Development of Avian and Human Influenza Analytical Reference Materials for Diagnostics and Surveillance

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Background and Introduction

Human and highly pathogenic avian influenza (HPAI) viruses pose a significant public health risk due to their potential for widespread illness and economic consequences. Early detection and control of outbreaks rely on effective surveillance and diagnostic testing.

ATCC® developed a comprehensive suite of quantitative synthetic analytical reference materials (ARMs) for HPAI virus serotypes H5N1, H5N6, H7N7, H7N9, and H9N2; human influenza A virus serotypes H1N1, H3N2, and H1N1 2009 pandemic; and Influenza B virus strains. Each synthetic ARM contains the complete sequences from segments 4, 5, 6, 7, and 8, including the HA, NP, NA, M1, M2, NS1, and NEP/NS1 genes, covering 50% of the influenza genome. These segments are key diagnostic targets for molecular tests and provide sufficient genomic context for assessing assay specificity. These ARMs are manufactured using a highly reliable synthetic biology technology, verified through next-generation sequencing, and quantified via Droplet Digital PCR (Bio-Rad Laboratories, Inc.). Furthermore, they do not contain any viable material and can be handled in a BSL-1 setting. As such, they are intended to serve as safe and reliable positive controls for molecular tests for surveillance and diagnostics.

The synthetic ARMs were experimentally evaluated using several published quantitative PCR assays, including those from the Centers for Disease Control and Prevention, the World Health Organization, the World Organization for Animal Health, and other highly cited sources. We conducted an *in silico* assessment of ARM compatibility with over 250 publicly available published assays. The synthetic products displayed equal performance to genomic RNA during all tests.

ATCC® Quantitative Synthetic Influenza Viral RNA

ATCC® Catalog Number	Influenza Subtype
ATCC® VR-3384SD™	B (Victoria)
ATCC® VR-3385SD™	B (Victoria)
ATCC® VR-3386SD™	H1N1
ATCC® VR-3387SD™	H3N2
ATCC® VR-3388SD™	H1N1 pdm09
ATCC® VR-3436SD™	H5N1
ATCC® VR-3437SD™	H7N9
ATCC® VR-3438SD™	H7N7
ATCC® VR-3439SD™	H5N6
ATCC® VR-3440SD™	H9N2

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

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

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 entire 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

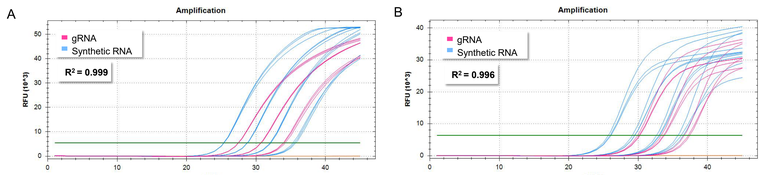

Figure 1: qPCR amplification curves generated with ATCC® VR-3436SD™ (subtype **H5N1**) (blue) and H5N1 gRNA (pink) using (A) a Hoffmann *et al*., 2016 assay targeting **HA,** and the (B) CDC Flu SC2 Multiplex assay targeting **M**.

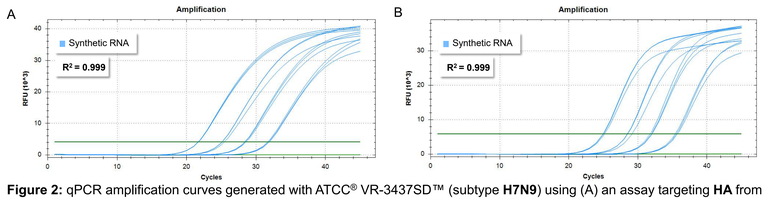

Figure 2: qPCR amplification curves generated with ATCC® VR-3437SD™ (subtype **H7N9**) using (A) an assay targeting **HA** from the WHO protocols for the Molecular Detection of Influenza viruses, and the (B) CDC Flu SC2 Multiplex assay targeting **M**.

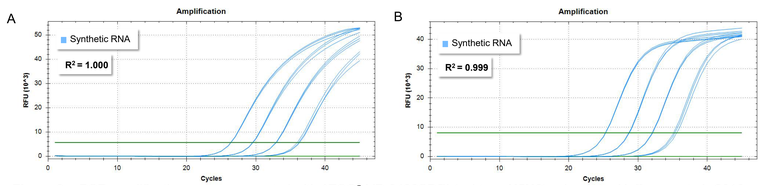

Figure 3: qPCR amplification curves generated with ATCC® VR-3439SD™ (subtype **H5N6**) using (A) a Hoffmann *et al*., 2016 assay targeting **HA,** and the (B) CDC Flu SC2 Multiplex assay targeting **M**.

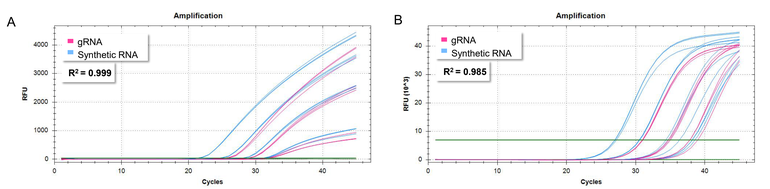

Figure 4: qPCR amplification curves generated with ATCC® VR-3440SD™ (subtype **H9N2**) using (A) a Hassan *et al*., 2022 assay targeting **HA**, and the (B) CDC Flu SC2 Multiplex assay targeting **M**.

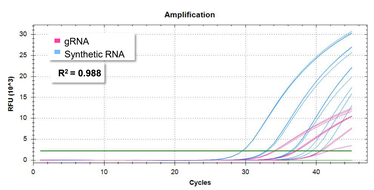

Figure 5: qPCR amplification of ATCC® VR-3436SD™ (blue) and H5N1 gRNA (pink) with the assay used by the FDA to test milk samples for H5 influenza virus contamination. The QIAGEN One-Step RT qPCR kit was used to achieve this amplification, per the FDA protocol. Cycling conditions were 50°C for 50 min 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.

Results (continued)

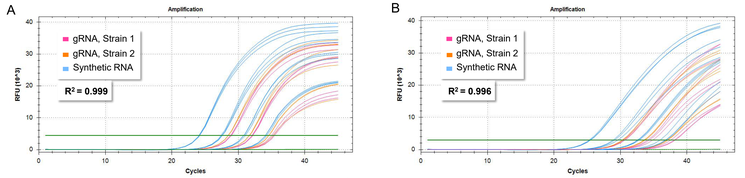

Figure 6: qPCR amplification curves generated with ATCC® VR-3388SD™ (subtype **H1N1 pdm09**) (blue) and gRNA from two different H1N1 pdm09 strains (pink & orange) using (A) an assay targeting **HA** and (B) an assay targeting **NA** from the WHO protocols for the Molecular Detection of Influenza viruses.

Table 1. Summary of qPCR assays tested with the quantitative synthetic influenza RNA products shown here.

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
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
H1N1 pdm09	WHO, Molecular Detection of Influenza viruses, 2021	HA
	WHO, Molecular Detection of Influenza viruses, 2021	NA

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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