**United States Department of Agriculture**
**Food Safety and Inspection Service**
**Office of Public Health Science**
**Laboratory QA/QC Division**
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# Laboratory Guidebook
# Notice of Change

Chapter **new**, revised**,** or archived: MLG 40.00

Title: **Avian Influenza Detection in the Chicken Heart Using Real-Time Reverse Transcriptase PCR**

Effective Date: 10/8/07

Description and purpose of change(s):

This new method utilizes a Real-Time Reverse Transcriptase (RT) PCR to identify Avian Influenza, and specifically, H5 subtype in the chicken heart. Viral RNA is extracted and purified from raw chicken heart. The Real-Time RT PCR assay amplifies the AIV Matrix gene, which is common to all Type A avian influenzas. A presumptive positive result requires a second Real-Time RT PCR assay to identify subtype H5 in the same sample extract.

**The methods described in this guidebook are for use by the FSIS laboratories. FSIS does not specifically endorse any of the mentioned test products and acknowledges that equivalent products may be available for laboratory use.**

# United States Department of Agriculture
# Food Safety And Inspection Service, Office of Public Health Science



## Procedure Outline


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## 40.1 Introduction

Avian Influenza (AI) is an economically and epidemiologically important disease of chicken, turkey and other fowl. The agent responsible for AI is a single-stranded RNA virus, avian influenza (AIV), which is a member of the *Orthomyxoviridae* family. As of 2005, there were at least 16 antigenically different hemagglutinin (H) subtypes and 9 different neuraminidase (N) subtypes. Avian Influenza Virus exists with one H subtype and one N subtype in any combination. The H5 and H7 subtypes have been identified to cause high pathogenicity avian influenza (HPAI) in birds with clinical signs ranging from sudden death to respiratory signs. Although most AI strains are classified as low-pathogenicity (LPAI), H5 and H7 subtypes have been designated as reportable. Due to the concern of animal to human transmission, AI, particularly H5 subtype, is important to both human and animal health.

The method described utilizes a Real-Time Reverse Transcriptase (RT) PCR to identify Avian Influenza, and specifically, H5 subtype in the chicken heart. Viral RNA is extracted

and purified from raw chicken heart. The Real-Time RT PCR assay amplifies the AIV Matrix gene, which is common to all Type A avian influenzas. A presumptive positive result requires a second Real-Time RT PCR assay to identify subtype H5 in the same sample extract. If either PCR assay provides positive results, confirmation will be determined from the reserve sample by Animal and Plant Health Inspection Services (APHIS) / National Veterinary Service Laboratory (NVSL) in Ames, Iowa.

## 40.2 Limit of Detection (LOD)

SEPRL-ARS-USDA (Das *et al*., unpublished manuscript) determined the live virus LOD in experimentally-infected tissue to be approximately 2.2 $Log_{10}$ $EID_{50}$/gm tissue (viral titer).

## 40.3 Safety

When working with diagnostic samples, use protective attire such as gloves and lab coats. Samples originating from an area (defined by APHIS or FSIS) with heightened concern for HPAI transmission should be handled with enhanced biosecurity, preferably with limited access to the laboratory area. Even though HPAI can be processed in BSL-2 laboratories, these laboratories must use the following BSL-3 work practices:

1. Any procedure generating aerosols or droplets should be performed in a Class II, HEPA filtered biological safety cabinet.
2. Laboratory workers should wear protective equipment, including disposable gloves, lab coats, head coverings and, where applicable, close-toed shoes and eye protection (safety glasses, surgical masks, or face shields) due to risk of aerosol/droplet exposure.
3. Centrifugation of specimens should be performed using sealed centrifuge rotors. After centrifuging, specimens should be unloaded from the rotors in a Class II, HEPA filtered biological safety cabinet.
4. Work surfaces and equipment should be decontaminated after specimens are processed using standard decontamination agents according to the manufacturer's recommendations or freshly prepared bleach solutions that are appropriate for biohazardous spillage. Several types of disinfectants including 70% alcohol, 10% sodium hypochlorite and peroxygen compounds will inactivate AIV by destroying the lipid envelope of the virus. Sodium hypochlorite and peroxygen compounds also degrades nucleic acids (Suarez et al., 2003).

5. Biological waste contaminated with suspect or confirmed HPAI should be treated as outlined in the WHO laboratory biosafety manual (http://www.who.int/csr/resources/publications/biosafety/Labbiosafety.pdf).

NOTE: Once samples have been treated with lysis buffer, the samples can be handled under normal BSL-2 conditions.

## 40.4 Quality Control Practices

### 40.4.1 General

Inactivated antigen preparation from NVSL (Ames, Iowa) is used as a positive extraction control. The inactivated antigen is produced in specific pathogen-free (SPF) embryonating chicken eggs inoculated by the allantoic sac route. The viral antigen is inactivated with beta-propriolactone, but contains viral RNA detectable by PCR. The crude antigens, 192-ADV H9/N2 and 190 ADV low path H5/N9, were prepared by NVSL.

### 40.4.2 Required Extraction Controls

a. H5N1 or H5N9 antigen, used in NVSL's Agar Gel Immunodiffusion testing, shall be used as a positive extraction control for the AIV matrix and H5 assays. The antigen contains inactivated virus that lacks the ability to replicate but contains RNA detectable by Real-Time RT PCR.
b. New Castle Disease Virus (NDV) available as inactivated antigen, or if unavailable, RNase-free water shall be used as an extraction negative control.

### 40.4.3 Required Real-Time RT PCR Controls

a. AIV matrix transcript (203 ADV 0601 is provided by NVSL) should be used as a PCR positive control for the AIV matrix screen.
b. H5 gene transcript (202 ADV 0704 provided by NVSL) should be used as a PCR positive for the H5 Real-Time RT PCR assay.
c. RNase-free water should be used as a No-Template control in the Real-Time RT PCR reactions.

### 40.4.5 Preparation of Extraction Controls

a. Prepare the control in lysing matrix tubes with 0.5 $\pm$ 0.05 g of heart tissue spiked with 40 µl of H5N1 or H5N9 antigen.
b. Prepare the negative control tissue the same way as the positive control tissue, but add NDV or RNase-free water instead of H5 antigen.

## 40.5 Equipment, Materials, Media, Reagents and Test Kits

### 40.5.1 Equipment

a. Cepheid® Smart Cycler II
b. Cepeid® Smart Cycler Tubes (25 µl volume)
c. Lysing Matrix A bead tubes for sample homogenization (Q-Biogene Cat#6910-500), or equivalent.
d. Vortex
e. Refrigerated microcentrifuge
f. Sterile RNase-free Pipet Tips with Filters
g. 1.5 ml Microcentrifuge Tubes
h. Gloves
i. Ambion® Magnetic Stand-96 (Ambion, Cat#10027)
j. Orbital Shaker for 96 well plates
k. Pipettors dedicated to RNA work
l. MoBIO® Vortex Adapter (MoBIO Cat#13000-V1-24), or equivalent
m. Sterile Scalpels
n. Sterile Forceps

### 40.5.2 Media, Reagents and Cultures

a. Chloroform, 99% pure (Sigma Cat#366927-100ml)
b. 2-Propanol, 99% pure (Sigma Cat#I9516-500)
c. Molecular Biology Grade Water (RNase-free water)
d. Transcribed AIV (Cat.# 203 ADV) and H5 RNA (Cat.# 202 ADV) as supplied by NVSL Ames, Iowa
e. Trizol® LS reagent (Invitrogen, Cat#10296-010)
f. RNase Inhibitor (Promega, Cat#N2511)
g. Cepheid® PCR Beads containing probes and primers
h. RNA/DNA decontaminant solution

### 40.5.3 Test Kits

a. Qiagen® OneStep RT-PCR Kit (Qiagen®, Cat#210210)
b. Ambion® MagMax™-96 AI/ND Viral RNA Isolation Kit (Ambion®, Cat#1835-plate kit)

## 40.6 Viral RNA Isolation Procedure: Preparation of Homogenate and Trizol Extraction of RNA

**NOTE: Before beginning analysis, frozen heart sample should be removed from ≤ -70$^0$ C and allowed to thaw. All reagent preparation must be done in a "clean area" Biosafety cabinet. Work with RNA or amplified product should not be done in this area. Keep all samples, reagents and enzymes on ice during <u>all</u> steps.**

A. Add 0.5 ± 0.05 gram of heart tissue to a sterile tube containing lysing matrix (glass beads) and 250 ± 2.5 µl of BHI broth. Add 750 ± 7.5 µl of Trizol® to the tubes.

B. Agitate the meat/beads mixture on MoBio® vortex adaptor and shake at maximum speed for approximately 10 minutes.

C. Hold the samples at room temperature for approximately 15 min. Add 200 ± 2.0 µl of chloroform and vortex the samples for approximately 15 seconds. Let the samples stand for at least 15 min at room temperature.

D. Centrifuge the samples for 15 - 20 min @ 12,000 rpm set at 4°C. Remove the aqueous layers from all tubes and place into clean RNase-free microcentrifuge tubes.

## 40.7 Viral RNA Purification Procedure: Ambion® Magnetic Bead Purification of Viral RNA

This procedure is designed for high-throughput loads. Paramagnetic beads with a nucleic acid binding surface bind nucleic acids and are detained with use of a magnet. Contaminants and cellular debris are washed away during wash steps. Make up solutions for day of use.

A. Follow kit instructions to prepare Wash Solutions I and II. Prepare Viral Lysis/Binding Solution as outlined in TABLE 1.

**TABLE 1:** Viral Lysis/Binding Solution Preparation

| Ingredients | Volume Per Sample |
|---|---|
| Lysis Binding Solution | 50.0 µl |
| Carrier RNA | 1.0 µl |
| Mix briefly, then add | |
| 2-Propanol | 50.0 µl |
| **Total Volume per Sample** | **101.0 µl** |

B. Prepare the Bead Re-suspension Mix as outlined in TABLE 2.

**TABLE 2:** RNA Binding Bead Re-Suspension

| Ingredients | Volume Per Sample |
|---|---|
| Bead Re-suspension Solution | 6.0 µl |
| RNase-Free Water | 4.0 µl |
| Mix briefly, then add | |
| RNA Binding Beads | 4.0 µl |
| Mix briefly, then add | |
| 2-Propanol | 6.0 µl |
| **Total Volume per sample** | **20.0 µl** |

C. Transfer 50 ± 0.5 µl of sample into the corresponding well on a 96 well processing plate supplied in the kit. Add 101 ±1.0 µl of prepared viral lysis/binding solution (TABLE 1) to each well containing sample. Shake the plate on a plate shaker at 550- 600 rpm for approximately 30 seconds.

D. Add 20 ± 0.2 µl of RNA Binding Beads solution (TABLE 2) to each well with sample. Shake the plate for approximately 4 minutes at 550-600 rpm.

E. Capture/pellet the RNA Binding Beads on a magnetic stand for approximately 2 minutes. Remove and discard the clear supernatant from the beads.

F. Remove the plate from the magnetic stand and add 100 ± 1.0 µl Wash Solution I mix to each well. Shake for approximately 30 seconds at 550 - 600 rpm.

G. Pellet the beads again on the magnetic stand for approximately 2 minutes and remove/discard the supernatant. Remove the plate from the magnetic stand. Add 100 ± 1.0 µl Wash Solution II Mix to each well. Shake for approximately 30 seconds at 550 -600 rpm.

H. Pellet the beads on the magnetic stand for approximately 2 minutes and remove/discard the supernatant. Remove the plate from the magnetic stand and repeat the wash with Wash Solution II. Shake for approximately 30 seconds at 550 - 600 rpm.

I. Pellet the beads again for approximately 2 minutes on the magnetic stand and discard the supernatant. Shake the plate vigorously for 2 - 5 minutes to dry the beads and remove ethanol.

J. Add 50 ± 0.5 µl of Elution Solution from the kit and shake for approximately 4 minutes at 1000 rpm.

K. Pellet the beads for approximately 2 minutes on the magnetic stand and transfer the RNA into an RNase-free sample tube for Real-Time RT PCR. Keep the sample, also referred to as extraction template at this point, on ice or in the refrigerator for immediate use. Store the sample at ≤ -70 C if not testing immediately.

## 40.8 Viral RNA Amplification and Detection: Real-Time RT PCR using Lyophilized Beads

PCR is performed using the SmartCycler system made by Cepheid®. The lyophilized beads contain primers, probes and an internal positive control (IPC).

- Each bead makes 4 reactions.
- Count the number of samples and controls and divide by 4 to determine the number of beads to reconstitute.
- All PCR runs must include a RNA gene transcript positive control and a no template control (NTC), usually RNase-free water.

- Do not combine the contents of One-step PCR kits. Record lot information for each sample if different.
- Dilute RNase Inhibitor (40U/µl) 1:3 with RNase-free water to get 13 U/µl Example**:** 2µl of RNase Inhibitor to 4µl of RNase-free water for final concentration of 13 U/µl**.**

**A.** Reconstitute each bead as outlined in TABLE 3.
**NOTE: Keep reagents on ice.**

**TABLE 3:** PCR Master Mix for four reactions.

| Ingredients of Master Mix | Volume per 4 reactions |
|---|---|
| # of Lyophilized beads | 1 |
| RNase-free water | 58.8 µl |
| dNTPs | 3.2 µl |
| Enzyme mix (RT + Taq) | 4.0 µl |
| RNase inhibitor (diluted) | 2.0 µl |
| **Total** | **68.0 µl** |

B. Add 17 µl of master mix to each SmartCycler tube in the cold block rack..

C. Add 8 µl of extraction template to each tube (total volume of 25 µl in each tube).

D. Pulse spin all tubes before loading the machine and then place samples in appropriate locations on the SmartCycler machine.

E. Create a run by adding sites for detection and choosing the program for AIV Matrix or the H5 assay. Select the viewable graphs including “FAM threshold”, “logFAM threshold”, “Texas Red threshold”, “logTexas Red threshold”, and “Temperature”. Name the run in the “Run Name ID” field. Select the “Start Run” button.

The run time for the assay is approximately 1.5 hours. Program the SmartCycler with the parameters shown in TABLE 4. Amplification of viral amplicon should be monitored in the FAM channel and the IPC should be monitored in the Texas Red Channel.

**TABLE 4:** Thermocycling Conditions for the two PCR tests.

| Target Gene | Cycle | Time | Temp | Step |
|---|---|---|---|---|
| AIV Matrix | 1 cycle | 30 min | 50°C | Reverse Transcription |
| | | 15 min | 95°C | |
| | 40 cycles | 20 sec | 94°C | Denaturation |
| | | 20 sec | 60°C | Annealing/Extension |
| H5 | 1 cycle | 30 min | 50°C | Reverse Transcription |
| | | 15 min | 95°C | |
| | 40 cycles | 1 sec | 94°C | Denaturation |
| | | 20 sec | 54°C | Annealing |
| | | 15 sec | 72°C | Extension |

**NOTE: Thermocycling conditions for the AIV Matrix contain only 2 steps. Turn Optics "On" for the annealing step of AIV Matrix and extension step of H5 assay. The Optics remain "Off" for all other steps. In the protocol setup, accept the default settings of all parameters except the following: The background maximum cycle should be "28" instead of the default "40". The Manual Threshold Fluorescence units should be set to "25" instead of the default "30". The lower threshold will decrease the chance of a false negative result.**

### 40.8.1 Interpretation of Sample Results

Both AIV Matrix and H5 gene targets, which are monitored in the FAM channel, are considered positive when the fluorescence crosses the cycle threshold ($C_T$) and negative if the fluorescence does not cross the $C_T$. However, for samples which have $C_T$ values $\geq 35.0$ on the matrix PCR assay, repeat the matrix PCR assay. If the second matrix PCR assay yields a positive result for that sample, then the sample is considered a suspect positive. Samples testing negative by the matrix PCR assay on the initial test or on the repeated matrix PCR assay (when initial test $C_T$ values are $\geq 35.0$) are considered screen negative so the H5 PCR assay is not performed. Samples testing positive by the matrix PCR assay, either on the initial test or the repeated test, are considered suspect positive so the H5 PCR assay is performed.

### 40.8.2 Interpreting and Troubleshooting Control Results

The performance criteria for the positive controls for a valid assay shall include a positive extraction control and a positive PCR control that are both positive with $C_T < 35.0$. Every valid assay shall also include a negative extraction control and a NTC in which the

fluorescence does not cross the cycle threshold. If any of the controls do not perform within the listed specifications, troubleshoot using the following criteria:

A. If a positive extraction control tests PCR negative, repeat the PCR assay. When a false negative occurs with a control that should be positive, this usually indicates either a deficient viral RNA extraction or PCR error. If the positive extraction control is negative on the repeated PCR assay, repeat the assay from the extraction step.

B. If a negative extraction control tests PCR positive, repeat the PCR assay. If a false positive occurs with a control that should be negative, this usually indicates either cross-contamination during extraction, pipetting error during PCR setup or high background fluorescence noise. If a suspected extraction error occurred, repeat the assay starting with the extraction step. If a suspected pipetting error occurred during PCR setup, repeat the assay from the PCR step.

C. If the positive PCR control is negative or is $< 35.0\ C_T$, repeat the PCR assay.

D. If the no template control (NTC) tests positive, repeat the PCR assay.

## 40.9 Confirmation by NVSL

FSIS reserve samples that are Real-Time RT PCR positive for AIV Matrix and/or H5 subtype assay are considered presumptive and forwarded to NVSL for viral isolation and further characterization. The number of samples sent to NVSL shall be determined by FSIS and NVSL. They are forwarded to NVSL following the “Transfer of Select Agents” guideline which states:

> FSIS may transfer select agent from other government or private laboratories. Both the shipper and receiver shall follow the transport requirements for the select agents listed in their APHIS permit. Prior to shipment, the APHIS transfer form shall be completed by the recipient and sender, and faxed to APHIS for shipment approval. APHIS will fax the form back to the sender and /or recipient with an approval authorization number after verification of the information on the form. This form is completed for each transfer of a select agent and must be kept as documentation for at least three years. Copies of this form, instructions for completing the form, and the Health and Human Services and USDA list of select agents are located on the APHIS website. The requirements for transfer of a select agent are stated in 42 CFR Part 73.

## 40.10 Selected References

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