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## Assay Performance Characteristics Summary Sheet

**Assay:** Avian Influenza Matrix 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 matrix assay was developed as a collaborative effort between the Southeast Poultry Research Laboratory (SEPRL), USDA, ARS and USDA, APHIS. This assay is intended to be used with RNA extracted from diagnostic samples for the detection of type A AI viral RNA. The AI rRT-PCR assay targets a conserved region of the matrix gene and will detect viral RNA from any type A influenza virus, regardless of species or hemagglutinin subtype. The assay will detect both highly pathogenic avian influenza (HPAI) and low pathogenicity avian influenza (LPAI) viruses but will not differentiate HPAI from LPAI.

The AI assay was initially validated on the Cepheid Smart Cycler with Qiagen One-Step<sup>®</sup> RT-PCR chemistry and Qiagen RNeasy<sup>®</sup> silica column and Trizol<sup>®</sup> RNA extraction. Subsequently, this assay has been shown to be equivalent when performed with Applied Biosystems (AB) 7500 and 7900 instruments with Ambion MagMAX<sup>™</sup> magnetic beads for the extraction of RNA. Ambion Ag-Path<sup>™</sup> chemistry has been shown to be equivalent for the AB7500 and Cepheid Smart Cycler instruments.

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

**Species:** All species

**Performance Characteristics:**

Analytical sensitivity and repeatability performance characteristics were determined using titered H5 and H7 AI viruses (representing 2 of 15 subtypes) obtained from the SEPRL reference collection. Analytical specificity performance characteristics were determined using representative avian, equine, swine and human H1-H15 AI viruses. Diagnostic performance characteristics were determined with chicken and turkey TR/OP swabs specimens collected during the 2002 outbreak of H7N2 LPAI in Virginia. Estimates of the diagnostic sensitivity and specificity for chickens and turkeys were evaluated by Elvinger<sup>1</sup> using Bayesian modeling.

Cepheid Smart Cycler: Spackman <sup>3</sup>	
Analytical Sensitivity	10 <sup>-1</sup> EID <sub>50</sub> / 10 <sup>3</sup> gene copies
Analytical Specificity	100%

**Total Samples Tested: n=>3,600**

Cepheid Smart Cycler	Cutoff Ct Value ≤45 (95% CI)
Diagnostic Sensitivity-turkeys	85.1 (71.9-95.7)
Diagnostic Specificity-turkeys	98.9 (98.0-99.5)
Diagnostic Sensitivity-chickens	86.3 (65.9-97.0)
Diagnostic Specificity-chickens	99.2 (98.9-99.6)

**Platforms:**

Cepheid Smart Cyclers, AB7500, AB7900

**Chemistries:**

Ambion MagMAX™: Cepheid Smart Cyclers, AB7500 and AB7900

Qiagen One Step® RT-PCR: AB7500, AB7900, Cepheid Smart Cyclers

**Diagnostic Performance Characteristics:**

Diagnostic performance characteristics were determined using TR/OP and CL swabs collected during the 2002 outbreak of LPAI H7N2 in Virginia and West Virginia and from LBM surveillance in NY, NJ and PA. The 2002 H7N2 data has been evaluated by Dr. Francois Elvinger<sup>1</sup> (Table 2), NVSL and SEPRL. The LBM data was evaluated by Dr. Erica Spackman<sup>2</sup>. The rRT-PCR procedure was compared to virus isolation for determination of the estimated diagnostic sensitivity and specificity relative to virus isolation.

Cepheid Smart Cycler	Cutoff Ct Value ≤45 (95% CI)	Data Source	Specimen Basis
Diagnostic Sensitivity-avian	66.7%*	2002 Spackman <sup>3</sup>	Per Specimen
Diagnostic Specificity-avian	94.9%	2002 Spackman <sup>3</sup>	Per Specimen
Diagnostic Sensitivity-poultry	95.1%	2002 Senne <sup>2</sup>	Per submission
Diagnostic Specificity-poultry	99.1%	2002 Senne <sup>2</sup>	Per submission

Total samples tested: n = >3,600 (2002 H7N2) and n = 1550 (LBM)

\* Cloacal swabs, which have been subsequently shown to be problematic for Qiagen silica column extraction, were included in the LBM validation study.

**Analytical Performance Characteristics:**

**Analytical Sensitivity:** Analytical sensitivity performance characteristics were determined using titrated H5 and H7 AI viruses (representing 2 of 15 subtypes) obtained from the SEPRL reference collection. Analytical performance characteristics have been shown to be reproducible.

Cepheid Smart Cycler	
Analytical Sensitivity	10 <sup>-1</sup> EID <sub>50</sub>
Analytical Specificity	100%

**Analytical Specificity:** The matrix gene primer and probe set was tested with RNA obtained from wild bird and poultry-origin influenza virus isolates representing 15 AI HA subtypes and isolates of human, equine, and swine origin 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/NY/14677-13/98	H6N2	+	DK/England/56	H11N1	+
Mal/NY/6750/78	H2N2	+	TY/PA/7975/97	H7N2	+	DK/LA/188B/87	H12N5	+
Env/NY/19019-6/98	H3N8	+	CK/PA/13552-1/98	H7N2	+	Gull/MD/704/77	H13N6	+
DK/Vic/9211-18-1400/92	H3N8	+	Quail/AR/16309/94	H7N3	+	Mallard/Gurjev/263/82	H14N5	+
DK/Alb/286/78	H4N8	+	CK/NY/8030-2/96	H7N2	+	Shearwater/W. Australia/2576/79	H15N6	+
CK/Puebla/8629-602/94	H5N2	+	CK/New South Wales/1688/97	H7N4	+	Aichi/68	H3N2	+
CK/MA/11801/86	H5N2	+	TY/NE/505577/07	H7N9	+	Equine/KY/211/87	H3N8	+
Avian/NY/31588-2/00	H5N2	+	TY/Ontario/6118/67	H8N4	+	Swine/MN/9088/89	H3N2	+
CK/NJ/17169/96	H5N2	+	CK/NY/1220/97	H9N2	+	Swine/IN/1726/89	H1N1	+
Mall/OH/184/86	H5N1	+	CK/Korea/96006/96	H9N2	+	Avian metapneumovirus	Subtype C	-
Swan/Mongolia/05	H5N1	+	CK/Germany/N/48	H10N7	+	Avian paramyxovirus	APMV-1-4, 6-9	-
CK/VN/NDVD/03	H5N1	+	TY/VA/31409/91	H10N7	+	Infectious Bronchitis	Mass	-
DK/Malaysia/97	H5N3	+	CK/NJ/15906-6/96	H11N1	+	Infectious Laryngotracheitis virus		-

### **Cepheid Smart Cycler:**

#### **Performance Characteristics:**

Analytical performance characteristics were determined using titered H5 and H7 AI viruses (representing 2 of 15 subtypes) obtained from the SEPRL reference collection. Analytical performance characteristics have been shown to be reproducible. The Ambion Ag-Path™ chemistry was compared to Qiagen One-Step® using a linear regression model and was shown to be approximately 1% more efficient in amplification resulting in lower Ct 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.

<b>Cepheid® SmartCycler: “Wet” Qiagen One-Step® RT-PCR</b>	
Analytical Sensitivity (titered virus)	10 <sup>-1</sup> EID <sub>50</sub>
Analytical Sensitivity (transcribed RNA)	10 <sup>2</sup> gene copies

***Intra-assay Variation:*** The absolute value of the intra-run replicates over the linear range of the assay on the Cepheid Smart Cycler was 0.15 with a standard deviation absolute difference of 0.12. Intra-run replicates of serially diluted AI viral RNA over the linear range of the assay demonstrated minimal (< 1.0 Ct) variation in cycle threshold values in 3 of 75 replicates, the remaining 3 replicates demonstrated a variability of < 2.5 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 matrix 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 ABI7500.

#### **ABI7500: Qiagen One-Step® RT-PCR “Wet” Chemistry**

Analytical Sensitivity	10 <sup>1</sup> gene copies
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***Intra-assay Variation:*** Intra-run replicates of serially diluted AI viral RNA over the linear range of the assay demonstrated minimal (< 1.0 Ct) variation in cycle threshold values.

***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: 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 matrix 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.

#### **ABI7900: Qiagen One-Step RT-PCR “Wet” Chemistry**

Analytical Sensitivity	10 <sup>1</sup> – 10 <sup>2</sup> gene copies
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***Intra-assay Variation:*** The absolute value of the intra-run replicates over the linear range of the assay on the AB7900 was 0.75 with a standard deviation absolute difference of 0.68.

***Inter-assay Variation:*** Inter-assay evaluation demonstrated less than 1 log of variability in the limit of detection (LOD).

#### **References:**

1. Elvinger, F., B. Akey, D. A. Senne, E. Spackman, D. Suarez. 2007. Characteristics of diagnostic tests used in the 2002 low pathogenicity avian influenza H7N2 outbreak in Virginia. *J Vet Diag Invest* submitted.
2. Senne, D. A. 2002. H7N2 LPAI Outbreak - VA 2002. Proceedings of the 53rd North Central Avian Disease Conference, Minneapolis. 6-8 October 2002.
3. 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.