

## 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.<sup>1</sup>
- 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.

---

<sup>1</sup> <http://www.who.int/csr/resources/publications/swineflu/realtimeptcr/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.<sup>1</sup>
- 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,<sup>2</sup> to one of the WHO Collaborating Centres for Reference and Research on Influenza (WHOCC).

Specimens with laboratory results indicative of influenza A that are untypable (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.<sup>3</sup>

### 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*, 3<sup>rd</sup> edition.<sup>4</sup>

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.<sup>5</sup>

### 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.

---

<sup>2</sup> [http://www.who.int/csr/resources/publications/swineflu/interim\\_guidance/en/index.html](http://www.who.int/csr/resources/publications/swineflu/interim_guidance/en/index.html)

<sup>3</sup> [http://www.who.int/csr/resources/publications/swineflu/storage\\_transport/en/index.html](http://www.who.int/csr/resources/publications/swineflu/storage_transport/en/index.html)

<sup>4</sup> [http://www.who.int/csr/resources/publications/biosafety/WHO\\_CDS\\_CSR\\_LYO\\_2004\\_11/en/](http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/)

<sup>5</sup> <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<sup>6</sup>

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<sup>7</sup> for diagnosis of influenza infection or one of the WHOCCs<sup>8</sup> for assistance in identifying the optimal primers to be used.

#### Materials required

- QIAamp<sup>®</sup> Viral RNA Mini Kit (QIAGEN<sup>®</sup>, Cat. No. 52904. Other extraction kits can be used after proper evaluation)
- OneStep RT-PCR Kit (QIAGEN<sup>®</sup>, 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

<sup>6</sup> 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: [todayiri@nih.go.jp](mailto:todayiri@nih.go.jp)  
<http://idsc.nih.go.jp/>

<sup>7</sup> [http://www.who.int/csr/disease/avian\\_influenza/guidelines/referencelabs/en/](http://www.who.int/csr/disease/avian_influenza/guidelines/referencelabs/en/)

<sup>8</sup> <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 ( $\mu$ l)
Water (molecular grade)	9.5
5X QIAGEN® RT-PCR buffer	5.0
dNTP mix (containing 10mM of each dNTP)	1.0
Forward primer (10 $\mu$ mol/l)	1.5
Reverse primer (10 $\mu$ mol/l)	1.5
QIAGEN® OneStep RT-PCR Enzyme mix (5 U/ $\mu$ l )	1.0
RNase Inhibitor (20U/ $\mu$ l)	0.5
<b>Total volume</b>	<b>20.0</b>

- Dispense 20 $\mu$ l of the master mix to each PCR reaction tube.
- Add 5 $\mu$ l sample RNA to the master mix. For control reactions, use 5 $\mu$ l of distilled water for negative control and 5 $\mu$ l 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 ( $\lambda = 302 \text{ nm}$ )
- Camera and Polaroid<sup>®</sup> 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  $\mu\text{l}$  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  $\mu\text{l}$  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  $\mu\text{l}$  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:**<sup>9</sup>  
**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:

---

<sup>9</sup> Department of Microbiology, Faculty of Medicine, University of Hong Kong, University Pathology Building Queen Mary Hospital, Hong Kong Special Administrative Region of China.

Reagent	Volume ( $\mu$ l)	Final concentration
Water	7.4	
5X PCR buffer (kit)	4.0	1X
dNTPs (kit)	0.8	400 $\mu$ M of each dNTP
5 $\mu$ M primer : HKU-SWF	2.4	0.6 $\mu$ M
5 $\mu$ M primer : HKU-SWR	2.4	0.6 $\mu$ M
RNase Inhibitor (20U/ $\mu$ l)	0.2	4 U
Enzyme mix (kit)	0.8	-
<b>Total</b>	<b>18.0</b>	

3. Dispense 18  $\mu$ l of master mix into each test tube.
4. Add 2  $\mu$ l of purified RNA to the above reaction mix.
5. Set the following RT-PCR conditions:

Step	Temperature ( $^{\circ}$ C)	Time (minute:second)	No. of cycle
Reverse transcription	50	30:00	1
	95	15:00	
Denaturation	94	0:30	} 40
Annealing	57	0:30	
Extension	72	0:20	
Post-PCR extension	72	7:00	1
Post-run	4	$\infty$	

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:<sup>10</sup>

### 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:

---

<sup>10</sup> 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](http://www.chp.gov.hk/files/pdf/CHP_Protocols_for_the_Detection_of_Human_Swine_Influenza.pdf)

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

3. Set the following RT-PCR conditions:

Temperature ( $^{\circ}$ C)	Time (minute:second)	No. of cycles
50	30:00	1
95	15:00	
94	0:30	} 45
55	0:30	
72	0:30	
72	7:00	1
10	$\infty$	

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

### Protocol No.3:<sup>11</sup>

#### 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	AATGTAGGATTTCTGAKCTTTGG

**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.

---

<sup>11</sup> 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: [todayiri@nih.go.jp](mailto:todayiri@nih.go.jp), <http://idsc.nih.go.jp/>.

## Group protocol No.1<sup>12</sup>

### 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.

---

<sup>12</sup> 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](mailto: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 H1HAR1087	CAACCAAATGAAAGCAAACACTAC AAACCGGCAATGGCTCCAAA
	HA-3'(H1)	H1HAF552 H1A2R1	TACCCAAACCTGAGCAAGTCCTAT GCATATTCTGCACTGCAAAGACCC
	NA-5'(N1)	H1N1F6 N1R1124	AGCAGGAGATTAATGAATCCAA TCTAAGTCTGTTACTTTTTAGTCCT
	NA-3'(N1)	N1F741 H1N1R1	ATAATGACCGATGGCCCCGAGTAAT GTAGAAACAAGGAGTTTTTTCAAC
Influenza A H3N2	HA-5'(H3)	H3A1F6 H3HAR1075	AAGCAGGGGATAATTCTATTAACC AACCGTACCAACCRCCACCATTCC
	HA-3'(H3)	H3HAF567 H3A2R1	CTGAACGTGACTATGCCAAACAAT AGTAGAAACAAGGGTGTTTTTAAT
	NA-5'(N2)	H3N2F1 H3N2R1104	AGCAAAAGCAGGAGTGAAAATGAA ATCCACACGTCATTTCCATCGTCA
	NA-3'(N2)	H3N2F387 H3N2R1	CATGCGATCCTGACAAGTGTTATC TTCTAAAATTGCGAAAGCTTATAT
Influenza A Matrix	Full gene	MF1 MR1027	AGCAAAAGCAGGTAGATATTGAAAGA AGTAGAAACAAGGTAGTTTTTTACTC
Influenza B	HA-5'	BHA1F1 BHAR1166	AATATCCACAAAATGAAGGCAATA ATCATTCTTCCCATCCTCCTTCT
	HA-3'	BHAF458 BHA2R1	AGAAAAGGCACCAGGAGGACCCTA GTAATGGTAACAAGCAAACAAGCA
	NA-5'	BNAF1 BNAR2	AGCAGAAGCAGAGCATATTCTTAG GATGGACAAATCCTCCCTTGATGC
	NA-3'	BNAF2 BNAR1	GCACTCCTAATTAGCCCTCATAGA CAGAAACAATTAAGTCCAGTAAGG
Influenza A H5N1	HA-5'(H5)	H5A1F1 H5R1265	AGCAAAAGCAGGGGTATAATC ACGGCCTCAAACCTGAGTGTTTCATT
	HA-3'(H5)	H5F417 H5A2R1	TTGAGAAAATWCAGATCATCCC AAGGGTGTTTTTAACATAACAATCT
	NA-5'(N1)	H5N1F4 H5N1R1112	AGCAAAAGCAGGAGATTAATGAAT TTCTCCCGATCCAAACACCATTGC
	NA-3'(N1)	H5N1F461 H5N1R1457	GACTGTCAAAGACAGAAGCCCTCA 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.

## 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 <sub>4</sub> *	1.0	1.0	
100mM dNTPs	0.9	0.9	25mM of each (G, A, T, C)
10 µmol/l Forward primer <sup>§</sup>	1.5	1.5	0.3 µmol/l final concentration
10 µmol/l Reverse primer <sup>§</sup>	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	
<b>Total</b>	<b>50.0</b>	<b>50.0</b>	

\* Supplied with the Pfx polymerase.

Thermal cycler BIORAD DNA Engine programme:

Temperature (°C)	Time (minute:second)	No. of cycles
50	30:00	
94	10:00	
94	0:05	} 40
55	0:05	
68	0:30	
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	
<b>Total</b>	<b>40.0</b>	

\* 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 <sub>4</sub> *	1.0	
100mM dNTPs	0.9	25mM of each (G, A, T, C)
10 µmol/l Forward primer <sup>S</sup>	1.5	
10 µmol/l Reverse primer <sup>S</sup>	1.5	
Pfx	0.5	Invitrogen, Cat. No. 11708-039
RT product	2.0	
<b>Total</b>	<b>50.0</b>	

\* 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	10:00	
55	5:00	
68	2:00	
94	0:05	} 39
55	0:05	
68	2:00	
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<sup>13</sup>

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<sub>2</sub> (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)-TTTGTGTTACGCTCACCGT-(TAMRA)-3' (5 µmol/l)
Influenza type A	Matrix (M)	FLUA-2P	5'-(FAM)-TGGATTCTTGATCGTCTTTTCTCAAATGCA-(TAMRA)-3 (5 µmol/l)

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

<sup>13</sup> 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 ( $\mu$ l) per reaction
10x PCR buffer I with 15 mmol/l MgCl <sub>2</sub>	2.0
Extra 25 mmol/l MgCl <sub>2</sub>	2.8
dNTPs (2.5 mmol/l)	8.0
Extracted RNA	4.2
Random hexamer 50 $\mu$ mol/l	1.0
RNAase inhibitor 20U/ $\mu$ l	1.0
Reverse transcriptase 50 U/ $\mu$ l	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  $\mu$ l 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 ( $\mu$ l)
PCR-grade H <sub>2</sub> O	7.6
MgCl <sub>2</sub> (25 mmol/l)	2.4
Primers and probe mix	3.0
"Hot Start" reaction mix	2.0
<b>Total volume</b>	<b>15.0</b>

### Each reaction:

Reagent	Volume ( $\mu$ l)
Master Mix	15.0
cDNA (from the RT product; step 1. above)	5.0

**PCR temperature-cycling conditions:**

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

## Real-time RT-PCR protocol 2<sup>14</sup>

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	CCMAGGTCGAAACGTAYGTTCTCTATC (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)-ATYTTCGGCTTTGAGGGGGCCTG- (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
<b>Total</b>	<b>20.0</b>

<sup>14</sup> 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  $\mu$ l of the reaction mixture into each RT-PCR reaction plate.
2. Add 5  $\mu$ l of the sample RNA to the reaction mixture. For control reactions, use 5  $\mu$ l of distilled water for negative control and 5  $\mu$ l 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	<b>1</b>
95	15:00	<b>1</b>
94	0:15	<b>} 45</b>
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	<b>1</b>
95	15:00	<b>1</b>
94	0:15 (ramp rate 1.5 °C/sec)	<b>} 45</b>
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<sup>15</sup>

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] TGGGTAATGTAACATTGCTGGCTGG [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:

<sup>15</sup> 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, $\mu$ l
Water H <sub>2</sub> O	NA	6.2
5X EZBuffer A	5X	5.0
Mn(OAc) <sub>2</sub>	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 $\mu$ M	0.4
Reverse Primer	50 $\mu$ M	0.4
Probe (FAM)	10 $\mu$ M	1.0
rTth Poly(2.5U/ $\mu$ l)	2.5 U/ $\mu$ l	1.0
UNG(1 U/ $\mu$ l)	1 U/ $\mu$ l	0.25
RNA template		4.0
<b>TOTAL</b>		<b>25.0</b>

3. Set the following RT-PCR conditions:

Detection Dye: FAM

Quencher Dye :TAMRA

Step	Temperature ( $^{\circ}$ C)	Time (minute:second)	No. of cycles
Reverse transcription	50	2:00	1
	60	40:00	
	95	5:00	
PCR	95	0:15	50
	55	1:00	

### 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).<sup>16</sup> If the test is run without controls, products should be confirmed

<sup>16</sup> 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<sup>17</sup>

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	swIH1F swIH1R swIH1P*	GACAAAATAACAAACGAAGCAACTGG GGGAGGCTGGTGTATAGCACC 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 <sub>2</sub> O	NA	5.5
2x PCR master mix* <sup>§</sup>	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
<b>Total mastermix</b>		<b>20.0</b>
RNA template		5.0
<b>TOTAL reaction volume</b>		<b>25.0</b>

\* Supplied in the Invitrogen kit.

<sup>§</sup> ROX reference dye (supplied with the Invitrogen kit) **must** be added to the master mix at the level recommended by the manufacturer.

<sup>17</sup> 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](mailto: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:**

<b>Step</b>	<b>Temperature (°C)</b>	<b>Time (minute:second)</b>	<b>No. of cycles</b>
Reverse transcription and activation of Taq	50	30:00	1
	95	02:00	
PCR	95	00:15	50
	55	00:30*	

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

## Protocol No. 4<sup>18</sup>

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<sub>2</sub> (25 mM) (vial 2)
  - PCR grade H<sub>2</sub>O (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.

<sup>18</sup> 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](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)
<b>Master Mix:</b>	
PCR-grade H <sub>2</sub> O	7.6
MgCl <sub>2</sub> (25 mM)	2.4
Primers and probes mix	3.0
“Hot Start” reaction mix	2.0
<b>Total volume</b>	<b>15.0</b>
<b>Each reaction:</b>	
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

<u>Temperature (°C)</u>	<u>Time (minute: second)</u>	<u>No. of cycles</u>
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<sup>19</sup>

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	TGTTCCACAATGTARGACCAT
Influenza A H1N1 pandemic virus	HA	NIID-swH1 Probe2*	5'-(FAM)-CAGCCAGCAATRTTRCATTACC-(MGB)-3'

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

<sup>19</sup> 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 ( $\mu$ l)
RNase-free water	3.75
2x QuantiTect <sup>®</sup> Probe RT-PCR Master Mix	12.5
Forward Primer (10 $\mu$ mol/l)	1.5
Reverse Primer (10 $\mu$ mol/l)	1.5
TaqMan MGB Probe (5 pmol/ $\mu$ l)	0.5
QuantiTect <sup>®</sup> RT Mix	0.25
<b>Total</b>	<b>20.0</b>

## Procedure

1. Dispense 20  $\mu$ l of the reaction mixture into each RT-PCR reaction plate.
2. Add 5  $\mu$ l of the sample RNA to the reaction mixture. For control reactions, use 5  $\mu$ l of distilled water for negative control and 5  $\mu$ l 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	<b>1</b>
95	15:00	<b>1</b>
94	0:15	<b>} 45</b>
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	<b>1</b>
95	15:00	<b>1</b>
94	0:15 (ramp rate 1.2 ° C/sec)	<b>} 45</b>
	1:15 (ramp rate 1.2 ° C/sec)	
56	Data collection	

## Realtime RT-PCR assays for the detection of pandemic (H1N1) 2009 virus, seasonal influenza viruses or avian (only group protocol No.2) influenza A viruses

### Group protocol No.1<sup>20</sup>

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

1. Influenza type A viruses (M gene)
2. Pandemic (H1N1) 2009 virus (H1 gene)
3. Seasonal influenza A (H1N1) and A (H3N2) viruses (H1h, N1h, H3h, N2h genes)

This protocol is to be used in case of suspicion of human infection by the pandemic (H1N1) 2009 virus.

The following testing strategy is recommended:

- RNA extraction.
- Amplification in parallel of M, H1 pandemic and GAPDH (to assess quality of the specimen and extraction procedure) genes.
- In a separate set, amplification of the H1h, N1h, H3h and N2h genes.
- All the above genes are tested in a single plex and not in a multiplex format.

### 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
- Smart Cycler® 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) pandemic 2009 virus (H1swl gene) were also validated under the following conditions:

RT-PCR Mix kit:

---

<sup>20</sup> Unité de Génétique Moléculaire des Virus Respiratoires. Institut Pasteur, 25 rue du Docteur Roux 75724, Paris Cedex 15, France. Email: [grippe@pasteur.fr](mailto:grippe@pasteur.fr); <http://www.pasteur.fr>

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

Real-time PCR equipments:

- 7500 Applied® Biosystems
- SmartCycler® Cepheid

### Primers and probes

If the sample is positive for M and negative for H1sw, we suggest use of the H1N1h and H3N2h sets of primers.

Type/subtype	Gene	Name	Sequences	Bases	PCR Product size	Reference
Seasonal H1N1	HA	H1h-678Fw	CACCCAGAAATAGCCAAAA	20		1
Seasonal H1N1	HA	H1h-840Rv	TCCTGATCCAAAGCCTCTAC	20	163 bp	1
Seasonal H1N1	HA	H1h-715probe	5'-Fam-CAGGAAGGAAGAATCAACTA 3'-BHQ-1	20		1
Seasonal H3N2	HA	H3h-177Fw	GAGCTGGTTCAGAGTTCCTC	20		1
Seasonal H3N2	HA	H3h-388Rv	GTGACCTAAGGGAGGCATAATC	22	211 bp	1
Seasonal H3N2	HA	H3h-306Probe	5'-Fam-TTTTGTGAACGCAGCAAAG 3'-BHQ-1	20		1
Seasonal H3N2	NA	N2h-1150 Fw	GTCCAMACCTAAYTCCAA	18		1
Seasonal H3N2	NA	N2h-1344 Rv	GCCACAAAACACAACAATAC	20	194 bp	1
Seasonal H3N2	NA	N2h-1290 probe	5'-Fam-CTTCCCCTTATCAACTCCACA-3'-BHQ-1	21		1
Seasonal H1N1	NA	N1h-1134 Fw	TGGATGGACAGATACCGACA	20		1
Seasonal H1N1	NA	N1h-1275 Rv	CTCAACCCAGAAGCAAGGTC	20	142 bp	1
Seasonal H1N1	NA	N1h-1206 probe	5'-Fam-CAGCGGAAGTTTCGTCAACAT 3'-BHQ-1	22		1
Pandemic H1N1	HA	GRswH1-349Fw	GAGCTAAGAGAGCAATTGA	19		1
Pandemic H1N1	HA	GRswH1-601Rv	GTAGATGGATGGTGAATG	18	253bp	1
Pandemic H1N1	HA	GRswH1-538Probe(-)	Fam 5'-TTGCTGAGCTTTGGGTATGA -3'-BHQ-1	20		1
Influenza type A		GRAM/7Fw	CTTCTAACCGAGTCGAAACGTA	23		2
Influenza type A		GRAM/161Rv	GGTGACAGGATTGGTCTGTCTTTA	25	202 bp	2
Influenza type A		GRAM probe/52/+	5'-Fam-TCAGGCCCTCAAAGCCGAG 3'-BHQ-1	21		2
Human internal control	GAPDH	GAPDH-6Fw	GAAGGTGAAGGTCGGAGT	18		3
Human internal control	GAPDH	GAPDH-231Rv	GAAGATGGTGATGGGATTTTC	20	226 bp	3
Human internal control	GAPDH	GAPDH-202Probe(-)	5'-Fam-CAAGCTTCCCCTTCTCAGCC-3'-BHQ-1	20		3

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

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

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

## Procedure

### LightCycler 1.5 or 2.0 (Capillaries) (ROCHE)

Reagent	Volume ( $\mu$ l)	Final concentration
H <sub>2</sub> O PPI	1.06	
Reaction mix 2x	10.0	3.0 mM Mg
MgSO <sub>4</sub> (50mM)	0.24	0.6 mM Mg
Forward Primer (10 $\mu$ M)	1.0	0.5 $\mu$ M
Reverse Primer (10 $\mu$ M)	1.0	0.5 $\mu$ M
Probe (10 $\mu$ M)	0.4	0.2 $\mu$ M
BSA non acetylated (10mg/ml)	0.5	0.25 mg/ml
Superscript III RT/Platinum Taq Mix	0.8	
<b>Total</b>	<b>15.0</b>	

### LightCycler 480 (96-well format) (ROCHE)

Reagent	Volume ( $\mu$ l)	Final concentration
H <sub>2</sub> O PPI	1.56	
Reaction mix 2x	10.0	3.0 mM Mg
MgSO <sub>4</sub> (50mM)	0.24	0.6 mM Mg
Forward Primer (10 $\mu$ M)	1.0	0.5 $\mu$ M
Reverse Primer (10 $\mu$ M)	1.0	0.5 $\mu$ M
Probe (10 $\mu$ M)	0.4	0.2 $\mu$ M
Superscript III RT/Platinum Taq Mix	0.8	
<b>Total</b>	<b>15.0</b>	

15  $\mu$ l of reaction mix + 5  $\mu$ l of RNA samples.

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

Reagent	Volume ( $\mu$ l)	Final concentration
H <sub>2</sub> O PPI	1.5	
Reaction mix 2x	12.5	3.0 mM Mg
Forward Primer (10 $\mu$ M)	2.0	0.8 $\mu$ M
Reverse Primer (10 $\mu$ M)	2.0	0.8 $\mu$ M
Probe (10 $\mu$ M)	1.0	0.2 $\mu$ M
ROX reference dye (diluted 1/10)	0.5	
Superscript III RT/Platinum Taq Mix	0.5	
<b>Total</b>	<b>20.0</b>	

20  $\mu$ l of reaction mix + 5  $\mu$ l of RNA samples

## Controls

Each real-time 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	0:10	} 50
	55	0:10	
	72	0:20	
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	
Amplification	95	0:15	50
	60	0:40	

## Sensitivity

### For the M real-time 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 pandemic real-time RT-PCR

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

### For the H3h and N2h real-time RT-PCR

Sensitivity of the H3h real-time RT-PCR is equivalent to that of the M real-time PCR ( $C_p \text{ H3h} \approx C_p \text{ M}$ ) but the sensitivity of the N2h real-time RT-PCR is lower ( $C_p \text{ N2h} \approx C_p \text{ M} + 5 C_p$ )

### For the H1h and N1h real-time RT-PCR

Sensitivity of the N1h real-time RT-PCR is equivalent to that of the M real-time PCR ( $C_p \text{ N1h} \approx C_p \text{ M}$ ) but the sensitivity of the H1h real-time RT-PCR is lower ( $C_p \text{ H1h} \approx C_p \text{ M} + 4 C_p$ ).

## Specificity

### For the H1 pandemic real-time RT-PCR

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

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

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

**NOTE: The H1 pandemic real-time RT-PCR does not detect the positive control from the CDC kit.**

### 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 100 µl of distilled water to obtain a solution at a concentration of  $10^9$  copies/µl. Store at -80°C.
- Dilute in H<sub>2</sub>O to prepare a master bank at  $2 \times 10^6$  copies/µl. Store at -80°C.
- From this, prepare a working bank of reagent at  $2 \times 10^4$  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](mailto:grippe@pasteur.fr).

### Interpretation of results

GAPDH reactions should give a  $C_p < 35$ ; 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 H1 pandemic and negative reactions for H1h, N1h, H3h, N2h: confirmed case for pandemic (H1N1) 2009 virus.

Positive reaction for M and negative for H1 pandemic 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 either N1h or H3h but negative for H1 pandemic and for H1h and N2h (usually seen for low virus load in specimen) may indicate an infection with seasonal virus; in this case, repeat reactions and/or repeat RNA extraction to determine subtypes.

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

## Group protocol No. 2<sup>21</sup>

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

1. Influenza type A viruses (M gene)
2. Pandemic influenza A (H1N1) 2009 virus (N1 gene)
3. Pandemic influenza A (H1N1) 2009 virus (HA gene)
4. Seasonal influenza A (H1N1) and A (H3N2) viruses (H1h, H3h, genes)
5. Avian influenza A (H5) viruses, including highly pathogenic H5N1 viruses
6. Avian influenza A (H7) viruses
7. Influenza type B viruses

### Materials required

1. ABI7500 machine
2. TaqMan EZ RT-PCR Core Reagents kit (Applied Biosystems, Cat. No. N8080236)
3. Amplification and detection is performed on ABI7500 machine with the TaqMan EZ RT-PCR Core Reagents kit (Applied Biosystems), Using 20 µl of RNA extract in an end volume of 50 µl

### Test validation

All tests have been optimized and validated to work in the platform described above. They have not been optimized for other platforms and other reagents. For instance, some of these tests do not work well with other reagents (e.g. Ampli-Taq Gold).

The influenza B virus test, the influenza A virus M-gene test, the human influenza A/H1 virus test, and the human influenza A/H3 virus test have been used successfully for several years in a diagnostic virology laboratory with human specimens. The influenza A virus M-gene test, the influenza A/H5 virus tests and the influenza A/H7 test have been used successfully for several years in a diagnostic virology laboratory with human and animal specimens.

The seasonal influenza A/H1 test does not detect the pandemic H1N1 2009 virus H1 sequences, only seasonal influenza A (H1N1) virus H1 sequences. The pandemic H1N1 2009 NA and HA tests do not detect seasonal influenza A (H1N1) NA or HA sequences, only N1 and H1 of the pandemic H1N1 2009 virus sequences. This has been validated using sets of numerous recent human H1N1 viruses and swine and the pandemic H1N1 viruses. In addition, the tests have proven successful in detecting sporadic cases of the pandemic H1N1 2009 viruses in The Netherlands.

---

<sup>21</sup> Dept Virology, Erasmus MC Rotterdam, Netherlands. ([www.virology.nl](http://www.virology.nl))

### Reaction mixture (EZ RT-PCR)

Reagent	Volume ( $\mu$ l)
DEPC water	4.5 $\mu$ l
5x Taqman EZ buffer	10.0 $\mu$ l
Manganese acetate (25 mM)	6.0 $\mu$ l
dNTP's	6.0 $\mu$ l
Primer/probe mix (1 $\mu$ l/reaction)	1.0 $\mu$ l
rTth DNA Polymerase (2,5U/ $\mu$ l)	2.0 $\mu$ l
Amperase UNG (1U/ $\mu$ l)	0.5 $\mu$ l
<b>Total mix volume</b>	<b>30.0</b>
Input RNA	20.0 $\mu$ l
<b>Total volume</b>	<b>50.0</b>

### Thermal Cycling conditions

Temperature ( $^{\circ}$ C)	Time (minute:second)	No. of cycles
50	02:00	1
60	30:00	1
95	05:00	1
94	00:20	} 40[a]
62	01:00	

[a] For pandemic (H1N1) 2009 NA and HA gene specific test, 45 cycles are used.

## Primers and probes

Type/subtype	Primer/probe <sup>22</sup>	Gene fragment	Oligonucleotide sequence	Quantity/reaction (pmol)
Flu B	Primer 1	M	GAGACACAATTGCCTACCTGCTT	10
Flu B	Primer 2	M	TTCTTTCCACCGAACCAAC	40
Flu B	Probe	M	AGAAGATGGAGAAGGCAAAGCAGAACTAGC	5
Flu type A	Primer 1	M	AAGACCAATCCTGTACCTCTGA	40
Flu type A	Primer 2	M	CAAAGCGTCTACGCTGCAGTCC	30
Flu type A	Probe	M	TTTGTGTTACGCTCACCGTGCC	5
Flu A (H1N1) seasonal	Primer 1	HA	GAATAGCCCCACTACAATTGGGTAA	10
Flu A (H1N1) seasonal	Primer 2	HA	GTAATTCGCATTCTGGGTTTCTT	10
Flu A (H1N1) seasonal	Probe	HA	AAGATCCATCCGGCAACGCTGCA	10
Flu A (H3) seasonal	Primer 1	HA	GATGTGTACAGAGATGAAGCATTAAACA	40
Flu A (H3) seasonal	Primer 2	HA	TAGGATCCAATCTTTGTATCCTGACTT	15
Flu A (H3) seasonal	Probe 1	HA	AGCTCAACACCTTTGATCTGGAACCGG	10
Flu A (H3) seasonal	Probe 2	HA	AGCTCAACGCCTTTGATCTGGAACCGG	10
Flu A (H5N1) HPAI, Set 1	Primer 1	HA	GAGAGGAAATAAGTGGAGTAAAATTGGA	30
Flu A (H5N1) HPAI, Set 1	Primer 2	HA	AAGATAGACCAGCTACCATGATTGC	2.5
Flu A (H5N1) HPAI, Set 1	Probe	HA	TTTATTCAACAGTGGCGAGTTCCTAGCACT	10
Flu A (H5N1) HPAI, Set 2	Primer 1	HA	GGAACTACCAAATACTGTCAATTTATTCA	40
Flu A (H5N1) HPAI, Set 2	Primer 2	HA	CCATAAAGATAGACCAGCTACCATGA	2.5
Flu A (H5N1) HPAI, Set 2	Probe	HA	TTGCCAGTGCTAGGGAACCGCCAC	10
Flu A (H7) AI viruses	Primer 1	HA	GGCAACAGGAATGAAGAATGTTCC	40
Flu A (H7) AI viruses	Primer 2	HA	AATCAGACCTTCCCATCATTTTC	15
Flu A (H7) AI viruses	Probe	HA	AGAGGCCTATTTGGTGCTATAGCGGGTTCA	10
Pandemic 2009 virus*	Primer 1	HA	GGAAAGAAATGCTGGATCTGGTA	30
Pandemic 2009 virus*	Primer 2	HA	ATGGGAGGCTGGTGTATATAGC	30
Pandemic 2009 virus*	Probe	HA	TGCAATACAACCTGTGACACCCCAAGGG	10
Pandemic 2009 virus	Primer 1	NA	ACATGTGTGTGCAGGGATAACTG	30
Pandemic 2009 virus	Primer 2	NA	TCCGAAAATCCCACCTGCATAT	30
Pandemic 2009 virus	Probe	NA	ATCGACCGTGGGTGTCTTTCAACCA	10

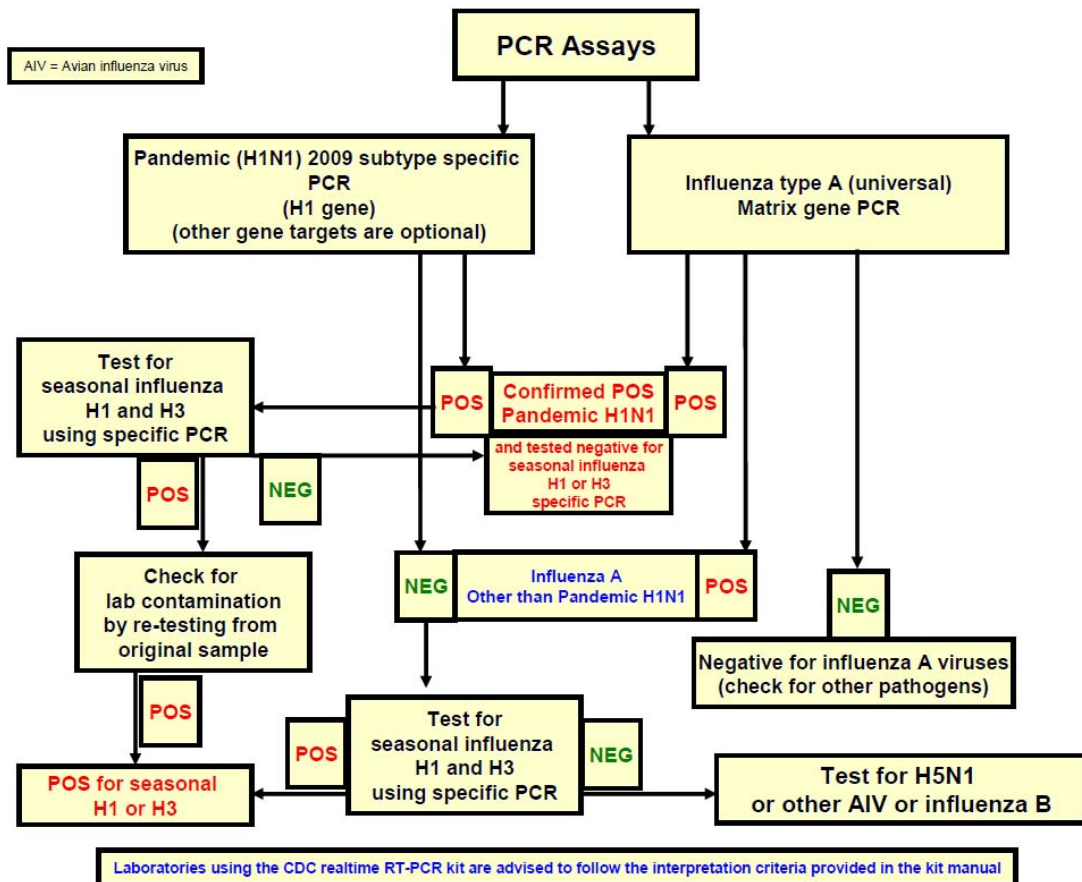
\* A new protocol which did not exist in the 18 August 2009 version of this document.

**Test result interpretation**

M gene pos, seasonal H1 pos, pandemic 2009 H1 and N1 neg: Human seasonal H1 influenza

M gene pos, seasonal H1 neg, pandemic 2009 H1 or N1 pos: pandemic (H1N1) 2009 virus

Annex 3:  
PCR testing algorithm and results interpretation



Laboratories using the CDC realtime RT-PCR kit are advised to follow the interpretation criteria provided in the kit manual

#### Annex 4: Collection of autopsy tissues in resource-limited settings

This document should be read as a supplement to existing autopsy guidelines recommended by the US Centers for Disease Control and Prevention (CDC) for submission procedures (see: <http://www.cdc.gov/h1n1flu/tissuesubmission.htm>) and safety requirements (see: [http://www.cdc.gov/h1n1flu/post\\_mortem.htm](http://www.cdc.gov/h1n1flu/post_mortem.htm).)

The information below specifically supplements the above CDC information when rapid freezing and storage methods such as dry ice are not readily available, and also, if consent has only been given for a limited or para-mortem examination.

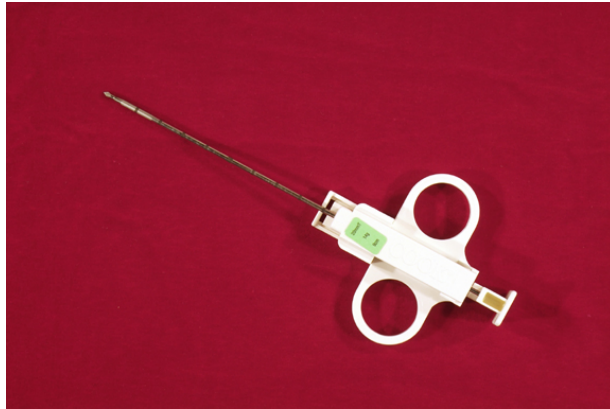
If consent has been given for a full autopsy/postmortem, then the relevant tissues should be ideally divided to be placed in appropriate containers for each of the following: (1) frozen tissue, (2) fixed in 70% alcohol (this gives better preservation of RNA than formalin), (3) placed in RNAlater Tissue Protect Tube (Qiagen®, Cat. No. 76163) for RNA preservation (see below), (4) virus transport medium for virus isolation and (5) 10% neutral buffered formalin.

For long term storage and transport, RNAlater (Ambion) Tissue Protect Tubes provide pre-measured volumes of RNAlater RNA Stabilization Reagent in re-closable tubes for convenient handling and sample storage. The reagent is **not** a fixative, but rather preserves RNA for up to one day at 37°C, seven days at 18–25°C, or four weeks at 2–8°C, allowing processing, transportation, storage, and shipping of samples without liquid nitrogen or dry ice. Alternatively, the samples can also be placed at –20°C or –80°C for archival storage. Morphological analysis, however, will be very difficult to interpret from tissues placed in this reagent.

#### Tissues to be sampled:

- Lung: all lobes, including affected areas
- Proximal and distal trachea
- Liver
- Brain
- Kidneys
- Nasopharynx (this is best done from a supratentorial approach with removal en bloc).

In the event that consent for a full autopsy is not forthcoming, the attending physicians should consider a para-mortem needle biopsy approach. In Hong Kong, during the SARS outbreak in 2003, this was used in 24 of the 39 post-mortem cases. These biopsies can be obtained percutaneously and apart from minor puncture wounds there is no disfigurement to the body and infectious risk to autopsy personnel is low.



Trucut biopsy needle that can be used for needle sampling of organs.

## LUNG

The area of the lung chosen for Trucut ideally should correspond to radiological signs of pneumonia. For negative control, a biopsy from a radiological normal area is also advised.

A minimum of four cores of tissue should be sampled, five if liquid nitrogen is available. It should be noted that samples involving fixation should be performed last so as to avoid potential cross contamination of specimen bottles.

Biopsy 1 (fresh): Half is put into viral transport medium, the other half is placed in OCT embedding media and frozen in liquid nitrogen, if it is available.

Biopsy 2 (fresh): \*Placed in RNAlater Tissue Protect Tube (Qiagen®, Cat. No. 76163) ,if it is available.

Biopsy 3 – 70% Ethanol: Ethanol is a superior fixative to formalin for RNA extraction but is inferior for morphology. This fixative can be used if the tissue needs to be transported over

distance and liquid nitrogen is not available. (If the same Trucut needle is used for all biopsies fixation in ethanol should be done first as even a small amount of formalin in ethanol will impair RNA extraction).

Biopsy 4 and 5 – 10% neutral buffered formalin: This will allow good morphology to determine the extent of lung damage, secondary bacterial infection, etc. It will also enable immunohistochemistry to be performed for viral antibodies and co-localization studies to determine the cells infected with the infectious agent. Formalin fixed tissue can be used for RNA extraction but is less optimal as a fixative in this respect than alcohol. One of the biopsy samples should be processed for paraffin embedding; the other will remain in formalin for future extraction of RNA/DNA or electron microscope examination. It should be recognized that the RNA that is from formalin fixed tissue extracted will be degraded and one should not aim at amplifying fragments larger than 60-120 bp. If freshly prepared, 2.5% glutaraldehyde in cacodylate buffer (0.1 M sodium cacodylate-HCl buffer pH 7.4) is available; a 1-2 mm sample from the core can be placed directly into this fixative for electron microscope examination.

#### **OTHER ORGANS**

Sampling of other organs, especially the spleen, bone marrow and liver, would be important (at least for standard histology), and for detecting the presence of virus (by culture, RT-PCR and viral antigen by immunohistology), and to look for haemophagocytosis or changes in these organs. To avoid possible PCR carryover contamination a new needle should be used for each organ. This is also important for determining the extent of disease dissemination.