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	30.6	33.6	(QIAGEN®, Cat. No. 129114)
10x Buffer*	7.5	7.5	
50mM $MgSO_4$*	1.0	1.0	
100mM dNTPs	0.9	0.9	25mM of each (G, A, T, C)
10 µmol/l Forward primer[$]	1.5	1.5	0.3 µmol/l final concentration
10 µmol/l Reverse primer[$]	1.5	1.5	0.3 µmol/l final concentration
RNAsin	0.5	0.5	(Promega, Cat. No. N2515)
SS III RT	1.0	1.0	(Invitrogen, Cat. No. 18080-085)
Pfx Polymerase*	0.5	0.5	(Invitrogen, Cat. No. 11708-039)
RNA	5.0	2.0	
Total	**50.0**	**50.0**	

* Supplied with the Pfx polymerase.

Thermal cycler BIORAD DNA Engine programme:

Temperature (°C)	Time (minute:second)	No. of cycles
50 94	30:00 10:00	
94 55 68	0:05 0:05 0:30	} 40
68	10:00	
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) **RT step**

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	12.1	(QIAGEN®, Cat. No. 129114)
5x buffer*	8.0	
0.1M DTT *	2.0	
RNAsin	2.0	(Promega, Cat. No. N2515)
100mM dNTPs	0.9	25mM of each (G, A, T, C)
30 µmol/l Uni12W or Buni 11W	3.0	
SS III RT	2.0	(Invitrogen, Cat. No. 18080-085)
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°C/5min. 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 (°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	35.1	
10x buffer*	7.5	
$MgSO_4$*	1.0	
100mM dNTPs	0.9	25mM of each (G, A, T, C)
10 µmol/l Forward primer$	1.5	
10 µmol/l Reverse primer$	1.5	
Pfx	0.5	Invitrogen, Cat. No. 11708-039
RT product	2.0	
Total	**50.0**	

* Supplied with the Pfx polymerase.

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 55 68	10:00 5:00 2:00	
94 55 68	0:05 0:05 2:00	} 39
94	0:05	
55	0:05	
68	10:00	
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 #28-9034-70). Manufacturer's instructions are followed, but 2 x 500 µl 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 100-200 ng 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 3700).

Annex 2:
Realtime RT-PCR analyses for the matrix gene (Influenza type A viruses)

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.

Realtime RT-PCR protocol 1[13]

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

Materials required

Reverse transcription

- 10x PCR buffer I with 15 mmol/l $MgCl_2$ (Applied Biosystems)
- Random hexamer 50 µmol/l (Applied Biosystems, Cat. No. 8080127)
- MuLV Reverse Transcriptase 50 U/µl (Applied Biosystems, Cat. No. 8080018)
- RNase Inhibitor 20 U/µl (Applied Biosystems, Cat. No. 8080119)
- LightCycler® – FastStart™ DNA Master HybProbe kit (Roche Applied Sciences, Cat. No. 03 003 248 001)

Realtime PCR

Primers and probes mix: Add equal volume of the following components to prepare primers and probes mix for the influenza type A, M gene.

Primers and probes

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

The working primer and probe mix is prepared by mixing the above 6 reagents in equal volumes.

[13] National Influenza Centre, Centre for Health Protection. 382 Nam Cheong Street, Shek Kip Mei Kowloon, Hong Kong Special Administrative Region of China. http://www.chp.gov.hk

Procedure

1. Perform the RT step using the reagents shown in the following table and instructions i-iii below it.

Reagent	Volume (µl) per reaction
10x PCR buffer I with 15 mmol/l $MgCl_2$	2.0
Extra 25 mmol/l $MgCl_2$	2.8
dNTPs (2.5 mmol/l)	8.0
Extracted RNA	4.2
Random hexamer 50 µmol/l	1.0
RNAase inhibitor 20U/µl	1.0
Reverse transcriptase 50 U/µl	1.0

i) Vortex and centrifuge the tube with the mixture briefly (~3 sec).
ii) Stand the tube at room temperature for 10 minutes and then incubate at 42 °C for at least 15 minutes.
iii) Incubate the tube at 95 °C for 5 minutes and then chill in ice.

2. Perform RT-PCR

i) Prepare Hot Start reaction mix by gently pipetting 60 µl of LightCycler-FastStart Reaction Mix HybProbe (vial 1b) into the LightCycler-FastStart Enzyme (vial 1a).
ii) For each test sample, positive and negative controls, prepare reagent mix with primers and probe mix as described in the following table:

Master Mix:

Reagent	Volume (µl)
PCR-grade H_2O	7.6
$MgCl_2$ (25 mmol/l)	2.4
Primers and probe mix	3.0
"Hot Start" reaction mix	2.0
Total volume	**15.0**

Each reaction:

Reagent	Volume (µl)
Master Mix	15.0
cDNA (from the RT product; step 1. above)	5.0

PCR temperature-cycling conditions:

Temperature (°C)	Time (minute:second)	No. of cycles
95	10:00	**1**
95	0:10	
56	0:15	**} 50**
72	0:10	
40	0:30	**1**

Real-time RT-PCR protocol 2[14]

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

Materials required

- QIAGEN• QuantiTect®, Probe RT-PCR kit (Cat. No. 204443)
- QIAGEN•2 x QuantiTect®, Probe RT-PCR Master Mix
- QIAGEN•QuantiTect®, RT Mix
- RNase-free water
- RNase Inhibitor (Applied Biosystems, Cat. No. N808-0119)
- Primers
- TaqMan® MGB Probe

Equipment

Chromo-4 Real-time PCR Detection system (BioRad)
LightCycler 2 (Roche) or LightCycler 480 (Roche)

Real-time PCR

Real-time PCR is performed by One-step RT-PCR using TaqMan• probe.

Primers and probes

Type/subtype	Gene fragment	Primer	Sequence
Influenza type A	Matrix (M)	MP-39-67For	CCMAGGTCGAAACGTAYGTTCTCTCTATC (10 μmol/l)
Influenza type A	Matrix (M)	MP-183-153Rev	TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA (10 μmol/l)
Influenza type A	Matrix (M)	MP-96-75ProbeAs	5' (FAM)-ATYTCGGCTTTGAGGGGGCCTG-(MGB)-3' (5 pmol/μl)

Reaction Mixture

Reagent	Volume (μl)
RNase-free water	3.75
2x QuantiTect® Probe RT-PCR Master Mix	12.5
Forward Primer (10 μmol/l)	1.5
Reverse Primer (10 μmol/l)	1.5
TaqMan MGB Probe (5 pmol/μl)	0.5
QuantiTect® RT Mix	0.25
Total	**20.0**

[14] Protocol provided by National Institute of Infectious Diseases (NIID), Center for Influenza Virus Research, Tokyo, Japan (WHO Collaborating Centre for Reference and Research on Influenza).

Procedure

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

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

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

4. Start the realtime RT-PCR program while the RT-PCR reaction plates are still *on ice*.

5. *Wait until the thermal cycler has reached 50 °C,* then place the RT-PCR reaction plates in the thermal cycler.

RT-PCR temperature-cycling conditions: 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 (data collection)	

RT-PCR temperature-cycling conditions: LightCycler 2 (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 (ramp rate 1.5 °C/sec) Data collection	

One step real-time RT-PCR for H1 gene of pandemic (H1N1) 2009 virus

RealTime RT-PCR Protocol 1[15]

This protocol is for realtime RT-PCR detection of pandemic (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 WHOCCs for assistance in identifying the optimal primers to be used.

Materials required

- QIAamp Viral RNA Mini Kit (QIAGEN® Cat. No. 52904)
- 750 RealTime PCR System (Applied Biosystems)
- TaqMan EZ RT-PCR Core Reagents (Applied Biosystems, Part No. N8080236)
- MicoAmp Fast Optical 96-well reaction plate (Applied Biosystems, Part No. 4346906)
- MicroAmp optical adhesive film (Applied Biosystems, Part No. 4311971)
- Ethanol (96–100%)
- Microcentrifuge (adjustable, up to 13 000 rpm)
- Adjustable pipettes (10, 20, 100, 200 µl)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes (0.2, 1.5 ml)
- Positive control (Swine influenza A virus A/SW/HK/PHK1578/03 or A/California/04/2009) (Available upon request)
- Primers and probe set

Primers and probes

Type/subtype	Gene fragment	Primer	Sequence
Influenza A H1N1 pandemic virus	HA	HKU-qSWF	GGGTAGCCCCATTGCAT
Influenza A H1N1 pandemic virus	HA	HKU-qSWR	AGAGTGATTCACACTCTGGATTTC
Influenza A H1N1 pandemic virus	HA	HKU-qSWP	5`-[FAM] TGGGTAAATGTAACATTGCTGGCTGG [TAMRA]-3`

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:

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

Component	Working concentration.	Volume, µl
Water H_2O	NA	6.2
5X EZBuffer A	5X	5.0
$Mn(OAc)_2$	25 mM	3.0
dATP •	10 mM	0.75
dCTP	10 mM	0.75
dGTP	10 mM	0.75
dUTP	20 mM	1.5
Forward Primer	50 µM	0.4
Reverse Primer	50 µM	0.4
Probe (FAM)	10 µM	1.0
rTth Poly(2.5U/µl)	2.5 U/µl	1.0
UNG(1 U/µl)	1 U/µl	0.25
RNA template		4.0
TOTAL		**25.0**

3. Set the following RT-PCR conditions:

Detection Dye: FAM
Quencher Dye :TAMRA

Step	Temperature (°C)	Time (minute:second)	No. of cycles
Reverse transcription	50 60 95	2:00 40:00 5:00	1
PCR	95 55	0:15 1:00	50

Interpretation of results

This assay can specifically detect samples with pandemic (H1N1) 2009) virus and, notably, some other swine H1 viral sequences, but not those with seasonal human H1N1. RNA samples extracted from seven human seasonal H1N1, two human seasonal H3N2, one human 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. One of the positive controls recommended in this assay is a swine H1 virus isolated in Hong Kong. This is designed to minimize the shipping and handling of A/California/04/2009-like H1N1 viruses to laboratories which do not have the recommended biosafety facility. Real-time RT-PCR assay specific for the pandemic (H1N1) 2009 virus, but not other swine viruses, has been developed (Poon et al., 2009).[16] If the test is run without controls, products should be confirmed

[16] Poon LL, Chan KH, Smith GJ, Leung CS, Guan Y, Yuen KY, Peiris JS. 2009.
Molecular Detection of a Novel Human Influenza (H1N1) of Pandemic Potential by Conventional and Real-Time Quantitative RT-PCR Assays. (In Press) Available online:
http://www.clinchem.org/cgi/content/abstract/clinchem.2009.130229v1

by sequencing and comparison with sequences in deposited databases. 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.

RealTime RT-PCR Protocol 2[17]

This protocol is a real-time RT-PCR to detect H1N1 pandemic virus (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 WHOCCs 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
Pandemic (H1N1) 2009	HA	swlH1F swlH1R swlH1P*	GACAAAATAACAAACGAAGCAACTGG GGGAGGCTGGTGTTTATAGCACC GCATTCGCAA"t"GGAAAGAAATGCTGG

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

Procedure

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

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

Component	Working Concentration.	Volume in μl
Water H_2O	NA	5.5
2x PCR master mix*$	5X	12.5
Forward primer	40 μM	0.5
Reverse primer	40 μM	0.5
Probe	10 μM	0.5
RT/DNA polymerase mix*		0.5
Total mastermix		**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.

[17] 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*	50

* Fluorescence data (FAM) is collected during the 55°C incubation step..

Protocol No. 4[18]

This protocol is a real-time RT-PCR to detect H1N1 pandemic 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 WHOCCs 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 13 000 rpm)
- Adjustable pipettes (10, 20, 100, 200 µl)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes (0.2, 1.5 ml)
- LightCycler – Fast Start DNA Master Hybridization Probes kit (Cat. No. 03003248001 or 12239272001) (Roche):
  - LC-Fast Start Enzyme (vial 1a)
  - LC-Fast Start Reaction Mix Hybridization Probes, 10 × conc. (vial 1b)
  - $MgCl_2$ (25 mM) (vial 2)
  - PCR grade H_2O (vial 3)

Primers and probes

Type/subtype	Gene fragment	Primer	Sequence
Influenza A H1N1 pandemic virus	HA	H1-sw-988f	AGA CTG GCC ACA GGA TTG AGG AAT (10 µmol/l)
Influenza A H1N1 pandemic virus	HA	H1-sw-1171r	CGT CAA TGG CAT TCT GTG TGC TCT (10 µmol/l)
Influenza A H1N1 pandemic virus	HA	H1-sw-1077p	5'-(FAM)-AGGGATGGTAGATGGATGGTACGG TT-(TAMRA)-3' (5 µmol/l)

* To prepare the primers and probe mix, add equal volumes (in µl) of each of the three reagents and mix.

Procedure

1. Perform RNA extraction of clinical specimens .

2. Perform cDNA synthesis on extracted RNA using the method described under realtime PCR protocol No.1 (Annex 2) page No. 19.

[18] Protocol provided by: Virology Division, Centre for Health Protection, Hong Kong SAR, China., (National Influenza Centre, WHO H5 Reference Laboratory).
http://www.chp.gov.hk/files/pdf/CHP_Protocols_for_the_Detection_of_Human_Swine_Influenza.pdf

3. Setting up of LightCycler 2.0 (see appendix I).

4. Preparation of "Hot Start" reaction mix by gently pipetting 60 μl of LC-Fast Start Reaction Mix Hybridization Probes (vial 1b) into the LC-Fast Start Enzyme (vial 1a).

5. For each test sample and positive and negative controls, prepare reagent mix according to the following instructions:

Reagent	Volume (μl)
Master Mix:	
PCR-grade H_2O	7.6
$MgCl_2$ (25 mM)	2.4
Primers and probes mix	3.0
"Hot Start" reaction mix	2.0
Total volume	**15.0**
Each reaction:	
Master Mix	15.0
cDNA	5.0

6. Place the carousel inside the cooling box and load capillaries into the carousel.

7. Pipette the Master Mix and cDNA into corresponding capillaries.

8. Close the capillaries and transfer the carousel into the LightCycler Carousel Centrifuge.

9. Centrifuge the capillaries in the LightCycler Carousel Centrifuge for 1 second at 400-x g (3000 rpm).

10. Place the carousel into the LightCycler and press Run to start the programme.

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 and channel denominator 640 from channel setting.

4. In the Fit Point screen, click on Noise band curve to see the result.

5. Under results, the column Cp displays the crossing points of the curves.

Appendix I

Setting up the LightCycler 2.0 for H1 (swine) real time RT-PCR

1. Switch on LightCycler 2.0, Carousel Centrifuge, computer and printer.

2. Type in the user name and password to log into Windows XP.

3. Click on **LightCycler 4.05 software** icon.

4. Type in the user name and password to login to the software.

5. Open the program **FluA.exp** on the front screen and an Experiment Kit Wizard will guide the whole experiment procedure.

6. Proceed to **self-test** from the Wizard.

7. After completion of the self-test, check the temperature-cycling condition.

PCR Temperature-cycling condition

Temperature (°C)	Time (minute: second)	No. of cycles
95	10:00	1
94	0:10	50
56	0:15	
72	0:10	
40	30	1

8. Edit the Sample List and enter the appropriate information into the fields labeled **Sample Name** and **Type** from the **Capillary View** of the software screen behind the Wizard.

9. Click on the button **Start Run** of the Wizard to start the run. During the run, the Wizard remains on the screen.

Protocol No.5[19]

This protocol is a real-time RT-PCR to detect H1N1 pandemic 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 WHOCCs for assistance in identifying the optimal primers to be used.

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

Materials required

- QIAGEN• QuantiTect®, Probe RT-PCR kit (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

Equipment

Chromo-4 Real-time PCR Detection system (BioRad)
LightCycler 2 (Roche) or LightCycler 480 (Roche)

Real-time PCR

Real-time PCR is performed by One-step RT-PCR using TaqMan• probe

Primers and probes

Type/subtype	Gene fragment	Primer	Sequence
Influenza A H1N1 pandemic virus	HA	NIID-swH1 TMPrimer-F1	AGAAAAGAATGTAACAGTAACACACTCTGT
Influenza A H1N1 pandemic virus	HA	NIID-swH1 TMPrimer-R1	TGTTTCCACAATGTARGACCAT
Influenza A H1N1 pandemic virus	HA	NIID-swH1 Probe2*	5'-(FAM)-CAGCCAGCAATRTTRCATTTACC-(MGB)-3'

* Updated probe; different from the previous version of 18 August 2009

[19] Protocol provided by WHO Collaborating Center for Reference and Research on Influenza and WHO H5 Reference Laboratory at National Institute of Infectious Diseases (NIID), Tokyo, Japan.

Reaction mixture

Reagent	Volume (μl)
RNase-free water	3.75
2x QuantiTect®Probe RT-PCR Master Mix	12.5
Forward Primer (10 μmol/l)	1.5
Reverse Primer (10 μmol/l)	1.5
TaqMan MGB Probe (5 pmol/μl)	0.5
QuantiTect®RT Mix	0.25
Total	**20.0**

Procedure

1. Dispense 20 μl of the reaction mixture into each RT-PCR reaction plate.
2. Add 5 μl of the sample RNA to the reaction mixture. For control reactions, use 5 μl of distilled water for negative control and 5 μl of appropriate viral RNAs for positive control.
3. Program the thermal cycler as shown in the table below.
4. Start the realtime RT-PCR program while the RT-PCR reaction plates are still ***on ice***.
5. Wait until the thermal cycler has reached 50 °C, then place the RT-PCR reaction plates in the thermal cycler.

RT-PCR temperature-cycling conditions: 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	

RT-PCR temperature-cycling conditions: LightCycler 2 (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 1.2 ° C/sec)	}45
56	1:15 (ramp rate 1.2 ° C/sec) Data collection	