THAI AGRICULTURAL STANDARD

TAS 10300-2008

DIAGNOSIS OF AVIAN INFLUENZA

National Bureau of Agricultural Commodity and Food Standards
Ministry of Agriculture and Cooperatives
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Thai Agricultural Standard (TAS) on Diagnosis of Avian Influenza prescribes diagnostic methods for approval of avian influenza free and uses for an avian influenza diagnostic manual in laboratory for proper and precise procedures. It includes sampling methods and laboratory diagnostic techniques i.e. virology, immunology and bio-molecular. It promotes poultry production system and poultry products to yield good quality products and safety for consumption both domestically and internationally. It is also essential for notifying country status of avian influenza to Thailand’s trading partners as well as the World Organization for Animal Health (OIE).

The establishment of this standard is based on the information of the following documents:

The Study project on the status of important contagious poultry diseases in Thailand conducted by National Bureau of Agricultural Commodity and Food Standards B.E. 2548 (2004) in cooperation with Faculty of Veterinary Medicine, Chulalongkorn University.


*Remark:*

The standard title has been revised from “Thai Agricultural Commodity and Food Standard (TACFS)” to “Thai Agricultural Standard (TAS)” in accordance with the enforcement of the Agricultural Standards Act B.E. 2551 (2008)
NOTIFICATION OF THE NATIONAL COMMITTEE ON AGRICULTURAL COMMODITY AND FOOD STANDARDS
SUBJECT: THAI AGRICULTURAL COMMODITY AND FOOD STANDARD: DIAGNOSIS OF AVIAN INFLUENZA
B.E. 2551 (2008)

The resolution of the 1/2551 session of the National Committee on Agricultural Commodity and Food Standards dated 11 August B.E. 2551 (2008) endorsed the Thai Agricultural Commodity and Food Standard entitled Diagnosis of Avian Influenza. This standard would be of benefits for quality improvement, facilitating trade and protecting consumers.

By virtue of the Cabinet Resolution on Appointment and Authorization of the National Committee on Agricultural Commodity and Food Standards dated 5 August B.E. 2551 (2008) the Notification on Thai Agricultural Commodity and Food Standard entitled Diagnosis of Avian Influenza is hereby issued as voluntary standard, the details of which are attached herewith.

Notified on 14 August B.E. 2551 (2008)

Mr. Somsak Prissana-nanthakul
Minister of Agriculture and Cooperatives
Chairperson of the National Committee on Agricultural Commodity and Food Standards
THAI AGRICULTURAL STANDARD
DIAGNOSIS OF AVIAN INFLUENZA

1 SCOPE

This Standard applies to defines criteria for diagnosis of avian influenza which is caused by influenza virus type A, subtype H5 and H7.

2 DEFINITIONS

For the purpose of this standard:

2.1 Avian Influenza means a severe contagious disease of poultry caused by influenza virus type A, subtype H5 and H7.
2.2 Avian means poultry including birds, chicken, ducks and geese.
2.3 Diagnosis means detection of sick or dead or suspected poultry to be infected with avian influenza virus by using laboratory methodologies together with history inquiries from related persons and clinical signs observation.
2.4 Laboratory Biosafety means a laboratory established with an appropriate system of construction, operation and examination as well as a safety operational procedures. Factors i.e. equipments, knowledge and technology shall be in place to prevent personnel, laboratory and environments from biohazards such as disease organisms, blood tissues, genetic materials and toxin which will enable to safe work with these hazardous substances.

3 DIAGNOSIS

Avian Influenza diagnostic methods in laboratory include virology, immunology and biomolecular which are indicated in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organization for Animal Health (OIE).

Avian Influenza is a severe contagious disease which is a notifiable disease in the Animal Epidemic Act (Annex A) and there is a high risk of virus spreading from the laboratory. In order to reduce such risk, the laboratory shall have a biosafety system covering building, equipments, and operational procedures, as well as training personnel for specific skills related to good laboratory practices, risk group of microorganisms, handling of samples or infected materials, and control of germs spreading. Laboratory biosafety system shall be regulary audited.

3.1 SPECIMEN COLLECTION AND SENDING SAMPLES

Samples collecting shall be appropriate using proper methods and sending to laboratory as soon as possible in order to have a precise and effective disease diagnosis in laboratory. The samples used for avian influenza diagnosis can be collected from sick animals or suspected animals to be infected with avian influenza or freshly dead carcasses before the post mortem autolysis occur.

3.1.1 Specimen collection from live poultry
(1) Secretion from respiratory system by using sterile stick with polyester on its tip to swab mucous secretion from choanal slit, trachea and then dip the swab mucous secretion
from choanal slit into viral transport media such as phosphate buffer saline (PBS) or other kinds of viral transport media which are added with antibiotics as prescribed in Annex B.

The collected cotton swab samples may be kept in the same viral transport media but not over than five sticks per tube.

(2) Excretion from cloaca by using sterile cotton swab on the inner wall of cloaca or the collection of fresh faeces at least 1 g. The collected cotton swab samples may be kept in the same viral transport media but not over than five sticks per tube.

3.1.2 Samples taken from dead birds shall be whole bird carcass or collected organs such as trachea, lungs, spleen, liver, pancreas, intestine, kidney, heart and brain, etc. and put into suitable container separately.

3.1.3 Samples shall be sent to the laboratory immediately or within 24 hours by keeping the samples in a tightly closed container to prevent spreading the disease with the controlled temperature at 4 °C (2 °C to 8 °C). The samples shall not be frozen.

3.2 Diagnosis of Avian influenza

Diagnosis of avian influenza subtypes H5 and H7 shall follow the standard methods which comprises two steps as the followings (details in Annex C):

(1) Virus isolation by inoculating the sample into hatching chicken eggs and by haemagglutination test (HA test) for detecting a property of red blood cells precipitation. This step shows the virus is virus influenza type A or other pathogens that have same property. If the HA test is positive then proceed to next step in (2).

(2) Virus identification: This step covers both testing for avian virus influenza type A and subtypes by AGID (Agar Gel Immunosorbent Assay) which is a precipitation in agar or by ELISA (Enzyme – Linked Immunosorbent Assay) or by other methods recommended by the OIE for confirmation as influenza virus type A. If the test is positive, then confirm for subtypes by using serum specific for H5 and H7 for haemagglutination inhibition test (HI test) to detect the inhibition of red blood cells precipitation.

Bio-molecular method may be used as prescribed in Annex G, for detecting genetic substances such as a reverse transcriptase polymerase chain reaction (RT – PCR) or a real time reverse transcriptase polymerase chain reaction (real – time RT – PCR).

3.2.1 Virus isolation and replication by the inoculation of hatching chicken eggs as stated in 3.1 and follow by using HA test method.

The following steps shall be operated in a biosafety laboratory.

(1) Sample of internal organs: The organs shall be placed in isotonic phosphate buffer saline (PBS) and finely minced to prepare as 10% to 20% (W/V) concentration. If samples are the collected cotton swabs kept in viral transport media as prescribed in Annex B, they shall be shaked vigorously by vortex mixer and separate the cotton swabs out.

(2) Bring the samples to centrifuge at 1 000 g for 10 minutes

(3) Bring the supernatant to add with penicillin 2 000 units/ml, streptomycin 2 mg/ml, gentamycin 50 μg/ml and mycostatin 1 000 units/ml and incubate for one hour at room temperature in order to get rid of contaminated bacteria. Other methods such as membrane filter size 0.22 μm may be applied for filtering bacteria out of the supernatant.
(4) Bring the samples from (3) at volume at least 200 μl and inoculate one sample into the allantoic sac of at least five embryonated specific pathogen free (SPF) fowl eggs, or specific antibody negative (SAN) eggs of 9 to 11 days incubation.

(5) The inoculated eggs are incubated at 35 °C to 37 °C for 4 to 7 days

(6) Daily check for a viable embryo for 4 to 7 days

(7) Eggs containing dead or dying embryos, and all eggs remaining at the end of the incubation period are chilled at 4 °C for one hour.

(8) The allantoic fluids are tested for haemagglutination activity by HA method as prescribed in Annex D.2.

(9) Fluids that give a negative reaction shall be repeated for second passag into at least one further batch of eggs and follow by steps (4) to (8).

3.2.2 Confirmation of influenza virus

A positive reaction of the HA activity by red blood cells precipitation (Figure E.1 and Figure E.2) indicates an availability of either influenza type A virus or Newcastle disease virus (NDV) or other organisms. The presence of influenza A virus shall be confirmed by AGID with known influenza type A antisera or by bio-molecular method to determine a type A genetic substance as prescribed in Annex G.

The other method is ELISA using a monoclonal antibody specific to influenza virus which shall be used for rapid diagnosis for avian influenza.

Next step is a subtypes H5 and H7 testing by using HI method which shall inhibit haemogglutination activity of red blood cells with antisera specific to subtypes H5 and H7 or with monoclonal antibody specific to subtypes H5 and H7 as prescribed in Annex D.3 or may use bio-molecular method to determine genetic substances of subtypes H5 and H7 as prescribed in Annex G.

4 Detection of antibody level by serological tests

Serological tests for antibody detections shall be beneficially used for determining an infection of influenza virus subtype H5 and H7 in case of no vaccination against avian influenza in poultry.

A standard serological test is HI method. Interpreting of results, if HI titre equal to or greater than log₂ 4 or 2⁴ or 1 : 16 when using antigen 4 HAU (haemagglutinating unit, HAU) means that there is a presence of antibodies against such a influenza virus subtype.
ANNEX A
EPIDEMIOLOGY, PATHOGENESIS AND CLINICAL SIGNS OF AVIAN INFLUENZA
(Section 3)

A.1 EPIDEMIOLOGY

Avian influenza or influenza of poultry is a severe contagious disease of poultry that can be transmitted to human. In some case, it causes high mortality rate in patients. It is caused by influenza virus type A which is a RNA virus of the family Orthomyxoviridae, genus influenza virus A. Influenza A viruses have different antigens on the envelope which are classified into subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens. Avian influenza is a disease in the listed diseases of the World Organization for Animal Health (OIE) as a notifiable avian influenza (NAI) that causes by influenza viruses subtypes H5 and H7 only. The disease has a great impact to poultry production and international trade, especially the subtype H5N1 which was reported and caused human death for the first time in Hong Kong in 1997, before spreading world wide and caused severe outbreaks in many countries in Asia. Thailand placed avian influenza in listed diseases under the Animal Epidemic Act (revised in 2004).

A.2 VIRAL CHARACTERISTICS

Influenza virus is a single strand RNA virus with envelope. Its outer layer is a lipid membrane from host cell comprising three glycoproteins. There are two important glycoproteins namely haemagglutinin and neuraminidase with the distinct spikes. The other one in less quantity is matrix protein 2 (M2) with its inner layer is a matrix protein 1 (M1) that keeps the virus in shape by wrapping 8 segments of ribonucleoprotein (RNP) inside and codified for 10 kinds of viral protein synthesis according to Table 1.

<table>
<thead>
<tr>
<th>Segments</th>
<th>Nucleotides</th>
<th>Polypeptides</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,341</td>
<td>polymerase PB2</td>
<td>Subunit of polymerase: Host cap binding and endonuclease</td>
</tr>
<tr>
<td>2</td>
<td>2,341</td>
<td>polymerase PB1</td>
<td>Catalytic subunit of polymerase</td>
</tr>
<tr>
<td>3</td>
<td>2,233</td>
<td>polymerase PA</td>
<td>Subunit of polymerase, active in vRNA synthesis</td>
</tr>
<tr>
<td>4</td>
<td>1,778</td>
<td>haemagglutinin HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>5</td>
<td>1,565</td>
<td>nucleoprotein NP</td>
<td>Nucleoprotein: Part of transcriptase complex</td>
</tr>
<tr>
<td>6</td>
<td>1,413</td>
<td>neuraminidase NA</td>
<td>Neuraminidase: release of virus</td>
</tr>
<tr>
<td>7</td>
<td>1,027</td>
<td>matrix protein M1</td>
<td>Matrix protein: Major component of virion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>matrix protein M2</td>
<td>Integral membrane protein: Ion channel</td>
</tr>
<tr>
<td>8</td>
<td>890</td>
<td>protein NS1 &amp; non structural</td>
<td>Anti-interferon protein. Effects on cellular RNA transport</td>
</tr>
<tr>
<td></td>
<td></td>
<td>protein NS2</td>
<td>RNP nuclear export</td>
</tr>
</tbody>
</table>
A.3 PATHOGENESIS

After poultry being infected with influenza virus, the virus will replicate in respiratory system, especially in the nasal cavity or in digestive tract before shedding into environment. Highly pathogenic influenza virus will penetrate into submucosa and replicate in endothelial cells of sinusoids. After the viruses escaped from the cells, they will spread through cardiovascular and lymphatic systems to various organs such as brain, skin. Occurrence of clinical signs and death of infected animals are results of internal organs failure. Influenza viruses can survive in tissues, secretions and faeces of infected animals as well as in water and environment. The viruses can be inactivated by heat at 56 °C for three hours or 60 °C for 30 minutes or in acidity by certain chemicals such as oxidizing agents, sodium dodecyl sulphate, lipid solvents, β- propiolactone, formalin and iodine compounds.

A.4 CLINICAL SIGNS

Avian influenza can be divided, on the basis of its pathogenesis (Annex F), into 2 kinds as highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) which HPAI is a vital disease of poultry, especially chicken. Besides, the clinical signs depend upon avian species, pathogenesis and other factors such as age, secondary infection, immunity level of infected animals, portal of entry, dosage, and period of infection etc, also including the external factors such as environment, stress, temperature. Generally, clinical signs found are as the followings:

1. Chicken and turkey have incubation period about 3 to 5 days. Chicken show signs of dullness, inappetite, rapid drop in egg production, swelling of face, comb and wattles, dehydrate, discharge from nose and mouth, red eyes or congestion. Nervous system signs, may be found. Mortality may be high up to 100%. However, clinical signs may not be found if the animal is suddenly death.

2. Mostly, birds do not show clinical signs or may be suddenly death.

3. Ducks and geese may not show clinical signs.

A.5 PATHOLOGY

Lesions found in infected animals are patchy haemorrhage on the shanks, ocular and nasal discharge, severe congestion of conjunctiva, subcutaneous edema in the head and neck areas, muscular congestion, haemorrhage in trachea with abnormal heavily mucous, petichial haemorrhage in inner sternum, heart, peritonium, abdominal fat, ovaries, pancreas, proventricular and gizzard mucosae, lymphoid tissue of intestinal tract, severe congestion in kidney. Lesions in respiratory tract may be variably found from non to very severe. In case of sudden death, the lesions may not be found. Microbiological features found in infected poultry are infarction of tissue and inflammation of inner organs which are generally found in brain, heart, lungs, pancrease, primary and secondary lymphoid organs.

These clinical signs and pathogenesis are not only typical for this disease. It may be differently diagnosed from other respiratory diseases such as Newcastle disease, infectious laryngotracheitis, infectious bronchitis and diseases caused by certain bacteria such as acute fowl cholera. Therefore, it is necessary to use laboratory diagnosis, especially the methods of virus isolation and identification.
A.6 TRANSMISSION

Poultry are infected by direct contact to secretions from infected poultry, especially faeces and secretion from respiratory system or transmit via feed, water, equipments, farm utensils, vehicles, contaminated shoes and apron, reservoirs. Avians that has high potential on transmission are duck, water fowl, and wild birds which may be infected without showing clinical signs but can replicate viruses and spread to other poultry.
ANNEX B
VIRAL TRANSPORT MEDIA FOR INFLUENZA VIRUS
(Section 3.1 and 3.2)

B.1 Viral transport media (VTM) are phosphate buffer saline (PBS), brain heart infusion broth, tryptose nutrient broth, peptose broth, minimal essential media (MEM) and M199, etc. In cases samples are from secretion of respiratory system, antibiotics such as penicillin 2 000 units/ml, streptomycin 2 mg/ml, gentamycin 50 μg/ml, mycostatin 1 000 units/ml are added. If the samples are cloacal swabs or fresh faeces, 5 folds of higher concentrations of antibiotics are added.

B.2 Chemicals used for PBS preparation for using as stock solution (10X)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>80 g</td>
</tr>
<tr>
<td>KCl</td>
<td>2 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>11.5 g</td>
</tr>
<tr>
<td>NH₂PO₄</td>
<td>2 g</td>
</tr>
<tr>
<td>Distilled water (D.W)</td>
<td>1,000 ml</td>
</tr>
</tbody>
</table>

(1) Mix chemicals with distilled water to dissolve and adjust acid–base equal to pH 7.0 to 7.4 to be stock solution (10X)
(2) Autoclave at 121 °C for 20 minutes
(3) Bring 100 ml stock solution to be diluted by mixing with distilled water until volume up to 1,000 ml as working solution then autoclave.
ANNEX C
STEPS OF AVIAN INFLUENZA DIAGNOSIS
(Section 3.2)

Step 1
Virus isolation

Step 2
Virus Identification

Virus isolation by inoculation of hatching chicken eggs

allantoic fluid

HA Test

HA positive

Testing for virus influenza type A (AGID or ELISA or BIO-MOLECULAR)

If negative means the virus is other haemagglutinating agents

If positive means the virus is influenza type A

HI test for detection of virus subtypes H5/H7

If positive means it is subtypes H5/H7

If negative means it is influenza Type A with other subtypes

HA negative, repeat another round (2nd passage)1/

------------------------------------------------------------------------------------------------------------------------------

1/ After repeating 2nd passage but still negative it means no influenza virus type A

2/ Detection of virus subtype H5/H7 may use bio-molecular methods i.e. reverse transcriptase polymerase chain reaction or RT-PCR and real time reverse transcriptase polymerase chain reaction or real-time RT-PCR which use primer / probe specific for subtypes H5/H7
ANNEX D
IMMUNOLOGICAL TEST FOR AVIAN INFLUENZA
(Section 3.2.1, 3.2.2 and 4)

D.1 PREPARATION OF CHICKEN RED BLOOD CELLS
(1) Collect blood from at least three mature chicken then add Alsever’s solution at proportion of 1 : 1.
(2) Wash red blood cells 3 times by using PBS and centrifugate at 800 g for five minutes.
(3) Adjust red blood cells concentration to 1% suspension in PBS.

D.2 TESTING FOR HAEMAGGLUTINATION ACTIVITY OF RED BLOOD CELLS BY HA TEST
(1) Dispense 25 μl of PBS into each well of a V-bottomed microtitre plate (96 wells).
(2) Place 25 μl of antigen, i.e. H5, in the first well of each row.
(3) Make a serial two fold dilution (1:2) respectively.
(4) Dispense a further 25 μl of PBS to each well.
(5) Dispense 25 μl of 1% (v/v) chicken red blood cells to each well.
(6) Allow the red blood cells to settle for about 40 minutes at room temperature or at 4 °C for 60 minutes or which time control red blood cells shall be settled to a distinct button.
(7) Read the results immediately. Wells that give HA positive (Figure F.2) shall be a complete HA of red blood cells at the bottom of the wells. The well with most diluted antigen that give positive represents 1 HA unit (HAU)
(8) Prepare 4 HAU antigen as the following:

Example : If the well with 1 HAU has antigen dilution equal to 1: 1 024
Antigen dilution can be calculated equal to 1 024/4 = 256
Thus the antigen shall be diluted in PBS at 1:256

Code of practice
Every time back titration shall be done for confirmation of antigen concentration by
(1) Dispense 25 μl of PBS to wells from one to four wells of each row.
(2) Dispense 25 μl of the prepared antigen to well number 1 of each row then dilute antigen respectively.
(3) Dispense 25 μl of PBS to every well.
(4) Dispense 25 μl of 1% red blood cells to every well. Shake the plate to evenly spread red blood cells and settle for about 40 minutes at room temperature.
(5) Read for result confirmation of 4 HAU which wells number 1 and 2 are positive and number three and four are negative.

D.3 HAEMAGGLUTINATION INHIBITION TEST (HI TEST)
(1) Dispense 25 μl of PBS into each well of a V-bottom microtitre plate
(2) Place 25 μl of each serum sample in the first well of the plate
(3) Make two fold dilution (1:2) of the serum across the plate
(4) Add 4 HAU of antigen in 25 μl to each well and shake.
(5) Leave for about 30 minutes at room temperature or 60 minutes at 4 °C
(6) Add 25 μl of 1% (v/v) to each well and gently mix.
(7) Allow the red blood cells to settle for about 40 minutes or when the red blood cells in the control wells (no antigen) settled to a distinct button or 60 minutes at 4 °C.

(8) To read the results, the well with HI positive means there is haemagglutination inhibition of red blood cells which precipitation of the red blood cells can be seen at the bottom of the wells or settled to a distinct button (Figure E.4) or when tilting the plate, the red blood cells stream at the same rate as the control wells (Figure E.5).

(9) To interpret the results, if HI titres equal to or more than \( \log_2 4 \) or \( 2^4 \) or 1:16 when using 4 HAU of antigen means there is antibody against such subtypes of influenza virus.

(10) The validity of results shall be assessed against both a negative control serum and a positive control serum every time.

Positive control serum means serum with known titre after tested the titre shall be the same or within one dilution

Negative control serum means serum with titre not more than \( \log_2 2 \) or \( 2^2 \) or 1:4

Remarks:

Samples of chicken sera rarely give agglutination of red blood cells but sera from species other than chicken i.e. native chicken, ducks, geese, may often cause agglutination of red blood cells as a non–specific haemagglutination. It is necessary to remove this property by

(1) Incubate serum for 30 minutes at 56 °C, or

(2) Mix same volume of sera and pack red blood cell (pack RBC) at room temperature for 30 minutes then centrifuge at 800 g for two to five minutes and the adsorbed sera are decanted before using the HI test.
ANNEX E
FIGURES OF AVIAN INFLUENZA DIAGNOSIS
(Section 3.2.2)

Figure E.1 Results of virus detection by HA test
Figure E.2 Positive results of HA test
A visible of red blood cells agglutination netting over the area

Figure E.3 Negative results of HA test
A visible of red blood cells sediment at the well bottom or settle to a distinct button
Figure E.4  Results of haemagglutination inhibition of red blood cells (RBCs) by HI test

When the plate is tilted, the RBCs of the positive wells stream at the same rate as the control wells.

Remark: Figure E.1 to E.5 are under the courtesy of Dr. Uthumporn Sristitnarakul
ANNEX F
INFLUENZA VIRUS PATHOGENICITY TEST
FOR AVIAN INFLUENZA
(Section A.4)

The objective of influenza virus pathogenicity test is to assess whether virus is virulent or non – virulent. The criteria for determining the virulent influenza virus are as the followings:

F.1 To determine pathogenicity of influenza virus by intravenous pathogenicity test (IVPT), one of the following two methods can be used:

(1) In case 4 to 8 weeks susceptible chickens are intravenous inoculated with 0.2 ml of a 1/10 dilution of an infected allantoic fluid, it shall be found at least six out of eight chickens died within 10 days.

or

(2) In case virus has an intravenous pathogenicity index (IVPI) more than 1.2 thereby intravenous inoculation with infected allantoic fluid with a HA titre more than 1 : 16 or log$_2$ 4 or $2^4$ which was diluted as 1 : 10 in sterile isotonic saline, then the 0.1 ml of the diluted virus is injected intravenously into each of ten 6 weeks SPF or SAN chicken. Birds are examined daily at the same time of injection for 10 days. Record shall be kept daily and the score shall be given as the followings:

- **Score 0** means normal birds
- **Score 1** means sick birds with only one of the clinical signs
- **Score 2** means severely sick birds with more than one of the following clinical signs: respiratory system, nervous system, depress, diarrhoea, cyanosis of the skin or wattles, oedema of the face and/or head.
- **Score 3** means dead birds, including the remaining severely sick birds unable to drink or eat which shall be humanely slaughtered.

Interpret the results: The intravenous pathogenicity index is the mean score per bird per observation over the 10 day period.

An index of 3.00 means that all birds died within 24 hours, and an index of 0.00 means that no bird showed any clinical sign during the 10 day observation period.

However, for all H5 and H7 viruses of low pathogenicity in chickens, the amino acid sequencing of the connecting peptides of the haemagglutinin shall be determined. If the genetic sequence is similar to that observed for other highly pathogenic AI isolates, the isolate being tested shall be deemed to be highly pathogenic.

F.2 Influenza virus classification by the OIE are 3 categories as the followings:

(1) All influenza viruses that meet the criteria in Annex F.1 are identified as highly pathogenic notifiable avian influenza (HPNAI) which shall be officially reported to the OIE.
(2) Influenza viruses subtypes H5 and H7 that are not virulent for chickens and do not have an haemagglutinin cleavage site amino acid similar to any of those that have been observed in HPNAI viruses are identified as low pathogenicity notifiable avian influenza (LPNAI) which shall be officially reported to the OIE.
(3) Influenza viruses other than those H5 and H7 subtypes that are not virulent for chickens are identified as low pathogenicity avian influenza (LPAI).
ANNEX G
SAMPLES FOR AVIAN INFLUENZA DIAGNOSIS BY BIO-MOLECULAR METHOD
(Section 3.2.3)

Diagnosis of the disease by this method is divided into 2 steps as follows:
(1) RNA extraction by general standard methods or RNA extracting test kit that its sensitivity and specificity is proved to be close or equivalent to the standard method.
(2) Confirmatory laboratory diagnosis of influenza virus subtype H5N1 by reverse transcription polymerase chain reaction (RT-PCR) and real-time reverse transcription polymerase chain reaction (real-time RT - PCR)

G.1 AVIAN INFLUENZA VIRUSES DIAGNOSIS BY RT – PCR Method

(1) Prepare master mix property as manufacturer instruction

Remark: This formula is for 1 sample which shall be done in the ice throughout the process.

Table F.1 Example of primer used for H5N1 avian influenza diagnosis (B.E. 2550-2551: 2007 – 2008)

<table>
<thead>
<tr>
<th>Direction</th>
<th>Sequence</th>
<th>PCR-product size (base pair: bp)</th>
<th>Specificity level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward reverse</td>
<td>5'-TGATCTTCTTTGAAATTTGACAG-3' 5'-TGTTGACAAAATGACCATCG-3'</td>
<td>276 bp</td>
<td>M gene (Influenza A)</td>
</tr>
<tr>
<td>Forward reverse</td>
<td>5'-GACTCAATGTCAGAAACCTTTA-3' 5'-CCACTTATTTTCCTCTCTCTGTATTAG-3'</td>
<td>189 bp</td>
<td>H5 gene</td>
</tr>
<tr>
<td>Forward reverse</td>
<td>5'-GGTTGAGTGCTGTTGGCTGGTC-3' 5'-TGTAGTAGTGTCTGTATATGACC-3'</td>
<td>131 bp</td>
<td>N1 gene</td>
</tr>
</tbody>
</table>

(2) Program the RT – PCR based on the example of primers in Table F.1 as the followings:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>reverse transcription</td>
<td>48 °C</td>
<td>45 min</td>
</tr>
<tr>
<td>initial denaturation</td>
<td>95 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>denaturation</td>
<td>94 °C</td>
<td>15 s</td>
</tr>
<tr>
<td>annealing</td>
<td>55 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>40 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>extension</td>
<td>72 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>final extension</td>
<td>72 °C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

(3) While the PCR machine is working, prepare 1.5% of agar rose gel in TBE buffer and warm until gel is melted then pour on to mould plate and wait until gel is cool and firm then place the gel on electrophoresis chamber contains TBE buffer at the level top over the gel.
(4) Take a mixer of 10 μl PCR product and 2 μl 6X loading dye to drop onto the prepared gel and use the first row of gel as DNA marker (100 bp).
(5) Sizing the PCR products by gel electrophoresis and dyeing with ethidium bromide 2 μg/ml in distilled water for 15 min
(6) Read by visible UV gel transmission to detect size of the PCR products.
(7) To interpret the results, if the tested viruses are influenza virus type A subtype H5N1, fluorescent band of DNA shall be visible (Figure G.1) in accordance with the primers used in Table G.1

![Ladder 1 2 3](image)

M 276 bp
H5 189 bp
N1 131 bp

Figure G.1 Result of influenza virus type A subtype H5N1 diagnosed by multiplex PCR, which give positive to influenza virus type A subtype H5N1, the PCR products of H5N1 and M gene size 189 bp 131 bp and 276 bp are visible respectively. In case of the absence of influenza virus type A (negative), there is no fluorescent band of M gene.

**Source:** Associate Professor Roongroje Thanawongnuwech

**G.2 AVIAN INFLUENZA DIAGNOSIS BY REAL–TIME RT–PCR**

(1) Materials and chemicals used
- **primer**
  - Inf A TH\textsubscript{3} F 5’ ATGCCCAAAATATGTTGAAATCA 3’
  - Inf A TH\textsubscript{5} A 5’ TTGTCTGCAGGGTACCCACTC 3’
- **probes**
  - Inf A TH 5 fl 5’ CAGGTATTTAAGGGAGGATGGC 3’
  - Inf A TH 5 lc 5’GGGAATGGTAGATGGTTGGTATGGGTACC 3’

RNA extraction: Magna Pure LC (Roche, Germany)
RT – PCR: Reagents from Roche, Germany in Light Cycler

(2) **PCR master mix** (15 μl/reaction)
- 2.7 x Light Cycler RNA Master hybridization probes 7.5 μl
- Distilled water 2.9 μl
- Mn (Oac)\textsubscript{2} 1.6 μl (2 mM)
- forward primer 1.0 μl (0.5 μM)
- reverse primer 1.0 μl (0.5 μM)
- probes TH5 fl 0.5 μl (0.4 μM)
- TH5 lc 0.5 μl (0.4 μM)
- Master Mix 15 μl
- RNA template 5 μl
- Total 20 μl/sample
(3) Conditions

- reverse transcription: 61 °C, 20 min
- Hot start: 95 °C, 2 min
- DNA amplification: 95 °C, 5 s (denaturation), 53 °C, 15 s (annealing), 72 °C, 8 s (extension); 65 cycles
- melting curve: 45 °C, 60 s
- cooling step: 40 °C, 30 s

(4) Primers

- forward (H5): 5’-ACTCCAATGGGGGCGATAAAC-3’
- reverse (H5): 5’-CAACGCGCCTCAAACTGAGTGT-3’
- forward (N1): 5’-AAGGGTTTTTCTTTAAATACGTC-3’
- reverse (N1): 5’-CCTGCCACCCATTTGGATCC-3’

(5) PCR products of H equal to 352 bp (Poddar, 2002) and N1 equal to 106 bp (Payungporn et al., 2004)

Conditions

- reverse transcription: 50 °C, 30 min, 1 cycle
- pre-denaturation: 95 °C, 15 min, 1 cycle
- denaturation: 94 °C, 15 s
- annealing: 55 °C, 15 s, 40 cycles
- extension: 72 °C, 30 s
- final-extension: 72 °C, 10 min, 1 cycle

Reaction

- Distilled water: 4 µl
- 5X buffer: 5 µl
- 5X Q solution: 5 µl
- dNTP: 1 µl
- enzyme: 1 µl
- primer H5 (N1) forward: 2 µl
- H5 (N1) reverse: 2 µl
- RNA: 5 µl
- total: 25 µl/reaction

Extract viral RNA: QIAamp Viral RNA Mini Kit (Cat. No. 52904)

Enzyme: One Step RT-PCR System (Qiagen)

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transcription-polymerase chain reaction (RT-PCR) for influenza A virus subtype H5N1 detection. Viral Immunol. 17:588-593

**ANNEX H**  
**UNITS**

The units and symbols used in this standard and the units of SI (International System of units or *Le Système International d’Unités*) recognized to be used are as follows:

<table>
<thead>
<tr>
<th><strong>Quantities</strong></th>
<th><strong>Units</strong></th>
<th><strong>symbols</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass</td>
<td>Gram</td>
<td>g</td>
</tr>
<tr>
<td></td>
<td>Milligram</td>
<td>mg</td>
</tr>
<tr>
<td>Volume</td>
<td>Milliliter</td>
<td>ml</td>
</tr>
<tr>
<td></td>
<td>Micro Liter</td>
<td>μl</td>
</tr>
<tr>
<td>Length</td>
<td>Centimeter</td>
<td>cm</td>
</tr>
<tr>
<td></td>
<td>Millimeter</td>
<td>mm</td>
</tr>
<tr>
<td></td>
<td>Micrometer</td>
<td>μm</td>
</tr>
<tr>
<td>Time</td>
<td>Second</td>
<td>s</td>
</tr>
<tr>
<td>Temperature</td>
<td>Degree Celsius</td>
<td>°C</td>
</tr>
<tr>
<td>Concentration</td>
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<td>mg/ml</td>
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<tr>
<td></td>
<td>Microgram per Milliliter</td>
<td>μg/ml</td>
</tr>
<tr>
<td></td>
<td>Unit per Milliliter</td>
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</tr>
<tr>
<td>Centrifugation</td>
<td>Gravity</td>
<td>g</td>
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</table>