WHO information for molecular diagnosis of influenza virus - update

It is strongly recommended that all un-subtypable influenza A specimens should be immediately sent for diagnosis and further characterization to one of the six WHO Collaborating Centres for Reference & Research on Influenza[1].

March 2014

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

This is a revision of the document published on the WHO website in November 2012 with updated molecular protocols. The protocols are in the annexes below:

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

Specimens

The most appropriate specimens for the diagnosis 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 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" recently published by WHO.[2]

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

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)2009 virus (formerly pandemic A(H1N1) 2009), A(H3N2), former seasonal A(H1N1) and the highly pathogenic avian influenza A(H5N1) virus;
- haemagglutinin gene specific for influenza B virus.

The following protocols are currently available:

— influenza A type-specific conventional and realtime-PCR (see Annexes 1 and 2);
— A(H1N1)2009 virus specific conventional and realtime-PCR (see Annexes 1 and 2);
— CDC realtime RT-PCR (rRT-PCR) protocol for the detection and characterization of A(H1N1) 2009;[3]
— former seasonal influenza A(H1N1), influenza A(H3N2), influenza B and avian influenza A(H5N1) realtime RT-PCR (see Annexes 1 and 2);
— influenza B lineages conventional and realtime RT-PCR (see Annexes 1 and 2);
— A(H7N9) conventional and realtime 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 Committee on influenza PCR. Members of this Committee are from the WHO H5 Reference laboratories.[4]

Interpretation of PCR results

- PCR — A sample is considered positive if results from tests using two different PCR targets (e.g. primers specific for universal M gene and A(H1N1)2009 haemagglutinin gene) are positive. If RT-PCR for multiple haemagglutinin (HA) targets (e.g. A(H3), and A(H1N1)2009) give positive results in the same specimen, the possibility of PCR contamination should first be excluded by repeating the 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 coinfection, which should be confirmed by sequencing or virus culture.
- CDC realtime-PCR assays — Results should be interpreted as described in the CDC H1N1 realtime assay manual.[2]
- A negative 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 PCR results are negative

[3] http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html
[4] http://www.who.int/influenza/gisrs_laboratory/h5_reflabs/list/en/index.html

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

Specimens with laboratory results indicative of influenza A that are un-subtypeable (i.e. negative for influenza A(H1), A(H3) and A(H5)) should be forwarded to a WHO CC as soon as possible for confirmation.

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

Standard and relevant IATA regulations for influenza specimen storage, packaging and shipping practices should be followed.[5]

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 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 neighbour.

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.[6]

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.

[5] http://www.who.int/csr/resources/publications/swineflu/storage_transport/en/index.html
[6] http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/

Validation

All protocols should always be validated in each laboratory 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 NIC evaluation exercises (external quality assessment programme) 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 level. RT-PCR should be performed in a space separated from that used for virus isolation techniques.

Equipment

Equipment should be used and maintained according to the manufacturer's recommendations.

Annex 1: Conventional RT-PCR protocols

A. Conventional RT-PCR analyses for the matrix gene of influenza type A viruses[7]

The following protocols are for conventional RT-PCR and gel electrophoresis of 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 laboratories[8] for diagnosis of influenza infection or one of the WHO CCs[9] 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 (Applied Biosystems, 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 fragment	Primer	Sequence
Influenza type A	Matrix (M)	M30F2/08 M264R3/08	ATGAGYCTTYTAACCGAGGTCGAAACG TGGACAAANCGTCTACGCTGCAG

Expected PCR product size is 244 bp

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:

[7] WHO Collaborating Centre for Reference and Research on Influenza. National Institute of Infectious Diseases. Gakuen 4-7-1, Musashi-Murayama-shi, Tokyo 208-001, Japan. Email: todagiri@nih.go.jp
http://idsc.nih.go.jp/

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

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

- 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

3. 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:

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

B) Sample loading:

i) Add 5µl of the gel loading buffer to each PCR tube.
ii) Load molecular weight marker to the first well of the agarose gel.
iii) Pipette 15µl of the PCR product in gel loading buffer to the gel.
iv) Close the lid on the chamber and attach the electrodes. Run the gel at 100V for 30–35 minutes.
v) Visualize the presence of marker and PCR product bands with a UV light.
vi) 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.

B. Conventional one step RT-PCR for A(H1N1)2009 HA gene[10]

The protocols and primers for conventional RT-PCR to detect A(H1N1)2009 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 H5 Reference laboratories or one of the WHO CCs 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)2009 virus	HA	HKU-SWF HKU-SWR	GAGCTCAGTGTCATCATTTGAA TGCTGAGCTTTGGGTATGAA

Expected PCR product size is 173 bp.

Procedure

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

[10] Department of Microbiology, Faculty of Medicine, University of Hong Kong, University Pathology Building Queen Mary Hospital, Hong Kong Special Administrative Region of China.

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	50 95	30:00 15:00	1
Denaturation Annealing Extension	94 57 72	0:30 0:30 0:20	40
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 173 bp. This assay can specifically detect samples with A(H1N1)2009, 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)2009 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.

C. Conventional one step RT-PCR for A(H5N1) HA gene

Protocol 1:[11]

This conventional RT-PCR protocol and primer set are designed to detect highly pathogenic H5N1 viruses in human specimens. 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
- 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.

Procedure

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

[11] Department of Microbiology, Faculty of Medicine, University of Hong Kong, University Pathology Building Queen Mary Hospital, Hong Kong Special Administrative Region of China.

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 (^{o}C)	Time (minute:second)	No. of cycles
Reverse transcription	50 95	30:00 15:00	1
Denaturation Annealing Extension	94 55 72	0:30 0:30 0:30	45
Post-PCR extension	72	7:00	1
Post-run	4	∝	

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.

Real-time RT-PCR format

The assay can be converted into a real-time RT-PCR format by including a hydrolysis probe (H5-Probe: FAM-ACCATKCCYTGCCAYCCYCCYTCT-3BHQ1).

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:[12]

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 members of the WHO Expert Committee on influenza PCR or one of the WHO CCs 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 on page 5 for detection of the universal M gene RT-PCR protocol developed by the US 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.

[12] 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 208-001, Japan.

D. Influenza B lineage-specific one-step conventional RT-PCR protocol[13]

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 fragment	Primer	Sequence
Influenza B Victoria lineage	HA	Bvf224	ACATACCCTCGGCAAGAGTTTC
		Bvr507	TGCTGTTTTGTTGTTGTCGTTTT
Influenza B Yamagata lineage	HA	BYf226	ACACCTTCTGCGAAAGCTTCA
		BYr613	CATAGAGGTTCTTCATTTGGGTTT

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.

[13] 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.

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

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

1. The expected PCR product sizes for B-Victoria lineage is 284 bp and for B-Yamagata lineage is 388 bp.
2. 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

E. Conventional RT-PCR assays for the detection of influenza A(H1N1, H3N2), influenza B and avian influenza A(H5N1) viruses[14]

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

1. A(H1N1)2009 viruses (H1 and N1 genes)
2. Influenza A(H3N2) viruses (H3 and N2 genes)
3. Influenza B viruses (HA and NA genes)
4. Avian influenza A(H5N1) (H5 and N1 genes)
5. 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 PCR products can be used for diagnosis of influenza and sequencing studies.

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 13 000 rpm)
- Adjustable pipettes (10, 20, 100, 200 µl)
- RNAsin (Promega #N2515)
- SS III RT (Invitrogen #18080-085)
- Pfx Polymerase (Invitrogen #11708-039)
- Water (QIAGEN®, Cat. No. 129114)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes (0.2, 1.5 ml)
- Thermocycler: BIORAD DNA Engine (BIORAD)
- Primer set

Procedure

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 sample extracts or 2µl in a 50µl reaction for grown virus extracts.

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

[14] WHO Collaborating Centre for Reference and Research on Influenza. National Institute for Medical Research. The Ridgeway, Mill Hill, London NW7 1AA, England. Email: whocc@nimr.mrc.ac.uk. http://www.nimr.mrc.ac.uk/wic/

Primers

Primer sets used for one-step RT-PCR for human influenza surveillance (London WHO CC; May 2011)

Type/subtype	Gene fragment	Primer	Sequence	PCR Product Size (bp)
Influenza A(H1N1)2009	HA-5'(H1)	H1F1 H1R1264	AGCAAAAGCAGGGGAAAATAAAAGC CCTACTGCTGTGAACTGTGTATTC	1264
	HA-3'(H1)	H1F848 HARUc	GCAATGGAAAGAAATGCTGGATCTG ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	945
	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	TATTCGTCTCAGGGAGCAGAAGCAGAGCATTTTCTAATATC TTCGTTGTGGAGTTCATCCAT	1361
	HA-3'	BHAF458 BHA2R1	AGAAAAGGCACCAGGAGGACCCTA GTAATGGTAACAAGCAAACAAGCA	1391
	NA-5'	BNAF1u BNAR2	TATTCGTCTCAGGGAGCAGAAGCAGAGCATCTTCTCA GATGGACAAATCCTCCCTTGATGC	1130
	NA-3'	BNAF2 BNAR1487	GCACTCCTAATTAGCCCTCATAGA TAAGGACAATTGTTCAAAC	1182
Influenza A(H5N1)	HA-5'(H5)	H5A1F1 H5R1265	AGCAAAAGCAGGGGTATAATC ACGGCCTCAAACTGAGTGTTCATT	1263*
	HA-3'(H5)	H5F417 H5A2R1	TTGAGAAAATWCAGATCATCCC AAGGGTGTTTTTAACTAACAATCT	1351*
	NA-5'(N1)	H5N1F4 H5N1R1112	AGCAAAAGCAGGAGATTAAAATGAAT TTCTCCCGATCCAAACACCATTGC	1104
	NA-3'(N1)	H5N1F461 H5N1R1457	GACTGTCAAAGACAGAAGCCCTCA GTAGAAACAAGGAGTTTTTTGAA	997
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

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.

* Size will vary slightly depending on the length of the encoded polybasic (amino acid) cleavage site.

Procedure

One-Step RT-PCR protocol

1. QIAGEN® OneStep RT-PCR kit (Cat. No. 210212) kit is sufficient for 100 x 50µl reactions following manufacturer's instructions.

2. Invitrogen reagent-based one-step protocol used at the National Institute for Medical Research (NIMR, London).

Reagent	Volume (µl) Clinical	Volume (µl) Virus	Final concentration
Water (molecular grade)	30.6	33.6	
10X Buffer*	7.5	7.5	
50mM $MgSO_4$*	1.0	1.0	
100mM dNTPs	0.9	0.9	25mM of each dNTP
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	
SS III RT	1.0	1.0	
Pfx Polymerase*	0.5	0.5	
RNA	5.0	2.0	
Total	**50.0**	**50.0**	

* Supplied with the Pfx polymerase.
† See above for primer pairings dependent on the type/subtype being amplified.

Thermal cycler BIORAD DNA Engine programme:

Temperature (°C)	Time (minute:second)	No. of cycles
50	30:00	1
94	10:00	1
94	0:05	40
55	0:05	
68	2:00	
68	10:00	1
4	Hold	

Two-step RT-PCR

For weak clinical samples (e.g. those with low Ct values [30 or higher] in qRT-PCR) an 'optimized' two-step protocol is used.

a) <u>RT step</u>

Type	Gene fragment	Primer	Sequence
Influenza A	all genes	uni12W	AGCRAAAGCAGG
Influenza B	all genes	Buni11W	AGCAGAAGCGS

For a 40 µl reaction:

Reagent	Volume (µl)	Final concentration
Water (molecular grade)	12.1	
5X buffer*	8.0	
0.1M DTT *	2.0	
RNAsin	2.0	
100mM dNTPs	0.9	25mM of each dNTP
30µmol/l Uni12W or Buni 11W	3.0	
SS III RT	2.0	
Template RNA	10.0	
Total	**40.0**	

* Supplied with the SS III RT.

Method

Mix primer and template in a thin-walled tube and incubate at 65^{o} C/5 min. Remove from heat source (DNAEngine) and allow to cool to room temperature. Centrifuge briefly before adding 27µl reaction mix, then mix and briefly centrifuge before thermal cycling using the programme:

Temperature (^{o}C)	Time (minute:second)
25	5:00
50	60:00
70	15:00

b) PCR step

For a 50 µl reaction:

Reagent	Volume (µl)	Final concentration
Water (molecular grade)	35.1	
10X buffer*	7.5	
$MgSO_4$*	1.0	
100mM dNTPs	0.9	25mM of each dNTP
10µmol/l Forward primer†	1.5	
10µmol/l Reverse primer†	1.5	
Pfx	0.5	
RT product	2.0	
Total	**50.0**	

* Supplied with the Pfx polymerase.

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

Method

Mix primers and template in a thin-walled tube, then add 45µl reaction mix. Mix and briefly centrifuge before thermal cycler programme:

Temperature (°C)	Time (minute:second)	No. of cycles
94	10:00	1
55	5:00	1
68	2:00	1
94	0:05	39
55	0:05	
68	2:00	
94	0:05	1
55	0:05	1
68	10:00	1
4	Hold	

Product analysis

Run 5µl each sample on a 0.8% (w/v) agarose gel made up with 1X TBE buffer and containing GelRed dye (Biotium, Cat. No. 41003-1) 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 NIMR is from GE Healthcare (illustra GFX PCR DNA and gel band purification kit No. 28-9034-70). 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 GeneQuant pro (Cat. No. 80-2114-98) 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 capillary based sequencers (MegaBACE 1000 or ABI X3730).

Annex 2: Realtime RT-PCR protocols

Realtime RT-PCR poses different challenges than conventional RT-PCR. In addition to the RT-PCR considerations described in Annex 1, specific considerations for realtime 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 discarded 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. Realtime RT-PCR test for the matrix gene of influenza type A viruses[15]

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

Materials required

Reverse transcription

- 10X PCR buffer I with 15mmol/l $MgCl_2$ (Applied Biosystems Cat No. 4379876)
- Random hexamer 50µmol/l (Applied Biosystems, Cat. No. 8080127)
- MuLV Reverse Transcriptase 50U/µl (Applied Biosystems, Cat. No. 8080018)
- RNase Inhibitor 20U/µl (Applied Biosystems, Cat. No. 8080119)

Realtime PCR

- LightCycler® – FastStart™ DNA Master HybProbe kit (Roche Applied Sciences, Cat. No. 03 003 248 001)

Primers and probes

Working primer and probe mix is prepared by adding equal volumes of the following six components:

Type/subtype	Gene fragment	Primer	Sequence
Influenza type A	Matrix (M)	FLUAM-1F	AAGACCAATCCTGTCACCTCTGA (10µmol/l)
		FLUAM-2F	CATTGGGATCTTGCACTTGATATT (10µmol/l)
		FLUAM-1R	CAA AGCGTCTACGCTGCAGTCC (10µmol/l)
		FLUAM-2R	AAACCGTATTTAAGGCGACGATAA (10µmol/l)
		FLUA-1P	5'-(FAM)-TTTGTGTTCACGCTCACCGT-(TAMRA)-3' (5µmol/l)
		FLUA-2P	5'-(FAM)-TGGATTCTTGATCGTCTTTTCTTCAAATGCA-(TAMRA)-3 (5µmol/l)

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

Procedure

1. Prepare master mixture for reverse transcription as below:

Reagent	Volume (µl)
10X PCR buffer I with 15mmol/l $MgCl_2$	2.0
Extra 25mmol/l $MgCl_2$	2.8
dNTPs (2.5mmol/l)	8.0
Random hexamer 50µmol/l	1.0
RNAase inhibitor 20U/µl	1.0
Reverse transcriptase 50U/µl	1.0
Total volume	**15.8**

2. Add 4.2µl viral RNA to the above mix.
3. Vortex and centrifuge the tube with the mixture briefly (~3 sec).
4. Stand the tube at room temperature for 10 minutes and then incubate at 42° C for at least 15 minutes.
5. Incubate the tube at 95° C for 5 minutes and then chill in ice.
6. Prepare master mixture for RT-PCR as below:

Reagent	Volume (µl)
Water (molecular grade)	7.6
$MgCl_2$ (25mmol/l)	2.4
Primers and probe mix	3.0
'Hot Start' reaction mix *	2.0
Total volume	**15.0**

* Prepare 'Hot Start' reaction mix according to Roche LightCycler - FastStart DNA Master HybProbes kit's instructions.

7. Add 5µL cDNA to the above mix.
8. Perform realtime RT-PCR according to the following conditions:

Step	Temperature (° C)	Time (minute: second)	No. of cycles
Initial PCR activation	95	10:00	**1**
Denaturation	95	0:10	**50**
Annealing	56	0:15	
Extension	72	0:10	
Cooling	40	0:30	**1**

Data analysis

1. When the run has completed, click Finish.
2. Click on Analysis in the Global Toolbar and select Absolute Qualification of the Analysis type for data analysis.
3. Select channel 530 from channel setting and 640 for channel denominator to read the results.

B. One-step realtime RT-PCR for H1 gene of A(H1N1)2009 virus

Protocol 1[16]

This protocol is a realtime RT-PCR to detect A(H1N1)2009 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 WHO H5 Reference Laboratories 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 fragment	Primer	Sequence
Influenza A(H1N1)2009	HA	swlH1F	GACAAAATAACAAACGAAGCAACTGG
		swlH1R	GGGAGGCTGGTGTTTATAGCACC
		swlH1P*	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.

[16] WHO Collaborating Centre for Reference and Research on Influenza. National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, England. Email: whocc@nimr.mrc.ac.uk. http://www.nimr.mrc.ac.uk/wic/

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 (°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°C incubation step.

Protocol 2[17]

This protocol is a realtime RT-PCR to detect A(H1N1)2009 viruses (HA gene) in specimens from humans. It is recommended that laboratories having concerns about identifying currently circulating viruses should contact one of the members of the WHO Expert Committee on influenza PCR or 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. 52904)
- 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）
- RealTime ready RNA Virus Master kit (Cat. No. 05619416001 for 100 reactions or 05992877001 for 1000 reactions) (Roche):
  - Enzyme Blend, 50 × conc. (vial 1, red cap)
  - Reaction Buffer, 5 × conc. (vial 2, colorless cap)
  - Water, PCR-grade (vial 3, colorless cap)

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

Primers and probes

Working primer and probe mix is prepared by adding equal volume of the following three components:

Type/subtype	Gene fragment	Primer	Sequence
Influenza A(H1N1)2009	HA	H1-sw-91F H1-sw-205R H1-sw-119P	GCATAACGGGAAACTATGCAA(10μmol/l) GCTTGCTGTGGAGAGTGATTC(10μmol/l) 5'-(FAM)-TTACCCAAATGCAATGGGGCTACCCC-(BBQ)-3'(10μmol/l)

Procedure

1. Perform RNA extraction of clinical specimens.

2. For each test sample and positive and negative controls, prepare master mixture as below:

Reagent	Volume (μl)
Master Mix:	
Water (molecular grade)	7.6
5X Reaction buffer	4.0
Primers and probes mix	3.0
Enzyme Blend	0.4
Total volume	**15.0**

3. Add 5μL viral RNA to the above mix.

4. Perform one-step real-time RT-PCR according to the following conditions:

Step	Temperature (°C)	Time (minute:second)	No. of cycles
Reverse transcription	50	8:00	1
Initial PCR activation	95	0:30	1
Denaturation	95	0:01	45
Annealing	56	0:20	
Extension	72	0:01	
Cooling	40	0:30	1

Data analysis

1. When the run has completed, click Finish.
2. Click on Analysis in the Global Toolbar and select Absolute Qualification of the Analysis type for data analysis.
3. Select channel 530 from channel setting to read the results.

C. Influenza B lineage specific one-step realtime RT-PCR[18]

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.

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 fragment**	**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'-CAGACCAAAATGCACGGGGAA**H**ATACC-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)

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

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^{o}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. Realtime RT-PCR assays for the detection of seasonal influenza viruses and H5N1 influenza viruses

Protocol 1[19]

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

1. Influenza type A viruses (M gene)
2. A(H1N1)2009 viruses (H1 and N1 genes)
3. Former seasonal influenza A(H1N1)
4. A(H3N2) viruses (H1h, N1h, H3h, N2h genes)
5. Influenza B viruses (HA and NA genes)

The following testing strategy is recommended:

- 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 genes.
- In case of suspicion of avian infection by the H5 virus, primers and probes are enclosed.

Materials required

- QIAamp Viral RNA (QIAGEN® mini Kit 50) (Qiagen®, Cat. No. 52904)
- SuperScript™ III Platinum® One-Step qRT-PCR System (Invitrogen, Cat. No. 11732-020)
- Superscript™ III Platinum® One-Step qRT-PCR system (Invitrogen, Cat. No. 11732-088)
- Non acetyled BSA 10% (Invitrogen, Cat. No. P2046)
- LightCycler 1.5 or 2.0 (Capillaries)
- LightCycler 480
- 7500 Real-Time PCR System, Applied Biosystems
- SmartCycler® Cepheid

Nucleic acid extraction

RNA is extracted from specimens using the QIAamp Viral RNA kit (QIAGEN® Mini Kit 50 ref 52904). RNA extracted from 200 µl of the original sample is eluted in 60 µl of elution buffer.

All primers and probes described below were validated under the following conditions using the above equipment and reagents mentioned in the materials required section above.

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

Primers and probes for the detection of influenza A viruses (M gene), GAPDH and the influenza A(H1N1) 2009 virus (H1swl gene) were also validated under the following conditions:

RT-PCR Mix kit:

- Invitrogen Superscript™ III Platinum® One-Step qRT-PCR system (Cat. No. 11732-088)

[19] 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

Real-time PCR equipment:

- 7500 Applied® Biosystems
- SmartCycler® Cepheid

Primers and probes

If the sample is positive for M and negative for H12009, the use of the H1N1h and H3N2h set of primers is suggested.

Type/subtype	Gene	Name	Sequences	Bases	PCR Product size	Reference
A(H3N2)	HA	H3h-319Fw H3h-377Rv H3h-358Probe	AGCAAAGCCTACAGCAA GACCTAAGGGAGGCATAA 5'-Fam-CCGGCACATCATAAGGGTAACA 3'-BHQ-1	17 18 22	59 bp	1
A(H3N2)	NA	N2h-1150b Fw N2h-1344 Rv N2h-1290 probe	GTCCAACCCTAAGTCCAA GCCACAAAACACAACAATAC 5'-Fam-CTTCCCCTTATCAACTCCACA-3'-BHQ-1	18 20 21	194 bp	1
Former seasonal A(H1N1)	HA	H1h-678Fw H1h-840Rv H1h-715probe	CACCCCAGAAATAGCCAAAA TCCTGATCCAAAGCCTCTAC 5'-Fam-CAGGAAGGAAGAATCAACTA 3'-BHQ-1	20 20 20	163 bp	1
Former seasonal A(H1N1)	NA	N1h-1134 Fw N1h-1275 Rv N1h-1206 probe	TGGATGGACAGATACCGACA CTCAACCCAGAAGCAAGGTC 5'Fam-CAGCGGAAGTTTCGTTCAACAT 3'-BHQ-1	20 20 22	142 bp	1
A(H1N1)2009	HA	GRswH1-349Fw GRswH1-601Rv GRswH1-538Probe(-)	GAGCTAAGAGAGCAATTGA GTAGATGGATGGTGAATG 5'Fam -TTGCTGAGCTTTGGGTATGA -3'-BHQ-1	19 18 20	253 bp	1
A(H1N1)2009	NA	GRswN1-975Fw GRswN1-1084Rv GRswN1-1045bProbe(-)	TCCACGCCCTAATGATAA TTCTCCCTATCCAAACAC 5'Fam -ATCCTTTTACTCCATTTGCTCC-3'BHQ-1	18 18 22	110bp	1
Influenza type A		GRAM/7Fw GRAM/161Rv GRAM probe/52/+	CTTCTAACCGAGGTCGAAACGTA GGTGACAGGATTGGTCTTGTCTTTA 5'Fam –TCAGGCCCCCTCAAAGCCGAG-3'-BHQ-1	23 25 21	202 bp	2
Influenza type B	HA	HA(B)-1102Fw HA(B)-1226Rv HA(B)-1125probe(+)	ATTGCTGGTTTCTTAGAAGG TTGTTTATRGCTTCTTGMGT 5'Fam- ATGGGAAGGAATGATTGCAGGT-3'-BHQ-1	20 20 22	125 bp	1
Influenza type B	NA	NA(B)-916Fw NA(B)-1069Rv NA(B)-probe(+)	TACACAGCAAAAAGACCC TCCACKCCCTTTRTCCCC 5'Fam-ACACCCCCAGACCAGATGA- 3'BHQ-1	18 18 19	254 bp	1
Influenza A(H5N1)	HA	H5-1544Fw H5-1683Rv H5d-1638Probe(+)	CCGCAGTATTCAGAAGAAGC AGACCAGCYAYCATGATTGC 5'Fam-AGTGCTAGRGAACTCGCMACTGTAG-3'BHQ-1	20 20 25	140 bp	1
Influenza A(H5N1)	HA	H5-1063Fw H5-1162Rv H5d-1088Probe(x)	TTTATAGAGGGAGGATGG GAGTGGATTCTTTGTCTG 5'Hex-TGGTAGATGGTTGGTATGGG-3'-BHQ-1	18 18 20	100 bp	1
Influenza A(H5N1)	NA	N1av-459Fw N1av-648Rv N1av-493Probe(+)	GTTTGAGTCTGTTGCTTGGTC GCCATTTACACATGCACATTCAG 5'Fam-CATGATGGCAYYAGTTGGTTGACAA-3'-BHQ-1	21 23 25	190 bp	1
Human internal control	GAPDH	GAPDH-6Fw GAPDH-231Rv GAPDH-202Probe(-)	GAAGGTGAAGGTCGGAGT GAAGATGGTGATGGGATTTC 5'-Fam-CAAGCTTCCCGTTCTCAGCC-3'-BHQ-1	18 20 20	226 bp	3

1. National Influenza Centre (Northern France), Institut Pasteur, Paris.
2. Wong et al., 2005, Journal of Clinical Pathology. 58:276-280.
3. National Influenza Centre (Southern France), CHU, Lyon.

Procedure

LightCycler 1.5 or 2.0 (Capillaries) (ROCHE)

Reagent	Volume (µl)	Final concentration
Water (molecular grade)	1.06	
Reaction mix 2X	10.0	3.0mM Mg
$MgSO_4$ (50mM)	0.24	0.6mM Mg
Forward Primer (10µM)	1.0	0.5µM
Reverse Primer (10µM)	1.0	0.5µM
Probe (10µM)	0.4	0.2µM
BSA non acetylated (10mg/ml)	0.5	0.25 mg/ml
Superscript III RT/Platinum Taq Mix	0.8	
Total	**15.0**	

LightCycler 480 (96-well format) (ROCHE)

Reagent	Volume (µl)	Final concentration
Water (molecular grade)	1.56	
Reaction mix 2X	10.0	3.0mM Mg
$MgSO_4$ (50mM)	0.24	0.6mM Mg
Forward Primer (10µM)	1.0	0.5µM
Reverse Primer (10µM)	1.0	0.5µM
Probe (10µM)	0.4	0.2µM
Superscript III RT/Platinum Taq Mix	0.8	
Total	**15.0**	

15µl of reaction mix + 5µl of RNA samples.

7500 Realtime PCR System (Applied Biosystems) or SmartCycler (Cepheid) Real-Time PCR machines

Reagent	Volume (µl)	Final concentration
Water (molecular grade)	1.5	
Reaction mix 2X	12.5	3.0mM Mg
Forward Primer (10µM)	2.0	0.8µM
Reverse Primer (10µM)	2.0	0.8µM
Probe (10µM)	1.0	0.2µM
ROX reference dye (diluted 1/10)	0.5	
Superscript III RT/Platinum Taq Mix	0.5	
Total	**20.0**	

20µl of reaction mix + 5µl of RNA samples.

Controls

Each realtime RT-PCR assay includes additional 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; and
- One negative amplification control.

LightCycler System

Amplification cycles	Temperature (° C)	Time (minute:second)	No. of cycles
Reverse transcription	45	15:00	**1**
Denaturation	95	3:00	**1**
Amplification	95 55 72	0:10 0:10 0:20	**50**
Cooling	40	0:30	**1**

7500 Applied or Smartcycler System

Amplification cycles	Temperature (°C)	Time (minute:second)	No. of cycles
Reverse transcription	50	2:00	1
Denaturation	95	15:00	1
Amplification	95 60	0:15 0:40	50

Sensitivity

For the M realtime RT-PCR
Sensitivity is about 100 copies of RNA genome equivalent per reaction (95% confidence level). This amount of target sequences is always detected; however, the probability to detect lower amounts of virus decreases accordingly. In our settings, samples containing 10 copies could be detected.

For the H1 and N1 2009 realtime RT-PCR
Sensitivity is comparable to that of the M real-time RT-PCR and comparable to the sensitivity of the CDC kit (Cp or Ct <36 for all positive specimens tested so far).

For the H3h and N2h realtime RT-PCR
Sensitivity of the H3h realtime RT-PCR is equivalent to that of the M realtime PCR (Cp H3h ≈ Cp M) but the sensitivity of the N2h realtime RT-PCR is lower (Cp N2h ≈ Cp M + 5 Cp).

For the H1h and N1h realtime RT-PCR
Sensitivity of the N1h realtime RT-PCR is equivalent to that of the M realtime PCR (Cp N1h ≈ Cp M) but the sensitivity of the H1h realtime RT-PCR is lower (Cp H1h ≈ Cp M + 4 Cp).

For the H5 and N1 avian real real-time RT-PCR
Sensitivity of the H5 realtime RT-PCR is equivalent to that of the M realtime PCR (Cp H5 ≈ Cp M) but the sensitivity of the N1 avian realtime RT-PCR is lower (Cp N1a ≈ Cp M + 5 Cp).

Specificity

For the H1 and N1 2009 realtime RT-PCR
The primers for the HA and NA of the A(H1N1)2009 virus did not detect former seasonal influenza A(H1N1), influenza A(H3N2), influenza B, or 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)2009 viruses, detection was positive for A/California/4/2009 and A/California/4/2009-like viruses.

NOTE: The A(H1N1)2009 realtime RT-PCR does not detect the positive control from the CDC kit. Detection with the N1 2009 set of primers is more robust than with the H1 2009 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 standard. 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 a negative strand. Each microtube contains 10^{11} copies of target sequences diluted in yeast tRNA and lyophilised.

Reconstitution of transcribed RNA

- Add 100µl of distilled water to obtain a solution at a concentration of 10^{9}copies/µl. Store at -80° C.
- Dilute in water 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 at 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 H12009 or N12009 and negative reactions for H1h, N1h, H3h, N2h: confirmed case for A(H1N1)2009 virus.

- Positive reaction for M and negative for H12009 or N12009 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 H12009 and N12009 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 H1 2009 and N1 2009 and positive reaction for either N1h, H3h may reflect a cross-contamination or a possible co-infection with both the A(H1N1)2009 virus and another seasonal virus: repeat RNA extraction and repeat reactions with all necessary precautions to avoid cross-contamination.

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

Protocol 2[20]

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

1. Influenza type A viruses (M gene)
2. A(H1N1)2009 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)

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)
  - −2 x QuantiTect®, Probe RT-PCR Master Mix
  - −QuantiTect®, RT Mix
- RNase-free water
- RNase Inhibitor (Applied Biosystems, Cat. No. N808-0119)
- Primers
- TaqMan® MGB Probe

Test validation

[20] 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 208-001, Japan.

Chromo-4 Real-time PCR Detection system (BioRad)
LightCycler 2.0 (Roche) or LightCycler 480 (Roche)

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 H1N1 pandemic virus	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(H5N1) viruses (Clade 1, 2, 3)	HA	H5HA-205-227v2-For	CGATCTAGAYGGGGTGAARCCTC
		H5HA-326-302v2-Rev	CCTTCTCCACTATGTANGACCATTC
		H5HA-205-227-For(2010) [1]	CGATCTAAATGGAGTGAAGCCTC
		H5HA-326-302-Rev(2010) [1]	CCTTCTCTACTATGTAAGACCATTC
		H5-Probe-239-RVa [2]	5'-(FAM)-AGCCAYCCAGCTACRCTACA-(MGB)-3'
		H5-Probe-239-RVb [2]	5'-(FAM)-AGCCATCCCGCAACACTACA-(MGB)-3'
Influenza type B	NS	NIID-TypeB TMPrimer-F1	GGAGCAACCAATGCCAC
		NIID-TypeB TMPrimer-R1	GTKTAGGCGGTCTTGACCAG
		NIID-TypeB Probe1	5'-(FAM)-ATAAACTTTGAAGCAGGAAT-(MGB)-3'

Procedure

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

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 detection, 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

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 realtime 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)

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

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

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

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

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

Test result interpretation

Each realtime 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
2.1	A/Indonesia/6/2005
2.1.1	A/chicken/Pekalongan/BPPV4/2003
2.1.2	A/chicken/Pangkal Pinang/BPPV3/2004
2.1.3	A/Indonesia/5/2005
2.1.3.2	A/Indonesia/NIHRD11931/2011
2.2	A/Turkey/12/2006
2.2.1	A/Egypt/321/2007
2.3.2	A/whooper swan/Hokkaido/1/2008
2.3.2.1	A/Hubei/1/2010
2.3.4	A/Anhui/01/2005
2.4	A/chicken/Guangxi/12/2004
2.5	A/chicken/Kyoto/3/2004

Protocol 3[21]

This protocol describes realtime RT-PCR procedures for the detection of different subtypes of influenza A viruses including A(H7N9) and B viruses).

1. Influenza type A viruses (M gene)
2. Influenza type A (H1N1(pdm)09), H3, H5 and H7) viruses (HA gene)
3. Influenza type B viruses (HA gene)

Materials required

- RealTime ready RNA Virus Master kit (Roche)
- Roche LightCycler Version 2.0 (Roche)

Primers and probes

Type/ subtype	Gene	Name	Sequence	Working concentration (µl)	Reference
Influenza type A	Matrix (M)	FLUAM-7-F	CTTCTAACCGAGGTCGAAACGTA	2	[1]
		FLUAM-161-R	GGTGACAGGATTGGTCTTGTCTTTA	2	
		FLUAM-49-P4	YAK–TCAGGCCCCCTCAAAGCCGAG–BBQ	2	
Influenza type B	HA	B84F	AGGGGAGGTCAATGTGACTG	2	[2]
		B185R	GGGCATAGTTTCCCTCTGGT	2	
		B141P-P2	YAK-TTTTGCAAATCTCAAAGGAACA-BHQ1	2	

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

A(H1N1)pdm09	HA	H1-sw-91F	GCATAACGGGAAACTATGCAA	3.3	[3]
		H1-sw-205R	GCTTGCTGTGGAGAGTGATTC	3.3	
		H1-sw-119P-1	FAM–TTACCCAAATGCAATGGGGCTACCCC–BBQ	3.3	
A(H3N2)	HA	H3-239F	ACCCTCAGTGTGATGGCTTCCAAA	2	[2]
		H3-400R	TAAGGGAGGCATAATCCGGCACAT	2	
		H3-342P	FAM–ACGCAGCAAAGCCTACAGCAACTGTT–BHQ1	2	
A(H5N1)	HA	H5-1012F	TGGGTACCACCATAGCAATGAGCA	4	[2]
		H5-1155R	AATTCCCTTCCAACGGCCTCAAAC	4	
		H5-1042P-P2	FAM–TGGGTACGCTGCAGACAAAGAATCCA–BHQ1	4	
A(H7N9)	HA	H7-F	TCACAGCAAATACAGGGAAGAG	3.3	[2]
		H7-R	CCCGAAGCTAAACCAGAGTATC	3.3	
		H7-P	FAM–TGACCCAGTCAAACTAAGCAGCGG–BBQ	3.3	

[1] Terrier O, Josset L, Textoris J, 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. Virology Journal 8:285.

[2] Virology Division, Public Health Laboratory Services Branch, Centre for Health Protection, Department of Health, Hong Kong SAR, China.

[3] Cheng PK, Wong KK, Mak GC, et al. 2010. Performance of laboratory diagnostics for the detection of influenza A(H1N1)v virus as correlated with the time after symptom onset and viral load. Journal of Clinical Virology 47:182-5.

Procedure

Prepare master mixture for realtime RT-PCR as below:

Reagent	Volume (μl)
Master Mix:	
Water (molecular grade)	7.6
5X Reaction buffer	4.0
Primers and probes mix	3.0
Enzyme Blend	0.4
RNA sample	5.0
Total	**15**

PCR temperature-cycling condition

1. **For amplification of (1) influenza A M gene; (2) influenza A H3 HA gene; (3) influenza A H7 HA gene; and (4) influenza B HA gene**

Step	Temperature (°C)	Time (minute:second)	No. of cycles
Reverse transcription	50	08:00	1
Initial PCR activation	95	00:30	1
Denaturation	95	00:01	45
Annealing	58	00:20	
Extension	72	00:05	
Cooling	40	00:30	1

2. **For amplification of influenza A H1N1pdm09 HA gene:**

Step	Temperature (°C)	Time (minute:second)	No. of cycles
Reverse transcription	50	08:00	1
Initial PCR activation	95	00:30	1
Denaturation	95	00:01	45
Annealing	56	00:20	
Extension	72	00:05	
Cooling	40	00:30	1

3. **For amplification of influenza A H5 HA gene:**

Step	Temperature (°C)	Time (minute:second)	No. of cycles
Reverse transcription	50	08:00	1
Initial PCR activation	95	00:30	1
Denaturation	95	00:10	45
Annealing	56	00:15	
Extension	72	00:10	
Cooling	40	00:30	1

Test result interpretation

Positive results are indicated by a sigmoidal curve when the cycle number was plotted against the fluorescence emission intensity.

Weak positive cases may show positive reaction for influenza A M gene real time RT-PCR and negative reactions for other subtyping real-time RT-PCRs or *vice versa*. Those weak positive cases should be confirmed by other means such as repeat RNA extraction, using a lower elution

volume during RNA extraction, obtaining a new specimen from the same patient or repeat testing in multiple wells.

The absence of positive signal may not rule out the influenza infection. Results should be interpreted together with clinical and epidemiological information.

Sensitivity and Specificity

1. Influenza A M gene real time RT-PCR

The influenza A M gene real time RT-PCR was used to test 68 respiratory specimens, where 21 were previously laboratory confirmed to be positive for influenza A either by viral culture or RT-PCR with another primer/probe set[22], while the remaining 47 were negative. The sensitivity and specificity for these 68 respiratory specimens were both 100%.

In addition, the specificity of the influenza A M gene real time RT-PCR was assessed by testing a variety of DNA and RNA respiratory viruses: influenza B virus, influenza C virus, adenovirus type types 1–7, respiratory syncytial virus groups A and B, parainfluenza virus types 1–4, rhinovirus, enterovirus, metapneumovirus, and herpes simplex virus types 1 and 2. None of the samples gave a positive signal.

2. Influenza B HA gene real time RT-PCR

The influenza B HA gene realtime RT-PCR was used to test 65 respiratory specimens, where 17 were previously laboratory confirmed to be positive for influenza B either by viral culture or RT-PCR with another primer/probe set[23], while the remaining 48 specimens were negative. The sensitivity and specificity for detecting those 65 respiratory specimens were both 100%.

In addition, the specificity of the influenza B HA gene real time RT-PCR was assessed by testing a variety of DNA and RNA respiratory viruses: pandemic influenza A(H1N1) 2009 virus, influenza A(H3N2) virus, influenza A(H5N1) virus, influenza A(H7N9) virus, influenza A(H9N2) virus, influenza C virus, adenovirus types 1-7, respiratory syncytial virus groups A and B, parainfluenza virus types 1-4, rhinovirus, enterovirus, metapneumovirus and herpes simplex virus types 1 and 2. None of the samples gave a positive signal.

3. Real-time RT-PCRs for HA genes of influenza A H1, H3, H5 and H7

The sensitivities of these four realtime RT-PCRs were shown to be comparable with the influenza A M gene real time RT-PCR by testing 10-fold serially diluted clinical isolates of respective viruses. For the H5 PCR, viruses belonging to clades 1, 2.1, 2.2, 2.3.2.1, and 2.3.4 have been evaluated. None of the realtime RT-PCRs gave a false positive signal for other subtypes.

[22] Cheng PK, Wong KK, Mak GC, et al. 2010. Performance of laboratory diagnostics for the detection of influenza A(H1N1)v virus as correlated with the time after symptom onset and viral load. Journal of Clinical Virology 47:182-5.
[23] van Elden LJ, Nijhuis M, Schipper P, et al. 2001. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. Journal of Clinical Microbiology 39:196-200.

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E. Realtime RT-PCR assays for human influenza A(H7N9) virus

Protocol 1[24]

The protocol is a realtime RT-PCR to specifically detect avian influenza A (H7N9) virus using realtime 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.

[24] 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**
   (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
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 realtime RT-PCR detection and use the following programme for cycling:

Temperature (^{o}C)	**Time (minute: second)**	**No. of cycles**
45 95 95 60	10:00 10:00 00:15 00:45	1
95 60	00:15 00:45	40

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[25]

This quantitative RT-PCR protocol and primer set are designed to detect human A(H7N9) viruses in human specimens. Suitable biosafety precautions should be made for handling suspected human 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
- TaqMan® EZ RT-PCR Core Reagents (Life Technologies, Cat. No. N8080236)
- Ethanol (96–100%)
- MicroAmp Fast Optical 96-well reaction plate (Life Technologies, Part No. 4346906)
- MicroAmp optical adhesive film (Life Technologies, Part No. 4311971)
- Microcentrifuge (adjustable, up to 13 000rpm)
- Adjustable pipettes（10, 20, 100, 200µl）
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes（0.5ml and 1.5ml）
- Thermocycler (Life Technologies, ViiA™ 7 Real-Time PCR System or Applied Biosystems 7500)
- Positive control (Available from HKU, e-mail: llmpoon@hkucc.hku.hk)
- Primer set

Primers and probes

Type/ subtype	Gene	Name	Sequence
A(H7N9)	HA	Human H7 for	ATAGATAGCAGGGCAGTTGG
		H7 rev	GATCWATTGCHGAYTGRGTG [1]
		H7 probe	FAM-CCYTCYCCYTGTGCRTTYTG-BHQ1 [1]

1. Journal of Clinical Microbiology. 48:4275

Procedure

1. Extract viral RNA from the clinical specimen by using 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:

[25] Centre of Influenza Research, School of Public Health, The University of Hong Kong, Hong Kong SAR, China, (WHO H5 Reference Laboratory)

Reagent per reaction	Volume (µl)
5x Taqman EZ Buffer	5.0
Manganese acetate (25mM)	3.0
dATP (10mM)	0.75
dCTP (10mM)	0.75
dGTP (10mM)	0.75
dUTP (20mm)	0.75
Forward primer (10µM)	0.5
Reverse primer (10µM)	0.5
Probe (10µM)	0.5
rTth DNA Polymerase (2.5U/ml)	1.0
UNG (1U/ml)	0.25
dH_2O	10.25
RNA sample	1.0
Total volume	**25.0**

3. Set the follow RT-PCR conditions:

Temperature (^{o}C)	Time (minute:second)	No. of cycles
50 60 95	02:00 30:00 05:00	1
94 60	00:20 01:00	40

Interpretation of results

Samples containing HA derived from human influenza A(H7N9) virus are expected to be positive in the assay. The absence of a positive signal from a reaction does not necessarily rule out the presence of influenza virus. Results should be interpreted together with the available clinical and epidemiological information.

Remarks

The forward primer is highly specific for the influenza A(H7N9) virus detected in humans and has ≥2 mismatches to other avian and equine H7 sequences. The reverse primer and probe, however, can bind to H7 HA of Eurasian lineage. It is therefore important to conduct the test using the indicated RT-PCR condition. In our evaluation, all RNA samples extracted from human A(H7N9) viruses (N=3) were positive in the test. By contrast, all the studied control samples (American H7, N=1; Eurasian H7, N=6; H1-H12, N=1 for each subtype; human respiratory samples from patients with seasonal influenza virus infection, N=5; and human respiratory samples from patients with other respiratory infection, N=5) were negative in the test.

Protocol 3[26]

This primer set is adapted from a previous published protocol from Tsukamoto and colleagues (*Journal of Clinical Microbiology.* 48:4275) and it is specific for Eurasian H7 HA (except the one from the human H7N9 virus). 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
- TaqMan® EZ RT-PCR Core Reagents (Life Technologies, Cat No. N8080236)
- Ethanol (96–100%)
- MicroAmp Fast Optical 96-well reaction plate (Life Technologies, Part No. 4346906)
- MicroAmp optical adhesive film (Life Technologies, Part No. 4311971)
- Microcentrifuge (adjustable, up to 13 000rpm)
- Adjustable pipettes (10, 20, 100, 200µl)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes (0.5ml and 1.5ml)
- Thermocycler (ViiA™ 7 Real-Time PCR System)
- Positive control (Available from HKU, e-mail: llmpoon@hkucc.hku.hk)
- Primer set

Primers and probes

Type/ subtype	Gene	Name	Sequence
A (H7N9)	HA	EA H7 for	ATMAATAGCAGRGCARTRGG [1]
		H7 rev	GATCWATTGCHGAYTGRGTG [1]
		H7 probe	FAM-CCYTCYCCYTGTGCRTTYTG-BHQ1 [1]

1. Journal of Clinical Microbiology. 48:4275

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)
5x Taqman EZ Buffer	5
Manganese acetate (25mM)	3

[26] Centre of Influenza Research, School of Public Health, The University of Hong Kong, Hong Kong SAR, China, (WHO H5 Reference Laboratory)

dATP (10mM)	0.75
dCTP (10mM)	0.75
dGTP (10mM)	0.75
dUTP (20mm)	0.75
Forward primer (10µM)	0.5
Reverse primer (10µM)	0.5
Probe (10µM)	0.5
rTth DNA Polymerase (2.5U/ml)	1
UNG (1U/ml)	0.25
dH_2O	10.25
RNA sample	1
Total volume	25

3. Set the follow RT-PCR conditions:

Temperature (°C)	**Time (minute:second)**	**No. of cycles**
50 52 95	02:00 06:00 05:00	1
95 52 60	00:10 00:20 00:32	40

Interpretation of results

H7, with the exception of human H7N9, viruses of Eurasian lineage are expected to be positive in the test. Samples containing HA derived from human influenza A(H7N9) virus or its closely related lineages are expected to be negative in the assay. The absence of the positive signal from a sample does not necessarily rule out the presence of influenza virus. Results should be interpreted together with the available clinical and epidemiological information.

Remarks

The degenerated forward primer can cross react with H7 HA of Eurasian lineage but it has two mismatches to the human H7N9 virus. The reverse primer and probe can bind to H7 HA of Eurasian lineage, including the one from human H7N9. It is important to conduct the test using the indicated RT-PCR condition. In our evaluation, all RNA samples extracted from human H7N9 viruses (N=3) were negative in the test. By contrast, all Eurasian avian H7 HA (N=6) were positive in the assay. All the control samples (American H7, N=1; H1-H6, N=1 for each subtype; H8-H12, N=1 for each subtype; human respiratory samples from patients with seasonal influenza virus infection, N=5; and human respiratory sample from patients with other respiratory infection, N=5) were negative in the test.

F. Realtime RT-PCR assays for human influenza A(H10N8) virus

Protocol 1[27]

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.

[27] 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 realtime RT-PCR detection and use the following programme for cycling:

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

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

3. 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.

4. 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

4. 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.

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

6. 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®).

Annex 3: Sequencing protocols

Protocol 1[28]

One-step conventional RT-PCR for amplification and sequencing of the HA and NA genes from avian influenza A(H7N9) virus

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

[28] 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	**22.0µl/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 H_2O, 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 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

9. 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

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

Additional RT-PCR protocols for the detection of influenza viruses and primers for full genome sequencing

Primer sequences for additional RT-PCR protocols and full genome sequencing may also be requested using the following link:
http://www.influenzacentre.org/flucentres_sequencing.htm