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332-2 2022-10-26

For research use only

Influenza A virus, subtype H5N1 PCR with reverse transcription Kit

REF **F3-P407-51/2EU**
R3-P407-23/4EU
R3-P407-S3/4EU

General information

Intended use:

The Kit is intended for detection of RNA specific to Influenza A virus subtype H5N1 in the human and animal biological samples.
The Kit can be used in scientific research practice.

Method:
Reverse transcription followed by real-time PCR qualitative assay.

Samples:
Nasal and guttur smears and lavage from human.
Tracheal, nasal, pharyngeal, cloacal smears and lavage, as well as fecal and visceral organs samples from dead and diseased animals.

RNA extraction:
The DNA-Technology's **PREP-NA DNA/RNA Extraction Kit** is recommended.

Features:
The RNA-IC control is intended for quality control of the all stages of the assay.
Positive control plasmid (C+) supplied with the kit is intended for specific PCR assessment.
We also recommend including in assay the negative control (C-) which is not supplied but very helpful for contamination control purposes.
The kit can be supplied in one of the two variants:
The Influenza A virus, subtype H5N1 FLASH PCR Detection Kit – is intended for end-point qualitative evaluation with an aid of fluorescence reader
and
The Influenza A virus, subtype H5N1 Real-Time PCR Detection Kit– for real-time qualitative evaluation with an aid of real-time thermal cyclers.

Devices:
The automatic analysis for Influenza A virus, subtype H5N1 (avian flu) PCR with reverse transcription Kit is available on "DNA-Technology" made Tercyc Thermal Cycler and Gene or Gene-4 Fluorescence Readers or DTlite[1] and DTprime[2] REAL-TIME Thermal Cyclers; the latest version of the software is available for download at https://www.dna-technology.com/software.
The Influenza A virus, subtype H5N1 (avian flu) PCR with reverse transcription Kit is also approved for use with iQ (Bio-Rad Laboratories) real-time thermal cyclers.

Time of analysis (including sample preparation procedure):
from 5 hours.

Number of tests:

48[3]/50[4] (including positive and negative control samples in each run).

Dye label detection channels

Fam	Hex	Rox	Cy5	Cy5.5
Influenza A-H5 virus	RNA-IC	-	-	-
Influenza A-N1 virus	RNA-IC	-	-	-

[1] - supported by 4S1, 4S2, 5S1, 5S2, 6S1, 6S2 instruments.
[2] supported by 4M1, 4M3, 4M6, 5M1, 5M3, 5M6, 6M1, 6M3, 6M6 instruments.
[3] – Real-time version (REF R3-P407-23/4EU, REF R3-P407-S3/4EU)
[4] – FLASH version (REF F3-P407-51/2EU)

Kit contents:

Reagent	Organoleptic parameters	Quantity	
Reverse Transcription Kit			
1. RT-buffer	Transparent colorless liquid	100 µL	1 tube
2. RT-H5N1 primers and dNTP's	Transparent colorless liquid	50 µL	1 tube
3. Reverse transcriptase	Transparent colorless viscous liquid	25 µL	1 tube
PCR-amplification Kit			
1. Virus A-H5 Paraffin sealed PCR-mix	Transparent colorless liquid under white wax layer	20 µL in each	48/50 tubes or 6 8-tubes strips
2. Virus A-N1 Paraffin sealed PCR-mix	Transparent colorless liquid under white wax layer	20 µL in each	48/50 tubes or 6 8-tubes strips
3. PCR-buffer	Transparent colorless liquid	500 µL	2 tubes
4. Taq-polymerase	Transparent colorless viscous liquid	50 µL	1 tube
5. Mineral oil	Transparent colorless viscous oily liquid	1.0 mL	2 tubes
6. Positive control H5	Transparent colorless liquid	75 µL	1 tube
7. Positive control N1	Transparent colorless liquid	75 µL	1 tube
Associated accessories:			
Strip's caps[5]			12 8-caps

Procedure

1 RNA extraction

⚠ Independently of DNA/RNA extraction kit used, a negative control sample should go through all stages of RNA extraction simultaneously with the RNA extraction from clinical samples.
Physiological saline solution can be used as a negative control sample in volumes as indicated in the instructions for use of extraction kits or negative control sample that is included in the corresponding extraction kit.

Perform sample preparation procedure according to the instruction to RNA-extraction kit (DNA Technology's **PREP-NA DNA/RNA Extraction Kit**).

⚠ Use RNase and DNase free pipette tips only.

Dissolve the RNA in 50 µL of buffer solution after drying-up when using **PREP-NA** kit.

The lysis buffer supplied with **PREP-NA** kit can contain the precipitate. Dissolve it at 65 °C for 10 min. prior to use.

2 Reverse transcription

2.1 Mark the required number of 0.5 mL tubes for each sample to be tested and for negative control (C-).
2.2 Thaw content of RT-buffer and RT-H5N1 primers and dNTP's tubes from Reverse Transcription Kit at room temperature (from 18 °C to 25 °C), then vortex thoroughly. Then spin briefly for 3-5 s.

⚠ The RT-buffer supplied with **PREP-NA** kit can contain the precipitate. Dissolve it at 18-25 °C prior to use.

2.3 Prepare RT-mix. Add to the one tube:
- 2.0 x (N+1) µL of RT-buffer,
- 1.0 x (N+1) µL of RT-H5N1 primers and dNTP's,
- 0.5 x (N+1) µL of reverse transcriptase,

N – is a quantity of the samples to be tested taking to account "C-".

Example: to test 5 samples, mark 6 tubes. Prepare RT-mix for 7 (6+1) tubes. Mix 14 µL of RT-buffer, 7.0 µL of RT-H5N1 primers and dNTP's and 3.5 µL of reverse transcriptase.

⚠ Reverse transcriptase should be kept out of freezer for as short time as possible.

2.4 Vortex the tube with RT-mix. Then spin briefly for 3-5 s.
2.5 Add 3.5 µL of RT-mix into each tube.
2.6 Add 16.5 µL of corresponding RNA sample, using new tip for each sample. Do not add RNA in negative control tube (C-).
2.7 Add 16.5 µL of negative control which passed all steps of RNA extraction procedure in "C-" tube.
2.8 Vortex the tubes and spin down the drops for 3-5 s.
2.9 Place tubes in thermostat and incubate at 40 °C for 30 min, then heat up to 95 °C and leave for 5 min.

⚠ Use "DNA-Technology" Gnom Programmable thermostat or similar thermostats with clamping cover.

2.10 Spin the tubes at RCF(g) 16000 for 30 s to collect drops.

The resulting cDNA preparation is ready for the PCR amplification.

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[5] - for detection kit packaged in strips REF R3-P407-S3/4EU

3 PCR amplification

The reagents and tubes should be kept away from direct sun light!

Strictly observe the completeness of the strips and caps to them. Do not use the caps for the strips of the other kits!

3.1 Mark two tubes with PCR-mixes (one with Virus A-H5 Paraffin sealed PCR-mix and one with Virus A-N1 Paraffin sealed PCR-mix) for each sample to be tested, for negative control (C-) and for positive control (C+).

Example: for simultaneous testing of 4 samples in one PCR run, mark 8 tubes for the samples, 2 for "C-" and 2 for "C+". The total number of tubes is 12.

Mark two additional tubes with Virus A-H5 Paraffin sealed PCR-mix and two with Virus A-N1 Paraffin sealed PCR-mix when using the Influenza A virus, subtype H5N1 FLASH PCR Detection Kit. These four tubes should be used as background while detection procedure (see Table 1). All remarks regarding background tubes hereafter should be concerned only in case of using FLASH version of the kit.

Table 1.The example of how to mark tubes

	Virus A-H5 Paraffin sealed PCR-mix	Virus A-N1 Paraffin sealed PCR-mix
Sample	√	√
"C-"	√	√
"C+"	√	√
Background tube 1 (applicaple to FLASH version of the kit)	√	√
Background tube 2 (applicaple to FLASH version of the kit)	√	√

3.2 Mix the PCR-buffer and Taq-polymerase thoroughly (3-5 sec), then spin briefly (1-3 sec).

Taq-polymerase should be got out from freezer just prior to use.

3.3 Prepare Taq-polymerase solution of PCR-buffer and Taq-polymerase. Add to the one tube:

- 10 x (N+1) µL of PCR-buffer,
- 0.5 x (N+1) µL of Taq-polymerase,

N – is a quantity of the samples to be tested taking to account "C-" and "C+".

Example: for testing of 4 samples, two "C-" and two "C+", marked tubes - 12, prepare mixture of PCR-buffer and Taq-polymerase for 13 (12+1) tubes: 130 µL PCR-buffer + 6.5 µL Taq-polymerase.

3.4 Vortex the tube thoroughly and spin down the drops for 3-5 s.

Taq-polymerase solution must be prepared immediately prior to use and should be used within two hours after preparation.

3.5 Add 10 µL of Taq-polymerase solution into each tube (except background tubes). Avoid paraffin layer break. Add 10 µL of PCR buffer to the background tubes.

3.6 Add one drop (~20 µL) of Mineral oil into each tube. Close tubes/strips.

Open the tube, add cDNA sample (or control sample), then close the tube before proceeding to the next sample to prevent contamination. In case of using tubes in strips, close the strip before proceeding to the next strip to prevent contamination.Use filter tips. Close tubes/strips tightly.

3.7 Add 5.0 µL of cDNA sample into corresponding PCR-tubes (2 test tubes for each sample). Avoid paraffin layer break. Do not add cDNA into the "C-", "C+" tubes.

3.8 Add 5.0 µL of negative control which passed all steps of RNA extraction and reverse transcription into "C-" and background tubes. Add 5.0 µL of corresponding positive control, into "C+" marked tubes. Avoid paraffin layer break.

3.9 Spin tubes/strips for 1-3 s to collect drops.

3.10 Set tubes in conventional or real-time thermal cycler depending on the type of kit.

3.11 When using the Influenza A virus, subtype H5N1 FLASH PCR Detection Kit run PCR considering volume equal to 35 µL (see Table 2).

3.12 When using the Influenza A virus, subtype H5N1 Real-Time PCR Detection Kit:

For DTlite and DTprime Thermal Cyclers: Launch the operating software for DT instrument[6]. Add corresponding test[7], specify the number and ID's of the samples, positive and negative control samples. Specify the position of the tubes/strips in the thermal unit (see 3.10) and run PCR (see Table 3).

For IQ cyclers: Turn on the device and the power supply of the device's optical part, leave to heat for 30 minutes. Run Software iCycler. Create and save a new protocol when the given type of the test for the first time. In subsequent productions select the saved protocol, install configuration of the plate (file with data of the sample ID's and their position in the plate) and run PCR according the volume of reaction mix (35 µL) (see Table 4).

[6] Please, apply to Operation Manual for DTprime and DTlite Real-Time PCR instruments PART II.
[7] Instructions for uploading "files with test parameters" can be found on "DNA-Technology's" website https://www.dna-technology.com/assaylibrary.

Table 2. The PCR program for Tercyc Thermal Cycler Precise algorithm (for the FLASH version of the kit)

Number of block	Thermocyclers with active temperature adjustment			Thermocyclers without active temperature adjustment			Number of cycles
	Temperature, °C	Time		Temperature, °C	Time		
		min	sec		min	sec	
1	94.0	1	30	94.0	1	30	1
2	94.0 64.0 72.0	0 0 0	5 5 5	94.0 64.0 72.0	0 0 0	50 50 50	5
3	94.0 64.0 72.0	0 0 0	1 5 5	94.0 64.0 72.0	0 0 0	50 50 50	40
4	10.0	…	…	10.0	…	…	Holding

Table 3. The PCR program for DTlite and DTprime Thermal Cyclers (for the real-time version of the kit)

Number of block	Temperature, °C	min	sec	Number of cycles	Optical measurement	Type of block
1	80.0	0	30	1		Cycle
	94.0	1	30			
2	94.0	0	30	5		Cycle
	64.0	0	15		√	
3	94.0	0	10	45		Cycle
	64.0	0	15		√	
4	94.0	0	5	1		Cycle
5	10.0	…	…	…		Holding

Table 4. The PCR program for iCycler iQ5 thermal cyclers (with dynamic well factor) (for the real-time version of the kit)

Cycle	Repeats	Step	Dwell time	Setpoint, ºC	PCR/Melt Data Acquisition
Well dynamic factor readout program(dynamicwf.tmo)					
1	1				
		1	00:30	80.0	
		2	01:30	94.0	
2	5				
		1	00:30	94.0	
		2	00:45	64.0	
3	2				
		1	00:30	80.0	Real Time
PCR program					
4	45				
		1	00:10	94.0	
		2	00:45	64.0	Real Time
5		…	…	10.0	storage

Note. When using the Influenza A virus, subtype H5N1 FLASH PCR Detection Kit the multiple usage of backgrounds of the same batch is allowed. The background tubes should be stored from 2 °C to 8 °C and out of light. Take the tubes out of refrigerator 1 hour before the run to let the content warm up to room temperature.

4 Data collection and data analysis

4.1 «FLASH» format: with the help of Gene and Gene–4 according to the device manual (the threshold values are 1.75–2.10 for a specific product and 2.50 for internal control).

4.2 «Real–time» format: on DTlite or DTprime Real-Time Thermal Cyclers or iCycler iQ cycler according to the device manuals. The interpretation should be performed according to table 5.

Table 5. Interpretation of PCR results

The result for FAM detection channel		The result for HEX detection channel (IC)	Interpretation
Virus A-H5 Paraffin sealed PCR-mix	Virus A-N1 Paraffin sealed PCR-mix		
Samples			
Cp/Ct defined	Cp/Ct defined	Not considered	Influenza A virus subtype H5N1 RNA is present in the sample
Cp/Ct defined	Cp/Ct undefined	Cp/Ct defined	Influenza A virus subtype H5N1 RNA is absent in the sample
Cp/Ct undefined	Cp/Ct defined	Cp/Ct defined	
Cp/Ct undefined	Cp/Ct undefined	Cp/Ct defined	
Cp/Ct undefined	Cp/Ct undefined	Cp/Ct undefined	Uncertain result
Positive control			
Cp/Ct defined	Cp/Ct defined	Not considered	Positive result The results are valid
Negative control			
Cp/Ct undefined	Cp/Ct undefined	Cp/Ct defined	Negative result The results are valid

Storage, shipping and handling requirements

All kit components, except tubes (or strips) with paraffin sealed PCR-mix and positive controls, must be stored at the temperatures from minus 18 °C to minus 22 °C during the storage period.

⚠ Multiple freezing of PCR-buffer and mineral oil is allowed.

The tubes (or strips) with paraffin sealed PCR-mix and positive controls must be stored from 2 °C to 8 °C and out of light during the storage period. Excessive temperature and light can be detrimental to product performance.

The kit has to be transported in thermoboxes with ice packs by all types of roofed transport at temperatures corresponding to storage conditions.

Transportation of the kit, except tubes (or strips) with paraffin sealed PCR-mix and positive controls, is allowed in termobox with ice packs by all types of roofed transport at temperatures up to 25 °C but no more than 5 days and should be stored at temperatures from minus 18 °C to minus 22 °C immediately on receipt.

It is allowed to transport the tubes (or strips) with paraffin sealed PCR-mix and positive controls in termobox with ice packs by all types of roofed transport at temperatures from 2 °C to 25 °C but no more than 5 days and should be stored at temperatures from 2 °C to 8 °C immediately on receipt.

Shelf-life – 12 months if all the conditions of transportation, storage and operation are met.

Contact our customer service department regarding quality issues with the kit:

8 800 200-75-15 (toll-free call for Russia)

+7 (495) 640-16-93 (chargeable call for CIS and foreign countries).

E-mail: hotline@dna-technology.ru, https://www.dna-technology.com

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Key to symbols

	Temperature limit		Consult instructions for use		Catalogue number
	Use-by date		Manufacturer		Batch code
	Date of manufacture		Contains sufficient for <n> tests		Keep away from sunlight
	Caution		Non-sterile		Do not reuse

Number: 332-2
2022-10-26