WHO information for the molecular detection of influenza viruses

It is strongly recommended that all un-subtypeable influenza A specimens of human origin, that may represent either seasonal viruses displaying significant genetic/antigenic drift or zoonotic viruses/ events with pandemic potential, should be immediately sent for detailed characterization to one of the six WHO Collaborating Centres (WHO CC)[1] for Reference & Research on Influenza.

February 2021

Revision* February 2021*:

- *Annex 4. Protocol 1 added: Multiplex RT-PCR assay for the detection of Flu SARS-CoV-2 (page 61)*
- *Annex 4. Protocol 2 added: Real-time RT-PCR for the initial screening of SARS-CoV-2, Influenza A, B and C viruses (page 64)*
- *Annex 4. Protocol 3 added: One-step real-time RT-PCR 4-plex assay for the detection and subtyping of influenza A, B, H1pdm09 and H3 viruses (page 66)*

This document provides information on molecular detection/diagnostic protocols updated as of the above date for surveillance of influenza viruses in humans.[1]

List of revisions of this document:

7th revision: February 2021 (current revision)
6th revision: January 2020
5th revision: November 2018
4th revision: July 2017
3rd revision: December 2016
2nd revision: May 2015
1st revision: March 2014
Version 1: November 2012

The protocols are described in the annexes 1, 2, 3, and 4.

[1] http://www.who.int/influenza/gisrs_laboratory/collaborating_centres/list/en/index.html

Contents

*Updated protocols

GENERAL GUIDELINES

Specimen selection

The most appropriate specimens for the detection of influenza are upper respiratory tract specimens. Samples should be taken from the deep nostrils (nasal swab), throat (oropharyngeal swab) and nasopharynx (nasopharyngeal swab). Nasopharyngeal aspirate and bronchial aspirate are also useful. Appropriate precautions should be taken in collecting specimens since this may expose the collector to respiratory secretions from patients.

Laboratory tests

Molecular detection/diagnostic techniques are rapid and sensitive methods for the detection and identification of influenza viruses, both for clinical samples and isolates. The reverse-transcription polymerase chain reaction (RT-PCR) allows template viral RNA to be reverse transcribed producing complementary DNA (cDNA) which can then be amplified and detected.

Protocols for influenza RT-PCR detection and subtyping of influenza are outlined below.

In addition to RT-PCR, other laboratory techniques are available for the detection, identification and characterization of influenza virus including **virus isolation in cell culture or fertilized chicken eggs, characterization** of the isolated virus by haemagglutination inhibition (HAI) testing, immunofluorescence detection of the virus in clinical specimens or isolates, rapid antigen tests and other molecular techniques.

Detailed and updated information on these methodologies are included in the "Manual for the laboratory diagnosis and virological surveillance of influenza" by WHO.2

Molecular diagnostics

The RT-PCR technique is used as a rapid and sensitive method for the detection of influenza viruses in both clinical samples and isolates.

The use of different target gene assays in the RT-PCR is most appropriate for correct identification of this virus. The following gene targets, among others, are important:

- type A influenza matrix gene;
- haemagglutinin gene specific for influenza A subtypes:
  - A(H1N1)pdm09 virus, A(H3N2), former seasonal A(H1N1), the highly pathogenic avian influenza A(H5N1) virus, low and highly pathogenic avian influenza A(H7N9) virus, and other subtypes associated with zoonotic events (e.g. H9N2, H7Nx, H5Nx, H10N8);
- type B influenza targeting matrix or NP or NS genes
- haemagglutinin gene specific for influenza B virus.

The following protocols are currently available:

- influenza A type-specific conventional and Real-time-PCR (see Annexes 1 and 2);
- A(H1N1)pdm09 virus specific conventional and Real-time-PCR (see Annexes 1 and 2);
- CDC Real-time RT-PCR (rRT-PCR) protocol for the detection and characterization of A(H1N1)pdm09;[3]
- former seasonal influenza A(H1N1), influenza A(H3N2), influenza B and avian influenza A(H5N1) Real-time RT-PCR (see Annexes 1 and 2);

[2] http://www.who.int/influenza/gisrs_laboratory/manual_diagnosis_surveillance_influenza/en/index.html

[3] http://www.who.int/csr/resources/publications/swineflu/Real-timeptpcr/en/index.html

- influenza B lineages conventional and Real-time RT-PCR (see Annexes 1 and 2);
- A(H7N9) conventional and Real-time PCR (see Annex 2).

For current PCR protocols for H7 viruses please contact the Department of Virology, Erasmus MC Rotterdam, Netherlands at http://www.virology.nl/

The majority of the molecular diagnostic procedures described in this document were developed by members of the WHO Expert Working Group (PCRWG) on influenza PCR. Members of this group include representatives from the WHO H5 Reference laboratories.[4]

Interpretation of RT-PCR results

RT-PCR

A sample is considered positive if results from tests using two different RT-PCR targets (e.g. primers specific for universal M gene and A(H1N1)pdm09 haemagglutinin gene) are positive. If RT-PCR for multiple haemagglutinin (HA) targets (e.g. A(H3), and A(H1N1)pdm09) give positive results in the same specimen, the possibility of RT-PCR contamination should first be excluded by repeating the RT-PCR procedure using a new RNA extract from the original specimen or an RNA extract from another specimen. If repeated positive results for multiple HA targets are obtained, this raises the possibility of co-infection, which should be confirmed by sequencing or virus culture.

CDC Real-time-RT-PCR assays

Results should be interpreted as described in the CDC H1N1 Real-time assay manual.[2]

A negative RT-PCR result does not rule out that a person may be infected with an influenza virus. Results should be interpreted in conjunction with the available clinical and epidemiological information. Specimens from patients whose RT-PCR results are negative but for whom there is a high suspicion of influenza infection should be further investigated and tested by other methods such as virus culture or serology.[1]

Referral for further characterization

Human specimens with laboratory results indicative of influenza A that are un-subtypeable (i.e. negative for seasonal influenza A(H1), A(H3)) or detected/diagnosed as non-seasonal HA subtypes (e.g. A(H5), A(H7), A(H9) or swine origin A(H1)) should be forwarded to a WHO CC as soon as possible for confirmation following the recommendations in the WHO guidance documents. [5,6]

Laboratories with no virus isolation capacity or required biosafety containment levels should forward the specimens to a WHO CC.

Applicable national and/or international regulations related to the transport of infectious substances should be followed. [7]

To allow optimal recovery of virus isolates from clinical specimens known to be influenza positive based on real-time RT-PCR, those with Ct values of up to 30 should be shipped to a WHO CC. Exceptions to this guidance are when specimens are associated with severe or fatal disease but have Ct values in the range 31–40, and specimens that are unsubtypeable (i.e. are influenza A

[4] http://www.who.int/influenza/gisrs_laboratory/h5_reflabs/list/en/index.html
[5] https://www.who.int/influenza/gisrs_laboratory/seasonal_sharing_guide/en/
[6] https://www.who.int/influenza/gisrs_laboratory/ivpp_sharing_guidance/en/
[7] http://www.who.int/ihr/capacity-strengthening/infectious-substances/en/

positive but not HA positive for a seasonal influenza subtype). All unsubtypeable specimens should be shipped to a WHO CC as soon as possible. When urgent detection/diagnosis for clinical needs is required, WHO CCs can provide this service, but possibly not in the most timely manner due to shipment times. Ideally, such a service may be provided by accredited hospital/private laboratories or the National Influenza Centre (NIC) of the country itself or a close neighbor.

Biosafety

Diagnostic laboratory work on clinical specimens and virus isolation from patients who are suspected of being infected with seasonal influenza virus should be conducted in BSL2 containment conditions with the use of appropriate personal protective equipment (PPE). All clinical specimen manipulations should be done inside a certified biosafety cabinet (BSC). Please refer to the WHO *Laboratory biosafety manual*, 3rd edition. [8]

Testing algorithms

The overall approach to influenza virus detection by RT-PCR should be considered in the context of the national situation; e.g. how many specimens can be handled (throughput), what gene sequence to target for RT-PCR, and whether to use concurrent or sequential testing for RT-PCR of M and HA genes.

Good laboratory practices

Standard protocols for all procedures should be in place and reviewed regularly. Ensuring that the recommended reagents are used and handled properly is critical, as reactions are complex and problems with a single reagent can have a significant effect on the results.

Validation

Each laboratory should validate the protocols to be used in their facility to ensure adequate specificity and sensitivity.

Quality assurance

Standard quality assurance protocols including the use of appropriate controls and good laboratory practices should be in place. Participation in the WHO External Quality Assessment Programme (EQAP)[9] is highly recommended to confirm that laboratories are achieving an adequate level of sensitivity and specificity in their tests.

Training of personnel

Familiarity with protocols and experience in correct interpretation of results are cornerstones for successful execution of the diagnostic tests.

Facilities and handling areas

Specimen and reagent handling facilities (including cold chains) with appropriate separation for different steps of RT-PCR must be in place to prevent cross-contamination. Facilities and equipment should meet the appropriate biosafety requirements. RT-PCR should be performed in a space separated from that used for virus isolation techniques.

Equipment

Equipment should be used and maintained (calibrated) according to the manufacturer's recommendations to ensure consistency between assays.

[8] http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/

[9] https://www.who.int/influenza/gisrs_laboratory/external_quality_assessment_project/en/

Annex 1: Conventional RT-PCR protocols

A.

Protocol 1: Conventional RT-PCR analyses for the matrix gene of influenza type A viruses[10]

The following protocols are for conventional RT-PCR and gel electrophoresis of RT-PCR products to detect influenza type A viruses (all subtypes) in specimens from humans. These protocols have been shown to be widely effective for the identification of influenza type A viruses when used with the reagents and primers indicated. For laboratories that have concerns about identifying currently circulating viruses, it is recommended that they contact one of the WHO H5 Reference laboratory11 or WHO CCs12 for diagnosis of influenza infection for assistance in identifying the optimal primers to be used.

Materials required

- QIAamp® Viral RNA Mini Kit (QIAGEN®, Cat. No. 52904. Other extraction kits can be used after proper evaluation)
- OneStep RT-PCR Kit (QIAGEN®, Cat. No. 210212)
- RNase Inhibitor 20U/µl (Thermo Fisher Scientific Inc., Cat. No. N8080119)
- RNase-free water
- Ethanol (96–100%)
- Microcentrifuge (adjustable up to 13 000 rpm)
- Adjustable pipettes (10, 20, 200, and 100 µl)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes (0.2, 1.5 ml)
- Thermocycler (PCR machine)
- Primer sets
- Positive control (may be obtained upon request from a WHOCC)

Primer sequences

Type/subtype	Gene	Primer	Sequence
Influenza type A	Matrix (M)	M30F2/08 M264R3/08	ATGAGYCTTYTAACCGAGGTCGAAACG TGGACAAANCGTCTACGCTGCAG

Expected PCR product size is 244 bp

[10] WHO Collaborating Centre for Reference and Research on Influenza. National Institute of Infectious Diseases. Gakuen 4-7-1, Musashi-Murayama-shi, Tokyo, Japan. Email: whocc-flu@nih.go.jp

[11] http://www.who.int/influenza/gisrs_laboratory/h5_reflabs/en/

[12] http://www.who.int/influenza/gisrs_laboratory/collaborating_centres/list/en/

Procedure

1 . Extract viral RNA from clinical specimen with QIAamp Viral RNA Mini Kit or equivalent extraction kit, according to manufacturer's instructions.

2. Perform one-step RT-PCR:

Take out the reagents from storage and thaw them at room temperature. After they are thawed out, keep them on ice.

Preparation of master mix (**operate on ice**)

- Add the following to microcentrifuge tubes and mix gently by pipetting the master mix up and down ten times. (Note: To avoid localized differences in salt concentration, it is important to mix the solutions completely before use).

Reaction without Q-Solution

Reagent	**Volume (µl)**
Water (molecular grade)	9.5
5X QIAGEN® RT-PCR buffer	5.0
dNTP mix (containing 10mM of each dNTP)	1.0
Forward primer (10µmol/l)	1.5
Reverse primer (10µmol/l)	1.5
QIAGEN® OneStep RT-PCR Enzyme mix (5U/µl)	1.0
RNase Inhibitor (20U/µl)	0.5
Total volume	**20.0**

Dispense 20µl of the master mix to each PCR reaction tube.

Add 5µl sample RNA to the master mix. For control reactions, use 5µl of distilled water for negative control and 5µl of appropriate viral RNAs for positive control.

Program the thermal cycler according to thermal cycling conditions.

Start the RT-PCR program while PCR tubes are still on ice. Wait until the thermal cycler has reached 50˚C, then place the PCR tubes in the thermal cycler.

Thermal cycling conditions

Type of cycle	**Temperature (°C)**	**Time (minute:second)**	**No. of cycles**
Reverse transcription	50	30:00	1
Initial PCR activation	95	15:00	1
Three step cycling: Denaturation	94	0:30	45
Annealing	50	0:30	
Extension	72	1:00	
Final Extension	72	10:00	1
Post-Run	4	Hold	

Agarose gel electrophoresis of RT-PCR products.

Prepare agarose gel, load PCR products and molecular weight marker, and run according to standard protocols. Visualize presence of marker under UV light. An example of the material required and the procedure is given below.

Materials required

- Agarose gel casting tray and electrophoresis chamber
- Power supply and electrode leads
- UV light box (λ = 302nm)
- Camera and Polaroid® film or use any digital gel documentation system
- Adjustable pipettes
- 2% agarose gel in 1X TAE buffer
- 1X TAE buffer
- Ethidium bromide (10mg/ml)
- 6x Gel loading buffer (GLB)
- Molecular weight marker

Procedure

A. Casting the agarose gel:

1. Place a gel-casting tray onto a gel-casting base. Insert a comb and level the base.
2. Prepare 2% agarose by weighing out 4g of agarose powder and dissolving it in 200ml 1X TAE buffer.
3. Dissolve the agar by heating in microwave oven.
4. Cool the melted agarose to about 60°C, then add 10µl of ethidium bromide.
5. Pour the melted agarose into the gel-casting tray.
6. Allow the gel to solidify at room temperature.
7. Remove the comb from the frame.
8. Place the tray into the electrophoresis chamber with the wells at the cathode side.
9. Fill the buffer chamber with 1X TAE at a level that can cover the top of the gel.

B. Sample loading:

1. Add 5µl of the gel loading buffer to each PCR tube.
2. Load molecular weight marker to the first well of the agarose gel.
3. Pipette 15µl of the PCR product in gel loading buffer to the gel.
4. Close the lid on the chamber and attach the electrodes. Run the gel at 100V for 30–35 minutes.
5. Visualize the presence of marker and PCR product bands with a UV light.
6. Document the gel picture by photographing it.

Interpretation of results

The size of PCR products obtained should be compared with the expected product size. Tests should always be run with a positive control.

Protocol 2: Conventional one step RT-PCR for A(H1N1)pdm09 HA gene[13]

The protocols and primers for conventional RT-PCR to detect A(H1N1)pdm09 viruses in specimens from humans are given below. It is recommended that laboratories having concerns about identifying currently circulating viruses should contact one of the WHO CCs or WHO H5 Reference laboratory for assistance in identifying the optimal primers to be used.

These assays were validated on the following working platforms:
GeneAmp PCR system 9700 (Applied Biosystems) Veriti 96-well thermal cycler (Applied Biosystems)

Materials required

- QIAamp Viral RNA Mini Kit (QIAGEN®, Cat. No. 52904)
- QIAGEN OneStep RT-PCR kit (QIAGEN®, Cat. No. 210212)
- RNase inhibitor 20U/µl (Applied Biosystems, Cat. No. N808-0119)
- Ethanol (96–100%)
- Microcentrifuge (adjustable, up to 13,000rpm)
- Adjustable pipettes (10, 20, 100, 200µl)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes (0.2, 1.5ml)
- Thermocycler (GeneAmp PCR system 9700, Applied Biosystems or Veriti 96-well thermal cycler, Applied Biosystems)
- Positive control (Swine influenza A virus A/SW/HK/PHK1578/03 or A/California/04/2009) (Available upon request from Hong Kong University)
- Primer set

Primers

Type/subtype	Gene fragment	Primer	Sequence
Influenza A(H1N1)pdm09 virus	HA	HKU-SWF HKU-SWR	TGAGCTCAGTGTCATCATTTGA TGCTGAGCTTTGGGTATGAA

Expected PCR product size is 174 bp.

[13] School of Public Health, University of Hong Kong, Hong Kong Special Administrative Region of China.

Procedure

1 . Extract viral RNA from clinical specimen with QIAamp Viral RNA Mini Kit or equivalent extraction kit according to manufacturer's instructions.

2. Prepare master mixture for RT-PCR as below:

Reagent	Volume (µl)	Final concentration
Water (molecular grade)	7.4	
5X PCR buffer (kit)	4.0	1X
dNTPs (kit)	0.8	400µM of each dNTP
5µM primer : HKU-SWF	2.4	0.6µM
5µM primer : HKU-SWR	2.4	0.6µM
Rnase Inhibitor (20U/µl)	0.2	4 U
Enzyme mix (kit)	0.8	-
Total	**18.0**	

3. Dispense 18µl of master mix into each test tube.

4. Add 2µl of purified RNA to the above reaction mix.

5. Set the following RT-PCR conditions:

Step	Temperature (°C)	Time (minute:second)	No. of cycles
Reverse transcription Initial PCR activation	50 95	30:00 15:00	1
Denaturation	94	0:30	40
Annealing	57	0:30	
Extension	72	0:20	
Post-PCR extension	72	7:00	1
Post-run	4	Hold	

6. Prepare 2% agarose gel, load PCR products and molecular weight markers, and run according to standard protocols. Visualize presence of marker and PCR product bands under UV light.

Interpretation of results

The expected size of PCR products for influenza H1 is 174 bp. This assay can specifically detect samples with A(H1N1)pdm09, but not those with former seasonal A(H1N1). RNA samples extracted from seven former seasonal A(H1N1), two A(H3N2), one human A(H5N1), seven avian influenza viruses (HA subtypes 4, 5, 7, 8, 9, and 10) and >150 nasopharyngeal aspirate samples from patients with other respiratory diseases were all negative in the assay. It should be noted that these assays can detect A(H1N1)pdm09 and some other swine H1 viral sequences. One of the positive controls recommended in this assay is a swine H1 virus isolated in Hong Kong. The absence of the correct PCR products (i.e. a negative result) does not rule out the presence of influenza virus. Results should be interpreted together with the available clinical and epidemiological information.

B.

Protocol 1: Conventional one step RT-PCR for (H5N1) HA gene[14]

This conventional RT-PCR protocol and primer set are designed to detect highly pathogenic H5N1 viruses in human specimens. Suitable biosafety precautions should be used for handling suspected H5 samples. Laboratories that have concerns about identifying currently circulating H5 viruses should contact one of the WHO CCs or a WHO H5 Reference laboratory for assistance in identifying the optimal primers to be used.

Materials required

- QIAamp Viral RNA Mini Kit (QIAGEN® Cat. No. 51104) or equivalent extraction kit
- QIAGEN OneStep RT-PCR kit (QIAGEN®, Cat. No. 210212)
- RNase inhibitor 20U/ml, (Applied Biosystems Cat. No. N808-0119)
- Ethanol (96–100%)
- Microcentrifuge (adjustable, up to 13,000 rpm)
- Adjustable pipettes (10, 20, 100, 200µl）
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes (0.2, 1.5ml）
- Thermocycler (Applied Biosystems 9700)
- Positive control (Can be obtained from HKU)
- Primer set

Primers

Type/subtype	Gene fragment	Primer	Sequence
Influenza A(H5N1) virus	HA	H5-918F H5-1166R	CCARTRGGKGCKATAAAYTC GTCTGCAGCRTAYCCACTYC

Expected PCR product size is 249 bp.

[14] School of Public Health, University of Hong Kong, Hong Kong Special Administrative Region of China.

Procedure

1. Extract viral RNA from clinical specimen with QIAamp Viral RNA Mini Kit or equivalent extraction kit according to manufacturer's instructions.

2. Prepare master mixture for one step RT-PCR as below:

Reagent	Volume(µl)	Final concentration
Water (molecular grade)	1.7	
5X PCR buffer (kit)	2.0	1X
dNTPs (kit)	0.4	400µM of each dNTP
5X Q-sol (kit)	2.0	1X
5µM primer : H5- 918F	1.2	0.5µM
5µM primer : H5- 1166R	1.2	0.5µM
Enzyme mix (kit)	0.4	-
Rnase Inhibitor (20U/µl)	0.1	10 U
Total	**9.0**	

3. Dispense 9µl of master mix into each test tube.

4. Add 1µl of purified RNA to the above reaction mix.

5. Set the follow RT-PCR conditions:

Step	Temperature (°C)	Time (minute:second)	No. of cycles
Reverse transcription Initial PCR activation	50 95	30:00 15:00	1
Denaturation	94	0:30	45
Annealing	55	0:30	
Extension	72	0:30	
Post-PCR extension	72	7:00	1
Post-run	4	Hold	

6. Prepare 2% agarose gel, load PCR products and molecular weight marker, and run according to standard protocols. Visualize presence of marker and PCR product bands under UV light.

Interpretation of results

The size of PCR products obtained should be compared with the expected product size. If the test is run without a positive control, products must be confirmed by sequencing and comparison with available sequences. The absence of the correct PCR products (i.e. a negative result) does not rule out the presence of influenza virus. Results should be interpreted together with the available clinical and epidemiological information.

Validation of PCR

The following highly pathogenic H5N1 viruses were found to be positive in the assay:

Clade	Virus
0	A/Hong Kong/483/1997
2.1.1	A/chicken/Hong Kong/YU324/2003
2.2	A/bar-headed goose/Qinghai/5/2005
2.3.1	A/duck/Hunan/139/2005
2.3.2	A/grey heron/Hong Kong/837/2004
2.3.2.1	A/grey heron/Hong Kong/779/2009
2.3.3	A/duck/Guiyang/3242/2005
2.3.4	A/chestnut-munia/Hong Kong/2442/2007
2.3.4.2	A/goose/Yunnan/6193/2006
2.3.4.3	A/blue-magpie/Hong Kong/1993/2007
2.4	A/chicken/Yunnan/6957/2003
2.5	A/goose/Shantou/239/2006
3	A/chicken/Hong Kong/YU562/01
4	A/chicken/Hong Kong/96.1/02
5	A/duck/Guangxi/1378/2004
7	A/goose/Yunnan/3315/2005
8	A/chicken/Hong Kong/86.3/2002
9	A/peregrine falcon/Hong Kong/D0028/2004

Protocol 2: Conventional RT-PCR to detect highly pathogenic H5N1 viruses[15]

The protocols and primers for conventional RT-PCR to detect highly pathogenic H5N1 viruses in specimens from humans are given below. It is recommended that laboratories having concerns about identifying currently circulating viruses should contact one of the WHO CCs or a member of the WHO Expert Committee on influenza PCR for assistance in identifying the optimal primers to be used.

Primers and probes

Type/subtype	Gene fragment	Primer	Sequence
Influenza A H5N1 virus	HA	H5-248-270F H5-671-647R	GTGACGAATTCATCAATGTRCCG CTCTGGTTTAGTGTTGATGTYCCAA

Expected PCR product size is 424 bp.

Procedure

Follow the same procedure and steps described for detection of the universal M gene RT-PCR protocol developed by National Institute of Infectious Diseases (NIID).

Interpretation

The size of PCR products obtained should be compared with the expected product size. Tests should always be run with a positive control.

[15] WHO Collaborating Centre for Reference and Research on Influenza and WHO H5 Reference Laboratory at National Institute of Infectious Diseases (NIID). Gakuen 4-7-1, Musashi-Murayama-shi, Tokyo, Japan. E-mail: whocc-flu@nih.go.jp

C.

Protocol 1: one-step conventional RT-PCR Influenza B lineage-specific[16]

This conventional RT-PCR protocol and primer set are designed to detect influenza B/Victoria/2/87 and B/Yamagata/16/88 lineages.

Materials required

- QIAGEN® RNeasy Mini Kit (Cat. No. 74104 or 74106)
- QIAGEN® OneStep RT-PCR Kit (Cat. No. 210212)
- Forward and reverse primers
- Molecular grade sterile distilled water (RNase and DNase free)
- Promega RNasin
- Positive control RNA
- Agarose 2%
- Cooler racks for 1.5ml tubes and 0.2ml PCR tubes
- 10µl and 200µl adjustable pipettes and aerosol barrier tips
- 0.2ml PCR tubes, slips, or plates
- Sterile, nuclease-free 1.5ml tubes
- Disposable powder-free gloves
- Microcentrifuge
- Vortex
- PCR thermocycler

Primers sequence

Type/subtype	Gene	Primer	Sequence
Influenza B Victoria lineage	HA	Bvf224 Bvr507	ACATACCCTCGGCAAGAGTTTC TGCTGTTTTGTTGTTGTCGTTTT
Influenza B Yamagata lineage	HA	BYf226 BYr613	ACACCTTCTGCGAAAGCTTCA CATAGAGGTTCTTCATTTGGGTT

Expected PCR product sizes are 284 bp and 388 bp, respectively.

Procedure

1. Extract viral RNA from clinical specimen with QIAGEN RNeasy Mini Kit or equivalent extraction kit according to manufacturer's instructions.

2. Prepare master mixture for one step RT-PCR as below:

[16] WHO Collaborating Centre for Reference and Research on Influenza, Chinese National Influenza Centre. National Institute for Viral Disease Control and Prevention. Chinese Centre for Disease Control and Prevention. 155 Changbai Road, Changping District. 102206 Beijing, China. http://www.cnic.org.cn

Reagent	Volume (µl)	Final concentration
PCR buffer	5.0	1
dNTPs	1.0	
Primer: BV HA F	0.5	10µM
Primer: BV HA R	0.5	10µM
Primer: BY HA F	0.5	10µM
Primer: BY HA R	0.5	10µM
Enzyme mix	1.0	
RNase Inhibitor	0.1	
Water (molecular grade)	10.9	
Total	**20ul**	

3. Dispense 20µl of master mix into each test tube.

4. Add 5µl of purified RNA to the above reaction mix.

5. Set the follow thermal cycling conditions:

Step	Temperature (°C)	Time (minute:second)	No. of cycles
Reverse transcription	60	1:00	1
	42	20:00	
	50	20:00	
Activation	95	15:00	1
Denaturation	94	0:30	35
Annealing	52	0:30	
Extension	72	1:00	
Post-PCR extension	72	10:00	1

6. Agarose gel electrophoresis of RT-PCR products: prepare 2% agarose gel, load PCR products and molecular weight marker, and run according to standard protocols. Visualize presence of marker and PCR product bands under UV light.

Interpretation of results

The expected PCR product sizes for B-Victoria lineage is 284 bp and for B-Yamagata lineage is 388 bp. There should be no cross reaction with the other influenza B lineage.

Limitations

1 . Analysts should be trained and familiar with testing procedures and interpretation of results prior to performing the assay.
2. A false negative result may occur if inadequate numbers of copies of the virus are present in the specimen due to improper collection, transport, or handling.

Validation of PCR:

The following viruses were used to validate these protocols:

B/Chongqing-Yuzhong/1384/2010 (B-Victoria)
B/Hubei-Wujiagang/158/2009 (B-Yamagata).

This PCR protocol and others are available in English on the CNIC web site at:
http://www.cnic.org.cn/chn/down/showdown.php?downid=663

D.

Protocol 1: Conventional RT-PCR assays for the detection of seasonal influenza A(H1N1), A(H3N2) and influenza B viruses[17]

This protocol describes conventional RT-PCR procedures for the detection of:

1. A(H1N1)pdm09 viruses (H1 and N1 genes)
2. Influenza A(H3N2) viruses (H3 and N2 genes)
3. Influenza B viruses (HA and NA genes)
4. Former seasonal influenza A(H1N1) viruses (H1 and N1 genes)

HA and NA genes are amplified as overlapping halves with the primer sets indicated below. Generated RT-PCR products can be used for detection of influenza and sequencing studies.

Materials required

- QIAamp Viral RNA Mini Kit (QIAGEN®, Cat. No. 52906)
- Ethanol Absolute (Fisher, Cat. No. E/0650DF/17)
- Microcentrifuge (adjustable, up to 13,000 rpm)
- Adjustable pipettes (10, 20, 100, 200, 1000 µl)
- RNAsin (Promega #N2515 or N2115)
- SS III one-step RT-PCR Platinum Taq HiFi kit (Invitrogen, Cat. No. 12574-035)
- Water (QIAGEN®, Cat. No. 129114)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes (0.2, 1.5 ml)
- Thermocycler: DNA Engine or DYAD (BIORAD)
- Primer set

Procedure

RNA Extraction

Follow the manufacturer's instructions and elute RNA in 50µl of the supplied buffer.

Use 5µl RNA in a 50µl one-step RT-PCR reaction for clinical specimen extracts or 2µl in a 50µl reaction for propagated virus extracts.

[17] WHO Collaborating Centre for Reference and Research on Influenza. Worldwide Influenza Centre, The Francis Crick Institute, 1 Midland Road, London NW1 1AT, United Kingdom. Email: whocc@crick.ac.uk

Primers

Primer sets used for one-step RT-PCR for human influenza surveillance (London WHO CC; July 2017 and January 2020*)

Type/subtype	Gene fragment	Primer	Sequence	PCR Product Size
Influenza A(H1N1)pdm09*	HA-5'(H1)	H1F1 H1R1264	AGCAAAAGCAGGGGAAAATAAAAGC CCTACTGCTGTGAACTGTGTATTC	1264
	HA-3'(H1)*	HAFA HARend	GGGAGAATGAACTATTACTGG AGTAGAAACAAGGGTGTTTTT	979
	NA-5'(N1)	N1F1 N1R1099	AGCAAAAGCAGGAGTTTAAAATG CCTATCCAAACACCATTGCCGTAT	1099
	NA-3'(N1)	N1F401 NARUc	GGAATGCAGAACCTTCTTCTTGAC ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	1073
Influenza A(H3N2)	HA-5'(H3)	H3A1F6 H3A1R1	AAGCAGGGGATAATTCTATTAACC GTCTATCATTCCCTCCCAACCATT	1127
	HA-3'(H3)	H3A1F3 HARUc	TGCATCACTCCAAATGGAAGCATT ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	863
	NA-5'(N2)	NAFUc H3N2R1095	TATTGGTCTCAGGGAGCAAAAGCAGGAGT TCATTTCCATCATCRAAGGCCCA	1095
	NA-3'(N2)	N2F387 NARUc	CATGCGATCCTGACAAGTGTTATC ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	1082
Influenza A Matrix	Full gene	MF1 MR1027	AGCAAAAGCAGGTAGATATTGAAAGA AGTAGAAACAAGGTAGTTTTTTACTC	1027
Influenza B*	HA-5'	BHAF1u BHAR1341	TATTCGTCTCAGGGAGCAGAAGCAGAGCATTTTCT AATATC TTCGTTGTGGAGTTCATCCAT	1361
	HA-3'	BHAF458 BHAREU*	AGAAAAGGCACCAGGAGGACCCTA ATATCGTCTCGTATTAGTAGTAACAAGAGCATTTTT	1391
	NA-5'	BNAF1u BNAR2	TATTCGTCTCAGGGAGCAGAAGCAGAGCATCTTCT CA GATGGACAAATCCTCCCTTGATGC	1130
	NA-3'	BNAF2 BNAR1487	GCACTCCTAATTAGCCCTCATAGA TAAGGACAATTGTTCAAAC	1182
Former seasonal Influenza A(H1N1)**	HA-5'(H1)	THAF2 SPHAR11	GCAGGGGAAAATAAAAACAACC TATTTTGGGCACTCTCCTATTG	990
	HA-3'(H1)	H1HAF552 HARUc	TACCCAAACCTGAGCAAGTCCTAT ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	1239
	NA-5'(N1)	H1N1F6 NASPR10	AGCAGGAGATTAAAATGAATCCAA CCTTCCTATCCAAACACCATT	1097
	NA-3'(N1)	N1F741 NARUc	ATAATGACCGATGGCCCGAGTAAT ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT	737

* updated primers in January 2020

HA and NA genes are amplified as overlapping halves with the primer sets indicated. Generated products can be used for diagnosis of influenza and sequencing studies.

** These primer sets are also of use for some A(H1N1) viruses from swine where the H1 and N1 genes have been introduced by reverse zoonosis.

One-Step RT-PCR protocol

1. For RT-PCR, all reactions are run on a BIORAD Peltier Thermal Cycler using thin-walled tubes with calculated (block) temperature control.

2. Invitrogen SS III one-step RT-PCR Platinum Taq HiFi kit (Cat. No. 12574-035) is sufficient for 100 x 50µl reactions following manufacturer's instructions.

3. Invitrogen reagent-based one-step protocol used at the London UK, WHO CC.

Reagent	Volume (µl) Clinical	Volume (µl) Virus	Final concentration
Water (molecular grade)	15.5	18.5	
2X Buffer*	25.0	25.0	
10 µmol/l Forward primer†	1.5	1.5	0.3µmol/l final concentration
10 µmol/l Reverse primer†	1.5	1.5	0.3µmol/l final concentration
RNAsin	0.5	0.5	
Enzyme mix*	1.0	1.0	
RNA	5.0	2.0	
Total	**50.0**	**50.0**	

* Supplied in the Invitrogen kit.

† See above for primer pairings dependent on the type/subtype being amplified.

Thermal cycler programme (BIORAD):

Temperature (°C)	Time (minute:second)	No. of cycles
42	30:00	1
50	10:00	1
94	2:00	1
94	0:30	40
55	0:30	
68	2:30	
68	10:00	1
4	Hold	

Product analysis

Run 5µl each sample on a 1.0% (w/v) agarose gel made up with 1X TBE buffer and containing GelRed dye (Biotium, Cat. No. 41003) according to manufacturer's instructions. Reactions should yield single bands and do not require gel purification.

Product clean-up

It is necessary to remove RT-PCR component reagents prior to gene sequencing. This is best done using a column DNA-capture/elute process and the system used at the London UK, WHO CC is from GE Healthcare (illustra GFX PCR DNA and gel band purification kit No. 28-9034-71 [columns] or 28-9034-45 [96-well plates]). Manufacturer's instructions are followed, but 2 x 500µl washes are used and, for sequencing purposes, products are usually eluted with either 50µl of water (QIAGEN®, Cat. No. 129114) or the 'pink' elution buffer supplied with the GE Healthcare kit.

Product quantification

Yields of DNA are measured using a NanoDrop 2000 spectrophotometer Thermo Scientific) and the equivalent of 100-200ng of DNA is used per sequencing reaction.

Gene sequencing

Performed using ABI BigDye® Terminator v1.1 Cycle Sequencing kits (Applied Biosystems, Cat. No. 4336774) and a capillary based sequencer (ABI X3730XL). The sequences of additional primers used in the Sanger sequencing process are available on request.

Annex 2: Real-time RT-PCR protocols

Real-time RT-PCR poses different challenges than conventional RT-PCR. In addition to the RT-PCR considerations described in Annex 1, specific considerations for Real-time RT-PCR include:

- Ensuring appropriate equipment, software, and fluorescent-based reagents are used and handled correctly.
- Ensuring appropriate training of personnel for interpretation of results (experience in recognizing true positives, interpreting controls/Ct value and aberrant fluorescence is crucial).
- Validation in the laboratory and optimization of reactions are essential to making quantitative determinations.
- There is little likelihood of contamination when reactions are disposed of after testing. However, many laboratories do further post-reaction analysis (e.g. restriction fragment length polymorphism using gels, sequencing, etc.) which can re-introduce contamination.

A.

Protocol 1: Real-time RT-PCR one-step triplex RT-PCR for the detection of seasonal influenza A, B and C viruses[18]

This protocol describes one-step real-time triplex RT-PCR procedures for the detection of influenza A, B and C viruses

Materials required

PCR kit	QuantiFast Pathogen, RT-PCR +IC Kit (Qiagen)
PCR reaction volume	10 µL
PCR instrument	LC480 (Roche)

Procedure

1. Prepare master mixture as below:

Reagent	Volume (µL) per reaction
QuantiFast Pathogen Master Mix, 5x	2
QuantiFast Pathogen RT Mix, 100x	0.1
Primer probe mix	Variable[a]
RNase-free water	Variable[a]
Total	5
Template RNA	5

[a] Depending on the concentration of primer-probe mix prepared. See the Primers and Probes Table for the final concentration of primers and probes for each target.

2. Perform real-time RT-PCR according to the following conditions:

Step	Temperature (°C)	Time
Reverse transcription	50	20 min
Initial PCR activation	95	5 min
2-step cycling (40 repeated cycles):		
Denaturation	95	15 sec
Annealing/extension	60	45 sec

[18] Microbiology Division, Centre for Health Protection, Hong Kong SAR, China, (National Influenza Centre, WHO H5 Reference Laboratory). http://www.chp.gov.hk/

Primers and probes used for detecting influenza A, B and C viruses

Virus (Target)	Name	Sequence[a]	Conc.[b]	Reference[c]
Influenza B virus (HA)	FLUBHA-940-F	AAATACGGTGGATTAAACAAAAGCAA	0.1	1
	FLUBHA-1109-R	CCAGCAATAGCTCCGAAGAAA	0.1	1
	FLUBHA-994-P4	Fam-CACCCATATTGGGCAATTTCCTATGGC-BHQ1	0.1	1
Influenza A virus (M)	FLUAM-7-F	CTTCTAACCGAGGTCGAAACGTA	0.1	2
	FLUAM-161-R	GGTGACAGGATTGGTCTTGTCTTTA	0.1	2
	FLUAM-49-P6	CFO560-TCAGGCCCCCTCAAAGCCGAG-BHQ1	0.1	2
Influenza C virus (NP)	CNP-1043-F	GCTTTGGACTTGCTTAT	0.1	own designed[d]
	CNP-1141-R	GACTCTGAAGTTTCCTATTT	0.1	own designed[d]
	CNP-1095-P3	Q670-CCCTCTTAAGTTGAGAAACAGAATG-BHQ2	0.1	own designed[d]

[a] CFO560, CAL Fluor Orange 560; Q670, Quasar 670.

[b] Final concentration of primers/probes (μM) in the master mixture.

[c] (1). van Elden LJ, et al. 2001. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J Clin Microbiol 39:196-200. (2). Terrier O, et al. 2011. Cellular transcriptional profiling in human lung epithelial cells infected by different subtypes of influenza A viruses reveals an overall down-regulation of the host p53 pathway. Virol J 8:285.

[d] Microbiology Division, Centre for Health Protection, Hong Kong SAR, China, (National Influenza Centre, WHO H5 Reference Laboratory). http://www.chp.gov.hk/

Protocol 2: One-step Real-time RT-PCR procedures for the detection of influenza A viruses

Materials required

PCR kit	QuantiFast Pathogen, RT-PCR +IC Kit (Qiagen)
PCR reaction volume	10 µL
PCR instrument	LC480 (Roche)

Procedure

1. Prepare master mixture as below:

Reagent	Volume (µL) per reaction
QuantiFast Pathogen Master Mix, 5x	2
QuantiFast Pathogen RT Mix, 100x	0.1
Primer probe mix	Variable[a]
RNase-free water	Variable[a]
Total	5
Template RNA	5

[a] Depending on the concentration of primer-probe mix prepared. See the Primers and Probes Table for the final concentration of primers and probes for each target.

2. Perform real-time RT-PCR according to the following conditions:

Step	Temperature (°C)	Time
Reverse transcription	50	20 min
Initial PCR activation	95	5 min
2-step cycling (40 repeated cycles):		
Denaturation	95	15 sec
Annealing/extension	60	45 sec

Primers and probes used for detecting influenza A, B and C viruses

Virus (Target)	Name	Sequence[a]	Conc.[b]	Reference[c]
Influenza A virus (M)	FLUAM-7-F	CTTCTAACCGAGGTCGAAACGTA	0.1	Terrier O, et al. 2011
	FLUAM-161-R	GGTGACAGGATTGGTCTTGTCTTTA	0.1	Terrier O, et al. 2011
	FLUAM-49-P6	CFO560-TCAGGCCCCCTCAAAGCCGAG-BHQ1	0.1	Terrier O, et al. 2011

[a] CFO560, CAL Fluor Orange 560; Q670, Quasar 670.
[b] Final concentration of primers/probes (µM) in the master mixture.
[c] Terrier O, et al. 2011. Cellular transcriptional profiling in human lung epithelial cells infected by different subtypes of influenza A viruses reveals an overall down-regulation of the host p53 pathway. Virol J 8:285.

B.

Protocol 1: One-step Real-time RT-PCR for H1 gene of A(H1N1)pdm09 virus[19]

This protocol is a Real-time RT-PCR to detect A(H1N1)pdm09 virus (HA gene) in specimens from humans. It is recommended that laboratories having concerns about identifying currently circulating viruses should contact one of the WHO CCs or a WHO H5 Reference Laboratory for assistance in identifying the optimal primers to be used.

Materials required

- QIAamp Viral RNA Mini Kit (QIAGEN®, Cat. No. 52904)
- 7500 Real-time PCR System (Applied Biosystems)
- Invitrogen SuperScript® III Platinum® one-step qRT-PCR System (No. 11732-088)

Primers and probes

Type/subtype	Gene	Primer	Sequence
Influenza A(H1N1)pdm09	HA	swlH1F swlH1R swlH1P*	GACAAAATAACAAACGAAGCAACTGG GGGAGGCTGGTGTTTATAGCACC GCATTCGCAA"t"GGAAAGAAATGCTGG

*Lower case "t" denotes position of quencher. Probes need to be labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and quenched internally at a modified "t" residue with BHQ1, with a terminal phosphate at the 3'-end to prevent probe extension by DNA polymerase.

Procedure

1. Extract viral RNA from clinical specimen with QIAamp Viral RNA Mini Kit or equivalent extraction kit according to manufacturer's instructions.

2. Prepare master mixture for RT-PCR as below:

Reagent	Volume (µl)	Final Concentration
Water (molecular grade)	5.5	
2X PCR master mix*†	12.5	5X
Forward primer	0.5	40µM
Reverse primer	0.5	40µM
Probe	0.5	10µM
RT/DNA polymerase mix*	0.5	
Total master mix	**20.0**	
RNA template	5.0	
TOTAL reaction volume	**25.0**	

* Supplied in the Invitrogen kit.
† ROX reference dye (supplied with the Invitrogen kit) **must** be added to the master mix at the level recommended by the manufacturer.

[19] School of Public Health, University of Hong Kong, Hong Kong Special Administrative Region of China

3. Assemble a master mix for the required number of samples (remember to make up more than required to account for pipetting losses).

4. Make 20µl aliquots of this and add the required RNA template. Briefly centrifuge the plates/tubes prior to loading the thermal cycler and running the thermal cycler programme.

Thermal cycler amplification programme:

Step	Temperature (^{o}C)	Time (minute:second)	No. of cycles
Reverse transcription and activation of Taq	50 95	30:00 02:00	1
PCR	95 55	00:15 00:30*	45

* Fluorescence data (FAM) is collected during the 55^{o} C incubation step.

Protocol 2: Real-time RT-PCR one-step duplex for the detection of Influenza type A subtype H1pdm09 and subtype H3[20]

This protocol describes one-step duplex real-time RT-PCR procedures for the detection of influenza A subtype H1pdm09 and subtype H3 viruses

Materials required

PCR kit	QuantiFast Pathogen, RT-PCR +IC Kit (Qiagen)
PCR reaction volume	10 µL
PCR instrument	LC480 (Roche)

Procedure

1. Prepare master mixture as below:

Reagent	Volume (µL) per reaction
QuantiFast Pathogen Master Mix, 5x	2
QuantiFast Pathogen RT Mix, 100x	0.1
Primer probe mix	Variable[a]
RNase-free water	Variable[a]
Total	5
Template RNA	5

[a] Depending on the concentration of primer-probe mix prepared. See the Primers and Probes Table for the final concentration of primers and probes for each target.

2. Perform Real-time RT-PCR according to the following conditions:

Step	Temperature (°C)	Time
Reverse transcription	50	20 min
Initial PCR activation	95	5 min
2-step cycling (40 repeated cycles):		
- denaturation	95	15 sec
- annealing/extension	60	45 sec

[20] Microbiology Division, Centre for Health Protection, Hong Kong SAR, China, (National Influenza Centre, WHO H5 Reference Laboratory). http://www.chp.gov.hk/

Primers and probes used for detecting influenza A subtype H1pdm09 and H3 viruses

Virus (Target)	Name	Sequence	Conc.[a]	Reference
A(H1)pdm09 HA gene	H1pdm-169-F	AAACTATGCAAACTAAGAGGGGT	0.1	own designed
	H1pdm-297-R	TGTTTCCACAATGTAGGACCA	0.1	own designed
	H1pdm-244-P	Q670–CCAGAGTGTGAATCACTCTCCACA–BHQ2	0.1	own designed
A(H3) HA gene	H3-266-F	ACCCTCAGTGTGATGGCTTTCAAA	0.1	own designed
	H3-373-R	TAAGGGAGGCATAATCCGGCACAT	0.1	own designed
	H3-315-P	FAM-ACGAAGCAAAGCCTACAGCAACTGTT-BHQ1	0.1	own designed
	H3-666-F	GCACAGGGAATCTAATTGCTCC	0.1	own designed
	H3-911-R	ATGCTTCCATTTGGAGTGATGCATTC	0.1	own designed
	H3-732P2	FAM-GATCAGATGCACCCATTGGCAAATGC-BHQ1	0.1	own designed

[a] Final concentration of primers/probes (µM) in the master mixture.
[b] Microbiology Division, Centre for Health Protection, Hong Kong SAR, China, (National Influenza Centre, WHO H5 Reference Laboratory). http://www.chp.gov.hk/

C.

Protocol 1: One-step Real-time RT PCR for the detection of Influenza B lineage[21]

Materials required

- QIAamp Viral RNA Mini Kit (QIAGEN®, Cat. No. 52904) or equivalent
- Water (molecular grade)
- Qiagen OneStep RT-PCR Kit (QIAGEN®, Cat. No. 210212)
- Primers and probes
- RNAse Inhibitor (40U/µl, e.g. TaKaRa Cat. No. 2310A)
- Adjustable pipettes and sterile, Rnase-free pipette tips with aerosol barrier
- Real-time thermocycler (method validated for Corbett Rotor Gene RG-3000/RG-6000)
- Positive control virus, Victoria/2/87 lineage, e.g. B/Shangdong/7/1997
- Positive control virus, Yamagata/16/88 lineage, e.g. B/Florida/4/2006

Test validation

The method had been tested against a wide variety of historical and recent influenza B reference viruses, including the lineage prototype strains B/Victoria/2/87 and B/Yamagata/16/88, and in all cases the method has clearly distinguished between the two lineages. The identity of a large number of viruses from patient specimens testing positive with the method has been verified by sequence analysis and antigenic characterization.

Furthermore, the method yielded consistently negative results against various influenza A viruses (former seasonal H1N1; 2009 pandemic H1N1; H3N2; H2N3; H5N1; H7N3; H9N2) as well as various non-influenza respiratory viruses (parainfluenza 1, 2, 3; RSV A and B; adenovirus) and human respiratory specimens that has tested negative for influenza B.

The test has been validated on Corbett Rotor Gene RG-3000 and RG-6000 instruments. Laboratories using different instruments should first critically and carefully examine the cycling conditions as they may *not* perform optimally on other thermocyclers.

[21] Department of Virology, Norwegian Institute of Public Health, P.O. Box 4404 Nydalen, N-0403 Oslo, Norway. Email: olav.hungnes@fhi.no

Primers and probes

Primers and probes have been designed to detect current influenza B viruses with probes reacting exclusively to B/Yamagata/16/88- or B/Victoria/2/87-lineage viruses, respectively. Since these probes carry different fluorophores, viruses can be discriminated by the colour of fluorescent emission from hydrolyzed probe.

Type/subtype	Gene	Primer	Sequence Degenerate nucleotides are indicated in **bold**
B	HA	BHA-188F*	AGACCAGAGGGAAACTATGCCC
B	HA	BHA-270R**	TCCGGATGTAACAGGTCTGACTT
B(Victoria lineage)	HA	Probe-VIC2	Yakima Yellow-5'-CAGACCAAAATGCACGGGGAAHATACC-3'-BHQ
B(Yamagata lineage)	HA	Probe-YAM2	FAM-5'CAG**R**CCAATGTGTGTGGGGA**Y**CACACC-3'-BHQ

* Schweiger et al. 2000 (Journal of Clinical Microbiology 38(4) 1552–1558)
** Watzinger et al. 2004 (Journal of Clinical Microbiology 42(11) 5189–5198)

Procedure

Each RT-PCR run should include, in addition to the specimen reactions, at least one negative control reaction and one positive control reaction for each of the targets, i.e. one Victoria-lineage positive control and one Yamagata-lineage positive control.

1. Extract viral RNA from clinical specimen with QIAamp Viral RNA Mini Kit or equivalent extraction kit according to manufacturer's instructions.

2. Prepare master mixture for RT-PCR as below. (Make sure to prepare some excess volume, e.g. corresponding to one extra reaction volume, to make up for dispensing losses.)

Reagent	Volume (µl)	Final concentration
Water (molecular grade)*	13.9	
Qiagen One-Step RT PCR buffer*	5.0	5X
Qiagen dNTP mix*	1.0	10mM each
BHA-188F forward primer, 500nM final	0.5	25 µM
BHA270R reverse primer, 500nM final	0.5	25 µM
probe-VIC2, 200 nM final	0.5	10µM
probe-YAM2, 200 nM final	0.5	10µM
RNAse Inhibitor, e.g. TaKaRa cat 2310A	0.1	ca 40U/µl
Qiagen 1-step RT PCR enzyme mix*	1.0	25X
Total master mix	**23.0**	
RNA template	2.0	
TOTAL reaction volume	**25.0**	

* Supplied in the Qiagen® OneStep RT-PCR Kit.

3. Dispense 23µl of master mix into each reaction vessel.

4. Add 2µl of purified specimen RNA to the individual reaction vessels.

5. Set the following thermocycling conditions:

Temperature (°C)	Time (minute:second)	No. of cycles
50° C	30:00	1
95° C	15:00	1
95° C	00:10	45
54° C*	00:40	
72° C	00:20	

* Fluorescence data is collected during the 54° C incubation step for probe-YAM2 (Green) and probe-VIC2 (Yellow). For the RotorGene thermocyclers, 5 sec is added for each fluorescence read, so step time is set to 30 sec + 2x5sec = 40 sec.

Interpretation of results

For a run to be valid, there should be no amplification in the negative control reaction. Each lineage specific control should display a positive reaction for the corresponding lineage, with a Ct value within the expected range established for the particular control material, and no amplification for the other lineage.

Provided that all controls meet stated requirements, a specimen is considered positive for the influenza B lineage for which there is a clear reaction growth curve that crosses the threshold line within 40 cycles.

Similarly, a specimen is considered negative in the influenza B lineage specific test if there is no growth curve crossing the threshold within 40 cycles for any of the influenza B lineages.

D.

Protocol 1: Real-time RT-PCR assays for the detection of seasonal influenza viruses and H5N1 influenza viruses[22]

August 2019: Modifications from the previous version include:

- detection/lineage of type A(H1N1)pdm09 (HA & NA)

This protocol describes procedures for the detection of influenza A viruses (M gene), the seasonal A(H3N2) viruses and A(H1N1)pdm09 (H3h, N2h, H1 and N1 genes) and the Type B viruses (detection and lineage - HA genes) by real-time RT-PCR. In case of suspicion of avian infection by the H5 virus, primers and probes are enclosed.

We suggest the following testing strategy:

- RNA extraction
- Amplification in parallel of M, and GAPDH (to assess quality of the specimen and extraction procedure) genes
- In a separate set, amplification of the HA and NA specific genes.

Material

Kits

QIAamp Viral RNA (QIAGEN mini Kit 50)	Ref: QIAGEN 52 904
SuperScript™ III Platinum® One-Step Quantitative RT-PCR System	Ref: Invitrogen 1732-020
Non acetyled BSA 10%	Ref: Invitrogen P2046

Primers and probes

Name	Sequences	Length (bases)	PCR product size	Ref.
Matrix (M) gene:				
GAPDH-6Fw	GAAGGTGAAGGTCGGAGT	18	226 bp	3
GAPDH-231Rv	GAAGATGGTGATGGGATTTC	20		3
GAPDH-202Probe(-)	CAAGCTTCCCGTTCTCAGCC [5']Fam [3']BHQ-1	20		3
GRAM/7Fw	CTTCTAACCGAGGTCGAAACGTA	23	202 bp	2
GRAM/161Rv	GGTGACAGGATTGGTCTTGTCTTTA	25		2
GRAM probe/52/+	TCAGGCCCCCTCAAAGCCGAG [5']Fam [3']BHQ-1	21		2
Seasonal viruses				
H1h-678Fw	CACCCCAGAAATAGCCAAAA	20	163 bp	1
H1h-840Rv	TCCTGATCCAAAGCCTCTAC	20		1
H1h-715probe	CAGGAAGGAAGAATCAACTA [5']Fam [3']BHQ-1	20		1
H3h-1070Fw	ATGGTTGGGAGGGAATG	17	98 bp	1
H3h-1167Rv	TGCTGCTTGAGTGCTT	16		1
H3h-1144dProbe	CTGCTGCTTGTCCTCTTCCCT [5']Fam [3']BHQ-1	21		1
N1h-1134 Fw	TGGATGGACAGATACCGACA	20	142 bp	1
N1h-1275 Rv	CTCAACCCAGAAGCAAGGTC	20		1
N1h-1206 probe	CAGCGGAAGTTTCGTTCAACAT [5']Fam [3']BHQ-1	22		1

[22] Unité de Génétique Moléculaire des Virus Respiratoires. Institut Pasteur, 25 rue du Docteur Roux 75724, Paris Cedex 15, France. Email: grippe@pasteur.fr; http://www.pasteur.fr

N2h-1150b Fw	GTCCAACCCTAAGTCCAA	18	194 bp	1
N2h-1344 Rv	GCCACAAAACACAACAATAC	20		1
N2h-1290 probe	CTTCCCCTTATCAACTCCACA [5']Fam [3']BHQ-1	21		1
A(H1N1)pdm09*				
GRswH1-60Fw	AGAAAAGAATGTAACAGTAACACACTCTGT	30	181 bp	5
GRswH1-240Rv	GTTTCCACAATGTAGGACCATG	22		5
GRswH1-180Probe(-)	CAGCCAGCAATGTTACATTTACC [5']Fam [3']BHQ-1	23		5
GRswH1-180cProbe(-)	CAGCCAGCAATGTTGCATTTACC [5']Fam [3']BHQ-1	23		5
GRswN1-1260Fw	AGACCTTGCTTCTGGGTTGA	20	121 bp	6
GRswN1-1380Rv	ACCGTCTGGCCAAGACCA	18		6
GRswN1-1320Probe(+)	ATCTGGACTAGCGGGAGCAGCAT [5']Hex [3']BHQ-1	23		6
Type B viruses + lineage				
HA(B)-444Fw	ACCCTACARAMTTGGAACYTCAGG	24	80 bp	4
HA(B)-524Rv	ACAGCCCAAGCCATTGTTG	19		4
HA(B)-501cProbe(-)	AAATCCAATTTTRCTGGTAG [5']Fam [3']MGBNFQ	20		4
HA(B)-501eProbe(-)	AAATCCGATTTTRCTGGTAG [5']Fam [3']MGBNFQ	20		4
HA(B)-499Probe(-)	ATCCGTTTCCATTGGTAA [5']Vic [3']MGBNFQ	18		4
Avian H5 viruses				
H5-1544Fw	CCGCAGTATTCAGAAGAAGC	20	140 bp	1
H5-1683Rv	AGACCAGCYAYCATGATTGC	20		1
H5d-1638Probe(+)	AGTGCTAGRGAACTCGCMACTGTAG [5']Fam [3']BHQ-1	25		1
H5-1063Fw	TTTATAGAGGGAGGATGG	18	100 bp	1
H5-1162Rv	GAGTGGATTCTTTGTCTG	18		1
H5d-1088Probe(x)	TGGTAGATGGTTGGTATGGG [5']Hex [3']BHQ-1	20		1
N1av-459Fw	GTTTGAGTCTGTTGCTTGGTC	21	190 bp	1
N1av-648Rv	GCCATTTACACATGCACATTCAG	23		1
N1av-493Probe(+)	CATGATGGCAYYAGTTGGTTGACAA [5']Fam [3']BHQ-1	25		1

** updated protocol (November 2019)*

1/ National Influenza Center (Northern-France), Institute Pasteur, Paris.

2/ National Influenza Center (Southern-France), CHU, Lyon.

3/ Wong et al., 2005, J. Clin. Pathol. 58;276-280

4/ Adapted primers from Biere et al., 2010 J. Clin. Microbiology. 48:1425-1427

5/ Adapted primers and probe from Japan NIC protocol

6/ Adapted primers and probe from Hoffmann et al., 2016 Scientific Reports 6, Article number: 27211 (2016)

NUCLEIC ACID EXTRACTION

RNA is extracted from specimens using the QIAamp Viral RNA kit (QIAGEN Mini Kit 50 ref 52904). RNA extracted from 140 µl of original sample, is eluted in 60 µl of elution buffer.

MIX PREPARATION FOR ALL SEPARATE PRIMER/PROBE COMBINATIONS

All primers and probes described below were validated under the following conditions.

RT-PCR Mix kit:

- Invitrogen Superscript™ III Platinum® One-Step qRT-PCR system (ref: 11732-088)

Real-time PCR equipment:

- LightCycler 480 (96)

Adjustments may be required for the use of other kits or other real-time PCR instruments.

Primers and probes for the detection were also validated under the following conditions.

RT-PCR Mix kit:

- Invitrogen Superscript™ III Platinum® One-Step qRT-PCR system (ref: 11732-088)

Mix :	**Vol (µl)**	**[final]**
H_2O PPI :	1.96	
Reaction mix 2X :	10	3 mM Mg
$MgSO_4$ (50mM) :	0.24	0.6 mM Mg
Forward Primer (10µM):	1	0.5 µM
Reverse Primer (10µM):	1	0.5 µM
Probe (10µM):	0.4	0.2 µM
SuperscriptIII RT/Platinum Taq Mix :	0.4	
Final volume	**15 µl**	

*: GRswN1-180's probe (180 et 180c) are mixed 50/50 before use. The mixture is then diluted in 10 µM.

Mix Type B viruses	**Vol (µl)**	**[final]**
H_2O PPI :	1.86	
Reaction mix 2X :	10	3 mM Mg
$MgSO_4$ (50mM) :	0.24	0.6 mM Mg
Forward Primer (10µM):	1.0	0.9 µM
Reverse Primer (10µM):	1.0	0.6 µM
*Probe FAM (10µM):	0.4	0.15 µM
Probe VIC (10µM):	0.1	0.1 µM
SuperscriptIII RT/Platinum Taq Mix :	0.4	
Final volume	**15 µl**	

*: Yamagata's probe (501c et 501e) are mixed 50/50 before use. The mixture is then diluted in 10 µM.

CONTROLS

Each real-time RT-PCR assay includes in addition of unknown samples:

- Two negative samples bracketing unknown samples during RNA extraction (negative extraction controls)

- Positive controls (in duplicate); when using in vitro synthesized transcripts as controls include five quantification positive controls (in duplicate) including 104, 103 and 102 copies of in vitro synthesized RNA transcripts.
- One negative amplification control.

AMPLIFICATION CYCLES (LIGHTCYCLER SYSTEM)

Steps	Temperature (°C)	Time	Cycles
Reverse transcription	45°C	15 min	x1
Denaturation	95°C	3 min	x1
Amplification	95°C	10 sec	x50
	60°C	10 sec	
Cooling	40°C	30 sec	x1

SENSITIVITY

For the M real-time RT-PCR

Sensitivity, in terms of 95% hit rate is about 100 copies of RNA genome equivalent per reaction (this amount of target sequences is always detected), the probability to detect lower amounts of virus decreases, but samples containing 10 copies could be detected.

For the H1 and N1 pdm real-time RT-PCR

Sensitivity is comparable to that of the M real-time RT-PCR and comparable to the sensitivity of the CDC kit (Cp <36 for all positive specimens tested so far).

For the H3h and N2h real-time RT-PCR

Sensitivity of the H3h real-time RT-PCR is equivalent to that of the M real-time PCR (Cp H3h ≈ Cp M) but the sensitivity of the N2h real-time RT-PCR is lower (Cp N2h ≈ Cp M + 5 Cp)

For the H5 and N1 avian real-time RT-PCR

Sensitivity of the H5 real-time RT-PCR is equivalent to that of the M real-time PCR (Cp N1h ≈ Cp M) but the sensitivity of the N1a real-time RT-PCR is lower (Cp H1h ≈ Cp M + 5 Cp)

SPECIFICITY

For the H1 and N1 pdm real-time RT-PCR

Limited testing so far showed no detection for seasonal influenza viruses (influenza A(H1N1), A(H3N2), B) nor for specimens known to be positive for other respiratory viruses (influenza C, RSV A, B, hBoV, hPIV1,3, hMPV, HRV, enterovirus, adenovirus, CMV, HSV, VZV).

For swine influenza viruses, detection was positive for A/sw/England/117316/86 (classical swine lineage) and negative for A/sw/England/502321/94 (H3N2).

For A(H1N1)pdm09 viruses, detection was positive for A/California/4/2009 as well as for more than 10 specimens positive for the novel A(H1N1)swl virus

NOTE: the H1pdm real-time RT-PCR does not detect the positive control from the CDC kit. Detection with the N1pdm set of primers is more robust than with the H1pdm set.

POSITIVE CONTROL FOR M AND GAPDH REAL-TIME RT-PCR

Positive control for M real-time RT-PCR is an *in vitro* transcribed RNA derived from strain A/Paris 650/06(H1N1). The transcript contains the Open Reading Frame of the M gene (from the ATG to nt 982) as negative strand. Each microtube contains 10^{11} copies of target sequences diluted in yeast tRNA, and lyophilised.

Positive control for GAPDH real-time RT-PCR is an *in vitro* transcribed RNA. The transcript contains the Open Reading Frame of the M gene (from nt 6 (ATG = 1) to nt 231) as negative strand. Each microtube contains 10^{11} copies of target sequences diluted in yeast tRNA, and lyophilised.

Reconstitution of transcribed RNA

Add 100 µl of distilled water to obtain a solution at a concentration of 10^9 copies/µl. Store at -80°C. Dilute in H_2O to prepare a master bank at $2x10^6$ copies/µl. Store at -80°C.

From this prepare a working bank of reagent at $2x10^4$ copies/µl in order to avoid freeze/thaw cycles. Working tubes may be stored at -20°C for less than one week.

Positive controls are available upon request (grippe@pasteur.fr)

INTERPRETATION OF RESULTS

GAPDH reactions should give a Cp < 35; if higher and otherwise negative results are obtained this may result from:

- poor quality of the specimen with insufficient number of cells ; obtain a new specimen for the same patient
- presence of inhibitors; repeat the procedure with dilutions of the extracted RNA (e.g. 1:10, 1:100) and/or repeat RNA extraction.

Positive reactions for M and H1pdm or N1pdm and negative reactions for H1h, N1h, H3h, N2h : confirmed case for A(H1N1)pdm09 virus

Positive reaction for M and negative for H1pdm and N1pdm and for H1h, N1h, H3h, N2h (usually seen for low virus load in specimen); repeat reactions and/or repeat RNA extraction

Positive reaction for M and negative for H1pdm and N1pdm but positive for either N1h, H3h and negative for H1h and N2h (usually seen for low virus load in specimen); infection with seasonal virus; repeat reactions and/or repeat RNA extraction to determine sub-types

Positive reaction for M and for H1pdm and N1pdm and positive reaction for either N1h, H3h may reflect a cross-contamination or a possible co-infection with both the novel A(H1N1)pdm09 virus and a seasonal virus; repeat RNA extraction and repeat reactions with all necessary precautions to avoid cross-contamination.

Negative reactions for M and positive reaction for HA-B confirmed case for type B virus (Yamagata or Victoria lineages).

Protocol 2: Real-time RT-PCR for Influenza A(H5) HA gene[23]

This Real-time RT-PCR protocol and primer set are designed to detect H5 viruses in avian specimens in Asia since the year of 2012. Suitable biosafety precautions should be made for handling suspected H5 samples. Laboratories that have concerns about identifying currently circulating H5 viruses should contact one of the WHO Collaborating Centres or WHO H5 Reference laboratories for assistance in identifying the optimal primers to be used.

Materials required

- QIAamp Viral RNA Mini Kit (QIAGEN® Cat. No. 51104) or equivalent extraction kit
- ThermoFisher Fast Virus 1-step Master Mix (Applied Biosystems®, Cat. No. 4444432)
- Ethanol (96–100%)
- Microcentrifuge (adjustable, up to 13,000 rpm)
- Benchtop centrifuge with 96-well plate adaptor
- Adjustable pipettes (10, 20, 100, 200µl)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortexer
- MicroAmp Fast Optical 96-Well Reaction Plate with Barcode (Applied Biosystems® Cat. no. 4346906)
- MicroAmp optical adhesive film (Life Technologies, Part No. 4311971)
- Real-time PCR system (Applied Biosystems® ViiA7 Real-Time PCR System, or equivalent)
- Positive control (Can be obtained from HKU)
- Primer and probe set

Primers and probe

Type/ subtype	Gene	Name	Sequence
Influenza A (H5Nx)	HA	H5-1201F	CARGGGAGTGGDTAYGCBGCAGA
		H5-1387R	ARAAGTTCAGCRTTRTARGTCCA
		H5-1285P	FAM-AARATGAACASTCARTTYGAGG-MGB
Influenza A (H5Nx) clade 2.3.4.4*	HA	H5.2344-1673F	TACCAAATAYTGTCAATTTATTCAAC
		H5.2344-1749R	GTAAYGACCCRTTRGARCACATCC
		H5.2344-1718P	FAM-CTGGCAATCATDRTGGCTGGTCT-BHQ1

* Provides improved sensitivity for clade 2.3.4.4 viruses

Procedure

1. Extract viral RNA from clinical specimen with QIAamp Viral RNA Mini Kit or equivalent extraction kit according to manufacturer's instructions.

2. Prepare master mixture for one step RT-PCR as below:

[23] School of Public Health, Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region of China.

Reagent	Volume (µl)	Final concentration
4X 1-step Master Mix (kit)	5	1X
50µM primer : H5-1201F	0.5	1.25µM
50µM primer : H5-1387R	0.5	1.25µM
50µM probe : H5-1285P	0.5	1.25µM
Water (molecular grade)	9.5	
Total	**16**	

3. Dispense 16µl of master mix into each reaction well.

4. Add 4µl of purified RNA to the above reaction mix.

5. Set the follow RT-qPCR conditions:

Step	Temperature (°C)	Time (min:sec)	No. of cycles
Reverse transcription	50 95	5:00 0:20	1
Denaturation	95	0:15	40
Extension (signal collection)	60	0:20	

Interpretation of results

Positive and negative controls should be included in the test. Before interpreting the data, these control reactions should be confirmed to have the expected results. An experimental reaction with a Ct value ≤38.0 is considered to be positive. A reaction with a Ct value greater than 38 should be re-tested. If possible, re-extract RNA from the original specimens and test the re-extracted RNA sample in the assay.

This test is intended to detect human H5 cases. It can detect both low and high pathogenic H5 viruses.

Protocol 3: Real-time RT-PCR procedures for the detection of [24]:

1. **Influenza type A viruses (M gene)**
2. **A(H1N1)pdm09 viruses (HA gene)**
3. **Former seasonal influenza A(H1N1) (HA gene)**
4. **A(H3N2) viruses (HA gene)**
5. **A(H5N1) viruses (Clade 1, 2, 3) (HA gene)**
6. **Influenza type B viruses (NS gene)**
7. **A(H7N9) viruses (HA gene)**
8. **A(H9N2) viruses (HA gene)**
9. **Influenza type B Victoria lineage viruses (HA gene)**
10. **Influenza type B Yamagata lineage viruses (HA gene)**

Extract viral RNA from clinical specimen as described in Annex 1: Conventional RT-PCR protocols.

Materials required

- QIAGEN® QuantiTect® Probe RT-PCR kit (Cat. No. 204443)*
  - 2x QuantiTect® Probe RT-PCR Master Mix
  - QuantiTect® RT Mix
- Thermo Fisher Scientific Inc. AgPath-ID™ One-Step RT-PCR Reagents (Cat. No. 4387424)*
  - 2x RT-PCR Buffer
  - 25x RT-PCR Enzyme Mix
- RNase-free water
- RNase Inhibitor (Thermo Fisher Scientific Inc., Cat. No. N808-0119)
- Primers
- TaqMan® MGB Probe

*Choose one kit of them

Test validation

Chromo-4 Real-time PCR Detection system (BioRad)*
LightCycler 2.0 (Roche) or LightCycler 480 (Roche)
ABI 7500 Fast (Applied Biosystems)

*Validated by QIAGEN® QuantiTect® Probe RT-PCR kit only

[24] WHO Collaborating Centre for Reference and Research on Influenza and WHO H5 Reference Laboratory at National Institute of Infectious Diseases (NIID). Gakuen 4-7-1, Musashi-Murayama-shi, Tokyo, Japan. E-mail : whocc-flu@nih.go.jp

Primers and probes

Type/subtype	Gene fragment	Primer	Sequence
Influenza type A	Matrix (M)	MP-39-67For	CCMAGGTCGAAACGTAYGTTCTCTCTATC
		MP-183-153Rev	TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA
		MP-96-75ProbeAs	5'-(FAM)-ATYTCGGCTTTGAGGGGGCCTG-(MGB)-3'
Influenza A H1N1pdm09	HA	NIID-swH1 TMPrimer-F1	AGAAAAGAATGTAACAGTAACACACTCTGT
		NIID-swH1 TMPrimer-R1	TGTTTCCACAATGTARGACCAT
		NIID-swH1 Probe2	5'-(FAM)-CAGCCAGCAATRTTRCATTTACC-(MGB)-3'
Former seasonal A(H1N1)	HA	NIID-H1 TMPrimer-F1	CCCAGGGYATTTCGCYGACTATGAG
		NIID-H1 TMPrimer-R1	CATGATGCTGAYACTCCGGTTACG
		NIID-H1 Probe1	5'-(FAM)-TCTCAAAYGAAGATACTGAACT-(MGB)-3'
A(H3N2) viruses	HA	NIID-H3 TMPrimer-F1	CTATTGGACAATAGTAAAACCGGGRGA
		NIID-H3 TMPrimer-R1	GTCATTGGGRATGCTTCCATTTGG
		NIID-H3 Probe1	5'-(FAM)-AAGTAACCCCKAGGAGCAATTAG-(MGB)-3'
A(H5) viruses (H5-1) (Clade 1, 2, 3)	HA	H5HA-205-227v2-For	CGATCTAGAYGGGGTGAARCCTC
		H5HA-326-302v2-Rev	CCTTCTCCACTATGTANGACCATTC
		H5HA-205-227-For(2010)	CGATCTAAATGGAGTGAAGCCTC
		H5HA-326-302-Rev(2010)	CCTTCTCTACTATGTAAGACCATTC
		H5-Probe-239-RVa2	5'-(FAM)-AGCCAYCCAGCTACRCTACA-(MGB)-3'
		H5-Probe-239-RVb2	5'-(FAM)-AGCCATCCCGCAACACTACA-(MGB)-3'
A(H5) viruses (H5-2) (Clade 2)	HA	H5HA-205-227-For(2014)	CGATCTTAATGGAGTGAAGCCCC
		H5HA-326-302-Rev(2014)	CCCTCTCCACGATGTAAGACCATTC
		H5-Probe-239-RVa2	5'-(FAM)-AGCCAYCCAGCTACRCTACA-(MGB)-3'
		H5-Probe-239-RVb2	5'-(FAM)-AGCCATCCCGCAACACTACA-(MGB)-3'
A(H7) viruses (Eurasian lineage)	HA	NIID-H7 TMPrimer-F1	TGTGATGAYGAYTGYATGGCCAG
		NIID-H7 TMPrimer-R1	ACATGATGCCCCGAAGCTAAAC
		NIID-H7 Probe1	5'-(FAM)ATCTGTATTCTATTTTGCATTGCYTC(MGB)-3'
A(H9) viruses	HA	NIID-H9 TMPrimer-F1	AATGTYCCTGTGACACATGCCAAAGA
		NIID-H9 TMPrimer-R1	AGRTCACAAGAAGGRTTGCCATA
		NIID-H9 Probe1	5'-(FAM)-CATYCCATTRTGCTCTGTGTGGAG-(MGB)-3'
Influenza type B	NS	NIID-TypeB TMPrimer-F1	GGAGCAACCAATGCCAC
		NIID-TypeB TMPrimer-R1	GTKTAGGCGGTCTTGACCAG
		NIID-TypeB Probe2	5'-(FAM)ATAAACTTYGAAGCAGGAAT(MGB)-3'
Influenza type B (Victoria lineage)	HA	F3vic v2	CCTGTTACATCTGGGTGCTTTCCTATAATG
		R3vic v2	GTTGATARCCTGATATGTTCGTATCCTCKG
		FAM-Type B HA Victoria	5'-(FAM)TTAGACAGCTGCCTAACC(MGB)-3'
Influenza type B (Yamagata lineage)	HA	F3yam v2	CCTGTTACATCCGGGTGCTTYCCTATAATG
		R3yam v2	GTTGATAACCTKATMTTTTCATATCCTCTG
		FAM-Type B HA Yamagata	5'-(FAM)TCAGRCAACTACCCAATC(MGB)-3'

Procedure

1. Prepare master mixture for Real-time RT-PCR as below:

For QIAGEN® QuantiTect® Probe RT-PCR kit

Reagent	Volume (µl)	Final Concentration
Water (molecular grade) [1]	3.65	
2x QuantiTectProbe® RT-PCR Master Mix	12.5	1X
Forward Primer (10µM)	1.5	0.6µM
Reverse Primer (10µM)	1.5	0.6µM
TaqMan MGB Probe (5pmol/µl) [2]	0.5	0.1µM
RNase Inhibitor (20U/µl)	0.1	
QuantiTect® RT Mix	0.25	
Total master mix	**20.0**	
RNA template	5.0	
TOTAL reaction volume	**25.0**	

[1] For the reaction of H5-1, the volume of RNase-free water is changed and primers, H5HA-205-227-For (2010) and H5HA-326-302-Rev(2010) are added as follows ;

RNase-free water	2.9µl
H5HA-205-227-For (2010) (10 µM)	0.375µl
H5HA-326-302-Rev (2010) (10 µM)	0.375µl

[2] For the reaction of H5 detection, a mixture of two probes is used.

H5-Probe-239-RVa	0.375µl
H5-Probe-239-RVb	0.125µl

For Thermo Fisher Scientific Inc. AgPath-ID™ One-Step RT-PCR Reagents

Reagent	Volume (µl)	Final Concentration
Water (molecular grade) [1]	2.9	
2X RT-PCR Buffer	12.5	1X
Forward Primer (10µM)	1.5	0.6µM
Reverse Primer (10µM)	1.5	0.6µM
TaqMan MGB Probe (5pmol/µl) [2]	0.5	0.1µM
RNase Inhibitor (20U/µl)	0.1	
25X RT-PCR Enzyme Mix	1.0	
Total master mix	**20.0**	
RNA template	5.0	
TOTAL reaction volume	**25.0**	

[1] For the reaction of H5-1, the volume of RNase-free water is changed and primers, H5HA-205-227-For (2010) and H5HA-326-302-Rev(2010) are added as follows ;

RNase-free water	2.15µl
H5HA-205-227-For (2010) (10 µM)	0.375µl
H5HA-326-302-Rev (2010) (10 µM)	0.375µl

[2] For the reaction of H5 detection, a mixture of two probes is used.

H5-Probe-239-RVa	0.375µl
H5-Probe-239-RVb	0.125µl

2. Dispense 20µl of the reaction mixture into each RT-PCR reaction plate.

3. Add 5µl of the sample RNA to the reaction mixture. For control reactions, use 5µl of distilled water for negative control and 5µl of appropriate viral RNAs for positive control.

4. Program the thermal cycler as shown in the table below.

5. Start the real-time RT-PCR program while the RT-PCR reaction plates are still ***on ice***.

PCR Temperature-cycling condition: Chromo-4 Real-time PCR Detection system (BioRad)

For QIAGEN® QuantiTect® Probe RT-PCR kit

Temperature (° C)	Time (minute:second)	No. of cycles
50	30:00	1
95	15:00	1
94 56*	0:15 1:00	45

* Fluorescence data is collected during 56°C incubation step.

PCR Temperature-cycling condition: LightCycler 2.0 (Roche) and LightCycler 480 (Roche)

For QIAGEN® QuantiTect® Probe RT-PCR kit

Temperature (°C)	Time (minute:second)	No. of cycles	Ramp Rate (˚C/sec)
50	30:00	1	Max
95	15:00	1	Max
94	0:15	45	1.5
56*	1:15		1

* Fluorescence data is collected during 56°C incubation step.

For Thermo Fisher Scientific Inc. AgPath-ID™ One-Step RT-PCR Reagents

Temperature (°C)	Time (minute:second)	No. of cycles	Ramp Rate (˚C/sec)
50	10:00	1	4.4
95	10:00	1	4.4
95	0:15	45	4.4
56*	0:30		2.2
72	0:15		4.4

* Fluorescence data is collected during 56°C incubation step.

PCR Temperature-cycling condition: ABI 7500 Fast (Applied Biosystems) (select Standard mode)

For QIAGEN® QuantiTect® Probe RT-PCR kit

Temperature (°C)	Time (minute:second)	No. of cycles
50	30:00	**1**
95	15:00	**1**
94	0:15	**45**
56*	1:15	

* Fluorescence data is collected during 56°C incubation step.

For Thermo Fisher Scientific Inc. AgPath-ID™ One-Step RT-PCR Reagents

Temperature (°C)	Time (minute:second)	No. of cycles
50	10:00	**1**
95	10:00	**1**
95	0:15	**45**
56*	0:30	
72	0:15	

* Fluorescence data is collected during 56°C incubation step.

Test result interpretation

Each Real-time RT-PCR assay includes:

- Positive controls; three or four 10-fold serial diluted each subtype-specific viral RNAs, including minimum detectable quantity
- Negative control; distilled water

When a Cp(Ct) value for a sample is lower than 40, and less than or equal to the Cp(Ct) value of each minimum detectable quantity positive control, the result indicates positive.

Subtyping of HA is determined when both the M gene and the corresponding HA gene are positive at the same time.

Validation of H5 PCR:

The following highly pathogenic H5N1 viruses were found to be positive in the assay:

Clade	Virus
1	A/Vietnam/1194/2004 (H5N1)
2.1	A/Indonesia/6/2005 (H5N1)
2.1.1	A/chicken/Pekalongan/BPPV4/2003 (H5N1)
2.1.2	A/chicken/Pangkal Pinang/BPPV3/2004 (H5N1)
2.1.3	A/Indonesia/5/2005 (H5N1)
2.1.3.2	A/Indonesia/NIHRD11931/2011(H5N1)
2.2	A/Turkey/12/2006 (H5N1)
2.2.1	A/Egypt/321/2007 (H5N1)
2.3.2	A/whooper swan/Hokkaido/1/2008 (H5N1)
2.3.2.1	A/Hubei/1/2010 (H5N1)
2.3.2.1	A/Vietnam/14011801/2014 (H5N1)
2.3.4	A/Anhui/01/2005 (H5N1)
2.3.4.4	A/chicken/Kumamoto/1-7/2014 (H5N8)
2.3.4.4	A/duck/Hyogo/1/2016 (H5N6)
2.4	A/chicken/Guangxi/12/2004 (H5N1)
2.5	A/chicken/Kyoto/3/2004 (H5N1)

Protocol 4: One-step Real-time RT-PCR for the detection of influenza A subtype H5, H7N9 and H9 viruses.

This protocol describes one-step real-time RT-PCR procedures for the detection of influenza A subtype H5, H7N9 and H9 viruses.[25]

Assay	Assay format	Target	Assay format
1	Singleplex	haemagglutinin gene - influenza virus type A subtype H5	Singleplex
2	Triplex	haemagglutinin gene - influenza virus type A subtype H7 neuraminidase gene - influenza virus type A subtype N9	Triplex
3	Singleplex	haemagglutinin gene - influenza virus type A subtype H9	Singleplex

Materials required

PCR kit	QuantiFast Pathogen, RT-PCR +IC Kit (Qiagen)
PCR reaction volume	10 µL
PCR instrument	LC480 (Roche)

Procedure

1. Prepare master mixture for reverse transcription as below:

Reagent	Volume (µL) per reaction
QuantiFast Pathogen Master Mix, 5x	2
QuantiFast Pathogen RT Mix, 100x	0.1
Primer probe mix	Variable[a]
RNase-free water	Variable[a]
Total	5
Template RNA	5

[a] Depending on the concentration of primer-probe mix prepared. See the Primers and Probes Table for the final concentration of primers and probes for each target.

2. Perform real-time RT-PCR according to the following conditions:

Step	Temperature (°C)	Time
Reverse transcription	50	20 min
Initial PCR activation	95	5 min
2-step cycling (40 repeated cycles):		
- denaturation	95	15 sec
- annealing/extension	60	45 sec

[25] Microbiology Division, Centre for Health Protection, Hong Kong SAR, China, (National Influenza Centre, WHO H5 Reference Laboratory). http://www.chp.gov.hk/

Primers and probes used for detecting influenza A subtype H5, H7N9 and H9 viruses

Assay	Virus (Target)	Name	Sequence[a]	Conc.[b]	Reference
1	A(H5) HA gene	H5-1012F	TGGGTACCACCATAGCAATGAGCA	0.1	own designed [c]
		H5-1155R	AATTCCCTTCCAACGGCCTCAAAC	0.1	own designed [c]
		H5-1042P-P2	CFO560–TGGGTACGCTGCAGACAAAGAATCCA–BHQ1	0.1	own designed [c]
2	A(H7) HA gene	H7-F	TCACAGCAAATACAGGGAAGAG	0.1	own designed [c]
		H7-R	CCCGAAGCTAAACCAGAGTATC	0.1	own designed [c]
		H7-P	FAM–TGACCCAGTCAAACTAAGCAGCGG–BHQ1	0.1	own designed [c]
	A(H7) HA gene	H7-Fe	CAATCACTGGACCACCTCAA	0.1	own designed [c]
		H7-Re	TCACGAATTTCCCAGGATAACA	0.1	own designed [c]
		H7-Pe	FAM-TGAGAGGCGAGAAGGAAGTGATGT-BHQ1	0.1	own designed [c]
	A(N9) NA gene	N9-F	GGAGTGGTTACAGTGGATCTTT	0.1	own designed [c]
		N9-R	CTTTATCCTCCTTGGGTCTTCC	0.1	own designed [c]
		N9-P2	Q670–AAACACGCTCGATAGCAGTCCC–BHQ2	0.1	own designed [c]
3	A(H9) HA gene	H9-1538-F	GGGTCAAGCTGGAATCTGA	0.1	own designed [c]
		H9-1651-R	TGGACATGGCCCAGAACAAGAA	0.1	own designed [c]
		H9-1567p2	Q670-TGTCGCCTCATCTCTTGTGVTTGCAA-BHQ2	0.1	own designed [c]

[a] CFO560, CAL Fluor Orange 560; Q670, Quasar 670.

[b] Final concentration of primers/probes (µM) in the master mixture.

[c] Microbiology Division, Centre for Health Protection, Hong Kong SAR, China, (National Influenza Centre, WHO H5 Reference Laboratory). http://www.chp.gov.hk/

E.

Protocol 1: Real-time RT-PCR assays for human influenza A(H7N9) virus[26]

The protocol is a Real-time RT-PCR to specifically detect avian influenza A (H7N9) virus using Real-time RT-PCR with specific primers and probes targeting the matrix, H7, and N9 genes.

Materials required

- Real-time fluorescence quantitative PCR analysis system
- Bench top centrifuge for 1.5mL Eppendorf tubes
- 10, 200, 1000µL pipettors and plugged tips
- Vortex
- QIAGEN® RNeasy Mini Kit
- AgPath one-step RT-PCR kit
- Primer set
- Other materials: RNase-free 1.5mL eppendorf tubes, RNase-free 0.2mL PCR tubes, powder-free disposables latex glove, goggles, headgear, shoe cover, tips for pipettors, β- thioglycol, 70% alcohol.

Primers and probes

The specific primers and probes for the H7 and N9 genes are summarized in the table below. In addition, the use of a primer and probe targeted M gene and house-keeping gene such a ribonucleoprotein (RNP) is recommended for typing all influenza A virus and internal control in the tests.

Type/ subtype	Gene	Name	Sequence	Note
A (H7N9)	HA	CNIC-H7F	5'-AGAAATGAAATGGCTCCTGTCAA-3'	Primer
		CNIC-H7R	5'-GGTTTTTTCTTGTATTTTTATATGACTTAG-3'	Primer
		CNIC-H7P	5'FAM-AGATAATGCTGCATTCCCGCAGATG-BHQ1-3'	Probe
	NA	CNIC-N9	5' -TAGCAATGACACACACTAGTCAAT-3'	Primer
		CNIC-N9R	5' -ATTACCTGGATAAGGGTCATTACACT-3'	Primer
		CNIC-N9P	5'FAM- AGACAATCCCCGACCGAATGACCC -BHQ1-3'	Probe
Influenza type A (Flu A)		InfA Forward	5' GACCRATCCTGTCACCTCTGA C 3'	Primer
		InfA Reverse	5' AGGGCATTYTGGACAAAKCGTCTA3'	Primer
		InfA Probe1	5' FAM-TGC AGT CCT CGC TCA CTG GGC ACG-BHQ1-3'	Probe
RNaseP		RnaseP Forward	5' AGATTTGGACCTGCGAGCG 3'	Primer
		RnaseP Reverse	5' GAGCGGCTGTCTCCACAA GT3'	Primer
		RnaseP Probe1	5'FAM-TTCTGACCTGAA GGCTCTGCGCG-BHQ1-3'	Probe

Note: FluA and RNase primer/probe sets were published from a WHO protocol provided by the US Centers for Disease Control and Prevention, Atlanta, USA.

[26] WHO Collaborating Center for Reference and Research on Influenza, Chinese Centers for Disease Control and Prevention, Beijing, China.

Procedure

1. Nucleic acid extraction
   The procedure is performed in a BSL-2 biohazard hood in the specimen preparation area according to the manufacturer's instructions. Elution of the RNA using a final volume of 50µL water is recommended.

2. Quality control parameters
   - Negative control: Sterile water is extracted as a negative control at the same time as the nuclear acid extraction of the other specimens.
   - Reagent blank control: RNase-free water
   - Positive control: RNA of the A(H7N9) virus provided. Internal positive control: RNP is recommended.

3. The reaction system preparation
   - Thaw the RT-PCR Master Mix, primers, and probes at room temperature in the reagent preparation area of the BSL-2 facility.
   - Prepare reaction mixture. Different primer pairs and probes should be prepared in the different tubes respectively. For each reaction:

Reagent	Volume (µL)
2× RT-PCR Master Mix	12.5
primer-forward (40µM)	0.5
primer-reverse (40µM)	0.5
Probe (20µM)	0.5
25xRT-PCR enzymes mix	1.0
Template RNA	5.0
RNase Free H2O	5.0
Total volume	25.0

4. Aliquot the reaction mixture into 0.2mL PCR tubes or a 96-well PCR plate as 20µL per tube and label clearly.
5. Add 5µL of the template RNA for the negative control, test specimens, or positive control into the separate tubes with the reaction mixture in a BSL-2 biohazard hood in the specimen preparation area.
6. Load the tubes in the PCR cycler for Real-time RT-PCR detection and use the following programme for cycling:

Temperature (°C)	Time (minute: second)	No. of cycles
45	10:00	1
95	10:00	
95	00:15	
60	00:45	
95	00:15	40
60	00:45	

Interpretation of results

The results are determined if the quality controls work.

1. The specimen is negative, if the value of Ct is undetectable.
2. The specimen is positive, if the Ct value is ≤38.0.
3. It is suggested that specimens with a Ct higher than 38 be repeated.

The specimen can be considered positive, if the repeat results are the same as before; i.e. the Ct value is higher than 38. If the repeat Ct is undetectable, the specimen is considered negative.

Criteria for quality control

1. The result of the negative control should be negative.
2. The Ct value of the positive control should not be more than 28.0.
3. Otherwise, the test is invalid.

Troubleshooting

1. False positives may be due to environmental contamination, if there is amplification detected in the negative control and reagent blank control. The unidirectional work flow must be strictly obeyed. The following measures should be taken should there be false positives: ventilate the laboratories, wash and clean the workbench, autoclave centrifuge tubes and tips, and use fresh reagents.
2. RNA degradation should be taken into consideration if the Ct value of the positive control is more than 30. All materials should be RNase-free.

Cautions

1. In order to avoiding nucleic acid cross-contamination, add the negative control to the reaction mixture first, then the specimen, followed by the positive control respectively.
2. Dedicated equipment for each area, including lab coats, pipettors, plugged tips and powder- free disposal latex gloves, are required.
3. Follow the instructions for maintenance of the incubator, PCR cycler, and pipettors. Calibration should be performed every 6 months.

Biosafety

The lysis of the specimen (500µL lysis buffer with 200µL clinical samples is recommended) should be to be carried out in a BSL-2 facility with BSL-3 level personal protection equipment. Subsequent procedures can be performed in a BSL-2 laboratory which has separate rooms, including a reagent preparation area, specimen preparation area, and amplification/detection area. The DNA-free area is the clean area and the area of amplified DNA is the dirty area. The work flow is from clean to dirty areas.

Protocol Use Limitations

These protocols were optimized using the quantitative one-step probe RT-PCR (AgPath one-step RT-PCR kit) which have been shown to produce comparable results on 96-well format thermocycler systems such as Stratagene QPCR instruments (MX3000® or MX3005®).

Protocol 2: Real-time RT-PCR assays for Eurasian H7 HA[27]

This assay is specific for Eurasian H7 HA. This assay might help to identify human viral infection caused by other Eurasian H7 viruses. Suspected human H7 cases that are negative for human H7N9 virus might be considered to be tested by this assay. Suitable biosafety precautions should be made for handling suspected H7 samples. It is recommended that laboratories having concerns about identifying human H7N9 viruses should contact one of the WHO CCs for assistance in identifying the optimal primers to be used.

Materials required

- QIAamp Viral RNA Mini Kit (QIAGEN®, Cat. No. 52906) or equivalent extraction kit
- ThermoFisher Fast Virus 1-step Master Mix (Applied Biosystems®, Cat. No. 4444432)
- Ethanol (96–100%)
- Microcentrifuge (adjustable, up to 13 000rpm)
- Benchtop centrifuge with 96-well plate adaptor
- Adjustable pipettes (10, 20, 100, 200µl)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortexer
- MicroAmp Fast Optical 96-well reaction plate (Life Technologies, Part No. 4346906)
- MicroAmp optical adhesive film (Life Technologies, Part No. 4311971)
- Real-time PCR system (ViiA™ 7 Real-Time PCR System)
- Positive control (Available from HKU, e-mail: llmpoon@hkucc.hku.hk)
- Primer and probe set

Primers and probe

Type/ subtype	Gene	Name	Sequence
Influenza A (H7Nx)	HA	H7-1603F	TTTAGCTTCGGGGCATCATGTTT
		H7-1674R	CAAATAGTGCACCGCATGTTTCCA
		H7-1646P	FAM-TGGGCCTTGTCTTCATATGTGTAAA-MGB

Procedure

1. Extract viral RNA from the clinical specimens by using the QIAamp viral RNA mini kit or equivalent extraction kit according to manufacturer's instructions.
2. Prepare master mixture for one step RT-PCR as below:

Reagent per reaction	Volume (µl)
4X 1-step Master Mix (kit)	5
10µM primer : H7-1603F	0.5
10µM primer : H7-1674F	0.5
10µM primer : H7-1646F	0.5
Water (molecular grade)	9.5
Total	16

[27] School of Public Health, The University of Hong Kong, Hong Kong SAR, China, (WHO H5 Reference Laboratory)

3. Dispense 16µl of master mix into each reaction well.

4. Add 4µl of purified RNA to the above reaction mix.

5. Set the follow RT-qPCR conditions:

Step	Temperature (^{o} C)	Time (minute:second)	No. of cycles
Reverse transcription	50	5:00	
	95	0:20	1
Denaturation	95	0:15	
Extension (signal collection)	60	0:20	40

Interpretation of results

Positive and negative controls should be included in the test. Before interpreting the data, these control reactions should be confirmed to have the expected results. An experimental reaction with a Ct value ≤38.0 is considered to be positive. A reaction with a Ct value greater than 38 should be re-tested. If possible, re-extract RNA from the original specimens and test the re-extracted RNA sample in the assay.

This test is intended to detect human H7 cases. It can detect both low and high pathogenic H7 viruses.

Protocol 3: Real-time RT-PCR for the detection of A(H7N9) influenza virus (M, H7 & N9 genes) [28]

Suitable biosafety precautions should be used for handling suspected A(H7N9) specimens.

We suggest the following testing strategy:

- RNA extraction
- Amplification in parallel of M, and GAPDH genes (to assess quality of the specimen and extraction procedure)
- In a separate set, amplification of the HA and NA specific genes.

Material

Kits

QIAamp Viral RNA (QIAGEN mini Kit 50) — Ref: QIAGEN 52 904

SuperScript™ III Platinum® One-Step Quantitative RT-PCR System — Ref: Invitrogen 11732-020

Primers and probes

The sequences from A/Shanghai/1/2013 (H7N9) virus served as template for assay design, i.e. for the design of the sets of primers and probe and for the design of the synthetic gene to be used as control (GISAID accession numbers: H7 EPI439486/ N9 EPI439487).

The table below provides a summary of the specific primers and probes targeting the H7 and N9 genes as well as primers and probes targeting the M gene for typing all influenza A viruses and a house-keeping gene such a GAPDH to be used as internal control in the tests.

Name	Sequences	Mers	PCR Product	Ref.
GAPDH-6Fw	GAAGGTGAAGGTCGGAGT	18	226 bp	3
GAPDH-231Rv	GAAGATGGTGATGGGATTTC	20		3
GAPDH-202Probe(-)	CAAGCTTCCCGTTCTCAGCC [5']Fam [3']BHQ-1	20		3
GRAM/7Fw	CTTCTAACCGAGGTCGAAACGTA	23	154 bp	2
GRAM/161Rv	GGTGACAGGATTGGTCTTGTCTTTA	25		2
GRAM probe/52/+	TCAGGCCCCCTCAAAGCCGAG [5']Fam [3']BHQ-1	21		2
H7N9 influenza virus				
GRavH7-155Fw	AACGAACAAACATCCCCA	18	186 bp	1
GRavH7-340Rv	CTTCATTCACGAATTTCCCA	20		1
GRavH7-311Probe(-)	ACATCACTTCCTTCTCGCCTCT [5']Fam [3']BHQ-1	22		1
GRavN9-67Fw	CTCATTGGAATGGCAAACCT	20	153 bp	1
GRavN9-219Rv	TTCCATTTGGATGTTGGTGA	20		1
GRavN9-121Probe(+)	TGCAATTGCTCACACTCACA [5']Fam [3']BHQ-1	20		1

References:

1/ National Influenza Center (Northern-France), Institut Pasteur, Paris.

2/ National Influenza Center (Southern-France), CHU, Lyon.

3/ Wong et al., 2005, J. Clin. Pathol. 58;276-280

[28] Unité de Génétique Moléculaire des Virus Respiratoires. Institut Pasteur, 25 rue du Docteur Roux 75724, Paris Cedex 15, France. Email: grippe@pasteur.fr; http://www.pasteur.fr

NUCLEIC ACID EXTRACTION

RNA is extracted from specimens using the QIAamp Viral RNA kit (QIAGEN Mini Kit 50 ref 52904). RNA extracted from 140 µl of original sample, is eluted into 60 µl of elution buffer.

MIX PREPARATION FOR ALL SEPARATE PRIMER/PROBE COMBINATIONS

All primers and probes described below for the detection of influenza A viruses (M gene), GAPDH and A(H7N9) (H7 and N9 genes) were validated under the following conditions.

RT-PCR Mix kit:

- Invitrogen Superscript™ III Platinum® One-Step qRT-PCR system (ref: 11732-088)

Real-time PCR equipments:

- LightCycler 480
- LightCycler 96

Prepare master mixture for real-time RT-PCR as below.

Mix :	**Vol (µl)**	**[final]**
H_2O PPI :	1.96	
Reaction mix 2X :	10	3 mM Mg
$MgSO_4$ (50mM) :	0.24	0.6 mM Mg
Forward Primer (10 µM):	1	0.5 µM
Reverse Primer (10 µM):	1	0.5 µM
Probe (10 µM):	0.4	0.2 µM
SuperscriptIII RT/Platinum Taq Mix :	0.4	
Final reaction mix volume:	**15 µl**	

15 µl of reaction mix + 5 µl of RNA samples

CONTROLS

Each real-time RT-PCR assay includes in addition of unknown samples :

- Two negative samples bracketing unknown samples during RNA extraction (negative extraction controls)
- Positive controls (in duplicate); when using *in vitro* synthesized transcripts as controls include five quantification positive controls (in duplicate) including 10^4, 10^3 and 10^2 copies of *in vitro* synthesized RNA transcripts.
- One negative amplification control.

AMPLIFICATION CYCLES (LIGHTCYCLER SYSTEM)

Detection Format:
LightCycler® 480 Instrument:

Fam 483-533 / Hex 523-568
LightCycler® 480 II Instrument: Fam 465-510 / Hex 533-580

1: Reverse Transcription (RT): transcription of the RNA to cDNA
2: Denaturation: sample denaturation and enzyme activation
3: Cycling: PCR-amplification of the target DNA
4: Cooling: cooling the instrument

Program Step	RT	Denaturation		Cycling		Cooling
Parameter						
Analysis Mode	None	None	Quantification mode			None
Cyles	1	1		50		1
Target [°C]	45	95	95	55	72	40
Hold [hh:mm:ss]	00:15:00	00:03:00	00:00:10	00:00:10	00:00:20	00:00:30
Ramp Rate [°C/s] 96	4.4	4.4	4.4	2.2	4.4	2.2
Acquisition Mode	None	None	None	None	Single	None

SENSITIVITY

For the M real-time RT-PCR

Sensitivity, in terms of 95% hit rate is about 100 copies of RNA genome equivalent per reaction (this amount of target sequences is always detected), the probability to detect lower amounts of virus decreases, but samples containing 10 copies could be detected.

For the H7 and N9 real-time RT-PCR

Sensitivity was verified using RNA extracted from a high titer virus stock of A/Anhui/ 1/2013(H7N9) virus. Sensitivity of the H7 and N9 real-time RT-PCR is equivalent to that of the M real-time PCR (Cp H7 or Cp N9 ≈ Cp M).

Expected Cp values with control synthetic RNA transcripts:

1E04 copies: Cp ≈ 28
1E03 copies: Cp ≈ 32
1E02 copies: Cp ≈ 36

SPECIFICITY

For the H7 and N9 real-time RT-PCR

Assay specificity was determined using:

- high-titer virus stock solutions of seasonal A(H1N1)pdm09, A(H3N2) and type B (Victoria and Yamagata lineages) influenza viruses, A/Turkey/England/63(H7N3) and A/chicken/HK/G9/97(H9N2) viruses.
- Samples from WHO EQAP-2011 including H5N1 clade 1, 2.1, 2.2 and 2.3.4 samples.
- clinical specimens (source NIC Northern-France) known to be positive for the following viruses:
  - influenza viruses : H1N1pdm09 (n=9), H3N2 (n=6), type B Victoria lineage (n=6), type B Yamagata lineage (n=12), type C (n=8)
  - other respiratory viruses: RSV A (n=8), RSV B (n=8), hMPV (n=11), HRV (n=7), hBoV(n=9).

Identities and virus RNA concentrations were re-confirmed by specific real-time RT-PCRs for each virus prior to testing.

To exclude non-specific reactivity of oligonucleotides among each other, all formulations were tested 24 times in parallel with assays containing water and no other nucleic acids except the provided oligonucleotides. In none of these reactions was any positive signal seen.

POSITIVE CONTROL FOR M, GAPDH, H7 AND N9 REAL-TIME RT-PCR

Positive control for M real-time RT-PCR is an *in vitro* transcribed RNA derived from strain A/Paris 650/06(H1N1). The transcript contains the Open Reading Frame of the M gene (from the ATG to nt 982) as negative strand. Each microtube contains 10^{11} copies of target sequences diluted in yeast tRNA, and lyophilized.

Positive control for GAPDH real-time RT-PCR is an *in vitro* transcribed RNA. The transcript contains the Open Reading Frame of the M gene (from nt 6 (ATG = 1) to nt 231). Each microtube contains 10^{11} copies of target sequences diluted in yeast tRNA, and lyophilized.

Positive control for H7 and N9 real-time RT-PCR is a combined (H7 and N9 genes) *in vitro* transcribed RNA. The transcript contains the Open Reading Frame of the H7 gene (from nt 12 (ATG = 1) to nt 427 and from nt 1528 to nt 1683) and the N9 gene (from nt 12 (ATG = 1) to 272) as negative strand. Each microtube contains 10^{11} copies of target sequences diluted in yeast tRNA, and lyophilized.

Reconstitution of transcribed RNA

Add 100 µl of RNase-free distilled water to obtain a solution at a concentration of 10^9 copies/µl. Store at -80°C.

Dilute in H_2O to prepare a master bank at $2x10^6$ copies/µl. Store at -80°C.

From this prepare a working bank of reagent at $2x10^4$ copies/µl in order to avoid freeze/thaw cycles. Working tubes may be stored at -20°C for less than one week.

Positive controls are available upon request (grippe@pasteur.fr)

INTERPRETATION OF RESULTS

GAPDH reactions should give a Cp < 35; if higher and otherwise negative results are obtained this may result from:

- poor quality of the specimen with insufficient number of cells ; obtain a new specimen for the same patient
- presence of inhibitors; repeat the procedure with dilutions of the extracted RNA (e.g. 1:10, 1:100) and/or repeat RNA extraction.

Positive reactions for M and H7 or N9 : confirmed case for A(H7N9) virus

Positive reaction for M and negative for H7 and N9 and for either seasonal influenza virus (might be observed for low virus load close to the detection limit); repeat reactions and/or repeat RNA extraction and/or obtain a new specimen for the same patient

Positive reaction for M and negative for H7 and N9 but positive for either seasonal influenza virus; infection with seasonal virus

Positive reaction for M and for H7 and N9 and positive for either seasonal influenza virus: may reflect a cross-contamination or a possible co-infection with both the novel A(H7N9) virus and a seasonal virus; repeat RNA extraction and repeat reactions with all necessary precautions to avoid cross-contamination.

AKNOWLEDGMENTS

We gratefully aknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database used for assay design (www.gisaid.org).

We are grateful to the originating laboratories for the A/Anhui/1/2013(H7N9) and A/chicken/HK/G9/97(H9N2) isolates used for validation of the sensitivity and specificity of the assay that were shared under the PIP Framework and respectively kindly provided by J. McCauley, WHO CC London and R. Webby, WHO CC Memphis.

F.

Protocol 1: Real-time RT-PCR assays for human influenza A(H10N8) virus[29]

To specifically detect avian influenza virus A(H10N8) virus using real-time RT-PCR with specific primers and probes targeting the matrix, H10 and N8 genes.

Materials required

- Real-time fluorescence quantitative PCR analysis system
- Bench top centrifuge for 1.5mL Eppendorf tubes
- 10, 200, 1000µL pipettors and plugged tips
- Vortex
- QIAGEN® RNeasy Mini Kit
- AgPath one-step RT-PCR kit
- The specific primers and probes for the H10and N8 genes are summarized in the table below. In addition, the use of a primer and probe targeted M gene and house-keeping gene such a RNP is recommended for typing all influenza A virus and internal control in the tests.
- Other materials: RNase-free 1.5mL eppendorf tubes, RNase-free 0.2mL PCR tubes, powder-free disposables latex glove, goggles, headgear, shoe cover, tips for pipettors, β- thioglycol, 70% alcohol

Primers and probes

The specific primers and probes for the H7 and N9 genes are summarized in the table below. In addition, the use of a primer and probe targeted M gene and house-keeping gene such a ribonucleoprotein (RNP) is recommended for typing all influenza A virus and internal control in the tests.

Type/ subtype	Gene	Name	Sequence
Influenza type A (H10N8)	HA	CNIC-H10F	5'- GCAGAAGAAGATGGRAAAGGR-3'
		CNIC-H10R	5'-GCTTCCTCTCTGTACTGTGWATG-3'
		CNIC-H10P	5'FAM-TGCATGGAGAGCATMAGAAACAACACCT-BHQ1-3'
	NA	CNIC-N8	5' –AGCTCCATTGTGATGTGTGG-3'
		CNIC-N8R	5' –AGGAAGAATAGCTCCATCGTG-3'
		CNIC-N8P	5'FAM- ACYATGAGATTGCCGACTGGTCA-BHQ1-3'
Influenza type A (FluA)		InfA Forward	5' GACCRATCCTGTCACCTCTGA C 3'
		InfA Reverse	5' AGGGCATTYTGGACAAAKCGTCTA3'
		InfA Probe1	5' FAM-TGC AGT CCT CGC TCA CTG GGC ACG-BHQ1-3'
RnaseP		RnaseP Forward	5' AGATTTGGACCTGCGAGCG 3'
		RnaseP Reverse	5' GAGCGGCTGTCTCCACAA GT3'
		RnaseP Probe1	5'FAM-TTCTGACCTGAA GGCTCTGCGCG-BHQ1-3'

**Note: FluA and RNase primer/probe sets were from published WHO protocol provided by CDC, Atlanta.*

[29] WHO Collaborating Center for Reference and Research on Influenza, Chinese Centers for Disease Control and Prevention, Beijing, China.

Procedure

1. **Nucleic acid extraction**
   The procedure is performed in a BSL-2 biohazard hood in the specimen preparation area according the manufacturer. Elution of the RNA using a final volume of 50µL water is recommended.

2. **Quality control parameters**
   Negative control: Sterile water is extracted as a negative control at the same time as the nuclear acid extraction of the other specimens.
   Reagent blank control: RNase-free water
   Positive control: RNA of the A(H10N8) virus
   Internal positive control: RNP is recommended.

3. **The reaction system preparation**
   (1) Thaw the RT-PCR Master Mix, primers, and probes at room temperature in the reagent preparation area of the BSL-2 facility.
   (2) Prepare reaction mixture. Different primer pairs and probes should be prepared in the different tubes respectively. For each reaction:

Reagent	Volume (µL)
2× RT-PCR Master Mix	12.5
primer-forward (40µM)	0.5
primer-reverse (40µM)	0.5
Probe (20µM)	0.5
25x RT-PCR enzymes mix	1.0
Template RNA	5.0
RNase Free H2O	5.0
Total volume	25.0

4. Aliquot the reaction mixture into 0.2mL PCR tubes or a 96-well PCR plate as 20µL per tube and label clearly.

5. Add 5µL of the template RNA for the negative control, test specimens, or positive control into the separate tubes with the reaction mixture in a BSL-2 biohazard hood in the specimen preparation area.

6. Load the tubes in the PCR cycler for Real-time RT-PCR detection and use the following programme for cycling:

Temperature (°C)	Time (minute: second)	No. of cycles
45	10:00	1
95	10:00	
95	00:15	
60	00:45	
95	00:15	40
60	00:45	

Interpretation of results

The results are determined if the quality controls work.

1. The specimen is negative, if the value of Ct is undetectable.
2. The specimen is positive, if the Ct value is ≤38.0.
3. It is suggested that specimens with a Ct higher than 38 be repeated.

The specimen can be considered positive, if the repeat results are the same as before; i.e. the Ct value is higher than 38. If the repeat Ct is undetectable, the specimen is considered negative.

Criteria for quality control

1. The result of the negative control should be negative.
2. The Ct value of the positive control should not be more than 28.0.
3. Otherwise, the test is invalid.

Troubleshooting

1. False positives may be due to environmental contamination, if there is amplification detected in the negative control and reagent blank control. The unidirectional work flow must be strictly obeyed. The following measures should be taken should there be false positives: ventilate the laboratories, wash and clean the workbench, autoclave centrifuge tubes and tips, and use fresh reagents.
2. RNA degradation should be taken into consideration if the Ct value of the positive control is more than 30. All materials should be RNase-free.

Cautions

1. In order to avoiding nucleic acid cross-contamination, add the negative control to the reaction mixture first, then the specimen, followed by the positive control respectively.

2. Dedicated equipment for each area, including lab coats, pipettors, plugged tips and powder- free disposal latex gloves, are required.

3. Follow the instructions for maintenance of the incubator, PCR cycler, andpipettors. Calibration should be performed every 6 months.

Biosafety

The lysis of the specimen (500µL lysis buffer with 200µL clinical samples is recommended) should be to be carried out in a BSL-2 facility with BSL-3 level personal protection equipment. Subsequent procedures can be performed in a BSL-2 laboratory which has separate rooms, including a reagent preparation area, specimen preparation area, and amplification/detection area. The DNA-free area is the clean area and the area of amplified DNA is the dirty area. The work flow is from clean to dirty areas.

Protocol Use Limitations

These protocols were optimized using the quantitative one-step probe RT-PCR (AgPath one-step RT-PCR kit) which have been shown to produce comparable results on 96-well format thermocycler systems such as Stratagene QPCR instruments (MX3000® or MX3005®).

Annex 3: Sequencing protocols

Protocol 1: One-step conventional RT-PCR for amplification and sequencing of the HA and NA genes from avian influenza A(H7N9) virus[30]

Materials Required

- QIAamp Viral RNA Mini Kit (QIAGEN® Cat. No. 52904）
- RNase-free water
- Ethanol (96-100%)
- MyTaq One-Step RT-PCR kit (Bioline BIO-65049)
- Microcentrifuge
- Adjustable pipettes（10, 20, 200, 1000µl）
- Sterile, RNase-free pipet tips with aerosol barrier
- Vortex
- Microcentrifuge tubes（0.2, 1.5ml）
- Thermal cyclers
- Primer sets
- E-gel (Invitrogen)

Primer sequences

Type/subtype	Gene fragment	Primer	Sequence
H7 Fragment I	H7	HAIM13F HAIM13R	TGTAAAACGACGGCCAGTATGAACACTCAAATCCTG CAGGAAACAGCTATGACCACAATTGGCATCAAC
H7 Fragment II	H7	HAIIM13F HAIIM13R	TGTAAAACGACGGCCAGTCATAGCTCCAGAC CAGGAAACAGCTATGACCTTATATACAAATAGTGCACC
	N9	NAIM13F NAIM13R	TGTAAAACGACGGCCAGTATGAATCCAAATCAGAAG CAGGAAACAGCTATGACCGTGAACACTACTGG
	N9	NAIIM13F NAIIM13R	TGTAAAACGACGGCCAGTCATACTAAGAACACAG CAGGAAACAGCTATGACCTTAGAGGAAGTACTC

For the H7 gene fragments, the expected product size is 860 bp and 890 bp, respectively.

For the N9 gene fragments, the expected product size is 720 bp and 740 bp, respectively.

Sequencing primers

M13F: TGTAAAACGACGGCCAGT
M13R: CAGGAAACAGCTATGACC

[30] RT-PCR protocols may be requested from the WHO CC for Reference and Research on Influenza, Melbourne, Australia.

Procedure

1. Extract viral RNA from clinical specimen using QIAamp viral RNA mini kit or equivalent extraction kit according to manufacturer's instructions.

2. Preparation of master mix for one step RT-PCR:

Reagent	Volume (µl)
2x MyTaq RT-PCR buffer	12.5µl
Forward Primer (20µM)	0.5µl
Reverse Primer (20µM)	0.5µl
RiboSafe RNase Inhibitor	0.5µl
RT/Taq	0.25µl
RNase free water	7.75µl
Total volume	**22.0 µl/test**

Mix gently by pipetting the master mix up and down gently and spin down briefly in a centrifuge.

3. Dispense 22µl of the master mix to each PCR reaction tube.

4. Add 3µl sample RNA to master mix in the PCR tube. Include proper positive control RNA and water for negative control.

5. Start the RT-PCR reaction using the following program:

Temperature (°C)	Time (minute:second)	No. of cycles
45	40:00	1
95	01:00	1
95 60 72	00:10 00:10 01:00	40
72	02:00	
7	Hold	Hold

6. Check 8µl PCR products on E-Gel according to manufacturer's instruction; take gel photo with a suitable Gel Doc system.

7. PCR product cleanup with ExoSAP-IT. Add 2µl of PCR product in a PCR tube containing 2µl of ExoSAP-IT and 3µl of H2O, mix well with gentle pipetting. Place the mixture in thermocycler and incubate at 37˚ C for 15 minutes, followed by 80˚ C for 15 minutes.

8. Each PCR products cleaned by ExoSAP-IT is to be sequenced from both ends using M13F and M13R primers. Prepare cycle sequencing master mixes with M13F or M13R primers, add 9µl

9. Master mix into corresponding wells of a 96-well PCR plate.

Reagent	Volume for 1 reaction (µl)
BigDye 5x buffer	2
BigDye 3.1 (1:5) dilution)	2
M13F or M13R primer (4µM)	1
Sterile MilliQ water	4
Total	**9**

10. Add 1µl of purified PCR product (5-20ng) into 9µl of M13F and M13R master mix, pulse spin the plate, place in thermocycle, and start the sequencing reaction using the following conditions:

Temperature (°C)	Time (minute:second)	No. of cycles
96 50 60	00:10 00:05 04:00	25
7	Hold	Hold

11. Clean up the sequencing reactions and load onto sequencer according to corresponding standard protocol.

Annex 4: Multiplex PCR protocols

Protocol 1: *Quadriplex RT-PCR assay for the detection of Influenza A, B, SARS-CoV-2, and RNaseP[31]

This method is a combination of two existing methods already validated in the laboratory:

- Influenza Real Time PCR Triplex A/B/RNP
- SARS-CoV-2 Real Time PCR (Duplex E Gene)

MATERIAL

Extraction

EasyMag , e-Mag Biomérieux + reagents

PCR Kit

SuperScript™ III Platinum® One-Step qRT-PCR System Ref: Invitrogen 11732- 088

PRIMERS AND PROBES

Influenza A (Gene Matrix) CDC protocol 2009

InfA Forward :	5' GACCRATCCTGTCACCTCTGAC 3'
InfA Reverse :	5' AGGGCATTYTGGACAAAKCGTCTA 3'
InfA Probe **FAM** :	5' 6-FAM/TGCAGTCCT/ZEN/CGCTCACTGGGCACG/IABkFQ 3'

Influenza B (van Elden et al., 2001)

InflB Forward :	5' AAATACGGTGGATTAAATAAAAGCAA 3'
InflB Reverse :	5' CCAGCAATAGCTCCGAAGAAA 3'
InflB Probe **HEX :**	5' HEX/CACCCATAT/ZEN/TGGGCAATTTCCTATGGC/IABkFQ 3'

Rnase P (internal control) **(Hummel et al., 2005)**

RP Forward :	5' AGATTTGGACCTGCGAGCG 3'
RP Reverse :	5' GAGCGGCTGTCTCCACAAGT 3'
RP Probe **ROX** :	5' 6-ROXN/TTCTGACCTGAAGGCTCTGCGCG/IABRQSP 3'

SARS-CoV-2 E Gene

E_Sarbeco_F1 :	5' ACAGGTACGTTAATAGTTAATAGCGT 3'
E_Sarbeco_R2 :	5' ATATTGCAGCAGTACGCACACA 3'
E_Sarbeco_P1 **Cy5** :	5' Cy5/ACACTAGCC/TAO/ATCCTTACTGCGCTTCG/IAbRQSp 3'

IABkFQ: 3' Iowa Black FQ
IAbRQSp: 3' Iowa Black RQ-Sp
ZEN & TAO: internal quencher in double quenched probes

NUCLEIC ACID EXTRACTION

RNA is extracted from respiratory samples using the EasyMAg or e-Mag
RNA extracted from 200 µl of original sample, is eluted in 50 µl of elution buffer.

All Assays used the same conditions.
Primer and probe, as well as optimized concentrations are below.
A 25µl reaction was set up containing 5µl of RNA template.

[31] National Influenza Centre, Brussels, Belgium

MIX PREPARATION

All primers and probes described below were validated under the following conditions.

Primer and Probes		[stock] µM	[final] nM
A	FP forward primer InfA	40	800
	RV reverse primer InfA	40	800
	Probe InfA (FAM)	10	200
B	FP forward primer InfB	50	1000
	RV reverse primer InfB	50	1000
	Probe InfB (HEX)	5	100
RP	FP forward primer RP	40	800
	RV reverse primer RP	40	800
	Probe InfA (ROX)	10	200
E gen	E-Sarbeco-F	40	400
	E-Sarbeco-R	40	400
	E-Sarbe co-P (Cy5)	10	200

RT-PCR Mix kit:

Invitrogen Superscript™ III Platinum® One-Step qRT-PCR system (ref: 11732-088)

Preparation of working master mix:

Reagents	x1 vol/reaction (µl)	x10 vol needed (µl)
Mastermix buffer	12.5	125
FP forward primer InfA	0.5	5
RV reverse primer InfA	0.5	5
Probe InfA (FAM)	0.5	5
FP forward primer InfB	0.5	5
RV reverse primer InfB	0.5	5
Probe InfB (HEX)	0.5	5
FP forward primer RP	0.5	5
RV reverse primer RP	0.5	5
Probe InfA (ROX)	0.5	5
E-Sarbeco-F	0.25	2.5
E-Sarbeco-R	0.25	2.5
E-Sarbe co-P (Cy5)	0.5	5
MgSO4	0.4	4
Enzyme mix	0.5	5
water	1.1	11
	Total volume	200

Distribute	20 µl per tube
Add	5 µl RNA template
Total volume	25 µl per tube

AMPLIFICATION CYCLES

Temperature (°C)	Time (minute:second)	No. of cycles
50	30:00	1
95	02:00	1
95 55	00:15 00:30	25

Flurochrome to detect: FAM, HEX, ROX, Cy5

Real-time PCR equipment:
Stratagene MX3500 or equivalent

CONTROLS

Negative Control

NPC (negative process control) is included every 10 samples (or every 5 samples depending on the epidemiological context)

NTC (negative template control)

Positive controls

PPC-A , PPC-B, PPC-COVID
PTC-A, PTC-B, PTC-COVID, PTC-RP

Prepared in ready to use aliquots and conserved at -80°C

Interpretation of results

Before interpreting the data, all control reactions should be confirmed to have the expected results. An experimental reaction with a Ct value ≤38.0 is considered to be positive. A reaction with a Ct value greater than 38 should be re-tested. If possible, re-extract RNA from the original specimens and test the re-extracted RNA sample in the assay.

REFERENCE

1. Corman VM, Landt O, Kaiser M, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill 2020;25.
2. Centers for Disease Control and Prevention. 2009. CDC protocol of realtime PCR for influenza A(H1N1)2009.
3. van Elden, L. J., M. Nijhuis, P. Schipper, R. Schuurman, and A. M. van Loon. 2001. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J.Clin.Microbiol. 39:196-200. doi:10.1128/JCM.39.1.196-200.2001 [doi].

Protocol 2: *Real time RT-PCR for the initial screening of SARS-CoV-2, Influenza A, B and C viruses[18]

Aim

A real time multiplex RT-PCR was developed to detect the influenza A, B, C and COVID-19 viruses. Selective samples (e.g. ILI) could be tested for surveillance purpose. Initial SARS-CoV-2 reactive samples should be confirmed with secondary test.

Reagents used

Roche NxtScript Master Mix, AptaTaq DNA polymerase and NxtScript RT

Real time PCR machine

Roche LC480 II (96 or 384 well format)

Primers and probe (5'-3'):

SARS-CoV-2 primer probe mix (in-house designed for SARS and SARS-related viruses)[1]		
Forward primer	Urbani-Faa	CTCACCTTATGGGTTGGGATTA
Reverse primer	Urbani-Raa	GTTTGCGAGCAAGAACAAGTG
Probe	Urbani-Paa	FAM-TGATAGAGCCATGCCTAACATGCT-BHQ1
Influenza A primer probe mix[2] (Ref 2)		
Forward primer	FLUAM-7-F	CTTCTAACCGAGGTCGAAACGTA
Reverse primer	FLUAM-161-R	GGTGACAGGATTGGTCTTGTCTTTA
Probe	FLUAM-49-P6	HEX-TCAGGCCCCCTCAAAGCCGAG-BHQ1
Influenza B primer probe mix[2] (Ref 1)		
Forward primer	FLUBHA-940-F	AAATACGGTGGATTAAACAAAAGCAA
Reverse primer	FLUBHA-1109-R	CCAGCAATAGCTCCGAAGAAA
Probe	FLUBHA-994-P4	Texas Red-CACCCATATTGGGCAATTTCCTATGGC-BHQ2
Influenza C primer probe mix[2] (in-house designed)		
Forward primer	CNP-1043-F	GCTTTGGACTTGCTTAT
Reverse primer	CNP-1141-R	GACTCTGAAGTTTCCTATTT
Probe	CNP-1095-P3	Q670-CCCTCTTAAGTTGAGAAACAGAATG-BHQ2

[1] equal volume of each primer (10µM) was mixed with equal volume of probe (10µM) to produce the SARS-CoV-2 in-use primer probe mix

[2] For influenza A, B and C, equal volume of each primer (10µM) was mixed with equal volume of probe (5µM) to produce each in-use primer probe mix.

Real time PCR master mix

Reagent	Volume (µl)
Master Mix:	
NxtScript 5X Master Mix	2
SARS-CoV-2 primer probe mix	0.9
Influenza A primer probe mix	0.6
Influenza B primer probe mix	0.6
Influenza C primer probe mix	0.6
Water	0.2
AptaTaq DNA polymerase (glycerol-free) 50U/µL	0.06
NxtScript RT Enzyme 85U/µL	0.05
Each reaction:	
Master Mix	5
Extracted RNA	5

Real time RT-PCR condition

Temperature (°C)	Time (minute:second)	No. of cycle
55	10:00	1
95	0:30	1
95	0:10	45
60	0:30	

References

1. van Elden LJ, et al. 2001. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J Clin Microbiol 39:196-200
2. Terrier O, et al. 2011. Cellular transcriptional profiling in human lung epithelial cells infected by different subtypes of influenza A viruses reveals an overall down-regulation of the host p53 pathway. Virol J 8:285

Protocol 3: *One-step real-time RT-PCR 4-plex assay for the detection and subtyping of Influenza A, B, (H1)pdm09 and H3 viruses[32]

This protocol describes a one-step 4-plex real-time RT-PCR assay for the detection of influenza A and influenza B viruses, and (H1)pdm09 and H3 subtype viruses. The assay is used primarily to underpin national surveillance of influenza viruses.

Following extraction of the viral RNA from test samples or controls, multiplex one-step RT-PCR is performed using an ABI QuantStudio 7 Flex thermocycler. Four sets of primers and five probes are used in this multiplex assay, which are specific for targeted regions of the matrix protein (M) of influenza A viruses and nucleoprotein (NP) of influenza B viruses, and the hemagglutinin (HA) genes of H3 and (H1)pdm09 influenza A virus subtypes.
Due to the multiplex format of the assay, four different fluorophores are used; the H3 and (H1)pdm09 probes are TaqMan® MGB Probes **which** incorporate a 5' reporter dye and a 3' non-fluorescent quencher (NFQ), with the MGB moiety attached to the quencher molecule. The AM and BNP probes are TaqMan QSY Probes, **which** incorporate a 5' reporter dye and a 3' QSY quencher. **These probes also allow mustang purple to be used as a passive reference dye, which is present in the TaqPath**1-Step Multiplex Master Mix.

Materials required

- TaqPath™ 1-Step Multiplex Master Mix (Thermofisher; A28525, A28526, A28527)
- Positive and negative controls
- Water (molecular grade)
- Applied Biosystems QuantStudio 7 Flex thermocycler
- Applied Biosystems MicroAmp Fast Optical 96-well Reaction Plate (0.1 ml)

Primers and probes

Type/subtype	Gene Target	Name	Sequence
Influenza A	M	AM Forward	5'- GAGTCTTCTAACMGAGGTCGAAACGTA - 3'[1]
		AM Reverse	5'- GGGCACGGTGAGCGTRAA - 3'[1]
		AM Probe	5' JUN - TCAGGCCCCCTCAAAGCCGAG - QSY 3'[2]
Influenza B	NP	BNP Forward	5'- GCAGCTCTGATGTCCATCAAGCT - 3'[1]
		BNP Reverse	5'- CAGCTTGCTTGCTTARAGCAATAGGTCT - 3'[1]
		BNP Probe	5' ABY - CCAGAYCTGGTCATYGGAGCCCAAAACTG - QSY 3'[1]
Influenza A(H1)pdm09	HA	AH1 Forward	5'- TTACCAGATTTTGGCRATCTAYT - 3'
		AH1 Reverse	5'- CCAGGGAGACTASCARTACCA - 3'
		AH1 (3) Probe	5' FAM - ACWGTYGCCAGTTC - MGBNFQ 3'
		AH1 (5) Probe	5' FAM - ACWGCYGCCAGTTC - MGBNFQ 3'
Influenza A(H3)	HA	AH3 Forward	5'- TGGGACCTTTTYGTTGAAMG - 3'
		AH3 Reverse	5'- CGGATGAGGCAACTAGTGAYCTA - 3'
		AH3 Probe	5' VIC - CCWACAGCAACTGTTAYC - MGBNFQ 3'

[1]Ellis J, Curran M. 2001 Simultaneous molecular detection and confirmation of influenza AH5, with internal control. Methods Mol Biol. 2011;665:161-81

[2]Terrier O, et al 2011. Cellular transcriptional profiling in human lung epithelial cells infected by different subtypes of influenza A viruses reveals an overall down-regulation of the host p53 pathway. Virol J 8:285
All other primers/probes designed in-house

[32] National Influenza Centre, Public Health England, UK

Test validation

This assay has been validated in a UKAS accredited laboratory in accordance with ISO 15189 standards, including measurement of diagnostic sensitivity, specificity, linearity and precision, and peer reviewed by the Public Health England Diagnostic Development Evaluations Unit. The assay has been validated using clinical samples known to be positive or negative for influenza A(H1)pdm09, A(H3) or B viruses. No cross-reactivity was observed between influenza types or subtypes, and in addition no cross-reactivity was seen with the clinical samples or virus isolates analysed containing other respiratory viruses. The assay may also detect the HA of swine H1N1, H1N2 or H3N2 viruses, since the HA gene of these viruses may be derived from human seasonal viruses.

Procedure

Following preparation of 150µl of specimen in the appropriate volume of lysis buffer for inactivation, nucleic acid was extracted using the Biomérieux NucliSENS easyMAG or eMAG automated system, or Perkin Elmer 360 Chemagic, and eluted in 100µl. Extracted RNA can be used directly in the influenza 4-plex assay described below.

Each RT-PCR run should include at least one negative and one positive control reaction for each of the targets.

1. Prepare master mix for RT-PCR as below for the required number of samples to be tested (n=x+1 to ensure excess volume);

Reagent	Final Conc (nM)	1rxn (µl)
Water		8.54
AM Forward (100uM)	900	0.18
AM Reverse (100uM)	900	0.18
BNP Forward (100uM)	400	0.08
BNP Reverse (100uM)	900	0.18
AH1 Forward 16R (100uM)	600	0.12
AH1 Reverse (100uM)	600	0.12
AH3 Forward (100uM)	400	0.08
AH3 Reverse (100uM)	500	0.1
AM probe (20uM)	250	0.25
BNP probe (20uM)	30	0.03
AH1 (3) & (5) probes (20uM)[1]	120	0.12
AH3 probe (20uM)	20	0.02
4X TaqPath 1-step Multiplex		5
RT-PCR Mix Per Reaction		15
RNA per reaction		5
Total reaction volume		20

[1]For (H1)pdm09 detection a mixture of two probes is used;

AH1 (3)	0.6µl
AH1 (5)	0.6µl

Keep reagents on ice and set up reactions in a cold block and protected from light.

2. In a 96-fast plate, aliquot 15µl of RT-PCR master mix into each well. Add 5µl of extracted RNA .

3. Run on an ABI QuantStudio 7 Flex real-time thermocycler. In the experiment properties set-up, select Fast 96-well (0.1ml), Standard Curve, TaqMan Reagents, FAST mode. Define FAM and VIC with NFQ-MGB, ABY and JUN with no quencher. Set passive reference to Mustang Purple. Assign Dyes to the wells.

4. Run the following cycling conditions and acquire data on the 60˚C extension cycle;

Step	Stage	Cycles	Temperature	Time for Fast systems
UNG incubation	1	1	25˚C	2 minutes
Reverse transcription	2	1	53˚C	10 minutes
Polymerase activation	3	1	95˚C	2 minutes
Amplification	4	40	95˚C	3 seconds
			60˚C	30 seconds

Interpretation of results

Positive and negative controls should be confirmed to have the expected results first before interpreting the data for test samples. Samples with RT-PCR Ct values ≤38 are considered positive. A reaction with a Ct >38 should be re-tested, if possible from the original sample.