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For Professional Use Only

AmpliSens® *Influenza virus* A/H1-swine-FEP PCR kit

Instruction Manual

AmpliSens®

EC REP

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1. INTENDED USE

AmpliSens® ***Influenza virus*** **A/H1-swine-FEP** PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection of *Influenza virus* A/H1N1(sw2009) RNA in clinical materials (nasal and oropharyngeal swabs, sputum or nasopharyngeal and tracheal aspirates, and autopsy material (fragments of affected parts of lungs)) by using end-point hybridization-fluorescence detection of amplified products.

It is recommended to combine nasal and oropharyngeal swabs in one tube. For this, after sampling, place the working ends of probes into one tube with 500 µl of Transport medium for storage and transportation of respiratory swabs and analyze as one sample.

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

2. PRINCIPLE OF PCR DETECTION

Influenza virus A/H1N1(sw2009) detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using specific *Influenza virus* A/H1N1(sw2009) primers. In Fluorescent End-Point PCR, the amplified product is detected by using fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. A multichannel rotor-type fluorometer is specially designed to detect fluorescence emission from the fluorophores in a reaction mixture after PCR. It allows detection of the accumulating product without re-opening the reaction tubes after the PCR run. **AmpliSens®** ***Influenza virus*** **A/H1-swine-FEP** PCR kit is a 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. **AmpliSens®** ***Influenza virus*** **A/H1-swine-FEP** 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 °C.

3. CONTENT

AmpliSens® ***Influenza virus*** **A/H1-swine-FEP** PCR kit is produced in 2 forms:

AmpliSens® *Influenza virus* A/H1-swine-FEP PCR kit variant FEP (0.5-ml tubes),

REF V55-50-R0,5-FEP-CE.

AmpliSens® *Influenza virus* A/H1-swine- FEP PCR kit variant FEP (0.2-ml tubes),

REF V55-50-R0,2-FEP-CE.

AmpliSens® *Influenza virus* A/H1-swine-FEP PCR kit variant FEP includes:

Reagent	*Description*	*Volume (ml)*	*Quantity*
PCR-mix-1-FEP/FRT *Influenza virus* A/H1-swine (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 dropper bottle
Positive Control cDNA *Influenza virus* A/H1-swine (C+)	colorless clear liquid	0.1	1 tube
Positive Control STI-88 (CS+)	colorless clear liquid	0.1	1 tube
TE-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C-)**	colorless clear liquid	1.2	1 tube
Internal Control STI-rec (IC) * **	colorless clear liquid	0.12	5 tubes

* must be used for thermocyclers without a constant-temperature cover.

** must be used in the extraction 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, RIBO-prep, REF K2-9-Et-50-CE protocols).

AmpliSens® *Influenza virus* A/H1-swine-FRT PCR kit is intended for 55 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

- RNA extraction kit.
- NucliSENS easyMAG automated system for DNA/RNA extraction (BioMérieux, France).
- Reagent kit to NucliSENS easyMAG automated system (NucliSens buffer for extraction 1, NucliSens buffer for extraction 2, NucliSens buffer for extraction 3, NucliSens lysis buffer, NucliSens magnetic silica) (BioMérieux, France).
- Reverse transcription kit.
- Transport medium.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile 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), GeneAmp PCR System 2700 (Applied Biosystems, USA), MaxyGene (Axygen, USA), Uno-2 (Biometra, Germany), or equivalent).
- Fluorometer (for example, ALA-1/4 (Biosan, Latvia) or equivalent).
- 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 pipette tips with aerosol barriers and use new tip for every procedure.
- Store and handle amplicons away from all other reagents.
- Thaw all components thoroughly at room temperature before starting detection.
- 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 accordance 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, 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 the manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

AmpliSens® *Influenza virus* A/H1-swine-FRT PCR kit is intended for the analysis of RNA extracted by RNA extraction kits from nasal and oropharyngeal swabs; sputum (or nasopharyngeal and tracheal aspirates), sectional material (fragments of affected lungs parts).

PREPARATION OF CLINICAL MATERIAL FOR RNA EXTRACTION

Swabs are used without pretreatment.

Sputum or tracheal aspirate. Use reagent Mucolysin (REF 180-CE) manufactured by FBIS CRIE for sputum pretreatment. See instruction manual to Mucolysin for proper use.

Treated sputum (100 µl) is used for RNA extraction. If it needs to repeat the test the rest of sputum can be frozen.

Autopsy material is homogenized with the use of sterile porcelain mortars and pestles. Then, prepare a 10 % suspension in a sterile saline or phosphate buffer. Transfer the suspension to a 1.5-ml tube and centrifuge it at 10,000 rpm for 5 min. The supernatant (100 µl) is used for RNA extraction. If it is necessary to repeat the test, the remaining sputum can be frozen.

7. WORKING CONDITIONS

AmpliSens® *Influenza virus* A/H1-swine-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,
- RIBO-prep, REF K2-9-Et-50-CE.

Extract DNA according to the manufacturer's instructions.

The volume of Internal Control STI-87-rec (IC) is 10 µl.

- NucliSENS easyMAG automated system can be used.

If RNA is extracted using the NucliSENS easyMAG automated system:

- EM-plus kit REF K2-15-96-CE (manufactured by FBIS CRIE) must be used
- Add 30 ml (the whole content of the bottle) of the **RT-G component from the EM-Plus kit** to the bottle with the NucliSens lysis buffer, close tightly the cap and **carefully** mix by turning upside down 7-10 times (this procedure is performed once for each reagent kit).

- Mix 10 µl of the **Internal Control (IC) sample with** 10 µl of **NucliSens magnetic silica** and 10 µl of **Component A** from the **EM-plus kit** with per one sample for RNA/DNA isolation in a new sterile tube using disposable tips with aerosol barriers.
- Set a sample volume as 0.1 ml or 1 ml
- Set the eluate volume as 50–60 µl (up to 100 µl)
- Both *On-board* and *Off-board* Lysis Buffer Dispensing and Lysis Incubation modes can be used

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 according to the manufacturer's instructions.

8.3. Preparing PCR

8.3.1. Preparing tubes for PCR

1. Prepare the required number of tubes with **PCR-mix-1-FEP/FRT *Influenza virus* A/H1-swine** and wax for amplification of cDNA from clinical and control samples.
2. Add **7 µl** of **PCR-mix-2-FL** to the surface of the 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/H1-swine.**
3. Add above **1 drop** of **mineral oil for PCR** (~ **25 µl**) if a thermocycler without a constant-temperature lid is used.
4. Prepare 2 tubes with **PCR-mix-1-FEP/FRT *Influenza virus* A/H1-swine** and mark them as **Background**. Add **17 µl** of **PCR-mix-Background** to the surface of the 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/H1-swine**. Add above **1 drop** of **mineral oil for PCR** if a thermocycler without a constant-temperature lid is used**.**
5. Add **10 µl** of c**DNA samples** obtained from clinical or control samples at the DNA extraction stage using tips with aerosol barrier.
6. 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 cDNA *Influenza virus* A/H1-swine** to the tube labeled C+ (Positive Control of Amplification).

CS+ - Add **10 µl** of **Positive Control STI-88** to the tube labeled CS+ (Positive Control of Amplification of IC)

C- - Add **10 µl** of the sample extracted from **Negative Control** to the tube labeled C- (Negative control of extraction).

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

8.2.1 Amplification

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

Table 1

***Influenza virus* A/H1N1(sw2009) cDNA amplification program**

	Thermocyclers with active temperature adjustment			Thermocyclers with block temperature adjustment		
	GeneAmp PCR System 2700 (Applied Biosystems), Gradient Palm Cycler (Corbett Research), MaxyGene (Axygen, USA)			**Uno-2 (Biometra)**		
Step	Temperature, °C	Time	Cycles	Temperature, °C	Time	Cycles
0	**95**	pause		**95**	pause	
1	**95**	5 min	1	**95**	5 min	1
2	**95**	10 s	42	**95**	25 s	42
	54	25 s		**54**	40 s	
	72	25 s		**72**	25 s	
3	**72**	1 min	1	**72**	1 min	1
4	**10**	storage		**10**	storage	

Amplification programs for different thermocycler models are described in Guidelines “End-Point PCR Detection of *Influenza virus* A/H1N1(sw2009) RNA” [2].

9. DATA ANALYSIS

Detection is performed using a fluorescence detector.

Please read the fluorescence detector Operating Manual before using this kit.

Detection can be conducted within 1 day after completion of amplification only if the tubes with the amplified product have been stored at 2–8 °C in a light-free area.

The fluorescent signal intensity is detected in two channels:

– the signal from the Internal Control STI-rec cDNA amplification product is detected in the FAM channel (or analogous, depending on the detector model);

– the signal from the *Influenza virus* A/H1N1(sw2009) cDNA amplification product is detected in the HEX channel (or analogous, depending on the detector model).

Prior to detection, all settings should be entered and saved. Refer to the **Guidelines** and the **Important Product Information Bulletin** for settings.

Interpretation of results

1. Principle of interpretation:
   - *Influenza virus* A/H1N1(sw2009) cDNA **is detected** in a sample if its signal in the HEX channel is greater than the specified threshold value of the positive result.
   - *Influenza virus* A/H1N1(sw2009) cDNA **is not detected** in a sample if the signal in the HEX channel is less than the specified threshold value of the negative result whereas the signal in the FAM channel is greater than the specified threshold value.
   - The result is **invalid** in a sample if the signal in the HEX channel is less than the specified threshold value of the negative result and the signal in the FAM channel is less than the specified threshold value.
   - The result is **equivocal** if the signal of a sample in the HEX channel is greater than the specified threshold value of the negative result but less than the threshold value of the positive result (the signal is between thresholds).

If the result is invalid or equivocal, PCR should be repeated once again.

2. The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification and the Negative Control of extraction are correct (Table 2).

Table 2

Results for controls

Control	Stage for control	Result of automatic interpretation		Interpretation
		FAM channel	HEX channel	
C–	DNA extraction	**>** threshold	**<** threshold	OK
NCA	Amplification	**<** threshold	**<** threshold	OK
C+	Amplification	**<** threshold	**>** threshold	OK
CS+	Amplification	**>** threshold	**<** threshold	OK

10. TROUBLESHOOTING

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

1. The absence of positive signal in C+ may indicate incorrect programming of the temperature profile of the thermocycler, incorrect configuration of PCR, noncompliance of the storage conditions for kit components with the manufacturer's instruction, or expiration of the reagent kit. Check programming of the thermocycler (see 8.3.2.), storage conditions, and the expiration date of reagents and repeat PCR once again for all samples.

2. If no signal was detected either in the channel for detection of the pathogen DNA or in the channel for detection of IC, the sample should be examined once again (PCR and detection). The same applies to the samples with equivocal results, because the fact that the specific signal does not exceed the threshold value is not sufficient to consider a sample as positive. If equivocal results are obtained in the second run, the analysis should be repeated starting from the DNA extraction stage.
3. Positive signal in C– and NCA indicates contamination of reagents or samples. In this case, the results of analysis must be considered as invalid. Repeat analysis and take measures to detect and eliminate the source of contamination.

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/H1-swine-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/H1-swine-FEP** PCR kit are to be stored at 2–8 °C when not in use. All components of the **AmpliSens® *Influenza virus* A/H1-swine-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/H1-swine is to be kept away from light.

13. SPECIFICATIONS

13.1. Sensitivity

The analytical sensitivity of **AmpliSens® *Influenza virus* A/H1-swine-FEP** PCR kit is following:

Clinical material	Nucleic acid extraction kit	Sensitivity, copies/ml
Nasal and oropharyngeal swabs [1]	RIBO-prep	$1x10^3$
	RIBO-sorb	$5x10^3$

13.2. Specificity

[1] Nasal and oropharyngeal swabs should be placed into the Transport Medium for Storage and Transportation of Respiratory Swabs (REF 957-CE, 958-CE, 959-CE).

The analytical specificity of **AmpliSens®** ***Influenza virus*** **A/H1-swine-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 deposited in gene banks by sequence comparison analysis. The clinical specificity of **AmpliSens®** ***Influenza virus*** **A/H1-swine-FEP** PCR kit was confirmed in laboratory clinical trials.

AmpliSens® ***Influenza virus*** **A/H1-swine-FEP** PCR kit determines the fragment of the *Influenza virus* A/H1N1 hemagglutinin gene, which is related to the North American strain of swine *Influenza virus*. The specific activity of the PCR kit was confirmed by analyzing A/California/04/2009(H1N1) and A/California/07/2009(H1N1) isolates provided by CDC.

Nonspecific reactions were absent is tests of 26 reference strains and isolates of epidemic *Influenza viruses* A/H1N1 isolated in 1977 to 2008 in the Russian Federation, Ukraine and Belarus Republics; *Influenza virus* A subtypes H13N2, H9N2, H8N4, H2N3, H2N9, H3N2, H3N8, H4N6, H11N6, H12N5, H1N1, H6N2, H10N7, H5N3, H7N1, H5N2, H5N3, and H2N2; *Influenza virus* B of Yamagata and Victoria strain, as well as in tests of strains and isolates of the main causative agents of human ARD and human genome nucleic acids.

14. REFERENCES

1. Handbook “Sampling, Transportation, and Storage of Clinical Material for PCR Diagnostics”, developed by Federal Budget Institution of Science “Central Research Institute for Epidemiology” of Federal Service for Surveillance on Consumers’ Rights Protection and Human Well-Being, Moscow, 2008.
2. Guidelines “End-Point PCR Detection of *Influenza virus* A/H1N1(sw2009) RNA”, developed by Federal Budget Institution of Science “Central Research Institute for Epidemiology” of Federal Service for Surveillance on Consumers’ Rights Protection and Human Well-Being, Moscow.

15. QUALITY CONTROL

In compliance with Federal Budget Institution of Science “Central Research Institute for Epidemiology” ISO 13485-Certified Quality Management System, each lot of **AmpliSens®** ***Influenza virus*** **A/H1-swine-FEP** 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		Sufficient for
IVD	*In vitro* diagnostic medical device		Expiration Date
VER	Version		Consult instructions for use
	Temperature limitation		Keep away from sunlight
	Upper limit of temperature	**NCA**	Negative control of amplification
	Manufacturer	**C–**	Negative control of extraction
	Date of manufacture	**C+**	Positive control of amplification
EC REP	Authorised representative in the European Community	**IC**	Internal control
CS+	Positive Control of Amplification of IC	**PCE**	Positive control of extraction
FBIS CRIE	Federal Budget Institution of Science “Central Research Institute for Epidemiology”		

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
13.12.10	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/H1-swine is to be kept away from light was added
	Key to Symbols Used	The explanation of symbols was corrected
23.06.11 RT	Cover page, text	The name of Institution was changed to Federal Budget Institution of Science “Central Research Institute for Epidemiology”