AmpliSens® *Influenza virus* A/B-FRT PCR kit

RUO

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number	Σ	Contains sufficient for <n> tests
LOT	Batch code		Use-by-Date
RUO	Research Use Only		Consult instructions for use
VER	Version		Keep away from sunlight
	Temperature limit	NCA	Negative control of amplification
	Manufacturer	C-	Negative control of extraction
	Date of manufacture	$C+_{A/B/STI}$	Positive control of amplification
	Caution	IC	Internal control

1. INTENDED USE

AmpliSens® *Influenza virus* A/B-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative detection and differentiation of RNA of *Influenza virus* A and *Influenza virus* B in the biological material (nasopharyngeal and oropharyngeal swabs; sputum / oropharyngeal or tracheal aspirate; bronchoalveolar lavage / bronchial washing fluid, autopsy material) and viral culture using real-time hybridization-fluorescence detection of amplified products. The material for PCR is RNA samples extracted from the test material and subjected to reverse transcription step.

There are no restrictions on the use of the medical device in different population and demographic groups.

Indications and contra-indications for use of the reagent kit

The reagent kit can be used with suspected influenza without distinction of form and presence of manifestation and for postmortem identification of *Influenza virus* A RNA and *Influenza virus* B RNA in autopsy material.

There are no contra-indications with the exception of cases when the material cannot be taken for medical reasons.

NOTE: For research use only. Not for diagnostic procedures.

2. PRINCIPLE OF PCR DETECTION

Principle of testing is based on the RNA extraction from test material together with the exogenous internal control sample (Internal Control-FL (IC)), RNA reverse transcription and amplification of cDNA fragments of the detected virus and cDNA of the Internal control with hybridization-fluorescence detection. Exogenous internal control (Internal Control-FL (IC)) allows to control all PCR-analysis stages of each individual sample and to identify possible reaction inhibition. RNA reverse transcription is performed with the use of TM-Revertase enzyme. Amplification of cDNA fragments is performed with the use of specific primers and Taq-polymerase enzyme.

In the real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes which bind specifically to the amplified product. 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/B-FRT PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by using chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

The PCR kit contains the system for prevention of contamination by amplicons using the enzyme uracil-DNA-glycosylase (UDG) and dUTP.

At the PCR stage 3 cDNA target are amplified simultaneously in one tube. The results of amplification are registered in the following fluorescence channels:

Table 1

Channel for fluorophore	FAM	JOE	ROX
cDNA-target	Internal Control-FL cDNA	*Influenza virus* B cDNA	*Influenza virus* A cDNA
Target gene	Artificially synthesized sequence	NS gene of *Influenza virus* B	M1 gene of *Influenza virus* A

3. CONTENT

AmpliSens® *Influenza virus* A/B-FRT PCR kit is produced in 2 forms:

variant FRT-100 F, REF R-V36-100-F-Mod(RG,iQ,Dt,CFX,SC)-CE;

variant FRT-100 F in bulk[1], REF R-V36-100-F-Mod(RG,iQ,Dt,CFX,SC)-CE-B.

Variant FRT-100 F includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-1-FL *Influenza virus* A/B	clear liquid from colorless to light lilac colour	1.2	1 tube
PCR-buffer-B	colorless clear liquid	0.6	1 tube
Polymerase (TaqF)	colorless clear liquid	0.06	1 tube
Positive Control cDNA *Influenza virus* A / *Influenza virus* B / STI ($C+_{A/B/STI}$)	colorless clear liquid	0.2	1 tube
TE-buffer	colorless clear liquid	0.2	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	2 tubes
Internal Control-FL (IC)**	colorless clear liquid	0.6	2 tubes

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

** add **10 µl** of **Internal Control-FL (IC)** during the RNA extraction procedure directly to the sample/lysis mixture (see **RIBO-prep** or **MAGNO-sorb** protocol).

Variant FRT-100 F is intended for 110 reactions (including controls).

4. ADDITIONAL REQUIREMENTS

For sampling and pretreatment

- Transport medium for storage and transportation of respiratory swabs.
- Reagent for pretreatment of viscous fluids (sputum, aspirates).
- 0.9 % of sodium chloride (sterile saline solution) or phosphate buffered saline (PBS) (137 mM sodium chloride; 2,7 mM potassium chloride; 10 mM sodium monophosphate; 2 mM potassium diphosphate; pH=7,5±0,2) for pretreatment of autopsy material or in case of viral culture testing.
- Flocked or fiber swabs for collecting specimens from kids and adults.
- Plastic container (50-60 ml) for storage and transportation of biological samples.
- Disposable tightly closed polypropylene 2.0-ml tubes for sampling and pretreatment.
- Sterile RNase-free pipette tips with aerosol filters (up to 100, 200 and 1000 µl).
- Sterile RNase-free pipette tips without aerosol filters (up to 200 µl).
- Sterile tools (individual for each sample) for homogenization (porcelain mortar and mallet) or homogenizer for pretreatment of viscera material.
- Desktop centrifuge up to 12,000 g (suitable for Eppendorf tubes).
- Vacuum aspirator with flask for removing supernatant.
- Vortex mixer.
- Tube racks.
- Pipettes (adjustable).
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir to throw off and inactivate the material.
- Disposable powder-free gloves and laboratory coat.

For RNA extraction, reverse transcription and amplification

- RNA/DNA extraction kit or Automatic station for RNA/DNA extraction based on magnetic beads with MAGNO-sorb Nucleic Acid Extraction kit.
- Set of consumables for the used automated station according to the manufacturer's recommendations.
- Reverse transcription kit.
- Sterile RNase-free pipette tips with aerosol filters (up to 10, 100 and 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with a rotor for 2-ml reaction tubes.
- PCR box.
- Real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia); Rotor-Gene Q (QIAGEN, Germany); CFX 96 (Bio-Rad, USA)).
- Disposable polypropylene 0.2- or 0.1-ml tubes for PCR:
  a) screwed or tightly closed 1.5-ml tubes for reaction mixture preparation;
  b) thin-walled 0.2-ml PCR tubes with optical transparent domed or flat caps or strips of eight 0.2-ml tubes with optical transparent caps if a plate-type instrument is used;
  c) thin-walled 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.
- Pipettes (adjustable).
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir for used tips.
- Disposable powder-free gloves and laboratory coat.

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 (specimens, 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 afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use the PCR kit if the internal packaging was damaged or its appearance was changed.
- Do not use the PCR kit if the transportation and storage conditions according to the Instruction Manual were not observed.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in biological cabinet in

[1] In bulk form contains unlabeled tubes. Tubes with identical reagent are packed in one bag with label.

compliance 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 inhalation of vapors, samples and reagents contact with the skin, eyes, and mucous membranes. Harmful if swallowed. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice if necessary.
- While observing the conditions of transportation, operation and storage, there are no risks of explosion and ignition.
- Safety Data Sheets (SDS) are available on request.
- The PCR kit is intended for single use for PCR analysis of specified number of samples (see the section "Content").
- The PCR kit is ready for use in accordance with the Instruction Manual. Use the PCR kit strictly for intended purpose.
- Use of this product should be limited to personnel trained in 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 in 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

AmpliSens® *Influenza virus* A/B-FRT PCR kit is intended for analysis of RNA extracted with RNA/DNA extraction kits from the biological material:
- nasopharyngeal and oropharyngeal swabs,
- sputum / oropharyngeal or tracheal aspirate,
- bronchoalveolar lavage / bronchial washing fluid,
- autopsy material,
- viral culture.

Sampling

NOTE: Swabs from the mucous membranes of the nasopharynx and oropharynx are taken with two dry sterile probes into one tube with transport medium in sequence: first from the mucous membrane of the lower nasal passage, and then from the oropharynx, and examined as one sample.
It is not recommended to use probes made of natural materials (wood, cotton).

Nasopharyngeal swab. Before sampling, make the patient blow his nose if it is filled with mucus. A dry sterile flocked swab is inserted along the lateral nasal wall horizontally into the lower nasal passage and removed along the lateral nasal wall. In total the probe should be inserted up to the half length from a nostril to an ear hole (3-4 cm for children and 5-6 cm for adults). After taking the material, place the working part of the probe into a tube with 0.5-1.0 ml of transport medium and, holding the tube lid, break off the probe handle so that it allows to close the tube tightly.

Oropharyngeal swab is taken with a sterile dry probe by rotary movements from the surface of tonsils, palatine glands and the posterior wall of the oropharynx. After taking the material, place the working part of the probe into the tube containing transport medium and a nasal swab. The end of the probe is broken off, holding the tube lid with the calculation that it allows to close the tube tightly. The tube is hermetically sealed, shaken to moisten both probes, and labeled.
Nasopharyngeal and oropharyngeal mucosal swabs can be stored prior to pretreatment:
- at the temperature from 2 to 8 °C – no more than 3 days;
- at the temperature from minus 24 to minus 16 °C – for 1 year.

Only one freeze–thaw cycle is allowed.

Sputum / oropharyngeal or tracheal aspirate. Sputum is collected into a sterile disposable plastic container. If it is not possible to obtain sputum or aspirate from the pharynx, saliva produced in the morning after deep coughing immediately after awakening is collected.
Sputum / oropharyngeal or tracheal aspirate can be stored prior to pretreatment:
- at the temperature from 2 to 8 °C – no more than 1 day;
- at the temperature from minus 24 to minus 16 °C - for 1 year.

Only one freeze–thaw cycle is allowed.

Bronchoalveolar lavage and bronchial washing fluid of at least 5-ml volume are collected into a disposable, tightly screwed polypropylene container.
Bronchoalveolar lavage and bronchial washing fluid can be stored prior to pretreatment:
- at the temperature from 2 to 8 °C – no more than 1 day;
- at the temperature from minus 24 to minus 16 °C - for 1 year.

Only one freeze–thaw cycle is allowed.

Autopsy material should be placed into a sterile disposable container and analyzed within 1 hour otherwise it should be frozen immediately after sampling.
Autopsy material can be stored prior to pretreatment:
- at the temperature from minus 24 to minus 16 °C - for 1 year.

Only one freeze-thaw cycle is allowed.

Viral culture of 1.0 ml volume is taken from the original vial and transferred to a test tube using a Pasteur pipette.
Viral culture can be stored prior to pretreatment:
- at the temperature from 18 to 25 °C – for 24 hours;
- at the temperature from 2 to 8 °C – for 7 days;
- at the temperature from minus 24 to minus 16 °C - for 1 year.

Only one freeze–thaw cycle is allowed.
It is allowed to transport the above material at temperature from 2 to 8 °C for 3 days.

Pretreatment

Nasopharyngeal and oropharyngeal swabs; sputum/oropharyngeal or tracheal aspirate; bronchoalveolar lavage/bronchial washing fluid, autopsy material are to be pretreated.
Pretreatment of viral culture is not required.

Nasopharyngeal and oropharyngeal swabs. Vortex the tube for 5 s to sediment drops from the interior wall of the tube cap. If the liquid level in the tube is below 0.5 ml, the volume should be increased by adding 0.5 ml of saline solution, vortex the tube for 5 s to sediment drops from the interior wall of the tube cap. If there is mucus in the sample, **Mucolysin** reagent should be added up to the mark of 1 ml. Incubate at the room temperature (from 18 to 25 °C) for 5 min (until visual clarification) stirring occasionally on vortex. Centrifuge the samples at 600 rpm for 5 min in case of precipitation. 100 µl of sample is used for RNA extraction. The residual sample should be frozen if it is necessary to repeat the analysis.

Sputum / pharyngeal or tracheal aspirate. To reduce viscosity of the material (if required), use a reagent for pretreatment of viscous fluids (for example **Mucolysin**, produced by FBIS CRIE) in accordance wtih manufacturer's instructions. The prepared sample (100 µl) is used for RNA extraction. The remained sample can be frozen for further use. Use **Mucolysin** reagent for viscous sputum / pharyngeal or tracheal aspirate pretreatment. In order to reduce viscosity in the container with the sample five times the amount of reagent **Mucolysin** relative to the amount of sample is added and incubated at room temperature (from 18 to 25 ° C) with periodic stirring for 10-20 minutes (until visual clarification). Transfer the sample to a 1.5-ml tube and centrifuge at 600 rpm for 5 min in case of precipitation. 100 µl of supernatant is used for RNA extraction. The residual sample should be frozen if it is necessary to repeat the analysis.

Bronchoalveolar lavage and bronchial washing fluid. The contents of the container are mixed by pipetting with a filter tip and 100 µl is taken for RNA extraction.

Autopsy material. Homogenize the material with a sterile porcelain mortar and pestle. Then, prepare 10 % suspension in sterile saline or phosphate buffer. Transfer the suspension into a 1.5-ml tube, centrifuge at 6,700 rpm for 5 min, and use the supernatant (100 µl) for RNA extraction. The remained suspension should be frozen if it is necessary to repeat the analysis.
The pretreated samples of nasopharyngeal and oropharyngeal swabs, sputum / pharyngeal or tracheal aspirate, bronchoalveolar lavage / bronchial washing fluids, autopsy material can be stored before the PCR-analysis:
- at the temperature from 18 to 25 °C – for 6 hours;
- at the temperature from 2 to 8 °C – for 1 day;
- at the temperature from minus 24 to minus 16 °C – for 1 year;

Only one freeze-thawing cycle is required.

Interfering substances and limitations of using test material samples

In order to control the efficiency of RNA extraction and amplification the Internal Control (Internal Control STI-87-rec (IC)) is used in the PCR kit. The Internal Control is added in each biological sample at the extraction stage. The presence of internal control signal after the amplification testifies the effectiveness of nucleic acid extraction and the absence of PCR inhibitors.
Potential interfering substances
Endogenous and exogenous substances that may be present in the biological material used for the study were selected to assess potential interference (see Table 2).
Model samples of nasopharyngeal and oropharyngeal mucosal swabs, sputum / pharyngeal or tracheal aspirate, bronchoalveolar lavage / bronchial lavage water, autopsy material without and with the addition of potentially interfering substances were tested. The concentration of each potentially interfering substance is listed in Table 2. Quality control samples (QCS) containing *Influenza virus* A RNA and *Influenza virus* B RNA were also added to the model samples, with the concentration of each target equal to $5x10^3$ GE/ml.
The results obtained with RIBO-prep and MAGNO-sorb extraction kits are presented in Table 6.

Table 2

Test material	Type of potential interferent	Potential interferent	Tested concentration in a sample	Interference presence	
				RIBO-prep	MAGNO-sorb
Nasopharyngeal and oropharyngeal swabs	Endogenous substances	Mucin	6 mg/ml	Not detected	Not tested
			9 mg/ml	Detected	Not detected
		Hemoglobin	0.21 g/ml	Not detected	Not detected
	Exogenous substances	Chlorhexidine aqueuos solution	2.5 %	Not detected	Not detected
Sputum / pharyngeal or tracheal aspirate	Endogenous substances	Mucin	6 mg/ml	Not detected	Not tested
			9 mg/ml	Detected	Not detected
Bronchoalveolar lavage / bronchial washing fluids	Endogenous substances	Mucin	6 mg/ml	Not detected	Not tested
			9 mg/ml	Detected	Not detected
		Hemoglobin	0.21 g/ml	Not detected	Not detected
Autopsy material	Endogenous substances	Hemoglobin	0.21 g/ml	Not detected	Not detected

7. WORKING CONDITIONS

AmpliSens® *Influenza virus* A/B-FRT PCR kit should be used at the temperature from 20 to 28 °C and relative humidity from 15 to 75 %.

8. PROTOCOL

8.1. RNA extraction

NOTE: Only sterile disposable plastic consumables with special RNase-free, DNase-free markings should be used for work with RNA.

It is recommended to use the following nucleic acid extraction kits:
- **RIBO-prep**,
- **MAGNO-sorb** in combination with "open type" automatic nucleic acid extraction stations.

The RNA extraction of each test sample is carried out in the presence of **Internal Control-FL (IC)**.

If using the RIBO-prep reagent kit extract the RNA according to the manufacturer's protocol.
The volumes of reagents and samples when the RNA is extracted by the RIBO-prep reagent kit:
Add **10 µl** of **Internal Control-FL (IC)** to each tube.
The volume of the test sample is **100 µl** except viral culture.
The volume of the test sample of viral culture is 90 µl of Negative Control (C–) and 10 µl of viral culture.
Add **100 µl** of **Negative Control (C–)** to the tube labeled C– (Negative Control of Extraction).
The volume of elution is **90 µl**.

If using the MAGNO-sorb reagent kit in combination with "open type" automatic nucleic acid extraction stations extract the RNA according to the manufacturer's protocol and corresponding extraction protocol.
The volumes of reagents and samples when the RNA is extracted by the MAGNO-sorb reagent kit in combination with "open type" automatic nucleic acid extraction stations:
The volume of **Internal Control-FL (IC)** is **10 µl** per sample.
The volume of the test sample is **100 µl** except viral culture.
The volume of the test sample of viral culture is **90 µl of Negative Control (C–)** and **10 µl of viral culture**.
Add **100 µl** of **Negative Control (C–)** to the tube labeled C– (Negative Control of Extraction).
The volume of elution is **100 µl**.

NOTE: It is recommended to carry out the reverse transcription just after obtaining the RNA samples. It is allowed to store the RNA samples at the temperature from 2 to 8 °C for 30 min, at the temperature from minus 24 to minus 16 °C for 1 week and at the temperature not more than minus 68 °C for 1 year. Only one freeze-thawing cycle is required.

8.2. Reverse transcription

NOTE: Only sterile disposable plastic consumables with special RNase-free, DNase-free markings should be used for work with RNA.

It is recommended to use the following kit for complementary DNA (cDNA) synthesis from RNA:
- **REVERTA-L**.

NOTE: Carry out the reverse transcription according to the manufacturer's protocol.

8.3. Preparing PCR

8.3.1 Preparing tubes for PCR

The total reaction volume is **25 µl** the volume of cDNA sample is **10 µl**.
The type of tubes depends on the PCR instrument used for analysis. Use disposable filter tips for adding reagents, cDNA and control samples into tubes.
1. Calculate the required quantity of each reagent for reaction mixture preparation. For one reaction:
   - **10 µl of PCR-mix-1-FL *Influenza virus* A/B**,

– **5.0 µl of PCR-buffer-B**,
– **0.5 µl of polymerase (TaqF)**.

2. Prepare the reaction mixture for the total number of test and control samples plus some extra reaction. See numbers of control samples in item 7.

The calculation for the required number of reactions can be performed according to Table 3.

Table 3

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[2]	**PCR-mix-1-FL *Influenza virus* A/B**	**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**

NOTE: Prepare the reaction mixture just before use.

3. Thaw the tube with **PCR-mix-1-FL *Influenza virus* A/B**. Thoroughly vortex the tubes with **PCR-mix-1-FL *Influenza virus* A/B, PCR-buffer-B** and **polymerase (TaqF)**, sediment the drops by vortex.
4. In a new tube prepare the reaction mixture. Mix the required quantities of **PCR-mix-1-FL *Influenza virus* A/B, PCR-buffer-B** and **polymerase (TaqF)**. Sediment the drops by vortex.
5. Take the required number of the tubes/strips for cDNA amplification of test and control samples.
6. Transfer **15 µl** of the prepared reaction mixture to each tube. Discard the unused reaction mixture.
7. Add **10 µl of cDNA samples** obtained at the RNA extraction and reverse transcription stages to the prepared tubes.
8. Carry out the control reactions:

C+$_{A/B/STI}$ – Add **10 µl** of **Positive Control cDNA *Influenza virus* A / *Influenza virus* B / STI (C+**$_{A/B/STI}$**)** to the tube labeled C+$_{A/B/STI}$

C– – Add **10 µl** of **the sample extracted from the Negative Control (C–) reagent** to the tube labeled C–

NOTE: It is necessary to carry out the control reaction for Negative Control of Amplification (NCA) for a new lot of reagent kit.

To rule out possible contamination, carry out an additional control reaction for Negative Control of Amplification:

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

8.3.2. Amplification

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

Table 4

Amplification program for *Influenza virus* A/B cDNA

Step	Rotor-type instruments[3]			Plate-type instruments[4]		
	Temperature, °C	**Time**	**Cycles**	**Temperature, °C**	**Time**	**Cycles**
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 и ROX fluorophores.

2. Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin.*
3. Insert tubes into the reaction module of the device.

NOTE: It is recommended to sediment drops from walls of tubes by short centrifugation (1–3 s) before placing them into the instrument.
Insert empty tubes at the edges of reaction module in case of incomplete filling of 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:

Table 6

Channel for the fluorophore	**FAM**	**JOE**	**ROX**
Amplification product	Internal Control-FL (IC) cDNA	*Influenza virus* B cDNA	*Influenza virus* A cDNA

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at a specific level that corresponds to the presence (or absence) of a *Ct* value of the cDNA sample in the corresponding column of the result grid.
Principle of interpretation is the following:

Table 7

Results interpretation for the test samples

***Ct* value in the channel for the fluorophore**			**Result**
FAM	**JOE**	**ROX**	
< boundary value	absent	absent	*Influenza virus* A and B RNA is **NOT detected**
determined or absent	< boundary value	determined or absent	*Influenza virus* B RNA **is detected**
determined or absent	determined or absent	< boundary value	*Influenza virus* A RNA **is detected**
absent or > boundary value	absent	absent	**Invalid***
determined or absent	> boundary value	> boundary value	**Equivocal****

* In case of **invalid** result, the PCR analysis should be repeated starting from the RNA extraction stage for the corresponding test sample.

** In case of **equivocal result** it is necessary to repeat PCR-study of the corresponding test sample, starting from the RNA extraction stage. If the same result was obtained once again, the sample is considered positive. If the negative result was obtained, the sample is considered equivocal and re-sampling of the material for analysis is recommended.

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed in the PCR kit.

The result of the analysis is considered reliable only if the results obtained for Positive and Negative Controls of amplification as well as for the Negative Control of extraction are correct (see the table below).

Table 8

Results for controls

Control	**Stage for control**	***Ct* value in the channel for fluorophore**		
		FAM	**JOE**	**ROX**
C–	RNA extraction	**<**boundary value	Absent	Absent
NCA	PCR	Absent	Absent	Absent
C+	PCR	**<**boundary value	**<**boundary value	**<**boundary value

Interpretation of some test samples is not possible if the results for the controls deviate from the results specified above (see *10.Troubleshooting*).

10. TROUBLESHOOTING

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

1. For the Positive Control of Amplification (C+):
   a) The *Ct* value determined in the channels for ROX and/or JOE fluorophores is absent or greater than the boundary value. It is not possible to interpret the results for samples in the corresponding channel. To obtain results for this channel, it is necessary to repeat amplification and detection for all samples.
   b) The *Ct* value determined in the channel for FAM fluorophore is absent or exceeds the boundary value. Interpretation of the results for the test samples is carried out according to Table 7.
2. For the Negative Control of Extraction (C–):
   a) The *Ct* value is determined in the channels for ROX and/or JOE fluorophores. The contamination of laboratory with amplification fragments or contamination of reagents / test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. PCR analysis (beginning with RNA extraction stage) should be repeated for all samples in which *Influenza virus* RNA was detected in the corresponding channel.
   b) The *Ct* value determined in the channel for FAM fluorophore is absent or exceeds the boundary value. Interpretation of the results for the tested samples is carried out according to Table 7.
3. For the Negative Control of Amplification (NCA):
   a) The *Ct* value is determined in the channels for ROX and/or JOE fluorophores. The contamination of laboratory with amplification fragments or contamination of reagents / test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. PCR analysis (beginning with RNA extraction stage) should be repeated for all samples in which *Influenza virus* RNA was detected in the corresponding channel.
   b) The *Ct* value is determined in the channels for FAM fluorophore. The contamination of laboratory with amplification fragments or contamination of reagents / test samples is probable at any stage of PCR analysis. It is not possible to interpret results for samples in which *Influenza virus* RNA was not detected. Measures for detecting and elimination of contamination source must be taken, PCR analysis should be repeated for such samples.
4. The *Ct* value is determined for the test sample, whereas the area of typical exponential growth of fluorescence (in the "raw" data view mode) is absent (the graphic looks like approximate straight line). It is necessary to check the correctness of selected threshold line level or parameters of base line calculation. If the result has been obtained with the correct level of threshold line (base line), or when analyzing the results in automatic mode, the amplification and detection should be repeated for this sample.

11. TRANSPORTATION

AmpliSens® *Influenza virus* A/B-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/B-FRT** PCR kit are to be stored at 2–8 °C when not in use (except for PCR-mix-1-FL *Influenza virus* A/B, PCR-buffer-B, and polymerase (TaqF)). All components of the **AmpliSens® *Influenza virus* A/B-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.

NOTE: PCR-mix-1-FL *Influenza virus* A/B, PCR-buffer-B, and polymerase (TaqF) are to be stored at the temperature from minus 24 to minus 16 °C.

NOTE: PCR-mix-1-FL *Influenza virus* A/B is to be kept away from light

[2] Number of test samples including the control of RNA extraction stage (N), controls of amplification, and one extra reaction (N+3+1).
[3] For example, Rotor-Gene 3000/6000 (Corbett Research), Rotor-Gene Q (QIAGEN GmbH).
[4] For example, CFX96 (Bio-Rad, USA).

13. SPECIFICATIONS

13.1. Analytical sensitivity (limit of detection)

Biological material	Pathogen agent	Analytical sensitivity (limit of detection), GE/ml[5]
Nasopharyngeal and oropharyngeal swabs, sputum / oropharyngeal or tracheal aspirate; bronchoalveolar lavage / bronchial washing fluid, autopsy materia, viral culture	*Influenza virus* A	1×10^3
	Influenza virus B	

The claimed features are achieved while respecting the rules specified in the section "*Sampling and Handling".*

13.2. Analytical specificity

The analytical specificity of **AmpliSens® *Influenza virus* A/B-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 reagent kit allows detection of cDNA fragments of *Influenza virus* A and *Influenza virus* B in the study of strains of Smorodintsev Research Institute of Influenza collection: A/Saint Petersburg/NIIG-252/19 (Influenza virus A (H3N2)), A/Kaliningrad/75/19 (Influenza virus A (H1N1)pdm09), B/Washington/02/19 (Influenza virus B Lineage Victoria), B/Yakutsk/NIIG-06/2019 (Influenza virus B Lineage Yamagata) in concentration no less than $1x10^3$ GE/ml.

Analytical specificity of the reagent kit was proven by testing of RNA/DNA samples of the following microorganisms as well as human genomic DNA:

- strains from the ATCC® collection (American Type Culture Collection, USA) in the concentration of no more than $1x10^9$ GE/ml and at least $1x10^6$ GE/ml: *Streptococcus pneumoniae* (ATCC® 49619™), *Staphylococcus aureus* subsp. *aureus*, Strain Seattle 1945 (ATCC® 25923™), *Pseudomonas aeruginosa* (ATCC® 9027™), *Moraxella catarrhalis* (ATCC® 8193™), *Neisseria mucosa* (ATCC® 19693™), *Enterococcus faecalis* (ATCC® 19433™), *Mycoplasma pneumoniae*, Strain PI 1428 (ATCC® 29085™), *Chlamydophila pneumoniae*, Strain CM-1 (ATCC® VR-1360™), *Legionella pneumophila* subsp. *pneumophila*, Strain Philadelphia 1 (ATCC® 33152™), *Staphylococcus epidermidis*, FDA Strain PCI 1200 (ATCC® 12228™), *Bacillus cereus*, Strain FDA 5 (ATCC® 10702™), *Human Respiratory Syncytial Virus*, Strain 9320 (ATCC® VR-955™), *Human Respiratory Syncytial Virus*, Strain A-2 (ATCC® VR-1540™), *Human Parainfluenza Virus* 1, Strain C35 (ATCC® VR-94™), *Human Parainfluenza Virus* 2, Strain Greer (ATCC® VR-92™), *Human Parainfluenza Virus* 3, Strain C243 (ATCC® VR-93™), *Human Rhinovirus* 17, Strain 33342 (ATCC® VR-1663™), *Human Adenovirus* 1, Strain Adenoid 71 (ATCC® VR-1™), *Human Coronavirus*, Strain OC43 (ATCC® VR-1558™), *Human Coronavirus*, Strain 229E (ATCC® VR-740™), *Human Coxsackievirus* B1, Strain Conn-5 (ATCC® VR-28™), *Human Echovirus* 4, Strain Pesascek (ATCC® VR-1734™), *Human Herpesvirus* 1, Strain HF (ATCC® VR-260™);
- strains from GCPM (State collection of pathogenic microorganisms) collection with concentration no more than $1x10^9$ GE/ml and at least $1x10^6$ GE/ml: *Haemophilus influenzae* 423, *Streptococcus pyogenes* Dick – I, *Corynebacterium pseudodiphtheriticum* №25, *Proteus mirabilis* 3177, *Klebsiella pneumoniae* 418, *Escherichia coli* M 17, *Salmonella typhimurium* 79, *Yersinia enterocolitica* 134, *Bordetella pertussis* 703 L 6, *Mycobacterium Bovis Ravenel* №700204;
- samples of the panel "CORONAVIRUS RNA SPECIFICITY PANEL" (Erasmus Medical Center - EMC, Rotterdam, The Netherlands) with concentration no more than $1x10^9$ GE/ml and at least $1x10^7$ GE/ml: severe acute respiratory syndrome *coronavirus* Strain HKU39849, Middle East respiratory syndrome *coronavirus*, *Human coronavirus*, Strain NL63;
- human DNA at a concentration of 0.2 mg/ml.

The nonspecific reactions were absent while testing RNA/DNA samples of the above-mentioned microorganisms/viruses, as well as human DNA.

The information about interfering substances is specified in the *Interfering substances and limitations of using test material samples*.

13.3. Reproducibility and repeatability

Repeatability and reproducibility were determined by testing positive and negative model samples. Positive samples were a quality control sample (QCS) containing *Influenza virus A* RNA and *Influenza virus B* RNA with a concentration of $5x10^3$ copies/ml, Negative control (C–) reagent was used as a negative sample.

Repeatability conditions included testing in the same laboratory, by the same operator, using the same equipment within a short period of time. Reproducibility conditions included testing in two independent laboratories, by different operators, on different days, on different equipment and different reagent kit series. The results are presented in Table 9.

Table 9

Sample type	Repeatability		Reproducibility	
	Number of samples	Agreement of results, %	Number of samples	Agreement of results, %
Influenza virus A	10	100	30	100
Influenza virus B	10	100	30	100
Negative	10	100	30	100

14. REFERENCES

1. Global Influenza Programme, WHO https://www.who.int/teams/global-influenza-programme/surveillance-and-monitoring/influenza-updates.

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 **AmpliSens® *Influenza virus* A/B-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
18.04.24 BA	Through the text	Corrections according to the template
	3. Content	The volume of TE-buffer was changed from 0.5 ml to 0.2 ml. Internal Control STI-rec (IC) (0.12 ml x 10 tubes) was changed to Internal Control-FL (IC) (0.6 ml x 1 tube), PCR-mix-1-FL-F *Influenza virus* A/B (0.2 ml x 5 tubes) was changed to PCR-mix-1-FL *Influenza virus* A/B (1.2 ml x 1 tube). Name of PCR-mix-2-FRT was changed to PCR-buffer-B Quantity of reactions (including controls) was changed (110 reactions instead of 100)
	4. Additional requirements	The section was actualized and updated with materials and instruments. Mx3000P, SmartCycler II, CFX 96 and iCycler iQ /iQ5 were deleted
	6. Sampling and handling	The information about sampling and handling was expanded. The subsection *Interfering substances and limitations of using test material samples* was added
	8. Protocol	NucliSENS easyMAG automated, RIBO-sorb were deleted. The section was updated with the information about extraction using RIBO-prep and MAGNO-sorb extraction kits
	9. Data analysis	Information on the correspondence of the amplification product and channels for the fluorophore, the principle of results interpretation for the test samples are presented in tables
	10. Troubleshooting	The section was rewritten
	13. Specifications	The "13.1. Analytical sensitivity (limit of detection)" and "13.2. Analytical specificity" subsections were actualized. The "13.3. Reproducibility and repeatability" subsection was added
	14. References	The section was actualized

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[5] Genome equivalents (GE) of the pathogen agent per 1 ml of a sample.