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Performance Assessment of Avian Influenza Virus Analytical Reference Materials for Diagnostic Surveillance: Subtypes H5N1, H7N9, H7N7, H5N6, and H9N2

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Background and Introduction

Avian influenza viruses (AIV) have become a public health issue in recent years and impose substantial burdens on the poultry and dairy industries. Reliable detection of new or emerging strains is dependent on the use of accurate analytical reference materials (ARMs); without them, diagnostic tests may yield false results that undermine surveillance and public health efforts. In response to recent outbreaks, ATCC® developed quantitative synthetic RNA products for some of the most concerning AIV subtypes: H5N1, H5N6, H7N7, H7N9, and H9N2. These were developed to serve as high quality ARMs suitable for use in BSL-1 facilities. In the following study, we experimentally verified the utility of the products with published quantitative PCR assays from sources such as the U.S. Centers for Disease Control and Prevention (CDC) and Food and Drug Administration (FDA) as well as published RT-LAMP assays and the BioFire FilmArray RP2.1 Respiratory Panel, demonstrating that these ARMs can serve as reliable and safe controls for molecular assays used in diagnostics and surveillance.

Table 1: ATCC® Synthetic RNA for Avian Influenza

ATCC® Item	Subtype	Reference Strain for Design
VR-3436SD™	H5N1	A/white-tailed eagle/Japan/OU-1/2022
VR-3437SD™	H7N9	A/Shanghai/4664T/2013
VR-3438SD™	H7N7	A/chicken/Wenzhou/334b/2013
VR-3439SD™	H5N6	A/goose/Guangdong/GS018/2015
VR-3440SD™	H9N2	A/ostrich/Yunnan/438/2014

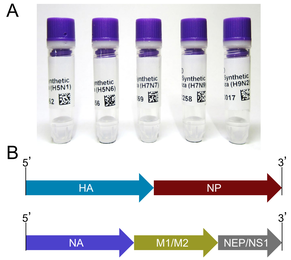

Figure 1: ATCC® Synthetic RNA for Avian Influenza. (A) Image of ATCC® synthetic RNA vials. (B) Graphical representation of each item's two synthetic transcripts, annotated with their corresponding genomic regions.

Materials and Methods

qRT-PCR

- Synthetic AIV RNA items were tested at concentrations ranging from 50-50,000 genome copies/reaction (GC/rxn), and when available, genomic RNA (gRNA) was tested at concentrations ranging from 50-5000 GC/rxn.
- The assays used were published by the FDA,[1] CDC,[2] or from highly cited papers.[3]
- Amplification was achieved using Invitrogen SuperScript III Platinum One-Step qRT-PCR Kit (Thermo Fisher Scientific) (Figures 2A-B, Figure 3) or QIAGEN One-Step RT qPCR Kit (Figure 2C) on the CFX Opus Real-Time PCR System (Bio-Rad).

RT-LAMP

- AIV A/H5 and A/H7 synthetic RNA items were evaluated with RT-LAMP assays from Ahn *et al.* 2019[4] using the WarmStart Colorimetric LAMP 2X Master Mix with UDG (New England Biolabs).

BioFire Film Array RP2.1 Panel

- Synthetic AIV RNA items were tested at 100×, 10×, and 3× limit of detection (LoD) for influenza A detection, which is reported in the kit details as 1.4 × 10^2 copies/mL.[5]

Results

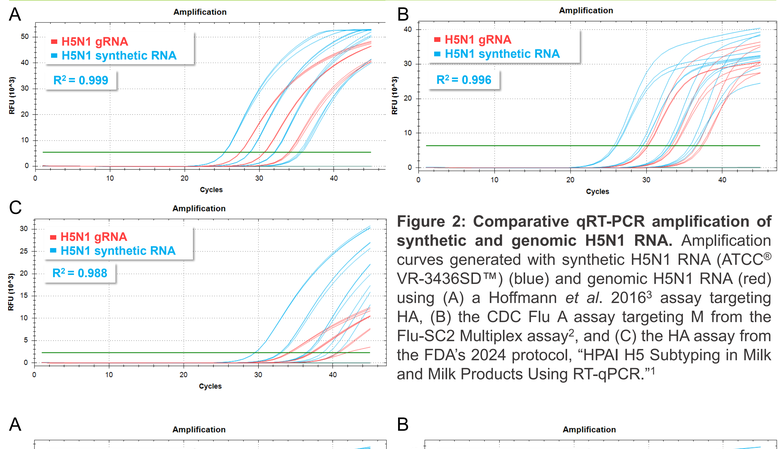

Figure 2: Comparative qRT-PCR amplification of synthetic and genomic H5N1 RNA. Amplification curves generated with synthetic H5N1 RNA (ATCC® VR-3436SD™) (blue) and genomic H5N1 RNA (red) using (A) a Hoffmann *et al.* 2016[3] assay targeting HA, (B) the CDC Flu A assay targeting M from the Flu-SC2 Multiplex assay[2], and (C) the HA assay from the FDA's 2024 protocol, "HPAI H5 Subtyping in Milk and Milk Products Using RT-qPCR."[1]

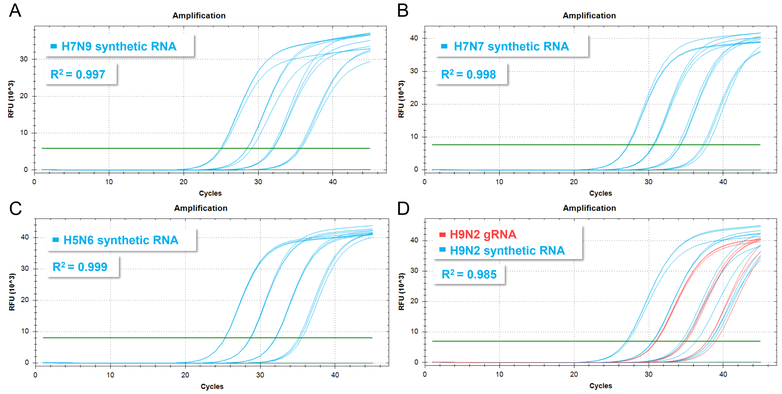

Figure 3: Comparative qRT-PCR amplification of the M gene across synthetic and genomic avian influenza RNA. Amplification curves generated using the CDC Flu A assay targeting M from the Flu-SC2 Multiplex assay[2] with (A) synthetic H7N9 RNA (ATCC® VR-3437SD™), (B) synthetic H7N7 RNA (ATCC® VR-3438SD™), (C) synthetic H5N6 RNA (ATCC® VR-3439SD™), and (D) synthetic H9N2 RNA (ATCC® VR-3440SD™) and genomic H9N2 RNA.

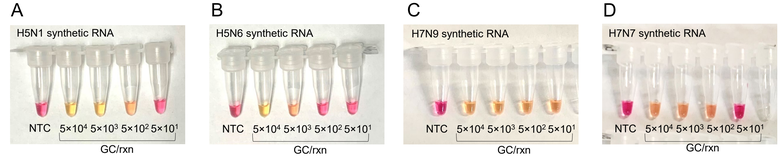

Figure 4: RT-LAMP results using assays from Ahn *et. al.* 2019.[4] The H5 assay from the paper was tested with (A) synthetic H5N1 RNA (ATCC® VR-3436SD™) and (B) synthetic H5N6 RNA (ATCC® VR-3439SD™), and the H7 assay was tested with (C) synthetic H7N9 RNA (ATCC® VR-3437SD™) and (D) synthetic H7N7 (ATCC® VR-3438SD™). From left to right, the PCR strip tubes contain a no-template control (NTC), 5 ×10^4 GC/rxn, 5 ×10^3 GC/rxn, 5 ×10^2 GC/rxn, and 5 ×10^1 GC/rxn of synthetic RNA.

Results (continued)

Table 2: Detection of ATCC® Synthetic Avian Influenza RNA Products with the BioFire Diagnostics FilmArray RP2.1 Respiratory Panel (bioMérieux)

ATCC® Item	Subtype	Diluent	× LOD		
			100	10	3
VR-3436SD™	H5N1	0.25 mg/mL Poly(A)	+	+	+
		Molecular-grade water	+	+	-
VR-3437SD™	H7N9	0.25 mg/mL Poly(A)	+	+	-
VR-3438SD™	H7N7	0.25 mg/mL Poly(A)	+	+	+*
VR-3439SD™	H5N6	0.25 mg/mL Poly(A)	+	+	+
VR-3440SD™	H9N2	0.25 mg/mL Poly(A)	+	+	+

*Detection failed on first attempt, successfully detected in retest of same sample

Conclusions

- ATCC® quantitative synthetic AIV RNA for subtypes H5N1, H7N9, H7N7, H5N6, and H9N2 function as reliable, well-characterized ARMs across multiple molecular detection platforms.
- These ARMs are compatible with numerous published qRT-PCR assays and RT-LAMP assays and performed robustly with the BioFire FilmArray RP2.1 Respiratory Panel.

ATCC Influenza Resources

References

1. FDA, HPAI H5 Subtyping in Milk and Milk Products Using RT-qPCR, 2024.
2. CDC, Research Use Only CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay Real- Time RT-PCR Primers and Probes, CDC, 2020.
3. Hoffmann B, *et al*. Riems influenza a typing array (RITA): An RT-qPCR-based low density array for subtyping avian and mammalian influenza a viruses. Sci Rep 6: 27211, 2016. PubMed: 27256976.
4. Ahn SJ, *et al*. BMC Rapid and simple colorimetric detection of multiple influenza viruses infecting humans using a reverse transcriptional loop-mediated isothermal amplification (RT-LAMP) diagnostic platform. Infect Dis 19(1):676, 2019. PubMed: 31370782.
5. BIOFIRE Respiratory Panel 2.1 (RP2.1). BFR0000-8579-03, 2023.

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