



Assay Performance Characteristics Summary Sheet

United States
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Marketing and
Regulatory
Programs

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Assay: Avian Influenza H5 real-time reverse transcriptase - polymerase chain reaction (rRT-PCR) assay

Disease: Avian Influenza (AI)

Type of Assay: rRT-PCR assay

Purpose of Assay: Screening (NAHLN), Confirmatory (NVSL reference laboratories)

Background Information: The AI USDA/NVSL rRT-PCR H5 assay was developed as a collaborative effort between the Southeast Poultry Research Laboratory (SEPRL), USDA, ARS and NVSL, APHIS. This assay is intended to be used with RNA extracted from diagnostic samples and viral cultures for the detection of H5 AI viral RNA. The H5 rRT-PCR assay targets a conserved region of the H5 hemagglutinin gene and has been shown to detect viral RNA from North American lineages of H5 AI viruses. The assay has been validated for detection of H5 in avian species and specimens. The assay will detect both highly pathogenic avian influenza (HPAI) and low pathogenicity avian influenza (LPAI) H5 viral RNA but will not differentiate HPAI from LPAI. Due to the diverse genetic coding of the H5 hemagglutinin gene, the H5 assay will not detect all lineages of H5, but has been shown to detect the Asian lineage of H5N1, as well as North American H5 lineage viruses. Specificity and sensitivity for detection of the Eurasian lineage of H5 was increased by modifying the assay to include both the North American (NA) and Eurasian (EA) forward primers in the original validated NA H5 assay.

The AI assay was initially validated on the Cepheid Smart Cycler with Qiagen One-Step[®] RT-PCR chemistry and Qiagen RNeasy[®] silica column and Trizol[®] RNA extraction procedures. Subsequently, this assay is shown to be equivalent when performed with the Applied Biosystems (AB) 7500 and 7900 instruments with Ambion MagMAX[™] magnetic beads for the extraction of RNA.

Sample Type: Tracheal/oropharyngeal (TR/OP) swabs, cloacal (CL) swabs, tissue, and tissue pools

Species: Avian

Performance Characteristics:

Analytical sensitivity performance characteristics were determined using titered H5 AI viruses obtained from the SEPRL reference collection and in-vitro transcribed RNA. Analytical specificity performance characteristic was determined using representative avian H5 AI viruses from the SEPRL and NVSL reference collections. The rRT-PCR procedure was compared to virus isolation for determination of the diagnostic sensitivity and specificity with migratory wild bird CL swabs specimens.

Cepheid Smart Cycler: Spackman ¹	
Analytical Sensitivity (transcribed RNA)	10 ⁻³ - 10 ⁴ gene copies or 100 fg RNA
Analytical Sensitivity (titered virus)	10 ¹ EID ₅₀

Total Samples Tested: n=>2179

Cepheid Smart Cycler	Cutoff Ct Value ≤40 (95% CI)
Diagnostic Sensitivity-wild birds	79%
Diagnostic Specificity-wild birds	89%

Platforms:

Cepheid Smart Cycler, AB7500, AB7900

Chemistries:

Qiagen One-Step[®] RT-PCR: Smart Cycler, AB7900, and AB7500

Ambion Ag-Path[™]: Smart Cycler AB7900 and AB7500

Diagnostic Performance Characteristics:

Diagnostic performance characteristics for wild birds was determined using CL swabs collected during the 2006 migratory wild bird surveillance conducted by the U.S. Departments of Agriculture (USDA) and Interior (DOI) and State Wildlife Agencies.

Cepheid Smart Cycler	Cutoff Ct Value ≤40	Data Source	Specimen Basis
Diagnostic Sensitivity- wild birds	79%	2006 wild bird surveillance	Per specimen
Diagnostic Specificity-wild birds	89%	2006 wild bird surveillance	Per specimen

Total samples tested: n= 2179

Analytical Performance Characteristics:

Analytical Sensitivity: Analytical sensitivity performance characteristics were determined using titered H5 AI viruses obtained from the SEPRL reference collection and H5 in-vitro transcribed RNA.

Cepheid Smart Cycler	
Analytical Sensitivity (transcribed RNA)	10 ³ - 10 ⁴ gene copies or 100 fg RNA
Analytical Sensitivity (titered virus)	10 ¹ EID ₅₀

Analytical Specificity: The H5 gene NA/EA assay was tested with RNA obtained from wild bird and poultry-origin influenza virus isolates representing 15 AI HA subtypes as well as isolates of poultry respiratory pathogens other than AI; avian paramyxovirus, infectious bronchitis virus, avian metapneumovirus, and infectious laryngotracheitis virus.

Isolate	Subtype	+/-	Isolate	Subtype	+/-	Isolate	Subtype	+/-
DK/NJ/7717-70/95	H1N1	-	CK/MA/11801/86	H5N2	+	CK/Germany/N/48	H10N7	-
Mal/NY/6750/78	H2N2	-	Avian/NY/31588-2/00	H5N2	+	CK/NJ/15906-6/96	H11N1	-
DK/Vic/9211-18-1400/92	H3N8	-	CK/NJ/17169/96	H5N2	+	DK/LA/188B/87	H12N5	-
DK/Alb/286/78	H4N8	-	DK/Malaysia/97	H5N3	+	Gull/MD/704/77	H13N6	-
MuteSwan/MI/451072-2/06	H5N1	+	Mallard/PA/454069/06	H5N4	+	Mallard/Gurjev/263/82	H14N5	-
Duck/PA/454069/05	H5N1	+	TY/WI/68	H5N9	+	Shearwater/W. Australia/2576/79	H15N6	-
Mall/OH/184/86	H5N1	+	CK/NY/14677-13/98	H6N2	-	Avian paramyxovirus	APMV 1-4, 6-9	-
Swan/Mongolia/05	H5N1	+	TY/PA/7975/97	H7N2	-	Infectious Laryngotracheitis virus		-
CK/VN/NDVD/03	H5N1	+	TY/Ontario/6118/67	H8N4	-	Infectious Bronchitis	Mass	-
CK/Puebla/8629-602/94	H5N2	+	CK/NY/1220/97	H9N2	-	Avian metapneumovirus	Colorado	-

Cepheid Smart Cycler:

Performance Characteristics:

Analytical performance characteristics were determined using titrated H5 AI viruses obtained from the SEPRL reference collection. Analytical performance characteristics have been shown to be reproducible. Qiagen One-Step® RT-PCR and Ambion Ag-Path™ chemistries have been validated for use with the Cepheid Smart Cycler. The Ambion Ag-Path™ chemistry was compared to the Qiagen One-Step® using a linear regression model and was shown to be approximately 1% more efficient in amplification resulting in lower Cts values and a slightly higher limit of detection.

Cepheid® SmartCycler: “Wet” Qiagen One-Step® RT-PCR	
Analytical Sensitivity (transcribed RNA)	10 ³ -10 ⁴ gene copies or 100 fg RNA
Analytical Sensitivity (titered virus)	10 ¹ EID ₅₀

Intra-assay Variation: Intra-run replicates of serially diluted H5 viral and in-vitro transcribed RNA over the linear range of the assay demonstrated minimal variation in cycle threshold values (50 replicates). A variation of approximately 1 log (3.33 Ct) was demonstrated in 2 of 40 replicates. The remaining replicates demonstrated a variability of < 2.0 Ct.

Inter-assay Variation: Inter-assay evaluation demonstrated less than 1 log of variability in the limit of detection (LOD) with Qiagen One-Step® RT-PCR chemistry and a slightly higher variability in the LOD with Ambion Ag-Path™ chemistry.

Applied Biosystems: AB7500

Performance Characteristics:

Assay modifications, length of time required for collection of fluorescence and amplification, required for the AB7500 did not alter the diagnostic or analytical specificity of the assay. Analytical sensitivity was determined using in vitro-transcribed H5 gene RNA. A standardized procedure (AVSOP1521) for AB software data interpretation was developed based on AI matrix, H7, H5, APMV-1 matrix and vNDV analytical tests. The Ambion Ag-Path™ chemistry was compared to the Qiagen One-Step® using a linear regression model and was shown to be approximately 1% more efficient in amplification resulting in lower Cts values and a slightly higher limit of detection. Qiagen One-Step® RT-PCR and Ambion Ag-Path™ chemistries have been validated for use with the Cepheid Smart Cycler.

ABI7500: Qiagen One-Step RT-PCR® “Wet” Chemistry	
Analytical Sensitivity	10 ² gene copies

Intra-assay Variation: Intra-run replicates of serially diluted H5 virus over the linear range of the assay demonstrated minimal (<1.5 Ct) variation in cycle threshold values (50 replicates).

Inter-assay Variation: Inter-assay evaluation demonstrated less than 1 log of variability in the LOD.

Applied Biosystems: AB7900:

Performance Characteristics:

Assay modifications, length of time required for collection of fluorescence and amplification, required for the AB7900 did not alter the diagnostic or analytical specificity of the assay. Analytical sensitivity was determined using in vitro-transcribed H5 gene RNA. A standardized procedure (AVSOP1523) for AB software data interpretation was developed based on AI matrix, H7, H5, APMV-1 matrix and vNDV analytical tests.

Analytical Sensitivity

10² gene copies

Intra-assay Variation: Intra-run replicates of serially diluted viral and H5 in-vitro transcribed RNA over the linear range of the assay demonstrated minimal (< 2.0 Ct) variation in cycle threshold values (40 replicates).

Inter-assay Variation: Inter-assay evaluation demonstrated approximately 1 log of variability in the LOD.

References:

1. Spackman, E., D. Senne, T. J. Meyers, L.L. Bulaga, L.P. Garber, M.L. Perdue, K. Lohman, L. T. Daum, D. L. Suarez. 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. 40.9:3256-3260.