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For Professional Use Only

AmpliSens® *Influenza virus* A H5N1-FEP

PCR kit

Instruction Manual

AmpliSens®

EC REP

Ecoli s.r.o., Studenohorska 12
841 03 Bratislava 47
Slovak Republic
Tel.: +421 2 6478 9336
Fax: +421 2 6478 9040

Federal Budget Institute of
Science “Central Research
Institute for Epidemiology”
3A Novogireevskaya Street
Moscow 111123 Russia

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1. INTENDED USE

AmpliSens® ***Influenza virus*** **A H5N1-FEP** PCR kit is an in vitro nucleic acid amplification test for qualitative detection of *Influenza virus* A RNA and identification of H5N1 subtype in clinical material (nasal and oropharyngeal swabs or washes, tracheal aspirate, feces, and autopsy material) by using end-point hybridization-fluorescence detection of amplified products.

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

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 **F**luorescent **E**nd-**P**oint PCR, the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that 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-FEP** PCR kit is qualitative test that contains the Internal Control (IC). It must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition. PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by separation of nucleotides and Taq-polymerase by using a wax layer. Wax melts and reaction components mix only at 95 ºC.

3. CONTENT

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

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

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

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

Reagent	*Description*	*Volume, ml*	*Quantity*
PCR-mix-1-FEP/FRT *Influenza virus* A 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 *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 extraction procedure as Negative Control of Extraction.

** add 10 µl of Internal Control STI-rec during the RNA extraction 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 *Influenza virus* A H5N1 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 *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-FEP PCR kit is intended for 55 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- RNA/DNA extraction 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 a 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 (0.5- or 0.2-ml; for example, Axygen, USA).
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –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 another suitable disinfectant.
- Avoid contact with the skin, eyes, and mucosa. If skin, eyes, or mucosa contact, immediately flush with water and seek medical attention.

- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- The laboratory process must be one-directional; it should begin in the Extraction Area and then 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 are 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 extraction kits from nasal and oropharyngeal swabs or washes, tracheal aspirate, feces, and autopsy material.

6.1. Samples obtained from humans

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

6.1.2. *Oropharyngeal swab samples* are obtained using a probe with a dry cotton swab. Obtain swabs by rotating the probe over the surface of tonsils, palatine arches, and posterior wall of pharynx after gargling the oral cavity with water. When material is obtained, place the working part of the probe with the cotton swab into a sterile disposable tube with 500 µl of sterile saline or phosphate buffer solution. Break off the terminal part of the probe or cut it off to allow tight closing of tube cup. Close the tube with the solution and the working part of the probe.

It is recommended to combine nasal and oropharyngeal swabs in a single tube. For this purpose, place the working parts of both probes into one tube containing 500 µl of transport medium and analyze them 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. *Oropharyngeal wash*. It is necessary to rinse the mouth with water before sampling. After that rinse the throat thoroughly with 8–10 ml of saline for 10–15 s. Collect the sample to a sterile tube using a 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 to a sterile container with a 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 ºC 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 to a sterile container.

6.2.2. *Cloak, pharyngeal, 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 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). Break off the terminal part of the probe or cut it off to allow tight closing of the tube cup. Close tube with solution and working area of the probe.

6.2.3. *Tracheal wash sample* is obtained using a sterile saline.

6.3. Samples obtained from other dead animals

6.3.1. *Visceral organs (fragments of trachea and lungs)* are collected to sterile disposable containers.

6.4. Preparation of clinical material

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

6.4.2. *Tracheal aspirate.* Mucolysin reagent REF 180-CE is additionally required. Treat samples according to manufacturer's instructions. The prepared solution (50 µl) is used for RNA extraction. The remaining sample can be frozen for further use.

6.4.3. *Autopsy material and visceral organs of animals* should be homogenized using a sterile porcelain mortar and pestle. Then, 10 % suspension in sterile saline or

phosphate buffer should be prepared. Transfer the suspension to a 1.5-ml tube and spin at 10000 rpm for 30 s. Use the supernatant for RNA extraction.

6.4.4. *Human feces*. Prepare fecal suspension from native feces that were not frozen.

Preparation of 10-20% fecal suspension (can be omitted for watery feces).

Take the required number of 1.5-ml tubes. Pipette 0.8 ml of phosphate buffer of sterile saline into each tube. Transfer 0.1 g (0.1 ml) of fecal sample to the tube using a disposable spatula and stir well on vortex to obtain a homogenous suspension.

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

Preparation of clarified fecal extract

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

6.4.5. *Bird droppings.* Use 4.0–5.0 g of droppings for analysis. Prepare a 10 % suspension in sterile saline, thoroughly resuspend, and decant for 10 min. Transfer the supernatant to an Eppendorf tube and spin at 12,000 rpm for 5 min. Use supernatant for RNA extraction.

7. WORKING CONDITIONS

AmpliSens® *Influenza virus* A H5N1-FEP PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. RNA extraction

It is recommended to use the following nucleic acid extraction kits:

- RIBO-sorb, **REF** K2-1-Et-50-CE.

Carry out the RNA extraction according to the manufacturer's instruction.

8.2. Reverse transcription.

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

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

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

8.3. Preparing PCR

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

Detection of *Influenza virus* A RNA

8.3.1. Preparing tubes for PCR

1. Prepare the required number 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 would not 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).

8.3.2. Amplification

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

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

Table 1

Programming thermocyclers at amplification of *Influenza virus* A cDNA

	Thermocyclers with active temperature adjustment:						Thermocyclers with block temperature adjustment: **Uno-2 (Biometra)**		
	Terzik (DNA-Technology)			**GeneAmp PCR System 2700 (Applied Biosystems) Gradient Palm Cycler (Corbett Research), MyCycler (Bio-Rad), MaxyGene (Axygen Scientific)**					
step	tempe-rature	time	cycles	tempe-rature	time	cycles	tempe-rature	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 s	42	**95 °C**	10 s	42	**95 °C**	25 s	42
	54 °C	10 s		**54 °C**	25 s		**54 °C**	40 s	
	72 °C	10 s		**72 °C**	25 s		**72 °C**	25 s	
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.

8.3.3. 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** (~ **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).

8.3.4. Amplification

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

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

Table 2

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

	Thermocyclers with active temperature adjustment:						Thermocyclers with block temperature adjustment: **Uno-2 (Biometra)**		
	Terzik (DNA-Technology)			**GeneAmp PCR System 2700 (Applied Biosystems), Gradient Palm Cycler (Corbett Research), MyCycler (Bio-Rad), MaxyGene (Axygen Scientific)**					
step	tempe-rature	time	cycles	tempe-rature	time	cycles	tempe-rature	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 s	42	**95 °C**	10 s	42	**95 °C**	25 s	42
	54 °C	10 s		**54 °C**	25 s		**54 °C**	40 s	
	72 °C	10 s		**72 °C**	25 s		**72 °C**	25 s	
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	

9. DATA ANALYSIS

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

Please read ALA-1/4 Operating Manual before use of this kit.

Program the detector according to manufacturer's manual and Guidelines.

9.1. 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. The results of analysis are considered reliable only if the results obtained for Positive and Negative Controls of amplification and Negative Control of extraction are correct (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 extraction	***Influenza virus* A** - neg	**+**	OK
NCA	Amplification	***Influenza virus* A** - nd	-	OK
$C+_A$	Amplification	***Influenza virus* A** - 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 extraction	**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

10. TROUBLESHOOTING

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

1. For samples (except for NCA) with the **nd** (invalid) result, PCR and detection should be repeated. If the same result is obtained once again, the sample should be examined starting from the RNA extraction stage (this option is applicable only for ***Influenza virus* A detection** test).
2. For samples with the **eq** (equivocal) result, repeat PCR and detection. If the same result is obtained once again, the samples should be considered as **positive**.
3. The absence of positive signal for positive controls of PCR (C_A+, C_{H5}+, C_{N1}+) may indicate incorrect programming of the temperature profile of the thermocycler, improper configuration of the PCR reaction, inappropriate storage of kit components, or expiration or reagents kit. Check the 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.
4. Positive signal detected in negative controls (C– or NCA) indicates contamination of reagents or samples. In such case, the results of analysis must be considered as invalid. Analysis should be repeated and measures to detect the source of contamination should be taken.

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

11. TRANSPORTATION

AmpliSens® A H5N1-FEP PCR kit should be transported at 2–8 ºC for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *Influenza virus* A H5N1-FEP** PCR kit are to be stored at 2–8 °C when not in use. All components of the **AmpliSens® *Influenza virus* A H5N1-FEP** 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.

PCR-mix-1-FEP/FRT *Influenza virus* A and PCR-mix-1-FEP/FRT *Influenza virus* A are to be kept away from light.

13. SPECIFICATIONS

13.1. Sensitivity

Analytical Sensitivity of **AmpliSens® *Influenza virus* A H5N1-FEP** PCR kit is not less than $5x10^3$ 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 Budget Institute of Science "Central Research Institute for Epidemiology") are used.

13.2. Specificity

The analytical specificity of **AmpliSens® *Influenza virus* A H5N1-FEP** PCR kit is ensured by selection of specific primers and probes as well as stringent reaction conditions. The primers and probes were checked for possible homologies to all sequences published in gene banks by sequence comparison analysis. The clinical specificity of **AmpliSens® *Influenza virus* A H5N1-FEP** PCR kit was confirmed in laboratory clinical trials.

14. REFERENCES

1. Manual "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, 2008.

15. QUALITY CONTROL

In accordance with Federal Budget Institute of Science "Central Research Institute for 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.

16. KEY TO SYMBOLS USED

REF	Catalogue number	Σ	Sufficient for
LOT	Batch code		Expiration Date
IVD	*In vitro* diagnostic medical device		Consult instructions for use
VER	Version		Keep away from sunlight
	Temperature limitation	**NCA**	Negative control of amplification
	Manufacturer	**C–**	Negative control of extraction
	Date of manufacture	**$C+_{A}$, $C+_{H5}$, $C+_{N1}$**	Positive control of amplification
EC REP	Authorised representative in the European Community	**IC**	Internal control
	Caution		

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
09.12.10	Cover page	The phrase “For Professional Use Only” was added
	Content	New sections “Working Conditions” and “Transportation” were added
		The “Explanation of Symbols” section was renamed to “Key to Symbols Used”
	Stability and Storage	The information about the shelf life of reagents before and after the first use was added
		Information that PCR-mix-1- FEP/FRT *Influenza virus* A is to be kept away from light was added
	Key to Symbols Used	The explanation of symbols was corrected
	Text	The name AmpliSens® *Influenza virus* A H5/N1-FEP was changed to AmpliSens® *Influenza virus* A H5N1-FEP
27.06.11 RT	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”