National Veterinary Services Laboratories

Testing Protocol

Real-Time RT-PCR for the Detection of Avian Influenza Virus and Identification of H5 and H7 subtypes in Clinical Samples

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Real-Time RT-PCR Test for Detection of Avian Influenza Virus.

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1. Introduction

1.1 Background

Avian Influenza (AI) is an economically important disease of chickens and turkeys and many other species. The etiologic agent of AI is the avian influenza virus (AIV), a member of the family *Orthomyxoviridae*, genus *Influenzavirus A*. There are currently 16 antigenically different hemagglutinin (H) and nine antigenically different neuraminidase (N) subtypes recognized; each virus possessing one H and one N, in any combination. Of the 16 H subtypes of AIV, only H5 and H7 viruses of certain lineages have caused HPAI in poultry, therefore, the detection and /or isolation of H5 or H7 subtype is considered serious. Depending on the age and type of bird and on environmental factors, clinical signs for HPAI may vary from sudden death to characteristic respiratory signs including excessive lacrimation, sinusitis, diarrhea, and edema of the head, wattles and comb. However, the vast majority of AIV isolates are low pathogenic AIV (LPAI) which may range from no obvious clinical signs to a mild respiratory disease. Therefore, the clinical signs for HPAI and LPAI are not considered pathognomonic. Due to the risk of a low virulent H5 or H7 becoming virulent by mutation in poultry hosts, all H5 and H7 viruses have also been classified as notifiable avian influenza (NAI). Highly pathogenic AIV as well as all H5 and H7 viruses are reportable to the World Organization for Animal Health (OIE).

The real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) technique was developed to assist in the rapid diagnosis of avian influenza infections in poultry. The technique utilizes a one step protocol with specific primers designed to amplify a portion of the genome that contains a target PCR sequence. Non-extendible fluorogenic hydrolysis/Taqman probes monitor the target PCR product formation at each cycle during the PCR reaction. The probes are labeled at the 5’ end with a reporter dye (e.g. FAM) and a quencher dye (e.g. blackhole quencher [BHQ-1]) at the 3’ end. The proximally located quencher dye absorbs the emission of the reporter dye as long as the probe is intact and not hybridized to the target. When the probe is hybridized to the target, the 5’ nuclease activity of Taq-polymerase will cause hydrolysis of the probe, separating the quencher from the reporter dye. This separation results in an increase in fluorescence emission of the reporter dye, which is detected spectrophomectrically and recorded. The

amount of fluorescence recorded is proportional to the amount of target template in the samples.

The AIV rRT-PCR procedure described in this protocol has three unique primer/probe combinations; one that targets the matrix gene and two that are subtype specific for H5 and H7 AIV's. The matrix primer/probe set is designed to detect all influenza A viruses, whereas the H5 and H7 subtyping primer/probe sets are designed to detect most North American H5 and H7 viruses. In addition, the H5 assay has been shown to successfully detect the Asian H5N1 virus. The matrix gene assay has a detection limit of 10 fg or approximately 1,000 copies of target RNA and can detect 0.1 50% egg infectious dose of virus. The H5- and H7-specific probe sets each have a detection limit of 100 fg of target RNA or approximately 10^3 to 10^4 gene copies. The diagnostic sensitivity and specificity of the matrix assay as compared to virus isolation, was shown to be 88.2% and 99.5%, respectively, based on the testing of >3,500 samples collected during the 2002 outbreak of H7N2 AIV in Virginia. When the same samples were compared by submission (all samples from the same premises) the sensitivity and specificity was shown to be 95.1% and 99.1%, respectively.

In addition to the standard wet mix rRT-PCR procedure, an alternative reagent bead procedure for use with the matrix and H5 assays has been described. The matrix reagent bead includes an internal control (IC) which utilizes the same forward and reverse primers as the viral target, but is detected with an IC sequence specific Texas Red 5' labeled probe. Diagnostic specimens which contain inhibitory substances give false negative results and should be reported as a "no test" by rRT-PCR. All false negative specimens should be tested by virus isolation. The H5 reagent bead has dual specificity, detecting both the Eurasian (EA) and North American (NA) lineages of H5 AIV. Both the EA and NA primer sets are detected with a FAM-labeled H5-HA probe. The bead procedure has been developed to identify specimens that contain inhibitory substances, to increase the specificity of the H5 assay and to aid in assay standardization. Reagent beads are distributed by the NVSL as a non-catalog reagent.

It is preferable to use the matrix primer/probe set to screen diagnostic samples because the matrix assay is more broadly reactive (detects all subtypes) and is more sensitive than the H5 or H7 primer/probe assays. Samples positive on the matrix assay can be tested to quickly

determine if H5 or H7 subtypes are involved. Virus isolation should be performed to confirm the rRT-PCR results for index cases involving AIV.

Tissues from more than one bird should not be pooled together. However, tracheal swabs from up to 5 birds from a single premise can be pooled together in 2-3.5 ml of brain heart infusion (BHI) broth, but should not be pooled with cloacal swabs. Tracheal swabs are the preferred specimen for the isolation of RNA. Validation data has demonstrated a decreased efficiency in the isolation of RNA from cloacal swabs due to the heavy load of organic material. The LPAI H7N2 virus which is currently circulating in the U.S. northeastern live bird market system is inconsistently shed in the feces. Therefore, it is often not possible to detect the virus in the feces or cloacal swabs by virus isolation or rRT-PCR. It should be emphasized that the rRT-PCR technique will detect viral nucleic acid from infectious as well as noninfectious virus. The rRT-PCR is not the test of choice to determine if infectious AIV is present in environmental samples.

The procedure described here is used in the Diagnostic Virology Laboratory (DVL) of the National Veterinary Services Laboratories (NVSL). The brands of equipment listed in the protocol are used in the DVL; however, comparable equipment may also be used. Laboratories using this protocol should follow quality assurance procedures as they pertain to equipment maintenance, receiving specimens, and recording/reporting results.

Any specimen from outside a USDA quarantine zone that is positive with the AIV (matrix) assay, regardless of the H5 or H7 assay results, should be referred to the National Veterinary Services Laboratories for further testing and characterization. Virus isolation and characterization is required to officially diagnose a foreign animal disease (FAD) such as HPAI.

There is recent evidence that some strains of AIV can cause infections in humans resulting in conjunctivitis and in rare cases illness and death. Personnel handling clinical samples or live virus should take appropriate safety precautions to avoid accidental introduction of the virus. Appropriate safety precautions should include wearing disposable gloves, laboratory coat, and safety glasses as well as handling all specimens in a class II biosafety cabinet.

1.2 Keywords

Avian Influenza virus (AIV), matrix gene (MA), real-time reverse-transcriptase polymerase chain reaction (rRT-PCR), room temperature (RT), biological safety cabinet (BSC).

1.3 Collaboration

This protocol was developed in cooperation with the Southeast Poultry Research Laboratory, Agricultural Research Service, USDA, Athens, GA.

2. Materials

2.1 Facilities/equipment/instrumentation

2.1.1 Surveillance samples originating from outside a known AIV quarantine zone can be processed in a biosafety level 2 (BSL-2) laboratory. However, samples originating from inside a known AIV quarantine zone or considered suspect for HPAIV, should be handled under increased biosecurity. This includes restricted access to the laboratory area where the clinical samples are being handled until the samples have been rendered non-infectious. Once a clinical sample has been treated with lysis buffer for RNA extraction, the sample can be moved to a less restrictive environment to complete the RNA extraction and rRT-PCR analysis. Once a sample is biologically amplified (isolated in cell or egg culture) and has been confirmed or is suspected of being a HPAI virus, the virus should be handled at a higher containment level, specifically BSL-3 or preferably biosafety level 3-ag.

2.1.2 Class II, HEPA filtered biological safety cabinets (BSC), minimum of 2, preferably 3, with UV germicidal lights are required to maintain sample integrity during processing and testing. In addition, the Trizol® and Qiagen® extraction procedures should be performed in a class II BSC that is connected to an external exhaust plenum to minimize exposure to organic chemical fumes.

2.1.3 Refrigerator (4 C $\pm$ 2°)

2.1.4 -20 C ($\pm$ 3°) freezer (not frost-free)

2.1.5 -70 C (± 5°) freezer

2.1.6 Microcentrifuge (non-refrigerated [International Equipment Co., MicroMax, Needham Heights, MA] and refrigerated [Hermle, Z 360K, Germany] or [Sorval® Heraeus Biofuge Fresco, Germany])

2.1.7 Vortex mixer

2.1.8 Assorted test tubes and Eppendorf tube racks

2.1.9 An integrated DNA/RNA amplification and detection instrument system that has the capability to detect specific sequences using hybridization probes. Instrumentation should be capable of exciting and detecting fluorescein-based probes (450-495nm, 500-550 nm, 565-590nm, and 630-750nm ranges). The current protocol was developed using the Cepheid Smart Cycler [(Cepheid® Smart Cycler, P#SC2500N1-1, Sunnyvale, CA). However, most if not all of the commercially available real-time PCR machines can detect the fluorescein-based probes, and likely can be used with this test. Based on past experience of transferring protocols between different machines, changes in cycling times and probe concentrations are often required to get equivalent sensitivity. Therefore, optimization of the assay on alternative machines is required. The optimization data needs to demonstrate that the alternative machine provides comparable sensitivity and limit of detection to the assay described in the current protocol with the Smart Cycler® instrument. This data should be available for review by APHIS.

2.1.10 25µl Smart Cycler® tubes (Catalog #900-0022 or 900-0003, Cepheid® Smart Cycler, Sunnyvale, CA)

2.1.11 Refrigerated PCR reaction tube holder and mini-microcentrifuge to spin reaction tubes. Both items are supplied with the Smart Cycler® real-time PCR system.

2.1.12 QiaVac® 24 vacuum manifold (Qiagen®, Valencia, CA) and vacuum pump with a capacity of 18-20 liter/min (Gast MFG Corp., St. Louis, MO). Note: Insufficient vacuum pressure may reduce RNA yield and purity. The vacuum manifold system with vacuum pump is optional, but is a highly recommended product for processing large numbers of samples for rRT-PCR.

2.2 Reagents/supplies

2.2.1 Molecular biology grade RNase-free sterile distilled water

2.2.2 *In vitro* transcribed AIV matrix gene positive control RNA and H5 and H7 *In vitro* transcribed control RNA (supplied by the NVSL, Ames, IA)

2.2.3 *Ethanol, absolute **(Caution: irritant, flammable)**

2.2.4 *Isopropanol, 99+% pure **(Caution: irritant, flammable)**

2.2.5 *Chloroform, 99+% pure **(Caution: toxic)**

2.2.6 *Trizol® LS reagent **(Caution: toxic in contact with skin and if swallowed; causes burns.)** (Cat. #10296-010 or 10296-028, Invitrogen, Carlsbad, CA)

2.2.7 Qiagen® RNeasy Extraction Kit (Cat. #74104 50 preps or #74106 250 preps, Qiagen®, Valencia, CA).

2.2.8 One-Step RT-PCR Kit (Cat. #210210 or 210212, Qiagen®, Valencia, CA). Many one-step RT-PCR kits are commercially available. However, the Qiagen® system has been tested extensively with good results. Other kits can be considered for use, but a minimum level of equivalency testing is required before substituting any reagents in the approved protocol. Currently, the only alternative kit that has been tested that has comparable test results to the Qiagen® system is the Superscript® One-Step RT-PCR System with Platinum Taq DNA Polymerase (Cat #10928-034 or 10928-042, Invitrogen®, Carlsbad, CA). Appropriate changes to optimize the protocol for use with alternative reagents or RT-PCR kits, including cycling parameters, must be supported by an equivalency evaluation as previously noted.

2.2.9 Hydrolysis probes and primers (Oligonucleotides) (Table 1) for the detection of AIV. Suggested sources: Biosearch Technologies (http://blackholequenchers.com) and Operon (http://oligos.qiagen.com). Suggested primers sources: Integrated DNA Technologies, (http://www.idtdna.com/),

or Operon (http://oligos.qiagen.com/). Other companies can be used to order both primers and probes. It is strongly recommended that the probes and primers be purified to a high level to reduce nonspecific reactions. Purification is typically performed with HPLC purification as the minimum.

2.2.10 Ambion MagMax®-96 Viral RNA Isolation Kit (Cat. # 1835-plate kit or 1929 for single tube kit) (Ambion®, Austin, TX 78744-1832)

Table 1. AIV real-time RT-PCR probe and primer sequences.

Specificity		Sequence
Matrix (any influenza)	M+25* 5′ Primer	5′-AgA TgA gTC TTC TAA CCg Agg TCg-3′
	M+64* Probe	5′-FAM-TCA ggC CCC CTC AAA gCC gA-TAMRA-3′
	M-124* 3′Primer	5′-TgC AAA AAC ATC TTC AAg TCT CTg-3′
H7- North American	H7+ 1244* 5′ Primer	5′-ATT ggA CAC gAg ACg CAA Tg-3′
	H7+1281* Probe[a]	5′-FAM-TAA TgC TgA gCT gTT ggT ggC-TAMRA-3′
	H7-1342* 3′Primer	5′-TTC TgA gTC CgC AAg ATC TAT Tg-3′
H5	H5+1456* 5′ Primer	5′-ACg TAT gAC TAT CCA CAA TAC TCA-3′
	H5+1637* Probe	5′-FAM-TCA ACA gTg gCg AgT TCC CTA gCA-TAMRA-3′
	H5-1685* 3′Primer	5′-AgA CCA gCT ACC ATg ATT gC-3′

*Refers to the nucleotide position where the 5′ end of the probe or primer anneals to the AIV genome. The FAM/TAMRA hybridizing probes were validated with the Smart Cycler I system. When using the Smart Cycler II system it is recommended that all hybridizing probes be labeled with FAM as a reporter dye and quenched with either Dabcyl or Black Hole™ quencher I. The Smart Cycler II system is not calibrated to use the TAMRA dye as a quencher dye. The TAMRA dye is read as background noise in channel 2.

2.2.11 RNase Inhibitor, 40 units/µl (Promega, catalog #N2511 or N2515, Madison, WI)

2.2.12 $MgCl_2$, 25 mM (Promega, catalog #A3511 or A3513, Madison, WI)

2.2.13 TE buffer pH 8.0, 1X, molecular grade (Promega #V6231 or V6232, Madison, WI)

2.2.14 14.3 M *β-mercaptoethanol (β-ME) (Sigma, M 6250, St. Louis, MO) **Caution: toxic – dispense in a fume hood, wear gloves**

2.2.15 Sterile, aerosol-resistant pipette tips of various sizes (1.0ml, 200-50µl, 100-10µl, 10-0.5µl)

2.2.16 1.5 ml microcentrifuge tubes (sterile)

2.2.17 Powder-free latex or nitrile gloves

2.2.18 Calibrated pipettors from 0.5 µl to 1000 µl, 2 complete sets, and one extra pipettor (5-40 µl) for DNA transfer

2.2.19 Ambion® Magnetic Stand-96 (Ambion Inc. catalog #10027, Austin, TX or an O-ring 96 well Magnetic-Ring Stand (Cat.#10050) (Ambion, Austin, TX). Product #10050 has 96 ring magnets that pellet beads in a donut shape. Product #10027 has 24 big magnetic rods that pellet beads to one side of the wells.

2.2.20 Orbital shaker for 96 well plates (Lab-Line Titer Plate Shaker Model #4625, Melrose Park, ILL)

2.2.21 5-250 µl 12 channel pipetting tool (Matrix Technologies Corp., catalog #2012, Hudson, NH)

2.2.22 Lyophilized AIV rRT-PCR matrix and H5 reagent beads have been prepared by Cepheid® (Sunnyvale, CA) for use with the matrix and H5 assays. Request for reagent beads should be submitted to the NVSL. Each bead is sufficient for four 25 µl reactions. Each bead consitists of assay specific primers and probe, KCl, $MgCl_2$, and HEPES buffer. In addition, the matrix bead includes an internal control (IC). The purpose of the IC is the detection of a "false" negative resulting from non-specific inhibition of PCR amplification. The matrix IC is 228 bases long in-vitro transcribed single stranded RNA. It contains sequences complementary to AI matrix forward primer at the 5'-end and AI matrix reverse primer at the 3'-end, and has a unique internal sequence for binding to the IC probe. The matrix bead includes a FAM-labeled probe for detection of the viral matrix gene, and the Cal-Flour Red 610 labeled probe for the IC.

The H5 reagent bead includes two forward primers, one for the detection of North American (NA) lineage H5 AIV and the other for the detection of the Eurasian (EA) lineage H5 AIV. The H5 bead includes one FAM-labeled H5 probe that will detect both the NA and EA amplicon. The beads are distributed in 0.5 ml micro-centrifuge tubes which can be used for reconstitution of the reagents. The beads are to be used with Qiagen® One-Step RT-PCR dNTP and enzyme. The beads can be stored in the foil packaging at 4C for 6 months. Do not remove from foil packaging till ready for use.

*Use caution when using these reagents. Refer to the individual Material Safety Data Sheet (MSDS) before handling any of these reagents.

3. Preparation for the test

For this procedure, it is critical to have separate preparation areas and equipment for nucleic acid extraction, "clean" procedures, and work with amplified nucleic acid. The "clean" area is used for preparing reagents for the PCR procedure. Amplified c-DNA or sample RNA should never be introduced into this area. One biological safety cabinet should be designated for "clean" work only. There should also be a separate set of "clean" pipettors and tips, RNase-free water, tubes for reagent preparation, racks, and ice container which are designated for "clean" use only and never leave the area. A -20 C freezer should also be designated as "clean" for storage of reagents. A second biological safety cabinet, set of pipettors, and other equipment and reagents should be used for extraction procedures. Ideally, a third biological safety cabinet should be used for transfer of RNA to amplification tubes. Latex/nitrile gloves, in particular, must be worn throughout the procedure and must be changed frequently. RNA is very labile and easily degraded by RNases that are ubiquitous, including on human skin. Gloves also help protect the reagents and samples from other contaminating agents and cross-contamination that can adversely affect results. Always change gloves after working with sample RNA or amplified DNA. Always wear fresh gloves when working with "clean" reagents. Protective eyewear, gloves, and lab coats should be worn as some of the reagents used are toxic. It is recommended that the Qiagen® and Trizol® extraction procedures be conducted

in a vented class II BSC.

3.1 Personnel qualifications/training

Personnel performing PCR procedures should be familiar with:

3.1.1 Preparation and proper handling of samples and reagents

3.1.2 Calibration, maintenance, and use of instruments included in this protocol

3.2 Preparation of equipment/instrumentation

Refrigeration equipment, incubators, centrifuges, pipettors and thermal cyclers are calibrated and certified according to the respective institution standard operating procedures.

3.3 Disinfectants

Several classes of disinfectants, e.g. iodophores, phenolics, quaternary ammonia compounds, 70% alcohol, 10% sodium hypochlorite, and peroxigen compounds, will inactivate AIV by destroying the lipid envelope of the virus. However, of the disinfectants listed above, only sodium hypochlorite (bleach) and peroxigen compounds (Vircon-S) have been shown to degrade nucleic acid as well as destroy infectivity of AIV. This is important when selecting a disinfectant to rid surfaces of contaminating nucleic acids.

3.4 Preparation of reagents/control procedures

3.4.1 Oligonucleotide primers

Prepare primers in a "clean" hood (see 3.0). Always wear latex/nitrile gloves when dispensing primers.

Dilute primers to 200 pmol/µl (200µM) in 1X TE for the stock dilution and 20 pmol/µl in RNase-free water for the working dilution. Aliquot primers in small volumes to avoid excessive freeze/thaw cycles. For short-term storage of primers (<2 week), 4 C is acceptable. For longer term storage, -20 C or colder is recommended. Store stock primer solutions at -20 C or -70 C. 10 pmol of the matrix forward and reverse primers, and 10 pmol of both the H5 and H7 forward and reverse primers in the subtyping assay are added per 25 µl reaction. See section 2.2.9.

3.4.2 Hydrolysis Probe

Prepare probe in a "clean" hood (see 3.0). Always wear latex/nitrile gloves when working with hydrolysis probes. Hydrolysis probes are light sensitive and should be protected from exposure to direct light. Diluted probes should be stored in amber sterile RNase free microcentrifuge tubes or tubes wrapped with foil.

Dilute probes to 120 pmol/µl (120µM) in 1X TE for the stock dilution and to 6 pmol/µl in RNase-free water for the working dilution. Aliquot probe in small volumes to avoid excessive freeze/thaw cycles. Store diluted probes at -20 C and stock probe solutions at -20 C or -70 C. Avoid excessive freezing and thaw cycles. Diluted probe should not be frozen/thawed more than 4 times. A total of 3 pmol of the matrix, H5 and H7 probe are added, respectively per 25 µl reaction.

3.4.3 Handling and Dilution of Primers and Probe

Lyophilized primers and probes must be centrifuged briefly to ensure that the DNA pellet is at the bottom of the tube before they are opened and reconstituted. TE buffer should be used for the initial reconstitution of lyophilized primers and probes. Quantitation information will be supplied for each oligonucleotide primer (oligo) by the manufacturer.

An example of calculation for oligo reconstitution:

You have 17786 pmol of oligo (will be on oligo information sheet from manufacturer).

Need 200pmol/µl for stock concentration.

Divide pmol of oligo by the pmol/µl needed or:

$$\frac{17786 \text{ pmol}}{200 \text{ pmol/µl}} = 88.9 \text{µl of 1X TE}$$

The calculation for the probe is the same, except divide the number of probe pmol by 120 pmol/µl. Mix gently by tapping the tube and allow the oligo to rehydrate for about 10 minutes before use.

Working stocks of primers should be 20 pmol/µl (20µM) and working stocks of probes should be 6 pmol/µl.

Dilute the primers 1:10 and dilute the probe 1:20 in nuclease free H_2O (do not use TE buffer) for the working stocks.

The background fluorescence of each lot of probe should be determined following receipt. If the background fluorescence exceeds 500 fluorescent units the probe should be discarded and a new lot requested from the manufacturer. Probes with high background fluorescence (excessive free floating dye) will reduce the quantity of fluorescent units available for diagnostic detection. The background fluorescence of each assay or run should be monitored to determine if there is an indication of probe degradation.

Additional information on fluorescent probe handling and storage can found at: www.operon.com, www.idtdna.com and www.idahotech.com.

3.4.4 Prepare 70% ethanol with 100% (absolute) ethanol and RNase-free water.

3.4.5 Prepare 80% ethanol with 100% (absolute) ethanol and RNase-free water.

3.4.6 β-Mercaptoethanol (β-ME) must be added to the RNeasy RLT lysis buffer before use. Add 10µl β-ME per 1 ml RLT buffer. RLT buffer is stable for 1 month after addition of β-ME. Be sure to date buffer after adding β-ME.

3.4.7 Add 100% (absolute) ethanol to the RNeasy RPE wash buffer according to the Qiagen® RNeasy kit directions.

3.4.8 The template for the positive controls is *in vitro* transcribed RNA from the AIV matrix, H5 and H7 protein gene. Transcribed RNA is available upon request from the NVSL, 1800 Dayton, Ave., Ames, IA. Request information is listed below. The transcribed RNA positive control should be diluted to a working or use concentration that will consistently give a target C_t of approximately 25.0. Dilute the stock RNA with sterile RNase free water to the suggested working dilution as described in the product insert sheet that is provided with the transcribed RNA. Run the diluted RNA on the rRT-PCR assay to determine if the positive

control has a C_t that is within the acceptable range (section 6.2).

Positive control transcribed RNA can be requested using the reagent codes listed below. Reagent request form 4-9 can be accessed through the NVSL website http://www.aphis.usda.gov/vs/nvsl/Home/sitemap.htm	
Reagent Code	Product Name
200 ADV	APMV-1 Transcribed RNA
201 ADV	AIV H7 Transcribed RNA
202 ADV	AIV H5 Transcribed RNA
203 ADV	AIV Matrix Transcribed RNA

3.4.9 Dilute RNase inhibitor to 13.3 units/µl with RNase free water.

3.5 Preparation of samples

Perform all procedures with potentially live agents in an approved Class II BSC with HEPA filtration. Always wear protective clothing, safety glasses and gloves when handling potentially infected tissues or live virus.

Pooled oropharyngeal/tracheal swabs (5 swabs/tube) are the specimen of choice for the PCR procedure and should be extracted using either the Qiagen® or Ambion® RNA extraction procedure. Appropriate tissue (spleen, lung, intestine) should be processed by preparing a 10-20% tissue homogenate and extracting RNA using the Trizol® extraction procedure. Alternatively, $5mm^3$ piece tissues are added to 2.0 ml of brain heart infusion broth (BHI), frozen solid, thawed and centrifuged. The supernatant from this tissue pool (250µl) is extracted using the Trizol® procedure. Validation/equivalency data indicate that cloacal swab specimens are less sensitive than oropharyngeal/tracheal swabs for the detection of AIV by rRT-PCR. The Ambion® and Trizol® extraction procedures are more sensitive for the isolation of RNA from cloacal swab pools (5 swabs/pool) than the Qiagen® extraction procedure. Cloacal swab specimens testing negative by rRT-PCR must be tested by virus isolation to determine if the specimen is negative for AI. However, if there is sufficient virus in the cloacal swab specimen it may be detected by rRT-PCR. Environmental swabs are inappropriate specimens for rRT-PCR and should be tested

by virus isolation. **Tissues from more than one bird should not be pooled together.**

All samples should be processed in a Class II BSC.

Samples received for testing are compared to accompanying paperwork to assure correct samples were received and that appropriate samples were submitted for the PCR test. Cases are logged into the logbook.

3.6 Preparation of matrix and H5 bead reagents

Each bead is sufficient for four 25 µl reactions. Reconstitute each bead (AI matrix or H5) according to the instructions in section 4.5. The reagent beads do not include dNTP's, RNase inhibitor or RT/PCR enzymes. Qiagen® enzyme mix and dNTP's as well as RNase inhibitor will need to be provided by the user.

4. Performance of the test

Before beginning the RT-PCR test, place "clean" pipettors, racks, tips, etc. into the "clean" hood. Similarly, place the sample equipment into a separate hood. Exposure to UV germicidal light for several hours or overnight may be beneficial for the degradation of contaminating RNA from pipettors and other equipment.

4.1 Extraction of RNA from swab specimens (Qiagen® RNeasy method)

4.1.1 Vortex swab specimen fluid and transfer 500µl of sample into the microcentrifuge tube labeled with the specimen number.

4.1.2 Place 500 µl of Qiagen® buffer RLT with β-ME into the microcentrifuge tube. Vortex well.

4.1.3 Pulse spin to eliminate liquid specimen in the lid after vortexing. Add 500µl 70% ETOH and vortex well. Centrifuge lysed swab specimen for 5 min. at 5,000 X g in a microcentrifuge at RT.

4.1.4 Transfer all of the lysed specimen supernatant to a RNeasy® Qiagen column that has been marked to identify the specimen. Centrifuge for 15 sec at $\geq$8,000 X g at RT*. Check to assure the entire specimen has

flowed through the column. Repeat until all of specimen has been applied to the column.

*Alternatively, a QiaVac® manifold can be used to pull the specimen and wash solutions through the collection columns. This will increase the efficiency and eliminate the need to centrifuge the columns at the steps 4.1.3, 4.1.4, 4.1.5, 4.1.6 and 4.1.7.

4.1.5 Add 700µl of RW1 buffer to the RNeasy column and centrifuge for 15 sec at ≥ 8,000 x g and place the column in a clean collection tube (the tube with RW1 flow through may be discarded).

4.1.6 Add 500µl RPE buffer to the RNeasy column and centrifuge for 15 sec at ≥8,000 x g. Discard flow through from the collection tube.

4.1.7 Repeat for a total of 2 washes with RPE buffer discarding flow through from the collection tube. Following the last RPE wash place the RNeasy column in a new 2 ml collection tube.

4.1.8 Centrifuge the empty RNeasy column an extra 2 minutes at full speed and discard the collection tube.

4.1.9 Place the RNeasy column in an elution tube or a 1.5 ml microfuge tube that has been marked with the specimen number and add 50µl RNase-free H_2O to the column. Do not touch the silica-gel membrane with the pipette tip. Incubate at room temperature for at least 1 minute. Elute RNA by centrifuging for 1 minute at ≥10,000 rpm. Discard RNeasy column.

4.1.10 Store at 4 C until specimen is tested on rRT-PCR. RNA should be stored at 4 C for as short of period as possible before testing. If the sample cannot be tested within 24 hours, it should be stored at -20 C or colder.

4.2 Trizol® LS Extraction for tissue samples

This procedure describes the extraction of total RNA from tissue using the Trizol® extraction reagent. The reagent is a mono-phasic solution of phenol and guanidine isothiocyanate. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase

and an organic phase. The RNA is recovered from the aqueous phase by precipitation with isopropanol.

Different systems for RNA isolation are commercially available and potentially may work as well or better than the method described here. Alternative methods of RNA extraction can be substituted if testing shows equivalency. This data should be available for review by APHIS.

Always wear protective clothing, eyewear, and latex gloves when working with extraction reagents.

4.2.1 Centrifuge tissue specimens (10-20% homogenates or tissue pools) at 1,500 x g for 30 min. Collect 250µl of the tissue supernatant and transfer to a 1.5 microcentrifuge tube. 750µl of Trizol® LS is added to the tube and sample is vortexed for 15 sec. Incubate at room temperature for 7 min.

4.2.2 Pulse spin to remove liquid from the tube lid.

4.2.3 Add 200µl 100% chloroform to the sample/Trizol homogenate. Vortex for 15 sec. Incubate at room temperature for 7 min.

4.2.4 Centrifuge at 12,000 x g for 15 min at room temperature.

4.2.5 Transfer 450µl of the upper aqueous layer to a separate microcentrifuge tube marked with sample number. **Caution: The transfer of organic phase material with the aqueous layer will inhibit the PCR reaction.** Add 500µl of 100% isopropanol. Invert tube several times to mix. Hold at room temperature for 10 min.

4.2.6 Centrifuge at 10,000 x g for 10 min at 4 C in a refrigerated microcentrifuge.

4.2.7 Decant liquid. Care should be taken to assure that the RNA pellet is not disturbed. Add 1.0 ml of 80% ethanol. Mix gently.

4.2.8 Centrifuge at 10,000 X g for 5 min at 4 C.

4.2.9 Decant ethanol. Invert tube on a clean tissue wipe and allow to air dry for a maximum of 10 min or until all visible signs of moisture are gone. It is

important not to let the RNA pellet over-dry, as this will decrease its solubility.

4.2.10 Hydrate pellet in 50µl of RNase free water and incubate at 4 C for 1 hr or overnight. Briefly vortex to resuspend pellet before pipetting.

4.3 High throughput magnet bead RNA extraction from swab specimens (Ambion® MagMax method)

This procedure describes the extraction of total RNA from swab specimens using the Ambion® extraction reagent. The MagMAX® Viral RNA Isolation Kit is designed for rapid high throughput purification of total RNA from oropharyngeal/tracheal and cloacal swab samples as well as cultured cells. The addition of guanidinium thiocyanate rapidly disrupts cellular membranes and inactivates cellular nucleases. Paramagnetic beads with a nucleic acid binding surface are added to the lysate to bind nucleic acids. The beads, containing the RNA, are then retained with the use of magnets and the supernatant containing cell debris and other contaminants is removed during the wash procedures.

Different systems for RNA isolation are commercially available and potentially may work as well as the described procedure. Alternative methods can be substituted when comparison testing of new and standard methods show equivalency. This data should be available for review by APHIS.

4.3.1 Preparation of Ambion® Viral Isolation Kit Components

Lysis/Binding Solution. See table below to determine the quantity of lysis/binding solution needed. Add 1.0 µl Poly (A) RNA (kit provided) per 50 µl lysis/binding and mix briefly. Following the addition of Poly (A) RNA add 50 µl of 100 % isopropanol to lysis/binding Poly (A) mixture to give a total volume of 101 µl per sample. Vortex well. **Lysis/binding solution is stable at room temperature for one month.** It is not recommended to store the prepared Viral Lysis/Binding Solution at 4° C or below as this may cause the carrier RNA to precipitate; if the solution is inadvertently stored at 4° C, warm it at 37° and shake to dissolve any precipitates before use.

Add carrier RNA to Lysis Solution and mix well before adding isopropanol, or carrier RNA may be very difficult to disperse.

1. Combine the following:	**1 well**	**1 Plate**	**4 Plates**
Viral Lysis/Binding Soln. Concentrate	50.0 µl	6.25 ml	25.0 ml
Carrier RNA	1.0 µl	125 µl	500 µl
2. MIX WELL, then add:			
100 % Isopropanol	50.0 µl	6.25 ml	25.0 ml
3. MIX WELL by vortexing			

4.3.1.1 Bead Resuspension Mix. Dilute the Bead Resuspension Solution with Nuclease-free Wateraccording to the table below. **Be sure to add 100% isopropanol to the mixture last or beads may clump together and be more difficult to enter into solution.**

Prepare only enough for the number of extractions being performed, or one plate at a time. Beads are only stable when mixed for one month. Store at RT.

Bead Resuspension Mix	Volume Per Well	Volume Per Plate
1. Bead Resuspension Solution	6.0 µl	750 µl
2. Nuclease-free Water	4.0 µl	500 µl
Mix briefly then add		
3. RNA Binding Beads	4.0 µl	500 µl
Mix briefly then add		
4. 100% Isopropanol	6.0 µl	750 µl
Mix well by vortexing Total Volume of Lysis/Binding Solution	20 µl	2500 µl

4.3.1.2 Wash Solution I Mix. Add 35 ml of 100% isopropanol to Wash Solution I concentrate. Mix well and date the bottle to indicate isopropanol was added. Store at RT.

4.3.1.3 Wash Solution II Mix. Add 80 ml 100% ethanol to Wash Solution II concentrate. Mix well

and date the label on the box to indicate ethanol was added. Store at RT.

4.3.2 Procedure for Extraction of RNA

4.3.2.1 Vortex swab specimen and transfer 50 µl of sample into the corresponding well on the 96 well processing plate (supplied with kit). The order of specimens in the extraction plate is stipulated by the order of specimens in the specimen rack. Each specimen in the specimen rack is identified with laboratory accession and sample number.

4.3.2.2 Place 101 µl of viral lysis/binding solution with Poly (A) RNA to each well containing sample. After addition of the lysis/binding solution, the processing plate may be removed from the BSC. All remaining steps can be performed on the deck at room temperature. Shake plate on the plate shaker at approximately 550-600 rpm for 30 sec.

4.3.2.3 Add 20 µl of RNA Binding Beads mix to each well. Shake plate on orbital shaker for 4 minutes at approximately 550-600 rpm.

4.3.2.4 Capture/pellet the RNA Binding Beads on a magnetic stand. Pellet the beads on the Ambion Magnetic Stand-96 for 2 minutes. The mixture should become transparent, indicating the capture is complete. Remove supernatant from beads (with plate still on magnet). Discard the supernatant.

4.3.2.5 Remove the plate from the magnetic stand.

4.3.2.6 Add 100 µl Wash Solution I Mix (with isopropanol added) to each well. Shake for 30 seconds at approximately 550-660 rpm. The RNA Binding Beads may not fully disperse during the step; this is expected, and it will not affect RNA purity or yield.

4.3.2.7 Pellet the beads for 1 minute and remove supernatant as in step 4.2.2.4.

4.3.2.8 Add 100 µl Wash Solution II Mix (with ethanol added) to each well. Shake for 30 seconds at approximately 550-600 rpm.

4.3.2.9 Pellet the beads for 30 seconds and remove supernatant. Discard the supernatant. Remove the processing plate from the magnetic stand.

4.3.2.10 Repeat steps 4.2.2.8-4.2.2.9 to wash a second time with Wash Solution II.

4.3.2.11 Shake vigorously for 2 minutes (shaker dial position 9 Lab Line) to briefly dry the beads. It is important to remove residual ethanol from the samples. Residual ethanol may affect RT-PCR efficiency. If necessary shake longer than 2 minutes to dry, but do not shake longer than 5.0 minutes total.

4.3.2.12 Add 50 µl of Elution Solution (RT) and shake for four minutes at approximately 1000 rpm.

4.3.2.13 Pellet the beads for 2 minutes and transfer the RNA into a sample tube, storage plate (kit provided), or rRT-PCR amplification plate.

4.3.2.14 Store at 4 C until specimen is tested on rRT-PCR. RNA should be stored at 4 C for as short of period as possible before testing. If the sample cannot be tested within 24 hours, it should be stored at -70 C.

4.4 Reverse-transcription and PCR

Two work areas are required for this procedure: a "clean" area with a dedicated BSC, freezer and supplies, and a thermal cycling area. Never introduce RNA/DNA material into the "clean" area and always change gloves before entering the "clean" area.

4.4.1 In the "clean" hood, prepare a master mix of the following reagents sufficient for the number of samples being tested. The amount given in the table is per sample.

This procedure was designed for the Cephied® Smart-Cycler (Cepheid®, Sunnyvale, CA).

Information on setting-up and programming the Smart Cycler can be found in the Smart Cycler user's manual. The conditions for the RT-PCR on the Smart Cycler are shown in tables 2 and 3.

Table 2. RT step thermocycling for Qiagen® one-step RT-PCR Kit.

RT Step	1 cycle	30 min.	50° C
		15 min.	95° C

Table 3. Thermocycling conditions for gene specific probe and primer sets.

Probe/Primer set		Step	Time	Temp
AIV matrix	45 cycles	denaturation	1 sec.	94° C
		annealing	20 sec.	60° C
H7	40 cylces	denaturation	1 sec.	94°C
		annealing	20 sec.	58°C
H5	40 cycles	denaturation	1 sec.	94°C
		annealing	20 sec.	57°C
		extension	5 sec.	72°C

Note: The fluorescence is detected at the annealing step.

4.4.2 The real-time RT-PCR reaction should be prepared with the following components and volumes using the appropriate primer and probe set and cycling conditions. Set-up the reactions with the reaction tubes in the cooling block and use aerosol resistant pipet-tips.

Prepare the reaction mix (everything but the template) by pipetting: H_2O, kit-supplied 5X reaction buffer, kit-supplied dNTP's, 25mM $MgCl_2$, and forward and reverse primers into a nuclease free microcentrifuge tube using the volumes per reaction for each reagent given in table 4. Frozen reagents, including $MgCl_2$ should be briefly vortexed and pulsed centrifuged prior to pipetting. Next, add the RNase inhibitor and enzyme. Add the probe last. Mix reagents and centrifuge briefly. Once the probe has been added to the reaction mix, minimize exposure to light.

4.4.3 Move the master mix to the RNA transfer hood and add the reaction mix (17µl) to the Smart Cycler tubes (add the mix to the bottom of the cup at the top of the reaction tube).

4.4.4 Add 8.0 µl of template RNA to the Smart Cycler reaction tube using a pipettor designated for RNA transfer. Close the lid of the tube and number the reaction tubes according to test worksheet. Transfer 8µl of diluted transcribed RNA into the positive control reaction tube and 8µl of clean RNase free water into negative control reaction tube. The transcribed RNA should be diluted by the user to a working dilution that will have a cycle threshold of approximately 25.0.

4.4.5 Centrifuge reaction tubes briefly to remove any air bubbles from the reading window of the PCR tubes. Bubbles in the reaction portion of the tube may be an indication of insufficient volume of RT-PCR master mix or the absence of sample RNA.

4.4.6 Insert reaction tubes into thermal cycler and select the designated PCR run protocol, start run and enter sample identification as well as positive and negative control information into the sample identification portion of the results table. Save run.

Table 4a. Real-time RT-PCR reaction mix volumes and conditions for type A influenza (MA gene), H5 and the H7 primer/probe set.

	Volume Per Reaction	Final Concentration
H_2O	6.95 µl	
5X buffer	5	1X
25mM $MgCl_2$	1.25	3.75 mM*
dNTP's (10 mM each)	0.8	320 µM ea. dNTP
Forward Primer (20 pmol/ul)	0.5	10 pmol/25µl
Reverse Primer (20 pmol/ul)	0.5	10 pmol/25µl
Rnase Inhibitor (13.3 units/ul)	0.5	0.266 units/µl
Enzyme Mix	1.0	
Probe (6pmol/ul)	0.5	0.12 µM
MM per rxn	17	
Template	8	
Total	25µl	

*Qiagen buffer already contains 2.5 mM $MgCl_2$ at 1X concentration

4.5 Reverse-Transcription and PCR for Matrix and H5 bead assay

4.5.1 Preparation of matrix and H5 bead reagents

Each bead is sufficient for four 25 µl reactions. Reconstitute each bead (AI matrix or H5) with the following:

RNase-free water:	60.0 µl	
dNTP Mix*	3.2 µl	
Enzyme Mix*	4.0 µl	
RNase Inhibitor+	2.0 µl	(26 units)
Total:	68.0 µl	

*From Qiagen OneStep RT-PCR Kit (Cat# 210210 or 210212)

+From Promega (Cat# N2511)

Distribute 17 µl of the above mix into 4 Smart Cycler/reaction tubes and add 8 µl of template (viral RNA). Centrifuge the tubes in Smart Cycler centrifuge.

4.5.2 Thermocycling Conditions for bead reagents

RT Step	1 cycle	30 min.	50° C	RT Step
		15 min.	95° C	
Probe/Primer set		**Step**	**Time**	**Temp**
AIV matrix	40cycles	denaturation	20 sec.	94° C
		annealing	20 sec.	60° C
H5	40 cycles	denaturation	1 sec.	94°C
		annealing	20 sec.	54°C
		extension	15 sec.	72°C

5. Data Analysis Settings for the Cepheid® Smart Cycler

The Smart Cycler software provides multiple methods for determining the cycle threshold (C_t). All data analysis options discussed below are selected from the **Analysis Settings** from the **View Results** screen. Changes in the standard default settings have been made to customize the analysis for the detection of AIV with the matrix, H5 and H7 primer/probe sets. Edit the Smart Cycler default analysis settings as described below so the raw data will be analyzed according to the analysis parameters described.

Curve Analysis – Accept the "**Primary curve"** setting – The C_t is detected and reported at the cycle where the primary curve crosses the threshold.

Usage – Accept the default setting "**Assay"** for the FAM channel.

Background Subtraction – Accept the default value of "**ON"**

Background Minimum – accept the default value of "**5"**(Min). The first cycle used to calculate the background if the background subtraction is **ON**.

Background maximum cycle – Enter **"28"** as the maximum cycle for the calculation of the background if the background subtraction is **ON.** The default is 40.

Threshold Settings – accept the default of "**Manual".**

Manual Threshold Fluorescence units – Enter **"25"** fluorescent units. It is critical that the C_t be above the background fluorescence. The closer the threshold is set to the background fluorescence the more sensitive the detection limit. However, if the threshold is set too close to the background fluorescence, background noise could cross the threshold and be reported incorrectly as a positive sample. By lowering the threshold fluorescence units from 30 (default value) to 25 the analytic sensitivity of the assay is increased. The possibility of reporting a false negative is reduced while the possibility of detecting a false positive is increased.

Boxcar Average – accept the default of "**0"**

5.1 Verification of test results

The only changes from the Cepheid® Smart Cycler default were the Max background and threshold settings. With these analysis settings a specimen will be called positive (crosses the C_t) when the fluorescence units exceeds 25 units. These settings were designed to optimize the discrimination of positive and negative specimens for this particular protocol, but the results from each run still need to be verified by the user. The curve should be in the log-linear phase when crossing the threshold. By lowering the Max background, the incidence of traces with a "V" shape is reduced. For samples that still have a "V" shaped trace, the Max background setting can be incrementally lowered to 15 until the early cycles in the trace are approximately horizontal and aligned with zero fluorescence (Figures 2a and 2b). When lowering the Max background keep in mind that you want as many cycles as possible used to calculate the background subtraction. This is a correction on how the software handles the data and it decreases the number of cycles used to calculate the background and correct the curve. If you have questions about the trace, look at the trace with the background subtraction off. This shows the raw fluorescence data, and can sometimes aid interpretation.

Background fluorescence should be monitored routinely. Probe degration is indicated by an increase in background fluorescence.

The fluorescence trace of each specimen should be reviewed before accepting the Smart Cycler positive/negative result. Any specimen whose fluorescence trace has a gradual increase in fluorescence units and approaches, but does not cross the C_t, should be sent to the NVSL for further testing. These specimens are referred to as "late risers" (LR) and on some occasions may indicate a sample near the threshold of detection. At this time the significance of "late riser" samples is not known and testing by virus isolation is recommended.

5.2 Interpretation of internal control results (matrix bead)

Assay systems for the bead reagents were optimized with the Cepheid® Smart Cycler II system. The results of amplification of viral RNA should be viewed on FAM channel and that of IC on Texas Red™/ROX™ channel (emission 606-650 nm). The C_t values corresponding to the IC should read between 34 and 38 (valid range) in the absence (negative control) or presence of template RNA. The IC C_t value for the valid range may increase depending on storage conditions and time. The beads should be stored in the foil packet at 4C for a maximum of 6 months. Non-optimal storage conditions may lead to degradation of the IC. Internal control results should be interpreted as questionable if the IC C_t is higher than 38. The negative (no template) control is used as the positive control for the IC. The C_t value should be evaluated on each run as the stability of the IC could change depending on handling and storage of beads. Since the IC and the viral RNA compete for the same set of primers the IC will only be detectable when the viral RNA is either limiting or absent in the sample. When the viral RNA has a C_t of 30 or lower, viral amplification (AIV specific) will out compete the IC.

Table 5a. Interpretation of Internal Control Results

IC Texas Red C_t	Viral FAM C_t	Interpretation of IC C_t	Interpretation of viral C_t
Negative	27.33	Negative –out competed	Positive
38.93	30.91	Negative – not valid	Positive
35.21	37.59	Positive – valid	Suspect
35.21	0.00	Positive – valid	True negative
0.00	0.00	Negative	False negative
38.0 or higher	0.00	No Test – non-valid C_t	No Test

The H5 bead reagents will detect both Eurasian H5 subtype viruses (for example the Asian H5N1) and North American H5 subtype viruses. Virus lineage can not be differentiated with this test.

6. Analysis of test results

6.1 Specificity of matrix, H5 and H7 assays

The matrix AIV assay is designed to detect all subtypes of AIV, whereas the H5 and H7 subtype-specific assays were designed to aid in the rapid identification of North American strains H5 and H7 AIV. The H5 assay has been demonstrated to successfully detect the Asian strain of H5N1 AIV. Because the matrix AIV test is more sensitive (approximately 10-fold) than either the H5 or the H7, the matrix AIV test is used to screen all samples. Samples positive by the matrix and either of the H5 or H7 assays provides strong evidence that the sample contains AIV RNA. However, samples positive by the matrix rRT-PCR but negative by the subype specific H5 and H7 assays could still contain H5 or H7 RNA if the quantity of RNA is below the detection limit of the assays, or if a subype other than H5 or H7 is causing the infection. These specimens should be considered to be suspect samples.

The matrix, H5 and H7 assays have been designed to err on the side of detecting false positive specimens. As a result, specimens with a C_t of 35 or higher are considered suspect positives. Samples with a C_t of 35 or higher should be repeated on the rRT-PCR.

If positive results, including those with C_t's above 35, are obtained with repeat testing the original specimen should be

transferred to NVSL for confirmational testing. Suspect samples as well as H5 and H7 positive specimens should be transferred to the NVSL as quickly as possible for confirmation and further testing. Additional samples may need to be collected for further testing. All suspect specimens and those only positive by the matrix rRT-PCR should be confirmed by virus isolation before reporting as positive. Any questionable samples should be re-tested. If results of the second test are unsatisfactory additional sampling from the flock or premises should be considered if possible.

Confirmation by virus isolation is required before a foreign animal disease (FAD) will be "officially" diagnosed.

6.2 Expected C_t for the transcribed RNA positive control

The transcribed RNA positive control should be diluted to a working concentration that will consistently give a target C_t of approximately 25.0. Any test run where the positive control has a C_t higher than 29.0 should be repeated to assure that test reproducibility is maintained. If the C_t of the positive control consistently runs lower than 20.0, recalibration/dilution of the positive control is suggested. Any time the positive control or any other growth curve C_t is lower than 14.0 the background subtraction can be skewed.

6.3 Recommendations for evaluating fluorograms

Evaluation of the fluorogram with the following conditions may be helpful in determining results manually. Importantly, each specimen should be analyzed independently:

6.3.1 Identification of weak positives.

Remove all reactions with greater than 100 units increase in fluorescence from the graph (this changes the scale, making it easier to identify weak positives). (Figures 1a and 1b). Looking at each sample alone will also help to identify weak positive reactions.

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Figure 1a. Example of a fluorogram from samples run on the Smart Cycler. Background subtraction is on. All analysis criteria are set to the customized default values. Note that scale is from 0 to 1000 fluorescence units (Y axis), making it difficult to evaluate weak positive samples.

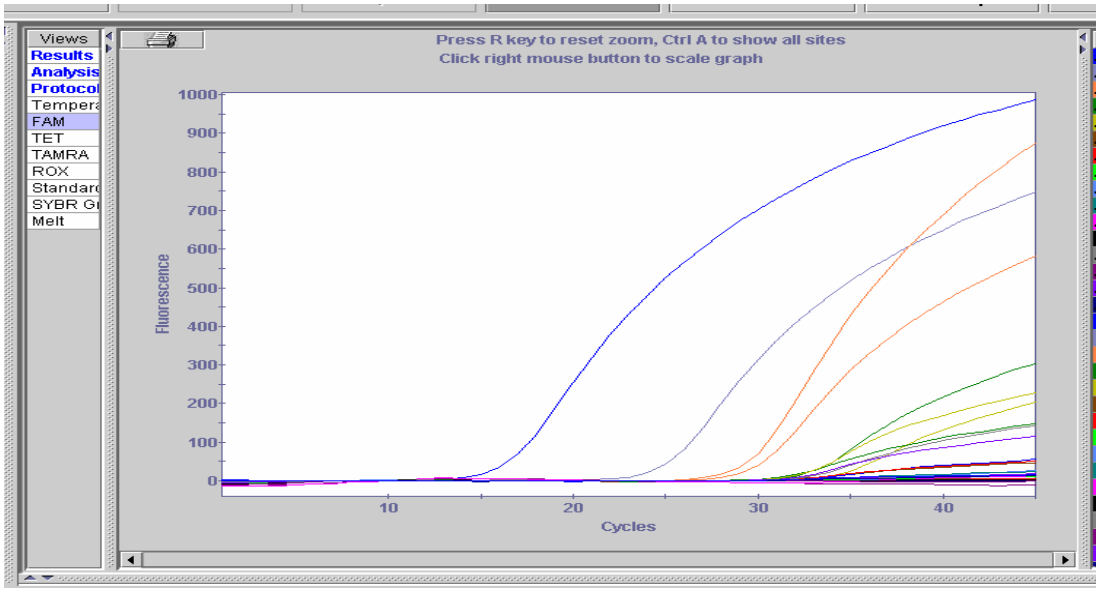
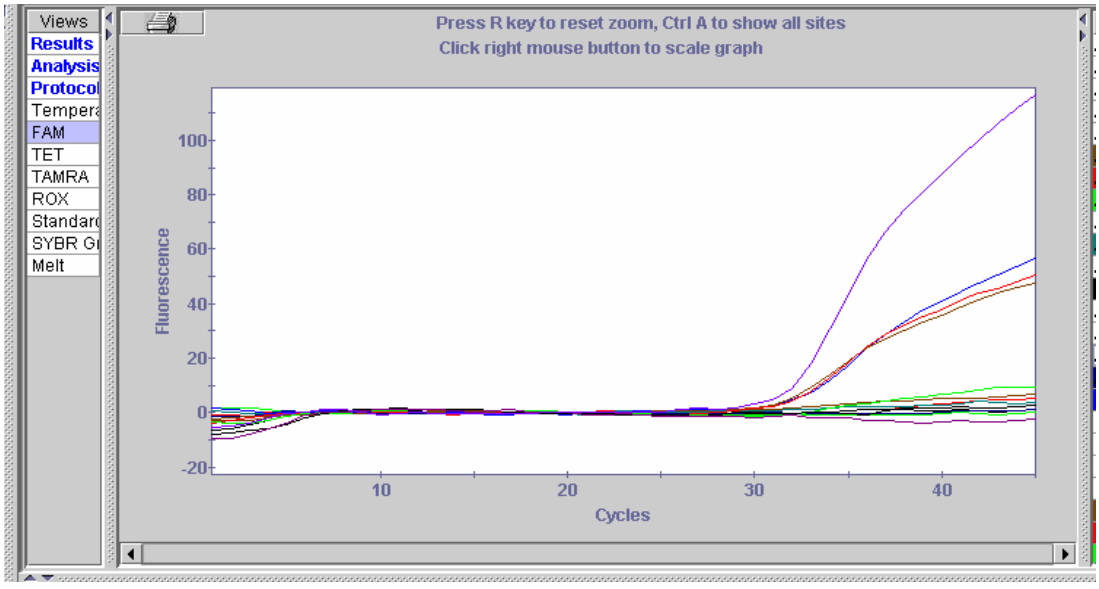


Figure 1b. Same fluorogram as figure 1a, however, all samples which increased greater than 100 units in fluorescence were removed from the graph. Note that the scale is from 0 to 120 fluorescence units (Y axis) making it easier to recognize weak positives.



6.3.2 If there are samples which have a "V" shaped fluorescence trace, lower the "background maximum cycles" (analysis settings table) until the early cycles are approximately horizontal and aligned with zero fluorescence (Figures 2a and 2b). The correct value for "background maximum cycles" is the cycle at the base of the "V", about 18 for figure 2a.

Figure 2a. Example of a "V" shaped fluorescence trace. The background maximum cycle is set to the default of 40 (circled). All other analysis criteria are set to the default values. The negative control is shown for reference (horizontal line at zero).

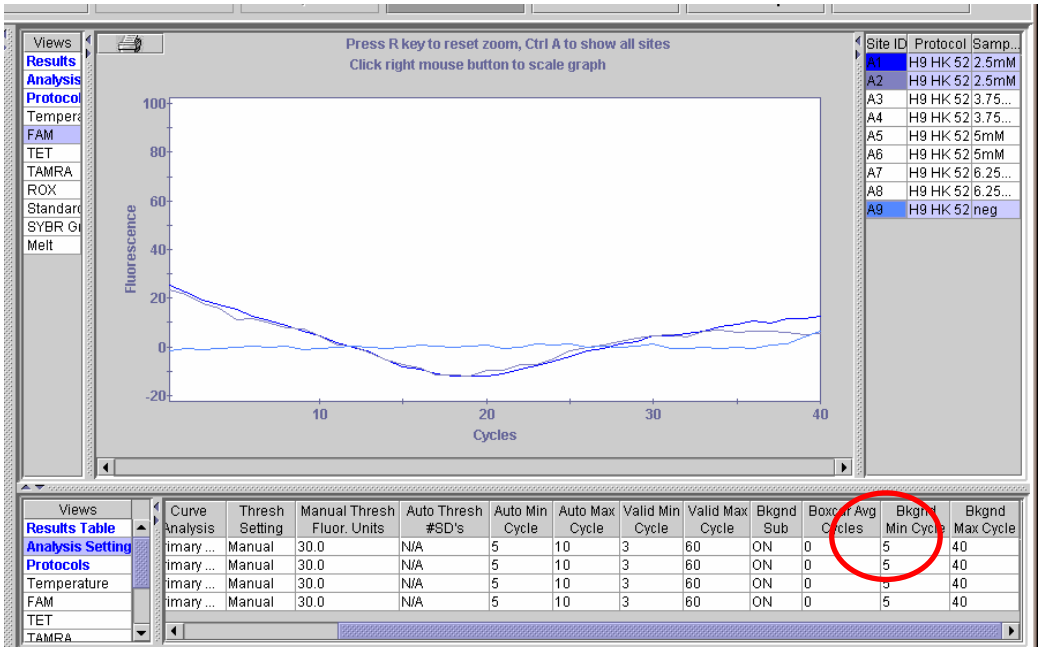
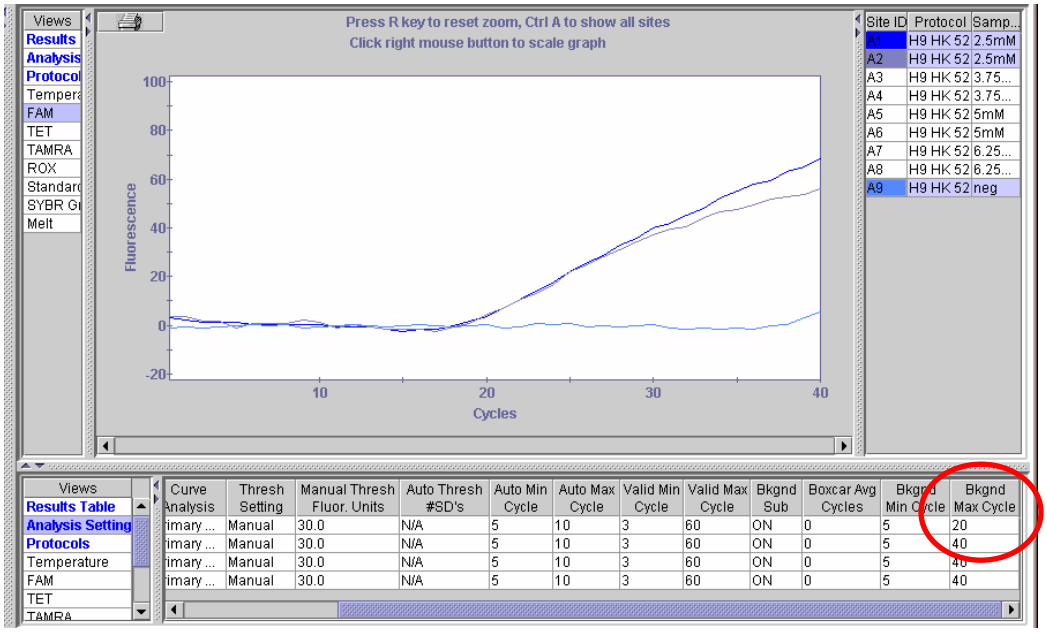


Figure 2b. Same fluorogram as figure 2a, however the background maximum cycles have been reduced to 20 (circled) to align the background fluorescence at 0 units. All other analysis criteria are set to the default values. The negative control is shown for reference (horizontal line at zero).



7. References

7.1 Office of International des Epizooties Manual of Standards for Diagnostic Tests and Vaccines, Fourth edition 2000. pp. 221-222.

7.2 Spackman, E., Senne, D.A., Myers, T.J. et al. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Micro. 40:3256-3260.

7.3 U.S. Department of Health and Human Services Public Health Service, Centers for Disease Control and Prevention and National Institutes of Health, Manual of Biosafety in Microbiological and Biomedical Laboratories, Fourth edition 1999.

7.4 Panigrahy, B., Senne, D.A., Pedersen, J.C., Avian Influenza virus subtypes inside and outside the live bird markets, 1993-2000: a spatial and temporal relationship. Avian Dis. 46:298-307.

8. Summary of Revisions

8.1 The recommended quencher dye was changed from TAMRA to BHQ-1. Cepheid is currently marketing only the Smart Cycler II system which is not calibrated for use with the TAMRA dye as a quencher.

8.2 The volume of BHI for the collection of swab pool specimens was changed from 2.0 ml per tube to 2-3.5 ml per tube. (Section 1.1)

8.3 The concentration of Rnase Inhibitor in Table 4a was corrected. Version 1510.01 listed the use dilution of Rnase Inhibitor as 6.65 units/50µl (Table 4a) and the final concentration of Rnase Inhibitor as 0.266 units. The correct use dilution of Rnase Inhibitor is 13.3 units/µl. The correct final concentration is still 0.266 units/µl.

8.4 The nucleotide sequence for primer H5-1685 (Table 1) was changed from 5'-AgA CCA gCT **AAC** ATg ATT gC-3' to 5'-AgA CCA gCT **ACC** ATg ATT gC-3'. The change was made to be consistent with that published by Spackman, et. al. J. Clin. Micro. 40:3256-3260.2002.

8.5 The Ambion® MagMax 96-well magnetic bead RNA isolation procedure was added as an approved method for the extraction of RNA from swab specimens. Equivalency validation conducted at the NVSL demonstrated equivalency between the Ambion® MagMax and Qiagen® RNeasy procedures.

8.6 The manual threshold fluorescence units was lowered from the default of 30 to 25 (Section 5.0). The threshold was lowered to increase analytical sensitivity. Analytical sensitivity testing to evaluate the change from 30 units to 25 units was conducted at the Southeast Poultry Research Laboratory prior to implementing the change.

8.7 Reagent codes and instructions for requesting positive control transcribed RNA were added to section 3.4.8.

8.8 Matrix and H5 reagent bead instructions for preparation (section 3.5), thermal cycling (section 4.5.1) and interpretation of results were included in the protocol. Reagent beads for the H7 assay are not available at this time.