced in 2 forms:

AmpliSens® *Influenza virus A H5N1-FEP* PCR kit (vials 0.5 ml), REF V33-50-R0,5-FEP-CE.

AmpliSens® *Influenza virus A H5N1-FEP* PCR kit (vials 0.2 ml), REF V33-50-R0,2-FEP-CE.

AmpliSens® *Influenza virus A H5N1-FEP* PCR kit includes:

Reagent	Description	Volume (ml)	Quantity
PCR-mix-1-FEP/FRT <i>Influenza virus A</i> ready-to-use single-dose test tubes (under wax)	colorless clear liquid	0.008	55 tubes of 0.5 or 0.2 ml
PCR-mix-2-FL	colorless clear liquid	0.77	1 tube
PCR-mix-Background	colorless clear liquid	0.5	1 tube
Mineral oil for PCR	colorless viscous liquid	4.0	1 vial
Positive Control cDNA <i>Influenza virus A</i> (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**	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 STI-rec 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 H5N1 subtype of *Influenza virus A*:

Reagent	Description	Volume (ml)	Quantity
PCR-mix-1-FEP/FRT <i>Influenza virus A H5N1</i> ready-to-use single-dose test tubes (under wax)	colorless clear liquid	0.008	55 tubes of 0.5 or 0.2 ml
Positive Control cDNA <i>Influenza virus A H5</i> (C _{H5+})	colorless clear liquid	0.1	1 tube
Positive Control cDNA <i>Influenza virus A N1</i> (C _{N1+})	colorless clear liquid	0.1	1 tube

AmpliSens® *Influenza virus A H5N1-FEP* PCR kit 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.
- Personal thermocyclers (for example, Gradient Palm Cycler (Corbett Research, Australia) or equivalent instrument).
- Fluorometer ALA-1/4 ("Biosan", Latvia) or equivalent instrument.
- Disposable polypropylene microtubes for PCR with 0.5 ml (0.2) capacity.
- 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, protect eyes while samples and reagents handling. 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 samples and unused reagents in compliance with local authorities requirements.
- Samples should be considered potentially infectious and handled in a biological cabinet in compliance 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® Influenza virus A H5N1-FEP PCR kit is intended for analysis of RNA extracted by using RNA isolation kits from nasal and throat swabs or washes; aspirate of trachea; feces; autopsy material.

6.1. Samples obtained from human.

6.1.1. *Nasal swab samples* are obtained by probe with dry cotton swab. Insert probe gently along the external nasal wall on 2–3 cm till the inferior nasal concha. Then move the probe slightly lower, insert in the inferior nasal meatus under the inferior nasal concha, rotate and remove along the external nasal wall. When material is obtained insert the working area of the probe with cotton swab to sterile disposable tube with 500 µl of sterile saline or phosphate buffer solution. Broke off the terminal part of the probe or cut it off to allow dense closing of tube cup. Close tube with solution and working area of the probe.

6.1.2. *Throat swab samples* are obtained by probe with dry cotton swab. Obtain smears by rotating the probe at the surface of tonsils, palatine arches, posterior wall of pharynx after gargling of oral cavity with water. When material is obtained insert the working area of the probe with cotton swab to sterile disposable tube with 500 µl of sterile saline or phosphate buffer solution. Broke off the terminal part of the probe or cut it off to allow dense closing of tube cup. Close tube with solution and working area of the probe.



It is recommended combining of nasal and throat swab samples in a single tube. For this purpose, place the effective parts of both probes in one tube containing 500 µl of transport medium and analysis as a single sample.

6.1.3. *Nasal wash.* Patient should sit with head tilted backward. Instill 3-5 ml of warm sterile saline solution into each nostril using disposable probe or syringe. Collect the sample from both nostrils in a single sterile tube using funnel. Only an autoclaved funnel should be used.

6.1.4. *Throat wash.* It is necessary to rinse the mouth with water before sampling. After that rinse the throat thoroughly with 8-10 ml of saline solution for 10-15 sec. Collect the sample in a sterile tube using funnel. Only an autoclaved funnel should be used.

6.1.5. *Fecal sample* (1.0 – 3.0 g) should be obtained from a sterile disinfected bedpan or a chamber-pot and transferred into a sterile container by disposable spatula.

6.1.6. *Autopsy sample* should be immediately placed in a sterile disposable container and frozen otherwise it should be examined within 1 hour from the time of sample collection. Store the samples at minus 68 °C for 1 year. Only one freeze-thaw cycle of clinical material is allowed.

6.2. Samples obtained from birds.

6.2.1. *Droppings* (4.0 – 5.0 g) are collected in a sterile container.

6.2.2. *Cloak, pharynx, tracheal swab samples* are obtained with dry sterile cotton swabs. The effective part of the probe is placed in a sterile disposable tube containing 500 µl of respiratory transport medium, sterile saline solution or phosphate buffer (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium monophosphate, 2mM potassium diphosphate; pH 7.5±0.2. Store phosphate buffer at 2-8°C for 1 year in a tightly sealed polypropylene tube). Broke off the terminal part of the probe or cut it off to allow dense closing of tube cup. Close tube with solution and working area of the probe.

6.2.3. *Tracheal wash sample* is obtained by sterile saline solution.

6.3. Samples obtained from other fallen animals.

6.3.1. *Visceral organs (fragments of trachea, lung)* are collected in sterile disposable containers.

6.4. Preparation of clinical material.

6.4.1. *Swabs and washes* are used without additional processing.

6.4.2. *Aspirate of trachea.* "Mucolysin" reagent [REF] 180 is additionally required. Perform treatment according to manufacturer instructions. Prepared solution (50µl) is used for RNA extraction. The rest of the sample can be frozen for further use.

6.4.3. *Autopsy material and visceral organs of animals* should be homogenized by a sterile porcelain mortar and pestle. After that 10% suspension should be prepared by adding sterile saline solution or phosphate buffer. Then transfer the suspension in a 1.5 ml tube and spin at 10,000 r/min for 30 sec. Use supernatant for RNA extraction.

6.4.4. *Human feces.* Prepare fecal suspension from native feces that weren't frozen.

Preparation of 10-20% fecal suspension (omit for watery feces).

Collect the required number of 1.5 ml tubes. Pipette 0.8 ml of phosphate buffer or sterile saline solution into each tube. Transfer 0.1 g (0.1 ml) of fecal sample in the tube using disposable spatula and stir well on vortex to ensure homogenous suspension.

If the material cannot be studied within 1 day and/or continuous storage is required then add glycerin (up to the final concentration of 10-15 %) in 10-20 % fecal suspension. Thoroughly homogenize samples with glycerin, incubate for 30-40 min, and then freeze.

Preparation of clarified fecal extract.

Vortex the tubes with prepared suspension (freshly made or frozen with glycerin) or liquid feces then spin at 10,000 g (12,000 r/min) for 5 min. Use supernatant for RNA extraction.

6.4.5. *Bird droppings.* Use 4.0 – 5.0 grams of droppings for analysis. Prepare 10% suspension with sterile saline solution, thoroughly re-suspend and decant for 10 min. Transfer supernatant in an Eppendorph tube and spin at 12,000 r/min for 5 min. Use supernatant for RNA extraction.

7. PROTOCOL.

7.1. RNA Isolation.

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

- "RIBO-sorb", [REF] K2-1-Et-50-CE.



Carry the RNA isolation according to the manufacturer's instruction.

7.2. Reverse transcription.

It's recommended to use the following kit for complementary DNA (cDNA) synthesis from RNA:

- "REVERTA-L", REF K3-4-50-CE.



Carry the reverse transcription procedure according to the manufacturer's instruction.

7.3. Preparing the PCR.

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

Detection of Influenza virus A RNA.

7.3.1. Preparing tubes for PCR.

1. Prepare the required quantity of tubes with PCR-mix-1-FEP/FRT *Influenza virus A* with wax for amplification of cDNA of 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*.
3. Add above 1 drop of mineral oil for PCR (about 25 µl).
4. Prepare 2 tubes with PCR-mix-1-FEP/FRT *Influenza virus A* and mark them as **Background**. Add 17 µl of PCR-mix-Background to the surface of wax layer of each tube, so that it wouldn't fall under the wax and mix with PCR-mix-1-FEP/FRT *Influenza virus A*. Add above 1 drop of mineral oil for PCR.
5. Using tips with aerosol filter add 10 µl of cDNA samples obtained from clinical or control samples at the stage of reverse transcription of RNA.
6. Carry out the control amplification reactions:

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

C_A + Add 10 µl of Positive Control cDNA *Influenza virus A* to the tube labeled C_A (Positive Control of Amplification).

7.3.2. Amplification.

Run the following program on the thermocycler (see Table 1). When the temperature reaches 95°C (pause regimen), insert tubes into the cells of amplifier and press the button to continue.

It is recommended to sediment drops from walls of tubes by short vortex (1–3 sec) before placing them in the thermocycler.

Table 1

Programming thermocyclers at amplification of *Influenza virus A* cDNA

step	Thermocyclers with active temperature adjustment:						Thermocyclers with block temperature adjustment: "Uno-2" (Biometra)		
	temperature	time	cycles	temperature	time	cycles	temperature	time	cycles
0	95 °C	pause		95 °C	pause		95 °C	pause	
1	95 °C	5 min	1	95 °C	5 min	1	95 °C	5 min	1
2	95 °C	10 sec	42	95 °C	10 sec	42	95 °C	25 sec	42
	54 °C	10 sec		54 °C	25 sec		54 °C	40 sec	
	72 °C	10 sec		72 °C	25 sec		72 °C	25 sec	
3	72 °C	1 min	1	72 °C	1 min	1	72 °C	1 min	1
4	10 °C	storage		10 °C	storage		10 °C	storage	

Identifying of H5N1 subtype of *Influenza virus A*.

For identifying of H5N1 subtype of *Influenza virus A*, use cDNA samples obtained at the stage of reverse transcription of RNA.

7.3.3. Preparing tubes for PCR.

1. Prepare the required quantity of tubes with PCR-mix-1-FEP/FRT *Influenza virus A H5N1* with wax for amplification of cDNA of

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 H5N1*.
3. Add above 1 drop of mineral oil for PCR (about 25 µl).
4. Prepare 2 tubes with PCR-mix-1-FEP/FRT *Influenza virus A H5N1* and mark them as **Background**. Add 17 µl of PCR-mix-Background 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*. Add above 1 drop of mineral oil for PCR.
5. Using tips with aerosol filter add 10 µl of cDNA samples obtained from clinical or control samples at the stage of reverse transcription of RNA.
6. Perform control amplification reactions:
 - NCA Add 10 µl of TE-buffer to the tube labeled NCA (Negative Control of Amplification).
 - C_{H5} + Add 10 µl of Positive Control cDNA *Influenza virus A H5* to the tube labeled C_{H5} (Positive Control of Amplification).
 - C_{N1} + Add 10 µl of Positive Control cDNA *Influenza virus A N1* to the tube labeled C_{N1} (Positive Control of Amplification).

7.3.4. Amplification.

Run the following program on the thermocycler (see Table 2). When the temperature reaches 95°C (pause regimen), insert tubes into the cells of amplifier and press the button to continue.

It is recommended to sediment drops from walls of tubes by short vortex (1–3 sec) before placing them in a thermocycler.

Table 2

Programming thermocyclers at amplification of *Influenza virus A H5N1* subtype cDNA

step	Thermocyclers with active temperature adjustment:						Thermocyclers with block temperature adjustment: "Uno-2" (Biometra)		
	temperature	time	cycles	temperature	time	cycles	temperature	time	cycles
0	95 °C	pause		95 °C	pause		95 °C	pause	
1	95 °C	5 min	1	95 °C	5 min	1	95 °C	5 min	1
2	95 °C	10 sec	42	95 °C	10 sec	42	95 °C	25 sec	42
	54 °C	10 sec		54 °C	25 sec		54 °C	40 sec	
	72 °C	10 sec		72 °C	25 sec		72 °C	25 sec	
3	72 °C	1 min	1	72 °C	1 min	1	72 °C	1 min	1
4	10 °C	storage		10 °C	storage		10 °C	storage	

8. DATA ANALYSIS.

Detection is conducted on ALA-1/4 fluorescence detector.



Please read Aladin Operating Manual before use of this kit.

Program the detector according to manufacturer's manual and Appendix 1.

8.1. Results interpretation

1. When the analysis is complete the results are automatically shown in the table in the manner of following indications:

pos – positive result;

neg – negative result;

eq – equivocal result (signal is in grey zone);

nd – invalid result (specific signal and IC signal are absent in the sample). This option is applicable only for *Influenza virus A* detection test.

2. Result of the analysis is considered reliable only if both Positive and Negative Controls of amplification as well as Negative

Control of extraction are passed (Table 3, 4).

Table 3

Results for controls of *Influenza virus A* detection

Control	Stage for control	Result of automatic interpretation on channel		Interpretation
		FAM (samples)	HEX (IC)	
C-	RNA isolation	<i>Influenza virus A</i> - neg	+	OK
NCA	Amplification	<i>Influenza virus A</i> - nd	-	OK
C _A +	Amplification	<i>Influenza virus A</i> - pos	-	OK

Table 4

Results for controls of H5N1 subtype identifying

Control	Stage for control	Result of automatic interpretation on channel		Interpretation
		FAM (samples)	HEX (samples)	
C-	RNA isolation	H5 – neg	N1 – neg	OK
NCA	Amplification	H5 – neg	N1 – neg	OK
C _{H5} +	Amplification	H5 – pos	N1 – neg	OK
C _{N1} +	Amplification	H5 – neg	N1 – pos	OK

9. TROUBLESHOOTING.

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

- The samples (except for NCA) with **nd** (invalid) result require repeating of PCR and detection. If the same result is detected in the second run, the sample should be examined starting from the stage of RNA extraction (this option is applicable only for *Influenza virus A* detection test).
- The samples with **eq** (equivocal) result require repeating of PCR and detection. If the same result is detected in the second run the samples should be considered as positive.
- No positive signal with positive controls of PCR (C_A, C_{H5}, C_{N1}) can indicate incorrect programming of the temperature profile of the thermocycler, improper configuration of the PCR reaction, inappropriate storing of kit components, or expiration of reagents kit. It is necessary to check programming of the thermocycler (see Table 1, 2) storage conditions, and the expiration date of the reagents and repeat PCR reaction once again for all samples.
- Positive signal detected in negative controls (C- or NCA) indicates the reagents or samples contamination. In such case results of analysis must be considered as irrelevant. Test analysis must be repeated and measures for detecting of contamination source must be undertaken.

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

10. STABILITY AND STORAGE.

All components of the **AmpliSens® Influenza virus A H5N1-FEP** PCR kit are to be stored between 2 °C and 8 °C, when not in use. They also must be stable until the expiry date stated on the label.

11. SPECIFICATIONS.

11.1. Sensitivity.

Analytical Sensitivity of **AmpliSens® Influenza virus A H5N1-FEP** PCR kit is not less than 5x10³ copies/ml.



The claimed analytical features of **AmpliSens® Influenza virus A H5N1-FEP** PCR kit are guaranteed only when additional reagents kits, "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-FEP** PCR kit is ensured 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-FEP** PCR kit was confirmed in laboratory clinical trials.

12. REFERENCES.

- Manual "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 accordance 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-FEP** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

14. SYMBOLS EXPLANATION.

	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