Development of an Avian Influenza H5N1, H7N9, H7N7, H5N6, and H9N2 Analytical Reference Material Set for Diagnostic Surveillance

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Background and Introduction

Highly pathogenic avian influenza (HPAI) viruses, especially H5N1 clade 2.3.4.4b, have become a major public health issue and have severely impacted the poultry and dairy industries. Accurate analytical reference materials (ARMs) are essential for reliable detection of new or emerging strains. Without reliable ARMs, diagnostic tests may yield false results, undermining surveillance and public health efforts. ARMs are vital for calibrating diagnostic assays to ensure they accurately detect these viruses and measure viral load.

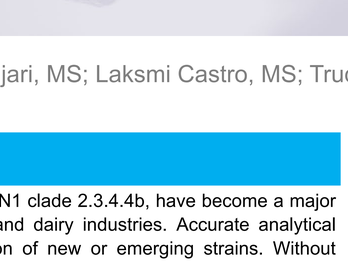
In response to the rapid outbreaks, ATCC® developed quantitative synthetic RNA for some of the most concerning HPAI virus serotypes: H5N1, H5N6, H7N7, H7N9, and H9N2. The H5N1 product is based on a recent clade 2.3.4.4b strain. Each ARM contains near complete sequences from segments 4, 5, 6, 7, and 8, covering 50% of the whole influenza genome, including key diagnostic targets. These ARMs are manufactured using reliable synthetic biology technology, verified by next-generation sequencing, quantitated via Droplet Digital PCR (Bio-Rad), and safe for use as positive controls in BSL-1 settings. We evaluated these ARMs using several published quantitative PCR assays and show that they can serve as reliable and safe controls for molecular assays used in diagnostics and surveillance.

ATCC® Quantitative Synthetic Avian Influenza Viral RNA

ATCC® Catalog Number	Influenza Subtype
ATCC® VR-3436SD™	H5N1
ATCC® VR-3437SD™	H7N9
ATCC® VR-3438SD™	H7N7
ATCC® VR-3439SD™	H5N6
ATCC® VR-3440SD™	H9N2

Applications

- Generation of a standard curve for quantitative PCR
- Positive control for qPCR assays
- Assay verification and validation studies
- Monitor assay-to-assay and lot-to-lot variation
- Molecular diagnostics assay development

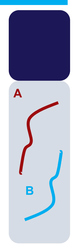


Breen M, et al. Viruses 8(7): 179, 2016. PubMed: 27347991

Materials and Methods

Quantitative Synthetic RNA

- We have implemented a two-transcript design to accommodate as many of the diagnostically relevant segments of the influenza genome as possible.
- The diagnostically relevant genome segments that we identified after a systematic literature review of over 260 influenza PCR assays were segments 4 (HA), 5 (NP), 6 (NA), 7 (M1/M2), and 8 (NEP/NS1). We have accommodated nearly the whole HA and NP genes on Transcript A and the whole M1/M2, NA, and NEP/NS1 genes on Transcript B.
- Both transcripts are quantified by Droplet Digital PCR (Bio-Rad) and fall within the range of 1×10^5 and 1×10^6 copies/µL.
- Here, we show qPCR data generated on the CFX Opus Real-Time PCR Systems (Bio-Rad). Amplification for Figures 1-5 was achieved using the Invitrogen SuperScript III Platinum One-Step qRT-PCR Kit.



Results

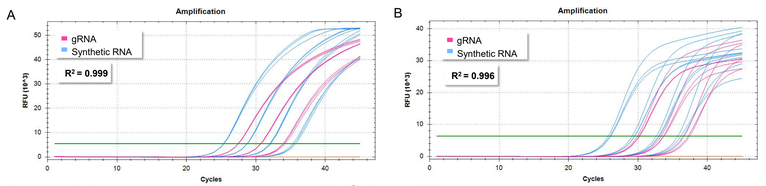


Figure 1: qPCR amplification curves generated with ATCC® VR-3436SD™ (subtype **H5N1**) (blue) and H5N1 gRNA (pink) using (A) a Hoffmann *et al.*, 2016 assay targeting **HA,** and the (B) CDC Flu SC2 Multiplex assay targeting **M**.

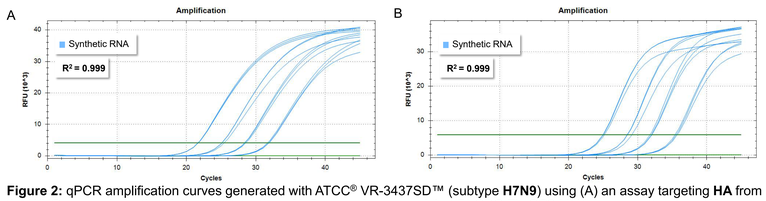


Figure 2: qPCR amplification curves generated with ATCC® VR-3437SD™ (subtype **H7N9**) using (A) an assay targeting **HA** from the WHO protocols for the Molecular Detection of Influenza viruses, and the (B) CDC Flu SC2 Multiplex assay targeting **M**.

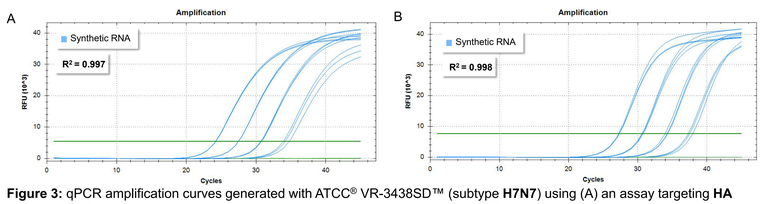


Figure 3: qPCR amplification curves generated with ATCC® VR-3438SD™ (subtype **H7N7**) using (A) an assay targeting **HA** from the WHO protocols for the Molecular Detection of Influenza viruses, and the (B) CDC Flu SC2 Multiplex assay targeting **M**.

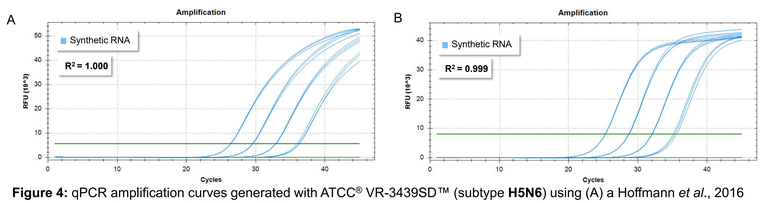


Figure 4: qPCR amplification curves generated with ATCC® VR-3439SD™ (subtype **H5N6**) using (A) a Hoffmann *et al.*, 2016 assay targeting **HA,** and the (B) CDC Flu SC2 Multiplex assay targeting **M**.

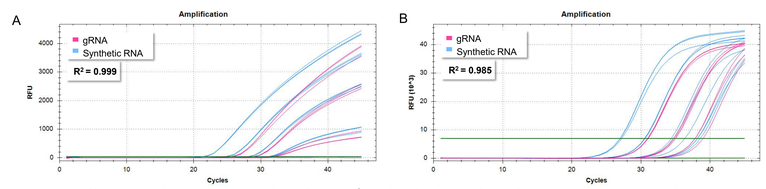


Figure 5: qPCR amplification curves generated with ATCC® VR-3440SD™ (subtype **H9N2**) using (A) a Hassan *et al.*, 2022 assay targeting **HA**, and the (B) CDC Flu SC2 Multiplex assay targeting **M**.

Results (continued)

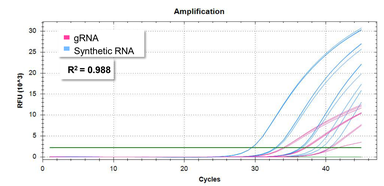


Figure 6: qPCR amplification of ATCC® VR-3436SD™ (blue) and H5N1 gRNA (pink) with the assay used by the FDA to test milk samples for H5 influenza virus contamination. The QIAGEN One-Step RT qPCR kit was used to achieve this amplification, per the FDA protocol. Cycling conditions were 50°C for 50 min and 95°C for 15 min, followed by 45 cycles of 95°C for 15 sec, 64°C for 1 min, and 90°C for 1 min and 10 sec. From these data, we show that VR-3436SD™ can be used as an analytical reference material.

Table 1. Summary of qPCR assays tested with the 5 quantitative synthetic avian influenza RNA products..

Influenza Subtype	Publication Source	Assay Target
H5N1	Hoffmann, *et al.,* 2016	HA
	CDC Flu SC2 Multiplex Assay, 2020	M
	FDA Milk Assay, 2024	HA
H7N9	WHO, Molecular Detection of Influenza viruses, 2021	HA
	CDC Flu SC2 Multiplex Assay, 2020	M
H7N7	WHO, Molecular Detection of Influenza viruses, 2021	HA
	CDC Flu SC2 Multiplex Assay, 2020	M
H5N6	Hoffmann, *et al.*, 2016	HA
	CDC Flu SC2 Multiplex Assay, 2020	M
H9N2	Hassan, *et al.*, 2022	HA
	CDC Flu SC2 Multiplex Assay, 2020	M

Conclusions

- Our data demonstrate that the ATCC® quantitative synthetic avian influenza viral RNA products can be used as reliable analytical reference materials for assay development, verification, and validation.
- The products can be used to generate a standard curve with qPCR assays to determine the viral load of samples.
- These analytical reference materials are compatible with numerous published assays and are shown here to serve as a useful controls for viral detection and quantification. Lists of known compatible assays from primary literature and public health organizations are available on each product page in the technical data sheet.

ATCC Influenza Resources

References

- CDC, Research Use Only CDC Influenza SARS-CoV-2 (Flu SC2) Multiplex Assay Real-Time RT-PCR Primers and Probes, CDC, 2020.
- Breen M, et al. Viruses 8(7): 179, 2016. PubMed: 27347991.
- FDA, HPAI H5 Subtyping in Milk and Milk Products Using RT-qPCR, 2024.
- WHO Information for the Molecular Detection of Influenza Viruses, 2021.
- Hassan KE, et al. Viruses 14(2): 415, 2022. PubMed: 35216008.
- Hoffmann B, et al. Sci Rep 6: 27211, 2016. PubMed: 27256976.

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