World Health Organization

Recommendations and laboratory procedures for detection of avian influenza A(H5N1) virus in specimens from suspected human cases[1]

Revised August 2007

Contents

1. General information and intended use of this document

Infection of avian populations with certain subtypes of avian influenza A virus poses continuing global human public health risks of (a) sporadic human zoonotic infections and (b) emergence of a pandemic influenza strain. These subtypes may be considered novel for humans as they are different from the currently circulating human seasonal influenza viruses[2] tested routinely in laboratories. Human infection with one of these novel highly pathogenic avian influenza (HPAI) A(H5N1) viruses, was first recognized during the 1997 outbreak in Hong Kong Special Administrative Region of China. Since 2003, outbreaks of HPAI A(H5N1) have occurred in poultry in Asia, Europe, and Africa and human infections with this subtype have continued to occur. The ongoing circulation of avian influenza A(H5N1)

[1] This document replaces the document *Recommended laboratory tests to identify avian influenza A virus in specimens from humans*, Revised March 2007 http://www.who.int/csr/disease/avian_influenza/guidelines/labtests/en/index.html

[2] "Novel" is used in this document to describe any influenza viruses that are not currently circulating seasonally in human populations, i.e. subtypes other than human origin influenza A(H1), A(H3), or B

viruses in poultry in areas of Asia and Africa and the continued human infections with this subtype, as well as the documentation of human infections with H9 and H7 subtypes of avian influenza, emphasize the need for diagnostic capacity for the rapid and sensitive detection of novel influenza infections in humans.

Procedures proposed in this document are intended for testing specimens from patients with suspected avian influenza A(H5N1) infection, as described in the document WHO guidelines for in*vestigation of human cases of avian influenza A(H5N1)* [3] . Only protocols for identification of the H5 subtype are included, but infection with seasonal human and other novel influenza viruses should always be considered. Investigation of suspected human infection with avian influenza A(H5N1) should also always be considered in the context of clinical and epidemiological background and according to the WHO case definition for human infections with avian influenza A(H5N1).[4] Relevant information, including patient history and exposure variables, should be recorded.

This document is intended for use by National Influenza Centres (NICs; members of the WHO Global Influenza Surveillance Network). Recommendations and procedures are in accordance with the WHO National Influenza Centres (NIC) role during interpandemic and pandemic alert phases as outlined in the WHO document *The role of National Influenza Centres (NICs) during interpandemic, pandemic alert and pandemic periods.*[5] Whenever possible, clinical specimens and/or virus isolates should be forwarded to a WHO H5 Reference Laboratory for confirmation of diagnosis if needed, or a WHO Collaborating Centre for Reference and Research on Influenza for full antigenic and genetic characterization, as well as antiviral susceptibility analysis.

Please note that the protocols in Annex A are subject to change over time, in response to ongoing changes in influenza viruses and/or diagnostic technologies. The latest version of this document (always available on the WHO web page[6]) should always be used.

2. Specimen collecting and handling

Specimen collection and handling directly impacts the validity of the laboratory result. Samples collected or handled inappropriately can lead to incorrect diagnostic results, even when testing procedures are followed correctly.

Specimens for H5N1 diagnosis should be collected according to WHO guidance available in the documents *Collecting, preserving and shipping specimens for the diagnosis of avian influenza A(H5N1) virus infection: Guide for field operations*[7] and *WHO guidelines for the storage and transport of human and animal specimens for laboratory diagnosis of suspected avian influenza A infection.*[8] Specimen collection should be done preferably before initiation of antiviral treatment.

"Golden Rule:" Clinical specimens from humans and from animals should NEVER be processed in the same laboratory. However they could be processed in the same institution if separation of

[3] Revised January, 2007 http://www.who.int/csr/resources/publications/influenza/WHO_CDS_EPR_GIP_2006_4/en/index.html
[4] http://www.who.int/csr/disease/avian_influenza/guidelines/case_definition2006_08_29/en/index.html
[5] Interim document, May 2007 http://www.who.int/csr/disease/avian_influenza/guidelines/RoleNICsMay07/en/index.html
[6] at http://www.who.int/csr/disease/avian_influenza/guidelines/labtests/en/index.html
[7] October 2006 http://www.who.int/csr/resources/publications/surveillance/WHO_CDS_EPR_ARO_2006_1/en/index.html
[8] January 2005 http://www.who.int/csr/disease/avian_influenza/guidelines/transport/en/index.html

working rooms for animal and human specimens is clear and strict. This is to eliminate risk of cross contamination of human and animal samples.

3. Laboratory requirements

- **Biosafety.** Processing of specimens and diagnostic testing should be carried out following WHO recommendations for laboratory biosafety[9] as well as specific biosafety recommendations available in the *WHO laboratory biosafety guidelines for handling specimens suspected of containing avian influenza A virus*.[10] Procedures that involve virus replication (virus isolation, micro-neutralization tests) should be carried out in biosafety level (BSL)-3 containment. Procedures that do not involve amplification of virus can be carried out in BSL-2 containment. All A(H5N1) virus isolates and specimens testing positive for this virus should be stored in an appropriate containment facility. Inventory of specimens, viruses and genetic materials should be kept and updated regularly.

- **Quality assurance.** Laboratories should have established, standard protocols for quality assurance and test validation including the appropriate use of positive and negative controls. With continued evolution of the H5N1 virus, it is essential that laboratories use test protocols that have been validated for most recent A(H5N1) virus strains. Optimization of test methods may be necessary if reagents or equipment used are different from original validated protocols. Training of personnel, appropriate design of facility and maintenance of equipment are other factors that may affect test results. Non-NIC laboratories conducting A(H5N1) virus testing on human samples should collaborate with a NIC[11] or a WHO H5 Reference Laboratory[12] to establish and validate protocols. Participation in the WHO External Quality Assessment Programmes[13] for the detection of subtype influenza A viruses using PCR is strongly recommended.

- **Diagnostic capacity.** Laboratories diagnosing avian influenza A (H5N1) virus in specimens from suspected human cases should be able to detect and identify seasonal influenza strains for exclusion purposes. Preferably, laboratories should be capable of identifying other novel influenza subtypes as well as other respiratory viruses.

4. Confirmation of results

During Interpandemic and Pandemic Alert periods as defined in the WHO Global Influenza *Preparedness Plan*,[14] laboratory results for suspected avian influenza infections in humans should be confirmed by an approved laboratory. In practice, specimen quality, stage of disease, clinical condition and epidemiological exposure should be taken into account in the

[9] WHO Laboratory Biosafety Manual - Third Edition, http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/
[10] January 2005 http://www.who.int/csr/disease/avian_influenza/guidelines/handlingspecimens/en/index.html
[11] List of National Influenza Centres http://www.who.int/csr/disease/influenza/centres/en/index.html
[12] List of WHO H5 Reference Laboratories: http://www.who.int/csr/disease/avian_influenza/guidelines/referencelabs/en/index.html
[13] WHO External Quality Assessment Project for the Detection of Subtype Influenza A Viruses by PCR, July 2007 http://www.who.int/csr/disease/avian_influenza/guidelines/eqa_project/en/index.html
[14] http://www.who.int/csr/resources/publications/influenza/WHO_CDS_CSR_GIP_2005_5/en/index.html

interpretation of test results, especially for a negative laboratory result in the context of clinical suspicion. Additional investigations may be considered on a case by case basis.

When receiving specimens from patients with suspected influenza A(H5N1) virus infection, NICs (and other laboratories) that **lack the capacity to confirm A(H5) infections** should:

a) Arrange forwarding of specimens to a NIC[15] with appropriate testing capacity or any WHO H5 Reference Laboratory for confirmatory testing;
and
b) Inform the WHO Office in the country and/or WHO Regional Office and/or WHO HQ Global Influenza Programme (whoinfluenza@who.int) that specimens or virus isolates are being forwarded to other laboratories for further identification or further characterization.

5. Available laboratory techniques for detection of influenza A viruses in humans

The following assays may be used to detect (and initially identify) both seasonal and novel influenza A viruses in specimens from humans. The assays vary in the expertise and infrastructure required rapidity, cost, and sensitivity/specificity.

- **Conventional reverse transcriptase polymerase chain reaction (RT-PCR) and real-time reverse transcriptase PCR assay.** PCR detects viral RNA present in either clinical specimens or virus cultures, and can be targeted at genes that are relatively conserved across all influenza A viruses (e.g. matrix gene) or to the haemagglutinin or neuraminidase genes which are subtype specific. Including the time taken for viral RNA extraction and for amplicon detection, the turn-around time of conventional RT-PCR assays is 6–8 hours. Real time RT-PCR methods can shorten this time interval to around 3–4 hours while providing increased sensitivity and possibility of quantitation of the viral target gene.

- **Other molecular detection systems.** These systems, mostly still under development, tend to be rapid. They include assays based on nucleic acid amplification and use various endpoint detection methods.

- **Virus culture.** Results are available in 2–10 days using either shell-vial or standard cell-culture methods. Positive influenza cultures may or may not exhibit cytopathic effects, thus a second step to specifically identify influenza viruses by immunofluorescence, haemagglutination – inhibition (HI) or RT-PCR is needed. An advantage of culture is that viruses are available for further characterization. Virus culture in appropriate cell lines can also detect other clinically important respiratory viruses.

  Most avian viruses grow readily in embryonated eggs and this is the option of choice for amplification of virus from avian hosts. However, highly pathogenic avian H5N1 viruses that infect humans are still quite virulent in eggs, killing them quickly. This makes standard egg culture amplification approaches more difficult. For this reason and

[15] List of National Influenza Centres http://www.who.int/csr/disease/influenza/centres/en/index.html

because of the biosafety concern, isolation of highly pathogenic H5N1 viruses is usually performed only in specially qualified and equipped laboratories.

- **Rapid antigen detection.** Viral antigen detection may be carried out by immunofluorescence or enzyme immunoassay (EIA) methods. The EIA based methods are simple and convenient to use. However, such tests are, at present, directed at conserved viral antigens (e.g. virus nucleoprotein, matrix protein) and detect all subtypes of influenza A viruses, whether of human or avian origin. Therefore these tests will not differentiate human virus subtypes H3N2 or H1N1 from avian influenza H5N1. Additionally, current viral antigen detection rapid tests, while being sensitive for the detection of human seasonal influenza viruses, appear to have low sensitivity for the diagnosis of avian influenza H5N1 (i.e. a negative result does not exclude H5N1 disease). Thus overall, presently commercially available antigen detection tests have limited utility for diagnosis of A(H5N1) disease in humans.

Examples of protocols for some available assays to detect influenza A(H5N1) viruses are described in Annex A. The laboratory protocols outlined in this document have proven to be valid for known H5N1 viruses when the listed reagents and platforms (and/or primers) are used together as presented, and in accordance with good laboratory practices. Presented protocols may require updates as new strains of H5N1 evolve.

6. Serological identification of antibodies against avian influenza A(H5N1) viruses

Serological tests available for the measurement of influenza A-specific antibody include the haemagglutination inhibition test (HI), enzyme immunoassay (EIA), and virus neutralization tests (VN). The microneutralization (MN) assay is the currently recommended test for the measurement of antibodies to highly pathogenic avian influenza A. However, it is impractical for routine diagnostic testing of clinical cases due to the constraints listed below. It is therefore most useful for research studies (e.g. for determination of extent of exposure in case contacts or at risk populations).

Optimally, paired sera, collected first during the acute phase of illness and then 14 days or later after the onset of illness, should be tested simultaneously. Retrospectively, infection with H5N1 is confirmed when one the following criteria are met:

- Fourfold or greater rise in antibody titre against A(H5N1) in paired sera (acute and convalescent) with the convalescent serum having a titre of 1:80 or higher.
- Antibody titre of 1:80 or more in a single serum collected at day 14 or later after onset of symptoms and a positive result using different serological assay (e.g. titre of 1:160 or greater in HI using horse red blood cell or an H5 –specific western blot).

Constraints of serological techniques:

- Antibodies take up to several weeks to develop and become detectable in serum.
- In general, standard panels of reagents for H5N1 and other novel strains are not widely available and results among the laboratories performing these tests vary widely.

- MN assay is technically difficult to perform.
- VN and MN assays require the use of live virus, thus can only be used in laboratories with Biosafety Level 3 containment facilities.
- Difficulties in determining endpoint in HI with horse red blood cells because of their small size. Adjustment of buffer concentration might overcome the problem.
- Non-specific cross reactivity in all serological assays may occur due to the previous infections with human influenza viruses or other factors. Appropriate serum adsorption before testing is recommended.

Annex A
Examples of protocols for some available assays to detect A(H5N1) viruses

The laboratory protocols outlined in this document have proven to be valid when the listed reagents and platforms (and/or primers) are used together as presented, and in accordance with good laboratory practices. The protocols described do not contain all the information required to perform these tests. Further details should be obtained from the references cited in this document, from a WHO H5 Reference Laboratory,[16] or from members of the WHO technical working group on PCR protocols (see Annex C). Presented protocols may require updates as new strains of H5N1 evolve.

All assays should be initially validated in each laboratory before starting to test clinical samples.

A1. Reverse transcriptase polymerase chain reaction (RT-PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) is a powerful technique for the identification of influenza virus genomes. The viral genome (consisting of single-stranded RNA) must first be extracted. Then, during reverse transcription, DNA copies of original RNA (cDNA) are synthesized using a reverse transcriptase (RT). Using polymerase in the next step, cDNA is amplified to high number of copies (PCR product). Finally, the cDNA products of amplification are detected with different techniques and can be further analysed by molecular genetic methods such as sequencing.

In conventional RT-PCR, the products are detected at the end of the reaction. With real time RT-PCR, products are detected as amplification is ongoing, allowing quantification. Conventional RT-PCR analysis is still widely in use, but more and more laboratories are changing to real time RT-PCR as costs decrease.

In both methods, the procedure for amplification requires a pair of oligonucleotide primers. These primer pairs are designed on the basis of the known sequences of different influenza genes (genes for haemagglutinin (HA), and neuraminidase (NA) are most often used) of influenza subtypes of interest, and therefore will specifically detect RNA of only one subtype. Other primers are targeted at genes that are relatively conserved across all influenza A viruses (e.g. matrix gene). With continuing evolution of H5 viruses, the genetic diversification of the H5N1 virus strains in the past two years has made recommendation of specific primer sets challenging. A WHO technical working group (Annex C) has been established to prepare the example protocols for conventional and real time RT-PCR primers to detect circulating strains of avian influenza.

In addition to the overall laboratory requirements of biosafety, quality assurance, and diagnostic capacity described in the body of this document, laboratories using RT-PCR to detect and diagnose avian influenza infections must consider the specific issues listed below. These can have profound effects on the likelihood of obtaining false negative or false positive results.

[16] List of WHO H5 Reference Laboratories:
http://www.who.int/csr/disease/avian_influenza/guidelines/referencelabs/en/index.html

- **Coverage of all H5N1 circulating clades causing human infection in all regions.** Variation in the genetic sequence of viruses used to design RT-PCR primer sequences can dramatically affect the cross reactivity and therefore the sensitivity of the test. Therefore, it is important to follow the extent of genetic variation in viruses from the various regions. The extent and nature of the variation in the circulating H5N1 strains are periodically analyzed by the WHO H5 Reference Laboratories in order to regularly evaluate and update the primers. Consulting WHO H5 Reference Laboratories or a member of the WHO technical working group (Annex C) is recommended before using the protocols in this document.

- **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 samples can be handled (throughput), what gene sequence to target for RT-PCR, whether to use concurrent or sequential testing for RT-PCR of M and HA genes.

- **Good laboratory practices.** Standard protocols for all procedures should be in place and reviewed regularly. Making sure that the recommended reagents are used and handled properly is critical, as reactions are complex and problems with a single reagent can have large effects.

- **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.

- **Quality assurance.** Standard quality assurance protocols and good laboratory practices should be in place. Participation in the NIC evaluation exercises (external quality assurance 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 allowing correct interpretation of results are cornerstones for any successful diagnostic.

- **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 space separate from that used for virus isolation techniques.

- **Equipment**. Equipment should be used and maintained according to manufacturer recommendations.

- **Interpretation of results.** A sample is considered to be positive when results from tests using two different PCR targets (e.g. primers specific for influenza A and H5 haemagglutinin) are positive. The negative PCR result does not rule out the infection of a person with influenza virus. Results should be interpreted together with the available clinical and epidemiological information. Specimens from patients negative on PCR but with a high suspicion of A(H5N1) infection should be further investigated and also be tested by other methods such as virus culture or serology to rule out influenza A(H5N1) infection. Testing for seasonal influenza strains as well as other non-typical subtypes of

influenza (e.g. H7, H9) and other respiratory viruses is recommended when results of tests for influenza A (H5N1) are not clear.

A.1.1 Conventional RT-PCR analyses

Conventional RT-PCR PROTOCOL 1[17]

The protocols and primers for conventional PCR and gel electrophoresis of products to detect avian influenza A(H5N1) viruses in specimens from humans are given below. These protocols have been shown to be widely effective for the identification of Clade 1, 2 and 3 H5N1 viruses when used with the reagents and primers indicated. It is recommended that laboratories having concerns about identifying currently circulating clades contact one of the members of the WHO Expert Committee on AI PCR (Appendix C) or one of the WHO H5 Reference Laboratories for assistance in identifying the optimal primers for their use.

Materials required

- QIAamp® Viral RNA Mini Kit（QIAGEN® Cat#51104）
- OneStep RT-PCR Kit（QIAGEN®, Cat#210212）
- RNase Inhibitor 20U/μl（Applied Biosystems Cat# N808-0119）
- RNase-free water
- Ethanol (96–100%)
- Microcentrifuge (adjustable, up to 13 000 rpm)
- Adjustable pipettes（10, 20, 200, 100 μl）
- Sterile, RNase-free pipette tips with aerosol barrier
- Vortex
- Microcentrifuge tubes（0.2, 1.5ml）
- Thermocycler
- Primers sets
- Positive control (Can be obtained upon request from a WHO H5 Reference Laboratory[18])

[17] Protocol provided by Government Virology Unit, Hong Kong SAR China, NIC

[18] http://www.who.int/csr/disease/avian_influenza/guidelines/referencelabs/en/

Primers

Gene	**H5**
Clade	1, 2, 3
Primers sequences	H5-1: GCC ATT CCA CAA CAT ACA CCC H5-3: CTC CCC TGC TCA TTG CTA TG
Expected product size	219 bp
Reference	Modified from Yuen et al. 1998 (Annex B)

Gene	**M**
Clade	1, 2, 3
Primers sequences	M30F: TTC TAA CCG AGG TCG AAA CG M264R2: ACA AAG CGT CTA CGC TGC AG
Expected product size	232 bp
Reference	NIID[19]

Gene	**N1**
Clade	-
Primers sequences	N1-1: TTG CTT GGT CGG CAA GTG C N1-2: CCA GTC CAC CCA TTT GGA TCC
Expected product size	616bp
Reference	Wright et al. 1995 (Annex B)

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

For H5 or N1 genes

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

5x QIAGEN® RT-PCR buffer	10 µl
dNTP mix	2 µl
5x Q-solution	10 µl
Forward primer (5 µM)	6 µl
Reverse primer (5 µM)	6 µl
Enzyme mix	2 µl
RNase inhibitor (20U/µl)	0.5 µl
Water (Molecular grade)	9 µl
Total	**45 µl**

b. Add 5 µl viral RNA

[19] Primers designed by Laboratory at National Institute of Infectious Diseases (NIID), Tokyo, Japan, WHO Collaborating Centre for Reference and Research on Influenza and WHO H5 Reference Laboratory

For M gene

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

5x QIAGEN® RT-PCR buffer	10µl
dNTP mix	2 µl
Forward primer (5 µM)	6 µl
Reverse primer (5 µM)	6 µl
Enzyme mix	2µl
RNase inhibitor (20U/µl)	0.5µl
Water (Molecular grade)	19µl
Total	**45µl**

b**.** Add 5 µl viral RNA

3. PCR temperature cycling conditions

H5, N1

Reverse transcription	30 min	50 °C
Initial PCR activation	15 min	95 °C
3-step cycling		
Denaturation	30 sec	94 °C
Annealing	30 sec	55 °C
Extension	30 sec	72 °C
Number of cycles	40	
Final extension	2 min	72 °C

M

Reverse transcription	30 min	50 °C
Initial PCR activation	15 min	95 °C
3-step cycling		
Denaturation	30 sec	94 °C
Annealing	30 sec	50 °C
Extension	30 sec	72 °C
Number of cycles	40	
Final extension	2 min	72 °C

4. 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 procedure is given below.

Materials required

- Agarose gel casting tray and electrophoresis chamber
- Power supply and electrode leads
- UV-light box (302nm)
- Camera and Polaroid film or computer connected to the camera
- Adjustable pipettes
- 2% agarose gel in 1× TAE
- 1× TAE buffer
- Ethidium bromide (10 mg/ml)
- 6 x Gel loading buffer (GLB)
- Molecular weight marker

Procedure

Cast an agarose gel

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

Sample loading

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

5. Interpretation of results

The size of PCR products obtained should be compared to the expected product size (given in tables above). If the test is run without a positive control, products must be confirmed by sequencing and comparison with available sequences.

Conventional RT-PCR PROTOCOL 2[20]

Materials required

Same as in Conventional RT-PCR PROTOCOL 1 described above.

Primers

Gene M (NIID)**:**
Clade 1, 2, 3
Primer sequences Type A/M264R2: 5'-ACAAAGCGTCTACGCTGCAG
Type A/M30F: 5'-TTCTAACCGAGGTCGAAACG
Expected product size: 232bp

Gene H5 (NIID)**:**
Clade 1, 2, 3
Primer sequences H5-248-270F: 5'-GTGACGAATTCATCAATGTRCCG
H5-671-647R: 5'-CTCTGGTTTAGTGTTGATGTYCCAA
Expected product size: 424bp

Gene N1 (NIID)**:**
Clade 1, 2, 3
Primer sequences N1-580-607F: 5'- TGAAGTACAATGGCATAATAACWGACAC
N1-891-918R: 5'- CCACTGCATATATATCCTATTTGATACTCC
Expected product size: 343bp

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

   2) 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: It is important to mix the solutions completely before use to avoid localized differences in salt concentration.)**

Reaction without Q-Solution

RNase-free water	9.5 µl
5 x QIAGEN® OneStep RT-PCR Buffer	5 µl
dNTP mix (containing 10mM of each dNTP)	1 µl
sense (+) primer (10 µM)	1.5 µl
antisense (-) primer (10 µM)	1.5 µl

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

QIAGEN®OneStep RT-PCR Enzyme Mix (5 U/µl)	1 µl
RNase Inhibitor (40 U/µl)	0.5 µl
Total volume	**20** µl **/test**

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

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

5) Program the thermal cycler according to Thermal cycling conditions.

6) Start the RT-PCR program while PCR tubes are still on ice. **Wait until the thermal cycler has reached at 50 ˚C.** Then place the PCR tubes in the thermal cycler.

3. Thermal cycling conditions

Reverse transcription	50 ˚C	30 min.
Initial PCR activation	95 ˚C	15 min.
Denaturation	94 ˚C	30 sec.
Annealing	50 ˚C	30 sec.
Extension	72 ˚C	1 min.
Number of cycles	40	
Final extension	72 °C for 10 min	
Hold at	4 °C	

4. 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 procedure is given in the Conventional RT-PCR PROTOCOL 1 described above.

5. Interpretation of results

The size of PCR products obtained should be compared to the expected product size (given in tables above). If the test is run without a positive control, products must be confirmed by sequencing and comparison with available sequences.

A.1.2 Real time RT-PCR analyses

Real time RT PCR poses different challenges compared to conventional RT-PCR. In addition to the RT-PCR considerations above, specific considerations for real time RT-PCR include:

- Ensuring appropriate equipment, software, and fluorescent-based reagents are used and handled correctly.

- Ensuring appropriate training of personnel for interpretation of results (experience in recognizing true positives, interpreting controls/ct value and aberrant fluorescence, etc., is crucial).
- Validation in the laboratory and optimization of reactions is 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) which can re-introduce contamination.

Real-time RT-PCR PROTOCOL 1[21]

Extract viral RNA from clinical specimen as described in section *A.1.1 Conventional RT-PCR analyses* above.

Materials required

Reverse Transcription

10X PCR buffer I with 15 mM $MgCl_2$ (Applied Biosystems)
Random hexamer 50 μM (Applied Biosystems)
MuLV Reverse Transcriptase 50 U/μl (Applied Biosystems)
RNase Inhibitor 20 U/μl (Applied Biosystems)

Real time PCR

LightCycler ® – FastStart™ DNA Master HybProbes kit (Roche)
Primers and probes mix: Add equal volume of the following components to prepare primers and probes mix for H5 and M

Primers and probes

Gene	**H5**
Clade	1, 2, 3
Primers sequences	H5-266F: 5'- TGCCGGAATGGTCTTACATAGTG -3' (5 μM)
	H5-1615F: 5'- GTGGCGAGCTCCCTAGCA -3' (5 μM)
	H5-347R: 5'- TCTTCATAGTCATTGAAATCCCCTG -3 (5 μM)
	H5-1695R: 5'- TCTGCATTGTAACGACCCATTG -3' (5 μM)
	H5-290P: 5'-(FAM)-AGAAGGCCAATCCAGTCAATGACCTCTGTTA-(TAMRA)-3' (2.5 μM)
	H5-1634P: 5'-(FAM)-TGGCAATCATGGTAGCTGGTCTATCCTTATGG-(TAMRA)-3' (2.5 μM)

[21] Protocol provided by, Government Virology Unit, Hong Kong SAR, China, WHO H5 Reference Laboratory

Gene	**M**
Clade	1, 2, 3
Primers sequences	FLUAM-1F: 5'-AAGACCAATCCTGTCACCTCTGA-3' (10 μM)
	FLUA-2F: 5'-CATTGGGATCTTGCACTTGATATT-3 (10 μM)
	FLUAM-1R: 5'-CAA AGCGTCTACGCTGCAGTCC-3' (10 μM)
	FLUAM-2R: 5'-AAACCGTATTTAAGGCGACGATAA-3' (10 μM)
	FLUA-1P: 5'-(FAM)-TTTGTGTTCACGCTCACCGT-(TAMRA)-3' (5 μM)
	FLUA-2P: 5'-(FAM)-TGGATTCTTGATCGTCTTTTCTTCAAATGCA-(TAMRA)-3' (5 μM)

Procedure

Perform RT

Reagent	**Volume (μl) per reaction**
10X PCR buffer I with 15 mM $MgCl_2$	2
Extra 25mM $MgCl_2$	2.8
2.5mM dNTPs	8
Random hexamer 50μM	1
RNAase inhibitor 20U/μl	1
Reverse transcriptase 50 U/μl	1
Extracted RNA	4.2

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

Perform real time PCR

(1) Preparation of "Hot Start" reaction mix by gently pipetting 60 μl of LC-FastStart™ Reaction Mix Hybridization Probes (vial 1b) into the LC-FastStart™ Enzyme (vial 1a).
(2) For each test sample and positive and negative controls, prepare reagent mix with primers and probe mix according to the following:

Master Mix:

Reagent	**Volume (μl)**
PCR grade H_2O	7.6
$MgCl_2$ (25 mM)	2.4
Primers and probe mix (H5 or M)	3

"Hot Start" reaction mix	2
Total volume	**15**
Each reaction:	
Master Mix	15
cDNA	5

PCR temperature-cycling conditions

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

Real-time RT-PCR PROTOCOL 2[22]

Extract viral RNA from clinical specimen as described in chapter *1.1 Conventional RT-PCR analyses* above.

Materials required

- QIAGEN® QuantiTect®, Probe RT-PCR kit (#204443):
  - 2 x QuantiTect®, Probe RT-PCR Master Mix
  - QuantiTect®, RT Mix
  - RNase free Water
- Primers
- Probe
- Equipment: Chromo-4 ™ Real-time PCR Detection system (BioRad)

Real-time PCR

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

Primers and probes:

Gene	**Type A (M)**
Clade	1, 2, 3
Primers sequences	MP-39-67For CCMAGGTCGAAACGTAYGTTCTCTCTATC (10 μM)
	MP-183-153Rev TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA (10 μM)
Probe sequences	MP-96-75ProbeAs FAM-ATYTCGGCTTTGAGGGGGCCTG-MGB (5pmol/μl)

Gene	**H5**
Clade	1, 2, 3
Primers sequences	H5HA-205-227v2-For CGATCTAGAYGGGGTGAARCCTC (10 μM)
	H5HA-326-302v2-Rev CCTTCTCCACTATGTANGACCATTC (10 μM)
Probe sequences	H5-Probe-239-RVa FAM-AGCCAYCCAGCTACRCTACA-MGB (5pmol/μl)
	H5-Probe-239-RVb FAM-AGCCATCCCGCAACACTACA-MGB (5pmol/μl)

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

Gene	**N1**
Clade	1, 2, 3
Primers sequences	N1-For-474-502-v2 TAYAACTCAAGGTTTGAGTCTGTYGCTTG (10 μM)
	N1-Rev-603-631-v2 ATGTTRTTCCTCCAACTCTTGATRGTGTC (10 μM)
Probe sequences	N1-Probe-501-525-v3 FAM-TCAGCRAGTGCYTGCCATGATGGCA-MGB (5pmol/μl)

Reaction Mixture

2x QuantiTect®Probe RT-PCR Master Mix	12.5 μl
Forward Primer (10μM)	1.5 μl
Reverse Primer (10μM)	1.5 μl
*Probe (5pmol/μl)	0.5 μl
QuantiTect®RT Mix	0.25 μl
RNase free Water	3.75 μl
Total	**20 μl**

**For the reaction of H5 detection, a mixture of two probes is used.*

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

Procedure

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

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

3) Program the thermal cycler according to the program outlined in below.

4) Start the Real-time RT-PCR program while RT-PCR reaction plates are still ***on ice***. ***Wait until the thermal cycler has reached at 50 ˚C.*** Then place RT-PCR reaction plates in the thermal cycler.

RT-PCR temperature-cycling conditions

Reverse transcription	50˚C 30min.	
	↓	
Denaturation	95˚C 15min.	
	↓	
PCR	94˚C 15sec. ⎫	
	56˚C 1min. ⎭	45 cycles

Real-time RT-PCR PROTOCOL 3[23]

Extract viral RNA from clinical specimens manually with the High Pure total RNA isolation kit (Roche Diagnostics, Almere, The Netherlands) or automatically with a MagnaPure LC system (Roche) and the MagnaPure LC Total nucleic acid isolation kit according to manufacturer's instructions.

Materials required

Reverse Transcription and Real time PCR

TaqMan® EZ-RT/PCR core reagents (Applied Biosystems, cat. nr. N808-0236)
Primers and probes (see list below)

Primers and probes

Gene	H5
Clade	Classical (Eurasian LPAI), 0, 1, 2
Primers sequences	RF 1151 5'-GGA-ACT-TAC-CAA-ATA-CTG-TCA-ATT-TAT-TCA-3'
	RF 1152 5'-CCA-TAA-AGA-TAG-ACC-AGC-TAC-CAT-GA-3'
	RF 1153 5'6-FAM-TTG-CCA-GTG-CTA-GGG-AAC-TCG-CCA-C-TAMRA-3'

Gene	M (modified from Ward et al., J. Clin. Virol. 29:179-188, 2004)
Clade	All influenza A viruses
Primers sequences	RF 1073 5'-AAG-ACC-AAT-CCT-GTC-ACC-TCT-GA -3'
	RF 1074 5'-CAA-AGC-GTC-TAC-GCT-GCA-GTC-C-3'
	RF 1293 5'6-FAM-TTT-GTG-TTC-ACG-CTC-ACC-GTG-CC-TAMRA-3'

Procedure

Perform one step RT-PCR. Use the Applied Biosystems TaqMan® EZ-RT/PCR core reagents kit and the following primers/probes:

Primer/Probe	Stock solution	Work solution
RF 1073	200 pmol/µl	30 pmol/µl
RF 1074	200 pmol/µl	40 pmol/µl
RF 1293	200 pmol/µl	20 pmol/µl
RF 1151	200 pmol/µl	40 pmol/µl
RF 1152	200 pmol/µl	40 pmol/µl
RF 1153	200 pmol/µl	10 pmol/µl

[23] Protocol provided by , Erasmus Medical Centre, National Influenza Centre,Rotterdam,The Netherlands

One step RT-PCR:

Reagent	Volume (µl)
Primer 1 (work solution)	1
Primer 2 (work solution)	1
Probe (work solution)	1
5 x EZ buffer	10
25 mM $Mn(OAc)_2$	6
dNTP (2.5 mM dATP, dCTP, dGTP, 5 mM dUTP)	6
rTth polymerase	2
Amperase	0.5
RNA	20 *
Bidest water	2.5 *

* This amount of input RNA is based on MagnaPure-purified RNA. For RNA isolated manually with the High Pure total RNA isolation kit (Roche), we use 5 µl RNA and 17.5 µl water.

Cover the plate with a special TaqMan® cover and remove side slips of the plate cover.

Spin the plate at 1000 rpm for 5 sec.

RT-PCR temperature-cycling conditions

Temperature (°C)	Time (minute:second)	No of cycles
50	2:00	1 x
60	30:00	
95	5:00	
94	0:20	50 x
60	1:00	

2. Virus culture with IFA, HA and HI identification

Virus isolation by culture is a sensitive technique with the advantage that virus is then available both for identification and for further antigenic and genetic characterization, drug susceptibility testing, and vaccine preparation. Preferred media for influenza virus growth include MDCK cells or embryonated chicken eggs. Unlike other influenza A strains, influenza A(H5N1) will also grow in other common cell lines such as Hep-2 and RD cells. Growth of human influenza A(H5N1) virus does not requires the addition of exogenous trypsin.

Identification and typing of a cultured influenza virus can then be carried out by PCR, by immunofluorescence (IFA) using specific monoclonal antibodies, or by haemagglutination (HA) and antigenic analysis (subtyping) by haemagglutination-inhibition (HI) using selected reference antisera.

Virus culture can only be recommended where BSL-3 facilities are available, and biosafety precautions must be taken when handling cell cultures potentially containing highly pathogenic avian influenza.

Materials required

- Madin-Darby Canine Kidney cells (MDCK), ATCC CCL34
- WHO Influenza Reagent Kit for the Identification of Influenza A(H5) Virus. Reagents for identification of A(H5N1) in cell culture include:
  - influenza A(H5N1) control antigen: inactivated virus
  - goat serum to A/Tern/South Africa/61/ H5
  - chicken pooled serum to A/Goose/Hong Kong/437-4/99

- WHO Influenza Reagent Kit (Annually distributed)
  - A(H1N1) and A(H3N2) reference antigens and reference antisera
- Receptor-destroying enzyme (RDE).
- Red blood cells (chicken, turkey, human type O, or guinea-pig red blood cells) in Alsever's solution.

Procedure

Standard laboratory cell-culture procedures should be followed for the propagation of cell cultures, the inoculation of specimens, and the harvesting of infected cells for IFA or culture supernatant for HA and HI testing (Lennette & Schmidt, 1995; WHO, 2002[24]), RT-PCR, or sequence analysis.

Standard HA and HI procedures should be followed, with the inclusion of all recommended controls. Specific details of these procedures are included in the *WHO manual on animal influenza diagnosis and surveillance*[25].

Interpretation of results

The highest dilution of virus that causes complete haemagglutination is the HA titration end-point. The last dilution of antiserum that completely inhibits haemagglutination is the HI end-point. The titre is expressed as the reciprocal of the last dilution.

Identification of the field isolate is carried out by comparing the results of the unknown isolate with those of the antigen control. An isolate is identified as a specific influenza A subtype if the subtype-specific HI titre is four-fold or greater than the titre obtained with the other antiserum.

Nonspecific agglutinins may be present in sera and may result in false-negative reactions; alternatively, some isolates may be highly sensitive to non-specific inhibitors in sera, resulting in false-positive reactions.

[24] http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf

[25] http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf

3. Immunofluorescence assay

Immunofluorescence assay (IFA) can be used for identification of influenza viruses in cell cultures. Performing IFA on inoculated cell cultures is preferable to testing clinical samples as it allows for the amplification of any virus present.

Materials required

- WHO Influenza Reagent Kit for the Identification of Influenza A(H5N1) Virus (1997–98, 2003 or 2004 version). The reagents in this kit for the immunofluorescence assay include:
  - influenza type A(H5)-specific monoclonal antibody pool
  - influenza A type-specific and influenza B type-specific monoclonal antibody pools
  - influenza A(H1) and an A(H3) subtype specific monoclonal antibodies
- Anti-mouse IgG FITC conjugate
- Microscope slides
- Cover slips, 24 x 60 mm
- Mountant
- Acetone
- Immunofluorescence microscope.

Procedure

This test should be performed in accordance with the instructions included in the WHO Influenza Reagent Kit. Epithelial cells are washed free of contaminating mucus by centrifugation, fixed, and stained with specific monoclonal antibodies. Infected respiratory epithelial cells in a clinical specimen are very labile and easily damaged; they should therefore be kept cold on ice during processing and not centrifuged at more than 500*g*. Control slides with influenza A(H3) and A(H1)-infected cells (and, when available, H5-infected cells) and uninfected cells should be included to allow appropriate control of monoclonal antibodies and conjugate and to assist with interpretation of specific staining.

Interpretation of results

Specific staining should be an intense intracellular apple-green fluorescence. Nuclear and/or cytoplasmic fluorescence may be observed. It is important to ensure that cell density is adequate. One or more intact cells showing specific intracellular fluorescence can be accepted as a positive result.

Because commercially available monoclonal antibodies for the subtyping of influenza A(H1) have been shown to cross-react with influenza A(H5) subtypes, including 2004 strains, confirmatory testing should be carried out using the monoclonal antibody provided in the WHO kit.

Annex B

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Annex C

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