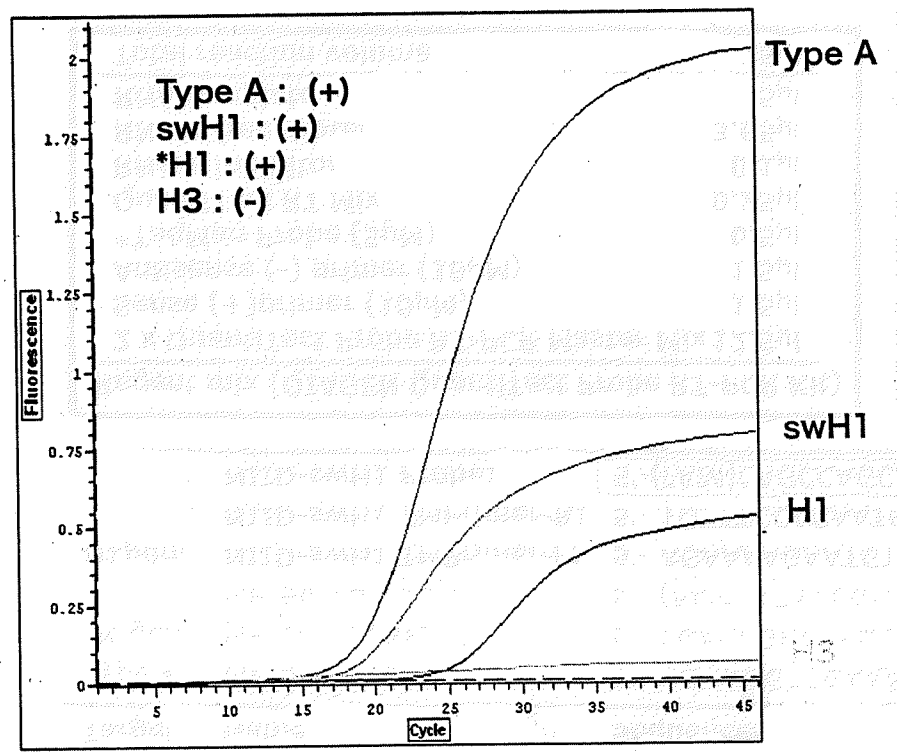
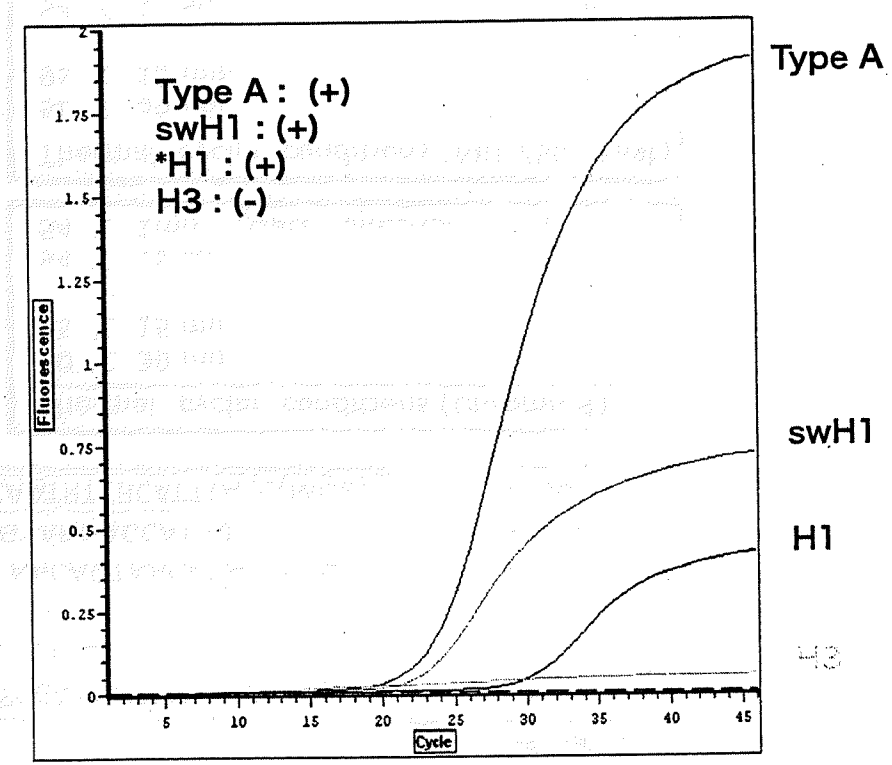
図4. リアルタイムRT-PCR実施例



陽性例1



陽性例2

*H1検出用プローブは新型H1N1pdmに対しても少し反応する

表3. 改良したA/H1N1pdm検出用 Real-time RT-PCR Primer & Probe の配列と反応条件

[ ] : 変更箇所

Target	Name	Sequences	Category
Type A (M gene)	MP-39-67For	5′- CCMAGGTCGAAACGTAYGTTCTCTCTATC -3′	Primer (+)
	MP-183-153Rev	5′- TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA -3′	Primer (-)
	MP-96-75ProbeAs	5′- (FAM) ATYTCGGCTTTGAGGGGGCCTG (MGB) -3′	Probe
H1pdm	**NIID-swH1 TMPrimer-F1**	**5′- AGAAAAGAATGTAACAGTAACACACTCTGT -3′**	**Primer (+)**
	NIID-swH1 TMPrimer-R1	**5′- TGTTTCCACAATGTARGACCAT -3′**	**Primer (-)**
	NIID-swH1 Probe1	**5′-(FAM)CAGCCAGCAATRTTRCATTTACC(MGB)-3′**	**Probe**

Regent mix (QIAGEN QuantiTect Probe RT-PCR Kit)	
2 x QuantiTect Probe RT-PCR Master Mix	12.5µl
Sense (+)primer (10µM)	1.5µl
Antisense (-) primer (10µM)	1.5µl
*TaqMan Probe (5µM)	0.5µl
QuantiTect RT Mix	0.25µl
RNsae inhibitor	0.1µl
RNase free Water	3.65µl
RNA template	5µl
Total reaction volume	25µl

Thermal cycler conditions (Chromo-4)

50 ℃ 30 min.
95 ℃ 15 min.
↓
94 ℃ 15 sec.
54 ℃ 1min. (Data collection)] x 45 cycles

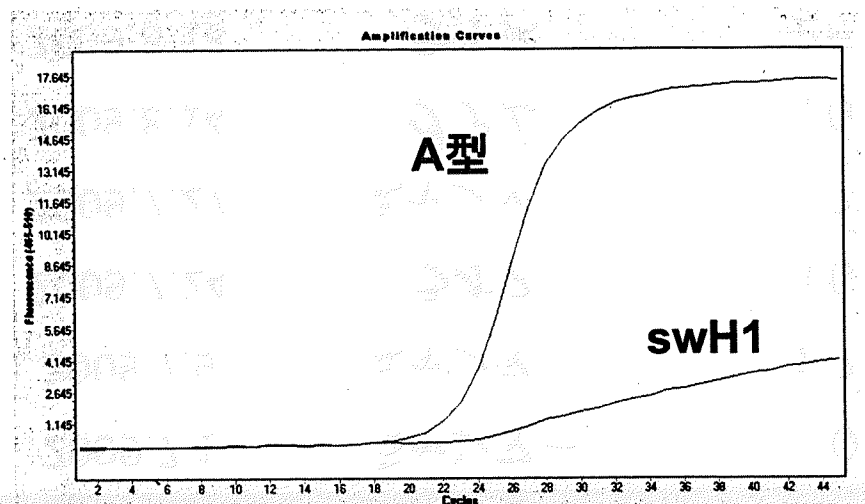
Thermal cycler conditions (ABI 7500 Fast)

50 ℃ 30 min.
95 ℃ 15 min.
↓
94 ℃ 15 sec.
54 ℃ 75 sec. (Data collection)] x 45 cycles

http://www.who.int/csr/resources/publications/swineflu/diagnostic_recommendations/en/index.htmlに記載

図5. 改良リアルタイムRT-PCR実施例

Reaction pattern by 1st version



Reaction pattern by 2nd version

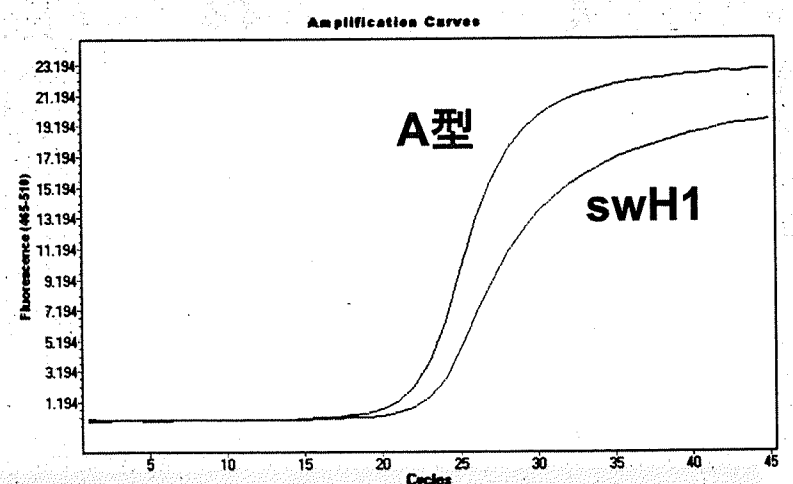


表4. 海外からの検査依頼

検体受取日	国名	送付検体数	新型陽性数	季節性陽性数
2009.7.1	ミャンマー	10	1	5
2009.7.9	ミャンマー	27	3	12
2009.7.24	ラオス	10	7	0
2009.7.27	ミャンマー	17	4	0
2009.8.14	グアム	10	8	0
2009.8.18	ラオス	26	22	2
2009.7.28	ミャンマー	8	6	0
2009.10.5	モンゴル	11	0	0
2009.12.21	モンゴル	10	10	0

表5. フィードバック例

Tube No. for test	Local Lab ID	Patient Ref#	Specimens	samples sent by (laboratory)	Received date of sample	NIID Results (Real-time RT-PCR)							
						FLU A		FLU A/swH1		FLU A/H1		FLU A/H3	
						Date of test	Result	Date of test	Result	Date of test	Result	Date of test	Result
090701-1	I-021/09	090701-001	Nasopharynges	Myanmar	01-Jul-09	01-Jul-09	-	01-Jul-09	-	01-Jul-09	-	01-Jul-09	-
090701-2	I-023/09	090701-002		Myanmar	01-Jul-09	01-Jul-09	-	01-Jul-09	-	01-Jul-09	-	01-Jul-09	-
090701-3	I-024/09	090701-003	Nasopharynges	Myanmar	01-Jul-09	01-Jul-09	-	01-Jul-09	-	01-Jul-09	-	01-Jul-09	-
090701-4	I-025/09	090701-004	Nasopharynges	Myanmar	01-Jul-09	01-Jul-09	+	01-Jul-09	-	01-Jul-09	-	01-Jul-09	+
090701-5	I-026/09	090701-005	Nasopharynges	Myanmar	01-Jul-09	01-Jul-09	+	01-Jul-09	-	01-Jul-09	-	01-Jul-09	+
090701-6	I-030/09	090701-006	Nasopharynges	Myanmar	01-Jul-09	01-Jul-09	-	01-Jul-09	-	01-Jul-09	-	01-Jul-09	-
090701-7	I-057/09	090701-007	Nasopharynges	Myanmar	01-Jul-09	01-Jul-09	+	01-Jul-09	-	01-Jul-09	+	01-Jul-09	-
090701-8	!-059/09	090701-008		Myanmar	01-Jul-09	01-Jul-09	+	01-Jul-09	-	01-Jul-09	-	01-Jul-09	+
090701-9	I-060/09	090701-009	Nasopharynges	Myanmar	01-Jul-09	01-Jul-09	+	01-Jul-09	+	01-Jul-09	+	01-Jul-09	-
090701-10	I-061/09	090701-010	Nasopharynges	Myanmar	01-Jul-09	01-Jul-09	+	01-Jul-09	-	01-Jul-09	-	01-Jul-09	+

World Health Organization

WHO information for laboratory diagnosis of pandemic (H1N1) 2009 virus in humans - revised

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

23 November 2009

This document provides information on the diagnostics available as of the above date for the human influenza A (H1N1) A/California/4/2009-like viruses. Further diagnostic information will be updated when available.

This is an update to the document published on WHO's website on 18 August 2009.

Updated protocols are:
1. Protocol No. 5 (by the WHO Collaborating Centre for influenza at the National Institute of Infectious Diseases, Japanm (WHO CC, NIID)) for real time RT-PCR specific for the pandemic H1N1 2009 virus HA gene: a new probe is used (NIID-swH1 Probe2).
2. Group protocol No. 2 (by Dept Virology, Erasmus MC Rotterdam, Netherland).

Specimens

Upper respiratory tract specimens, as recommended for seasonal influenza investigation, are the most appropriate. Samples should be taken from the deep nostrils (nasal swab), nasopharynx (nasopharyngeal swab), nasopharyngeal aspirate, throat or bronchial aspirate. It is not yet known which clinical specimen gives the best diagnostic yield. Appropriate precautions should be taken in collecting specimens since this may expose the collector to respiratory secretions from patients.

There is, as yet, no information on the diagnostic value of non-respiratory specimens, e.g., stool samples.

Acute and convalescent serum specimens should be used for the detection of rising antibody titres.

Laboratory tests

Molecular diagnostics

Molecular diagnostics are currently the method of choice for pandemic (H1N1) 2009 virus.

The use of different target gene assays is more appropriate for correct identification of this virus. The following gene targets are important: type A influenza matrix gene; haemagglutinin gene specific for pandemic (H1N1) 2009 virus and haemagglutinin gene specific for seasonal influenza A H1/H3.

The following protocols are currently available:
— influenza A type-specific conventional and realtime-PCR (see Annexes 1 and 2);
— pandemic (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 pandemic (H1N1) 2009.[1]
— Seasonal influenza A (H1N1 and H3N2) and avian influenza A (H5, H7 and H9) realtime RT-PCR (see Annex 2)

Sequence analyses of the type A influenza matrix gene PCR product using the primers in the WHO protocols (see Annex 1) will differentiate between M genes of pandemic and seasonal H1N1 viruses; however, additional analysis should be performed to confirm the origin of the virus.

Virus isolation and typing using haemagglutination inhibition or immunofluorescence

Current protocols for virus isolation of seasonal influenza viruses using MDCK cells and egg inoculation can be used, although their sensitivity remains to be determined (see section on Biosafety below).

Turkey, chicken, guinea pig and human red blood cells will agglutinate with the pandemic (H1N1) 2009 virus.

Polyclonal antibodies specific for subtype H1 seasonal influenza viruses from the WHO influenza reagent kit will **not** react in the haemagglutination inhibition (HAI) test with the current pandemic (H1N1) 2009 virus.

Results obtained using the WHO (HAI) kit H1 monoclonal antibodies should not be taken as conclusive and further verification is recommended.

Rapid tests or immunofluorescence

The sensitivity and specificity of rapid-point-of-care or immunofluorescence tests designed for direct detection of influenza A viruses are currently being evaluated. Of point-of-care antigen detection tests evaluated so far, their analytical sensitivity for the novel H1N1 virus is comparable with their sensitivity for detecting seasonal influenza. It should be noted that these tests have appreciably lower sensitivity than RT-PCR for both novel H1N1 and seasonal H1N1 or H3N2 viruses. It should be emphasized that these tests will not differentiate seasonal influenza from pandemic (H1N1) 2009 virus.

Serology

HAI and microneutralization tests using pandemic (H1N1) 2009 virus are expected to be able to detect antibody responses following infection.

[1] http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html

Pathology

Since the pandemic (H1N1) 2009 virus is a new virus to humans, little is known about the pathological changes associated with infection in severe cases at this stage. It is important to collect autopsy tissue samples from fatal cases for pathological studies. Guidance for specimen collection, storage and shipment is provided by CDC in the following link: http://www.cdc.gov/h1n1flu/tissuesubmission.htm.

Additional information for autopsy in resource-limited settings is provided in Annex 4.

Interpretation of laboratory 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 swine H1 haemagglutinin gene) are positive but the PCR for human H1 + H3 is negative. If RT-PCR for multiple haemagglutinin (HA) targets (i.e. H1, H3, and H1-pandemic) give positive results in the same specimen, the possibility of PCR contamination should first be excluded by repeating PCR procedure using new RNA extract from the original specimen or RNA extract from another specimen. If repeated positive results for multiple HA targets are obtained, this raises the possibility of co-infection, which should be confirmed by sequencing or virus culture. Annex 3 shows a flowchart for use in interpreting PCR results.

- CDC realtime PCR assays — Results should be interpreted as described in the CDC H1N1 realtime assay manual.[1]

- A negative PCR result does not rule out that a person may be infected with pandemic (H1N1) 2009 virus. Results should be interpreted in conjunction with the available clinical and epidemiological information. Specimens from patients whose PCR results are negative but for whom there is a high suspicion of pandemic (H1N1) 2009 infection should be further investigated and tested by other methods such as virus culture or serology, to rule out pandemic (H1N1) 2009 infection (see flowchart in Annex 3).

- Serology — A four-fold or greater rise in specific pandemic (H1N1) 2009 antibody titres indicates recent infection with the virus.

- Sequencing — Sequence analyses of the type A influenza matrix gene PCR product using the primers in the WHO protocols (see Annex 1) will differentiate between M genes of pandemic and seasonal H1N1 viruses, however, additional analysis should be performed to confirm the origin of the virus.

- Virus isolation — Identification and typing of a cultured influenza virus can be carried out by PCR, indirect fluorescent antibody (IFA) testing using specific NP monoclonal antibodies, or HA and antigenic analysis (subtyping) by HAI using selected reference antisera.

Referral for confirmation and further characterization

Laboratories with no capacity for diagnosis of influenza A viruses are recommended to send representative specimens from suspect cases of pandemic (H1N1) 2009, according to case definition guidance by WHO,[2] to one of the WHO Collaborating Centres for Reference and Research on Influenza (WHOCC).

Specimens with laboratory results indicative of influenza A that are untysubpeable (i.e. negative for influenza A (H1) and A (H3)) and are not confirmed according to the WHO criteria should be forwarded to a WHOCC 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.[3]

Biosafety

Diagnostic laboratory work on clinical specimens from patients who are suspected cases of being infected with pandemic (H1N1) 2009 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.[4]

Virus isolation currently requires higher biosafety containment measures. Please refer to the document *WHO Laboratory biorisk management for laboratories handling human specimens suspected or confirmed to contain influenza A (H1N1) causing the current international epidemics* for recommended guidance.[5]

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, NP and HA genes.

Good laboratory practices

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

Validation

All protocols should always be validated in each laboratory to ensure adequate specificity and sensitivity using the same controls that are employed in each run.

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[2] http://www.who.int/csr/resources/publications/swineflu/interim_guidance/en/index.html
[3] http://www.who.int/csr/resources/publications/swineflu/storage_transport/en/index.html
[4] http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/
[5] http://www.who.int/csr/resources/publications/swineflu/LaboratoryHumanspecimensinfluenza/en/index.html

Quality assurance

Standard quality assurance protocols and good laboratory practices should be in place. Participation in the National Influenza Centres (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 separate 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 analyses for the matrix gene of influenza type A viruses

Conventional RT-PCR protocol[6]

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. It is recommended that laboratories that have concerns about identifying currently circulating viruses contact one of the WHO reference laboratories[7] for diagnosis of influenza infection or one of the WHOCCs[8] 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)
- Primers sets
- Positive control (May be obtained upon request from a WHOCC)

Primers sequence

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

Expected 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

[6] 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/
[7] http://www.who.int/csr/disease/avian_influenza/guidelines/referencelabs/en/
[8] http://www.who.int/csr/disease/influenza/collabcentres/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 (10 µmol/l)	1.5
Reverse primer (10 µmol/l)	1.5
QIAGEN® OneStep RT-PCR Enzyme mix (5 U/ 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	
Initial PCR activation	95	15:00	
Three step cycling:			
Denaturation	94	0:30	45
Annealing	50	0:30	
Extension	72	1:00	
Final Extension	72	10:00	

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 = 302 nm)
- Camera and Polaroid® film or use any digital gel documentation system
- Adjustable pipettes
- 2% agarose gel in 1× TAE buffer
- 1× TAE buffer
- Ethidium bromide (10 mg/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 4 g of agarose powder and dissolve it in 200ml 1× TAE buffer. Dissolve the agar by heating in microwave oven.
iii) Cool the melted agarose to about 60 °C, then add 10 µl 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 1× TAE at a level that can cover the top of the gel.

B) Sample loading:

i) Add 5 µl of the gel loading buffer to each PCR tube.
ii) Load molecular weight marker to the first well of the agarose gel.
iii) Pipette 15 µl of the PCR product/GLB 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. If the test is run without a positive control, products must be confirmed by sequencing and comparison with available sequences.

Protocol No.1:[9]
One step conventional RT-PCR for pandemic (H1N1) 2009 HA gene

The protocols and primers for conventional RT-PCR to detect pandemic (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 members of the WHO Expert Committee on influenza PCR or one of the WHOCCs 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 13 000 rpm)
- Adjustable pipettes (10, 20, 100, 200 μl)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes (0.2, 1.5 ml)
- 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 and probes

Type/subtype	Gene fragment	Primer	Sequence
Influenza A H1N1 (2009 pandemic virus)	HA	HKU-SWF	GAGCTCAGTGTCATCATTTGAA
	HA	HKU-SWR	TGCTGAGCTTTGGGTATGAA

Expected size : 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.

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

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

Reagent	Volume (μl)	Final concentration
Water	7.4	
5X PCR buffer (kit)	4.0	1X
dNTPs (kit)	0.8	400 μM of each dNTP
5 μM primer : HKU-SWF	2.4	0.6μM
5 μM 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 18 μl of master mix into each test tube.

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

5. Set the following RT-PCR conditions:

Step	Temperature (°C)	Time (minute:second)	No. of cycle
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	∞	

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

Interpretation of results

The expected size of PCR products for influenza H1 is 173bp. This assay can specifically detect samples with H1N1 pandemic, 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. It should be noted that these assays can detect H1N1 pandemic 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. 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 facilities. If the test is run without controls, products should be confirmed 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.

Protocol No.2:[10]
One step conventional RT-PCR for pandemic (H1N1) 2009 HA gene

The protocols and primers for conventional RT-PCR to detect pandemic (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 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)
- 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)
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes (0.2, 1.5 ml)
- 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)
- Primer set

Primers and probes

Type/subtype	Gene fragment	Primer	Sequence
Influenza A H1N1 2009 pandemic virus	HA	H1-sw-434f	CGA ACA AAG GTG TAA CGG CAG CAT
	HA	H1-sw-905r	GCA CCC TTG GGT GTT TGA CAA GTT

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:

[10] 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

Reagent	Volume (µl)	Final concentration
5X PCR buffer (kit)	10.0	1X
dNTPs (kit)	2.0	400 µM of each dNTP
5X Q-sol (kit)	10.0	1 X
5 µM primer : H1-sw-434f	6.0	0.5µM
5 µM primer : H1-sw-905r	6.0	0.5µM
Enzyme mix (kit)	2.0	-
RNase Inhibitor (20U/µl)	0.5	10 U
Water	8.5	
Total	**45.0**	
RNA template	5.0	

3. Set the following RT-PCR conditions:

Temperature (°C)	Time (minute:second)	No. of cycles
50 95	30:00 15:00	1
94 55 72	0:30 0:30 0:30	} 45
72	7:00	1
10	∝	

4. Perform gel electrophoresis as described before. PCR product size is 472 bp for pandemic (H1N1) 2009.

Protocol No.3:[11]

One step conventional RT-PCR for pandemic (H1N1) 2009 HA gene

The protocols and primers for conventional RT-PCR to detect pandemic (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 members of the WHO Expert Committee on influenza PCR or one of the WHOCCs for assistance in identifying the optimal primers to be used.

Primers and probes

Type/subtype	Gene fragment	Primer	Sequence
Influenza A H1N1 pandemic 2009 virus	HA	NIID-swH1 Conv-F1	TGCATTTGGGTAAATGTAACATTG
	HA	NIID-swH1 Conv-R1	AATGTAGGATTTRCTGAKCTTTGG

Expected product size : 349bp

Procedure

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

Interpretation

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.

[11] 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. Email: todagiri@nih.go.jp, http://idsc.nih.go.jp/.

Group protocol No.1[12]

Conventional RT-PCR assays for the detection of seasonal influenza A (H1N1, H3N2), influenza B and avian influenza A (H5N1) viruses

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

1. Seasonal influenza A (H1N1) viruses (H1 and N1 genes)
2. Seasonal influenza A (H3N2) viruses (H3 and N2 genes)
3. Seasonal influenza B viruses (HA and NA genes)
4. Avian influenza A (H5N1) (H5 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)
- 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 50 µl of the supplied buffer.

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

[12] 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 and probes

Primer sets used for one-step RT-PCR for seasonal influenza and H5N1 surveillance (London WHOCC; April 2009).

Type/subtype	Gene fragment	Primer	Sequence
Influenza A H1N1	HA-5'(H1)	H1A1F1	CAACCAAAATGAAAGCAAAACTAC
		H1HAR1087	AAACCGGCAATGGCTCCAAA
	HA-3'(H1)	H1HAF552	TACCCAAACCTGAGCAAGTCCTAT
		H1A2R1	GCATATTCTGCACTGCAAAGACCC
	NA-5'(N1)	H1N1F6	AGCAGGAGATTAAAATGAATCCAA
		N1R1124	TCTAAGTCTGTTACTTTTAGTCCT
	NA-3'(N1)	N1F741	ATAATGACCGATGGCCCGAGTAAT
		H1N1R1	GTAGAAACAAGGAGTTTTTTCAAC
Influenza A H3N2	HA-5'(H3)	H3A1F6	AAGCAGGGGATAATTCTATTAACC
		H3HAR1075	AACCGTACCAACCRTCCACCATTC
	HA-3'(H3)	H3HAF567	CTGAACGTGACTATGCCAAACAAT
		H3A2R1	AGTAGAAACAAGGGTGTTTTTAAT
	NA-5'(N2)	H3N2F1	AGCAAAAGCAGGAGTGAAAATGAA
		H3N2R1104	ATCCACACGTCATTTCCATCGTCA
	NA-3'(N2)	H3N2F387	CATGCGATCCTGACAAGTGTTATC
		H3N2R1	TTCTAAAATTGCGAAAGCTTATAT
Influenza A Matrix	Full gene	MF1	AGCAAAAGCAGGTAGATATTGAAAGA
		MR1027	AGTAGAAACAAGGTAGTTTTTTACTC
Influenza B	HA-5'	BHA1F1	AATATCCACAAAATGAAGGCAATA
		BHAR1166	ATCATTCCTTCCCATCCTCCTTCT
	HA-3'	BHAF458	AGAAAAGGCACCAGGAGGACCCTA
		BHA2R1	GTAATGGTAACAAGCAAACAAGCA
	NA-5'	BNAF1	AGCAGAAGCAGAGCATATTCTTAG
		BNAR2	GATGGACAAATCCTCCCTTGATGC
	NA-3'	BNAF2	GCACTCCTAATTAGCCCTCATAGA
		BNAR1	CAGAAACAATTAAGTCCAGTAAGG
Influenza A H5N1	HA-5'(H5)	H5A1F1	AGCAAAAGCAGGGGTATAATC
		H5R1265	ACGGCCTCAAACTGAGTGTTCATT
	HA-3'(H5)	H5F417	TTGAGAAAATWCAGATCATCCC
		H5A2R1	AAGGGTGTTTTTAACTAACAATCT
	NA-5'(N1)	H5N1F4	AGCAAAAGCAGGAGATTAAAATGAAT
		H5N1R1112	TTCTCCCGATCCAAACACCATTGC
	NA-3'(N1)	H5N1F461	GACTGTCAAAGACAGAAGCCCTCA
		H5N1R1457	GTAGAAACAAGGAGTTTTTTGAA

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.