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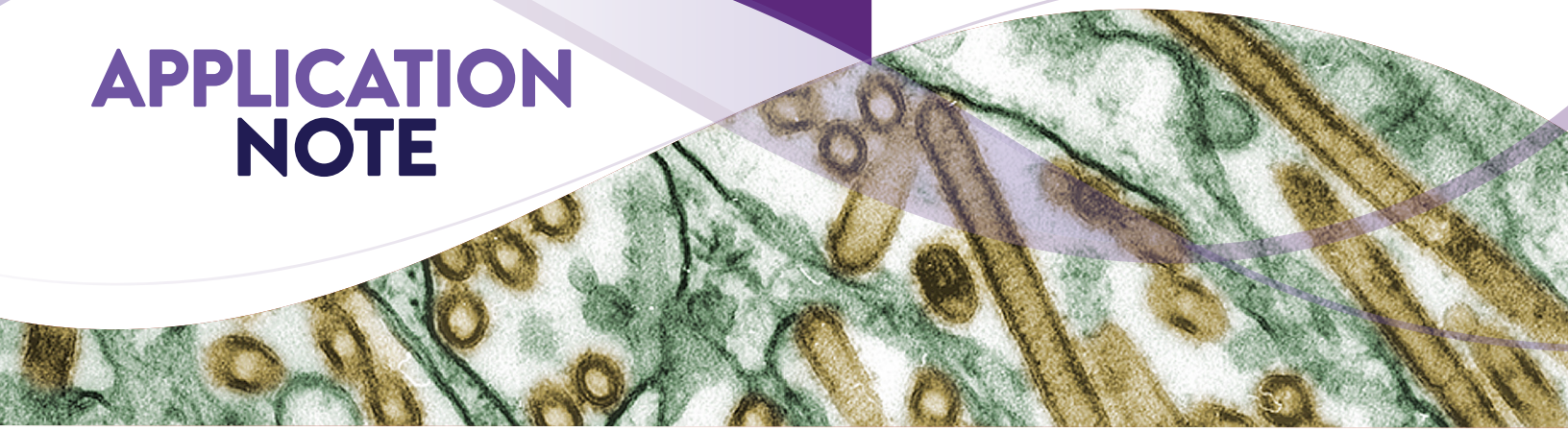
APPLICATION NOTE



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

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Abstract

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. To meet this need, ATCC® developed quantitative synthetic RNA ARMs for some of the most concerning AIV subtypes: H5N1, H5N6, H7N7, H7N9, and H9N2. These ARMs are manufactured using reliable synthetic biology technology, verified by next-generation sequencing, quantified via digital-based PCR, and designed for safe use in BSL-1 settings as reliable positive controls for molecular tests. Here, we demonstrate the functionality of the ARMs as positive controls across several published quantitative PCR assays and commercially available platforms.

Introduction

Avian influenza viruses (AIV) are an ongoing public health issue and a source of significant economic losses. Although AIVs do not typically infect humans, sporadic cases have occurred due to exposure to intermediate hosts, most notably pigs, where more adaptable variants can emerge.[1,2] Human AIV infection can present with typical influenza symptoms such as fever, cough, sore throat, and muscle aches, and in some cases conjunctivitis or gastrointestinal distress. In severe cases, illness may progress to include difficulty breathing, pneumonia, and death.[3]

In the United States, a few cases of infection with AIV subtype H5N1 have been reported among dairy workers, most of whom presented primarily with conjunctivitis.[4] Outbreaks of H5N1 in humans have been documented since 1997, mainly in southeast Asia, and are often associated with severe disease and case-fatality rates as high as 50%.[5] Other AIV subtypes have also caused concern. Human infections with H7N9, for instance, have been documented in China since 2014; like H5N1 infections, these infections are frequently associated with severe clinical cases. Other AIV subtypes that have caused human infections within recent history include H3N8, H5N6, H7N3, H7N7, and H9N2.[5,8]

Beyond human health, AIVs exert substantial economic impact. In the United States, H5N1 has affected more than 130 million birds since 2022,[5] contributing to major economic losses in the poultry and egg industries.[6] These outbreaks have been attributed to a specific subgroup of H5N1 viruses designated clade 2.3.4.4b. This H5N1 clade has also been the cause of AIV outbreaks in dairy cows since 2024, which has resulted in reduced milk output and subsequent economic impact. Since then, the United States Department of Agriculture

(USDA) and Food and Drug Administration (FDA) have been testing milk samples for the presence of H5N1 RNA to monitor public health risk.[7]

To support surveillance and public health efforts, high-quality analytical reference materials (ARMs) are essential for calibrating diagnostic assays, ensuring accuracy, and improving reliability, while reducing the risk of false results. In response to recent outbreaks, ATCC® developed quantitative synthetic RNA 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 reputable sources such as the Centers for Disease Control and Prevention (CDC), World Health Organization (WHO), and World Organization for Animal Health (WOAH), 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.

Materials and Methods

To develop the synthetic AIV RNA ARMs for subtypes H5N1, H5N6, H7N7, H7N9, and H9N2, we performed a systematic literature review of over 260 influenza PCR assays to identify diagnostically relevant genome segments. We implemented a two-transcript design in each ARM to accommodate segments 4 (HA gene), 5 (NP gene), 6 (NA gene), 7 (M1/M2 genes), and 8 (NEP/NS1 genes), which, in total, account for ~50% of the influenza genome. The transcript sequences of each ARM closely match the sequences of genome segments from specific strains of the respective subtypes (Table 1), with the H5N1 synthetic RNA using a clade 2.3.4.4b genome as the design reference. One transcript of each ARM contains nearly the entire HA and NP genes, and the other contains the complete NA, M1/M2, and NEP/NS1 genes. Both transcripts were quantified by digital-based PCR and fall within a range of 1×10^5 and 1×10^6 genome copies/µL. Further, each ARM was manufactured under ISO 13485 guidance and stabilized in a proprietary storage buffer, which enables a 5-year-long shelf life and ensures consistent results.

We evaluated the ARMs using published quantitative reverse transcriptase PCR (qRT-PCR) assays from public health organizations such as the CDC, WHO, and WOAH, or from highly cited papers (Table 2). All oligonucleotides were ordered from Integrated DNA Technologies, and TaqMan probes were tagged with 6-FAM and double-quenched. The qRT-PCR data were generated on the CFX Opus Real-Time PCR System (Bio-Rad). Amplification of qRT-PCR was achieved using the Invitrogen SuperScript III Platinum One-Step qRT-PCR Kit (Thermo Fisher Scientific) for all assays except one; for the assay from the FDA's 2024 protocol "HPAI H5 Subtyping in Milk and Milk Products Using RT-qPCR,"[7] we used the QIAGEN One-Step RT qPCR kit to achieve amplification per the FDA protocol. Cycling parameters and oligonucleotide concentrations emulated conditions reported in each source publication. Synthetic RNA was 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. We obtained gRNA for subtypes H5N1 (BEIR NR-59885) and H9N2 (BEIR NR-43016) from BEI Resources (Manassas, VA).

In addition to qRT-PCR verification, we assessed the synthetic products' performance by reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) using the WarmStart Colorimetric LAMP 2X Master Mix with UDG (New England Biolabs). We tested H5- and H7-specific RT-LAMP assays from Ahn *et al.* 2019[9] with 50-50,000 GC/rxn of synthetic RNA. The H5 assay was tested with synthetic H5N1 RNA (ATCC® VR-3436SD™) and synthetic H5N6 RNA (ATCC® VR-3439SD™), and the H7 assay was tested with synthetic H7N9 RNA (ATCC® VR-3437SD™) and synthetic H7N7 RNA (ATCC® VR-3438SD™). We also verified product compatibility of all five synthetic AIV RNA products with the BioFire Film Array RP2.1 Respiratory Panel at 100×, 10×, and 3× limit of detection (LoD) for influenza A detection. The samples for qRT-PCR and RT-LAMP and most samples for FilmArray testing were diluted in 0.25 mg/mL Poly(A) buffer prepared from Roche lyophilized Poly(A) (Millipore Sigma product # 10108626001). Select samples were diluted in molecular grade water for FilmArray testing to assess the diluent impact on detection.

Table 1: ATCC® synthetic AIV RNA

ATCC® Item	Influenza A 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

Table 2: Published qRT-PCR assays used to experimentally verify ATCC® synthetic AIV RNA ARMs

ATCC® Item	Influenza A Subtype	Publication Source	Assay Target
VR-3436SD™	H5N1	Hoffmann *et al*., 2016[10]	HA
		CDC Flu SC2 Multiplex Assay, 2020[11]	M
		FDA Milk Assay, 2024[7]	HA
		WHO, Molecular Detection of Influenza viruses, 2021[12]	NA
VR-3437SD™	H7N9	WHO, Molecular Detection of Influenza viruses, 2021[12]	HA
		CDC Flu SC2 Multiplex Assay, 2020[11]	M
VR-3438SD™	H7N7	WHO, Molecular Detection of Influenza viruses, 2021[12]	HA
		CDC Flu SC2 Multiplex Assay, 2020[11]	M
VR-3439SD™	H5N6	Hoffmann *et al*., 2016[10]	HA
		CDC Flu SC2 Multiplex Assay, 2020[11]	M
VR-3440SD™	H9N2	Hassan *et al*., 2022[13]	HA
		CDC Flu SC2 Multiplex Assay, 2020[11]	M

Results

To evaluate the performance and utility of the ATCC® quantitative synthetic AIV RNA (Table 1) in qRT-PCR applications, we conducted experiments using published assays (Table 2) from either public health organizations or influential papers. For each synthetic AIV RNA preparation, we verified performance using an HA-targeted qRT-PCR assay specific to the corresponding subtype (i.e., H5, H7, H9) as well as the universal influenza A assay from the CDC Flu-SC2 Multiplex,[11] which targets the M gene.

Comparative amplification results for qRT-PCR assays targeting HA from Hoffmann *et al*. 2016[10] (Figure 1A) and M from the CDC Flu-SC2 Multiplex[11] assay (Figure 1B), demonstrate efficient and comparable performance of both synthetic and genomic H5N1 RNA. For the H5N1 RNA ARM, we also tested an HA assay from the FDA protocol for milk testing[7] (Figure 1C), which is used to screen milk for H5 RNA during the ongoing outbreaks of clade 2.3.4.4b in dairy cows. Here, we observed that the Cq values for the synthetic RNA and gRNA were comparable despite the discrepancy in fluorescent signal between them. For the H7N9, H7N7, or H5N6 ARMs, though we did not have corresponding gRNA available for comparison, we observed efficient and linear amplification of the synthetic RNA ARMs with HA and M assays (Figures 2-4). For the H9N2 RNA, we again see comparable amplification performance between the synthetic RNA and gRNA (Figure 5).

We also experimentally verified the performance of the synthetic avian flu RNA ARMs from H5 and H7 subtypes with RT-LAMP assays according to the paper by Ahn *et al*., 2019[9] (Figure 6). The positive RT-LAMP reactions resulted in a color change of the phenol red pH indicator from pink to yellow due to decreased pH caused by polymerase activity, allowing visual confirmation of amplification. For the H5 assays, we observed positive RT-LAMP reactions down to 500 GC/rxn for synthetic H5N1 RNA (ATCC® VR-3436SD™) and 5000 GC/rxn for synthetic H5N6 RNA (ATCC® VR-3439SD™) after 60 minutes of LAMP at 65°C; whereas for the H7 assays, we observed detection down to 50 GC/rxn for H7N9 RNA (ATCC® VR-3437SD™) and 500 GC/rxn for synthetic H7N7 RNA (ATCC® VR-3438SD™) after 60 minutes of LAMP at 65°C.

Finally, all five synthetic avian flu RNA products were assessed for compatibility with the BioFire FilmArray RP2.1 Respiratory Panel at 100×, 10×, and 3× LoD for influenza A detection (Table 3). When the synthetic RNA ARMs were diluted in a 0.25 mg/mL Poly(A) buffer, we were able to detect as low as 3× LoD (LoD = 140 copies/mL for influenza A detection per the RP2.1 handbook[14]) for all five synthetic RNA products. Although the synthetic H7N7 RNA (ATCC® VR-3438SD™) sample at 3× LoD was not initially detected, the retest of the same sample was successfully detected, thus indicating some stochasticity at this low concentration. Synthetic H5N1 RNA (ATCC® VR-3436SD™) was tested with dilutions prepared in molecular grade water as well as 0.25 mg/mL Poly(A) and we observed a loss in sensitivity as compared to the same RNA diluted in Poly(A). The 3× LoD samples prepared in molecular grade water could not be detected initially or after sample retest; however, the RNA diluted in molecular grade water to 10× LoD could still be detected. It should be noted that the RP2.1 panel can distinguish between human flu subtypes A/H1, A/H3, and A/H1-2009 but not between any avian flu subtypes, so all successful detections were categorized as "Influenza A – No subtype detected."

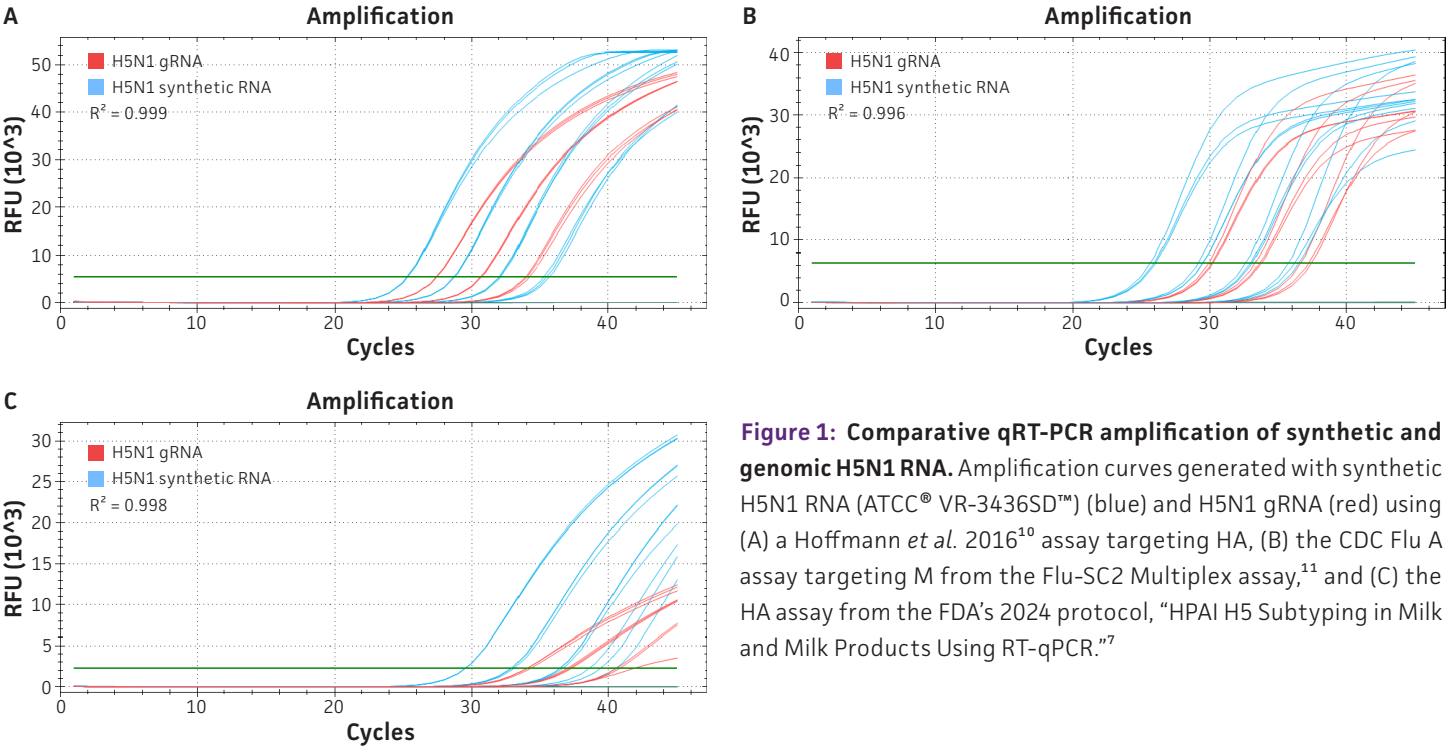

Figure 1: Comparative qRT-PCR amplification of synthetic and genomic H5N1 RNA. Amplification curves generated with synthetic H5N1 RNA (ATCC® VR-3436SD™) (blue) and H5N1 gRNA (red) using (A) a Hoffmann *et al.* 2016[10] assay targeting HA, (B) the CDC Flu A assay targeting M from the Flu-SC2 Multiplex assay,[11] and (C) the HA assay from the FDA's 2024 protocol, "HPAI H5 Subtyping in Milk and Milk Products Using RT-qPCR."[7]

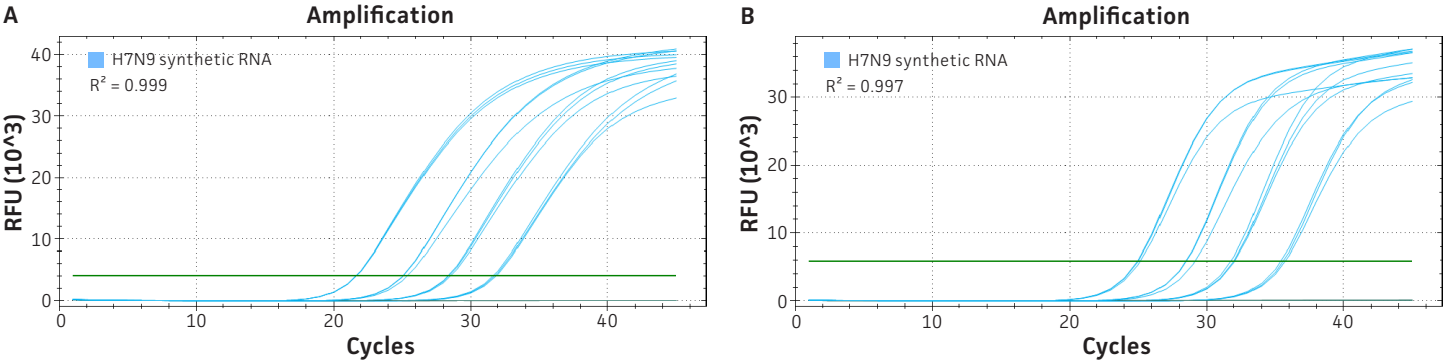

Figure 2: qRT-PCR amplification of synthetic H7N9 RNA. Amplification curves generated with synthetic H7N9 RNA (ATCC® VR-3437SD™) using (A) an assay targeting HA from the WHO protocols for the Molecular Detection of Influenza viruses[12] and (B) the CDC Flu A assay targeting M from the Flu-SC2 Multiplex assay.[11]

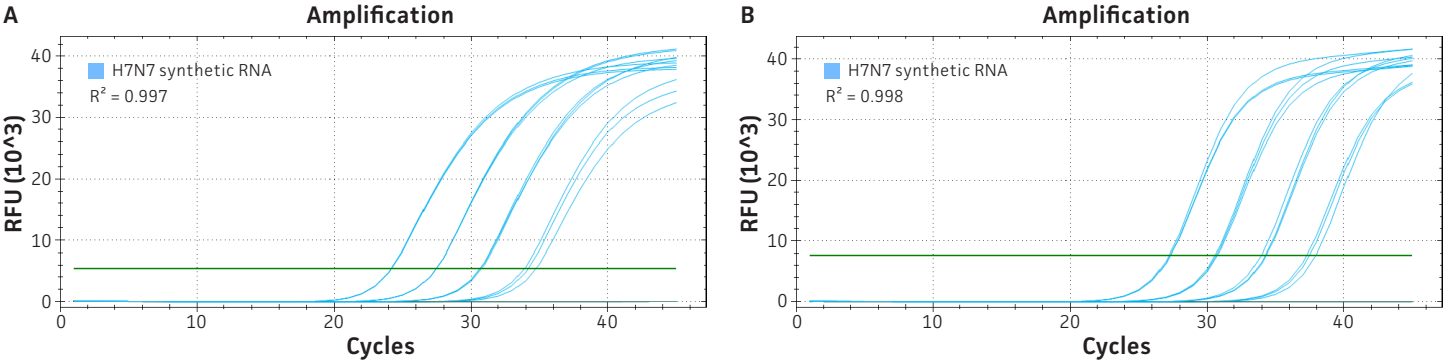

Figure 3: qRT-PCR amplification of synthetic H7N7 RNA. Amplification curves generated with synthetic H7N7 RNA (ATCC® VR-3438SD™) using (A) an assay targeting HA from the WHO protocols for the Molecular Detection of Influenza viruses[12] and (B) the CDC Flu A assay targeting M from the Flu-SC2 Multiplex assay.[11]

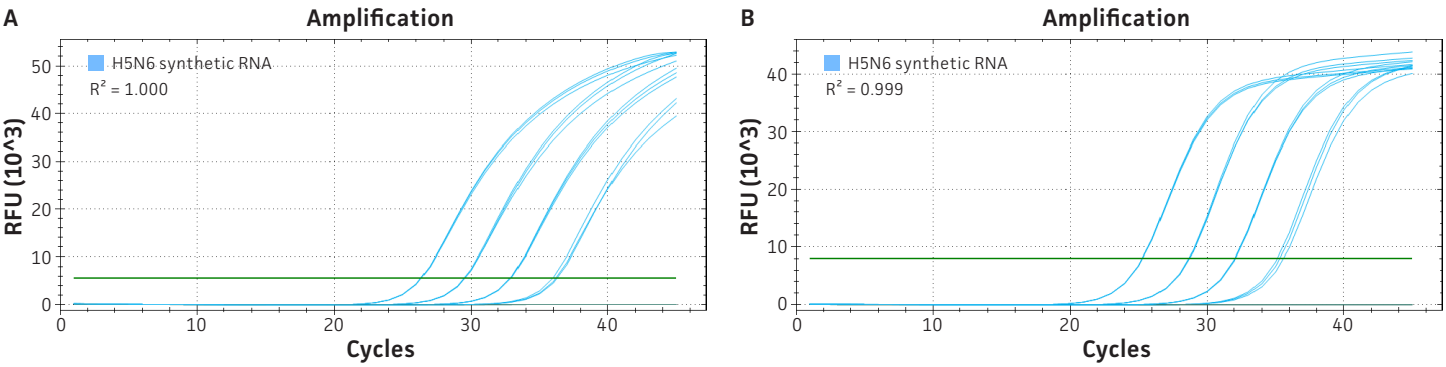

Figure 4: qRT-PCR amplification of synthetic H5N6 RNA. Amplification curves generated with synthetic H5N6 RNA (ATCC® VR-3439SD™) using (A) a Hoffmann *et al.* 2016[10] assay targeting HA and (B) the CDC Flu A assay targeting M from the Flu-SC2 Multiplex assay.[11]

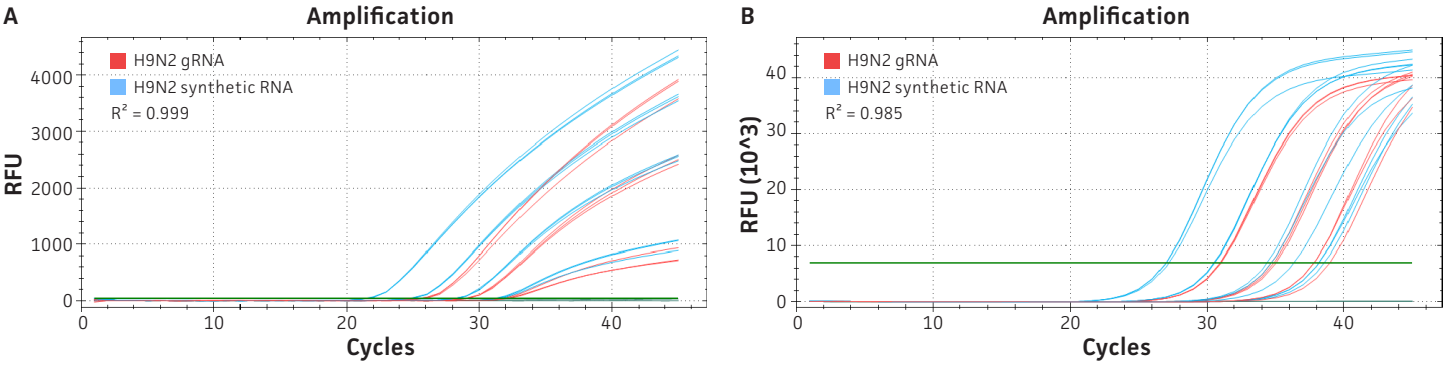

Figure 5: Comparative qRT-PCR amplification of synthetic and genomic H9N2 RNA. Amplification curves generated with synthetic H9N2 RNA (ATCC® VR-3440SD™) (blue) and H9N2 gRNA (red) using (A) a Hassan *et al.* 2022[13] assay targeting HA and (B) the CDC Flu A assay targeting M from the Flu-SC2 Multiplex assay.[11]

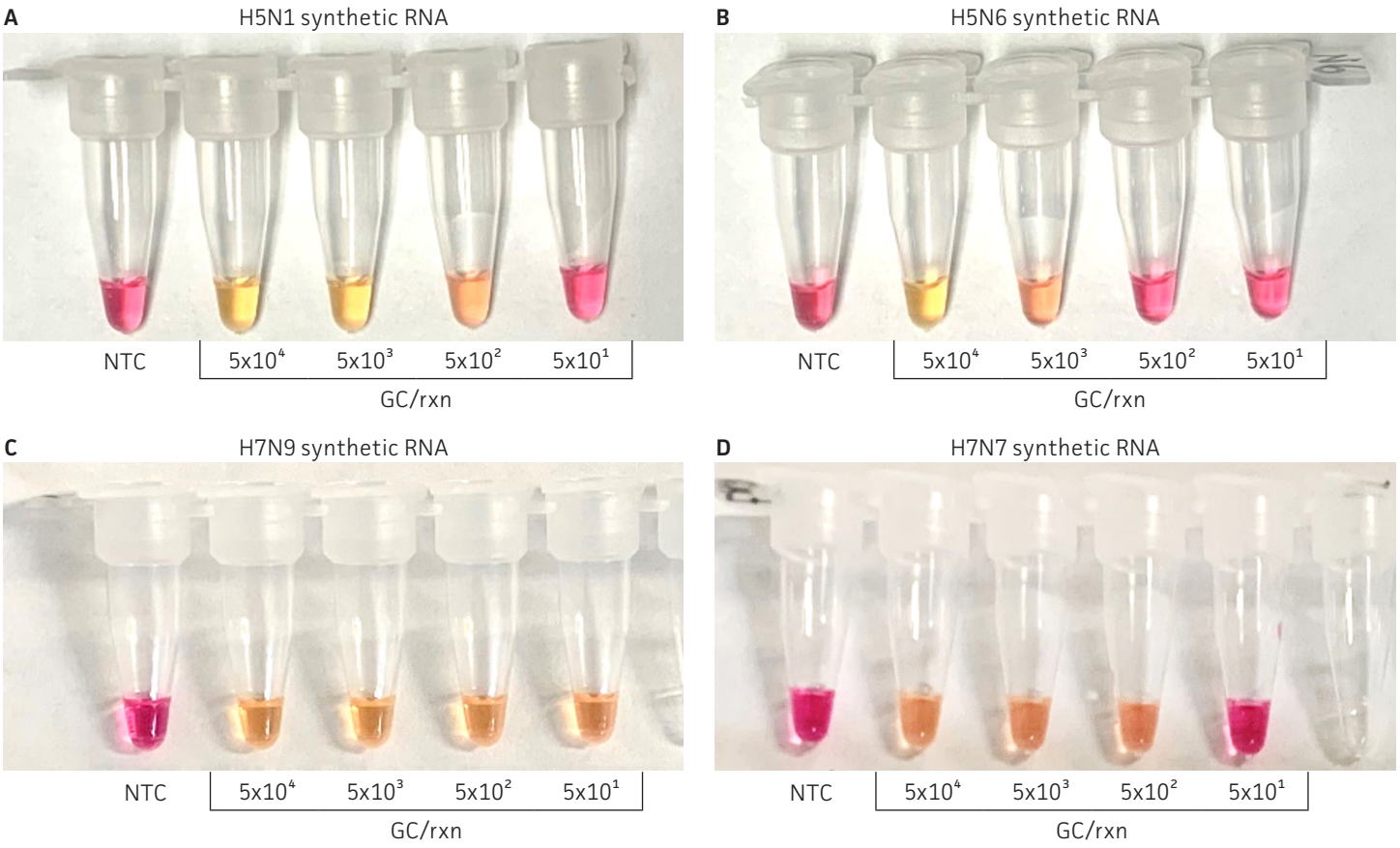

Figure 6: LAMP results using assays from Ahn *et al*. 2019.[9] The H5 assay from the paper was tested with the (A) synthetic H5N1 RNA (ATCC® VR-3436SD™) and (B) synthetic H5N6 RNA (ATCC® VR-3439SD™), and the H7 assay was tested with the (C) synthetic H7N9 RNA (ATCC® VR-3437SD™) and (D) synthetic H7N7 RNA (ATCC® VR-3438SD™). From left to right the tubes contain a no-template control (NTC) and synthetic RNA at 5 ×10^4 GC/rxn, 5 ×10^3 GC/rxn, 5 ×10^2 GC/rxn, and 5 ×10^1 GC/rxn. With the WarmStart Colorimetric LAMP 2X Master Mix with UDG (New England Biolabs), positive RT-LAMP reactions result in a color change of the phenol red pH indicator from pink to yellow due to decreased pH in the presence of polymerase activity.

Table 3: Detection of ATCC® synthetic AIV RNA products with the BioFire Diagnostics FilmArray RP2.1 Respiratory Panel (bioMérieux)

			× Limit of Detection		
ATCC® Item	**Influenza A Subtype**	**Diluent**	**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 the first attempt but was successful when the same sample was retested.

Conclusions

This study demonstrates that 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. Their compatibility across diverse assay formats underscores their utility as safe, stable positive controls for assay development, verification, and validation.

In addition to this experimental verification, we conducted extensive *in silico* evaluations to map theoretical compatibility of the synthetic RNA constructs with PCR assays from primary literature and public health organizations. These comprehensive findings are available within the technical data sheets located on each product page. Collectively, our findings highlight the value of ATCC synthetic AIV RNA ARMs in strengthening influenza diagnostic preparedness and surveillance capacity.

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