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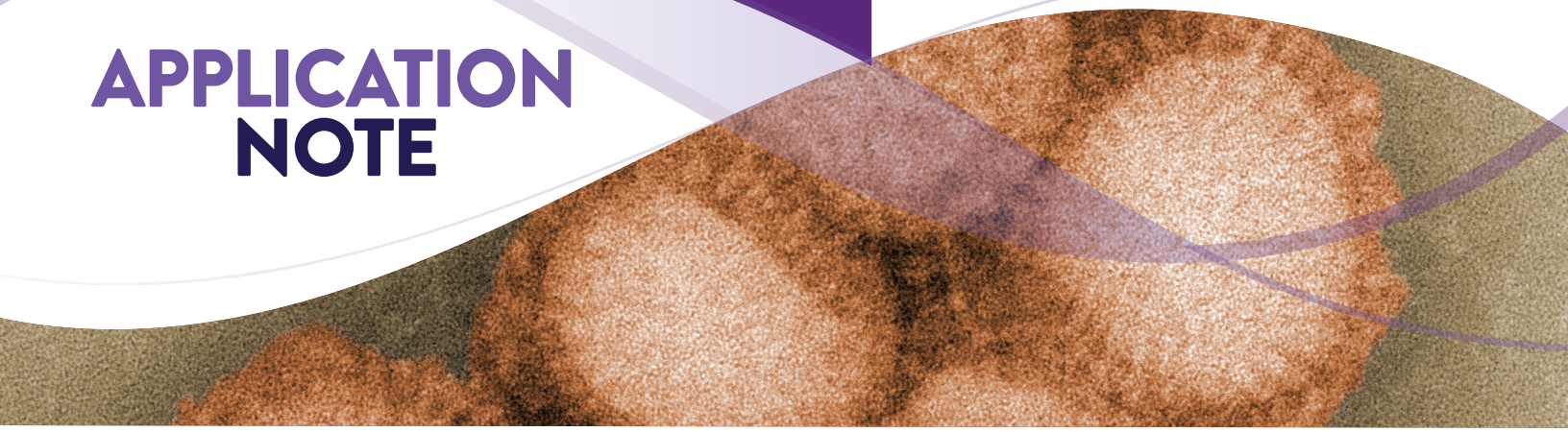
APPLICATION NOTE



Performance Assessment of ATCC® Oseltamivir-Resistant Influenza A/H1N1pdm09 Strains as Analytical Reference Materials for Diagnostic Surveillance

Authors: Holly A. Asbury, BS; Jason Bose, BS; Kyle Young, MBA; Victoria Knight-Connoni, PhD; Leka Papazisi, DVM, PhD

Abstract

Antiviral resistance in influenza viruses poses a significant public health concern, as it can compromise the effectiveness of frontline therapeutics. Oseltamivir (commonly sold under the brand name Tamiflu) is a neuraminidase inhibitor widely used to treat influenza A and B infections. Resistance to oseltamivir typically arises from mutations in the neuraminidase (NA) gene, which reduce the drug's binding affinity or enzymatic function. Quantitative RT-PCR offers a sensitive and cost-effective approach for detecting resistance-associated mutations, making it well suited for surveillance and assay development. ATCC® maintains oseltamivir-resistant Influenza strains—such as Influenza A virus H1N1pdm09 strain A/Washington/29/2009 (ATCC® VR-1988™) and Influenza A virus H1N1pdm09 strain A/Connecticut/11/2023 (ATCC® VR-3441™)—that can serve as positive controls for mutation-specific assays. Here, we experimentally verified the performance of these oseltamivir-resistant strains in mutation-specific quantitative RT-PCR assays and compared their amplification profiles with those obtained using quantitative synthetic Influenza A virus H1N1pdm09 RNA (ATCC® VR-3388SD™) as a control.

Introduction

Influenza viruses pose a major public health concern. In the Unites States alone, seasonal influenza is estimated to cause tens of millions of illnesses each autumn and winter, leading to tens of thousands of deaths annually.[1] In many cases, patients recover without medical intervention; however, in severe cases—such as when patients are hospitalized or at high risk of complications—clinicians may treat influenza with antiviral drugs.[2] There are two main classes of anti-influenza drugs approved in the United States: M2 ion channel blockers, which include amantadine and rimantadine, and neuraminidase inhibitors (NAIs), which include oseltamivir and zanamivir.[3] Oseltamivir (brand name: Tamiflu) is an orally administered antiviral drug commonly prescribed for the treatment of influenza A and B. Influenza viruses can develop resistance to oseltamivir through neuraminidase (NA) gene mutations that reduce drug binding or enzymatic activity.[4] Several amino acid replacement mutations have been associated with oseltamivir resistance, including H275Y and S247N.

Quantitative RT-PCR (qRT-PCR) is an effective and widely used method for detecting these mutations due to its high sensitivity and cost-effectiveness. Although the overall prevalence of oseltamivir-resistant influenza viruses is expected to be low, recent detections of influenza A/H1N1pdm09 strains with reduced oseltamivir susceptibility have been reported through the World Health Organization (WHO) and United States Centers for Disease Control and Prevention (CDC) surveillance systems.[5] Ongoing surveillance of resistance mutations is therefore critical for containing the spread of these strains, particularly given the evidence that oseltamivir resistance mutations can persist in wild bird populations, potentially driven by environmental exposure to oseltamivir drug metabolites in sewage and river water.[6] ATCC® maintains oseltamivir-resistant strains that can aid surveillance efforts by serving as analytical reference materials for mutation detection. Influenza A virus H1N1pdm09 strain A/Washington/29/2009 (ATCC® VR-1988™) is resistant to oseltamivir and carries the H275Y

mutation in the NA gene, which confers resistance. This strain is also resistant to amantadine and rimantadine and contains the S31N mutation in the M2 gene. Influenza A virus H1N1pdm09 strain A/Connecticut/11/2023 (ATCC® VR-3441™) is resistant to oseltamivir and harbors the I223V and S247N mutations in the NA gene (Table 1).

In this study, we demonstrate that these oseltamivir-resistant strains can be used as positive controls in mutation-specific qRT-PCR assays, supporting their utility in influenza antiviral resistance surveillance and assay validation.

Materials and Methods

Influenza A virus H1N1pdm09 strains A/Washington/29/2009 (ATCC® VR-1988™) and A/Connecticut/11/2023 (ATCC® VR-3441™) were selected because they contain specific mutations conferring resistance to oseltamivir (Table 1). gRNA from the oseltamivir-resistant strains was extracted via EZ1 Advanced XL automated nucleic acid extraction system (QIAGEN) and quantified by digital-based PCR. A quantitative synthetic reference material (ATCC® VR-3388SD™) was chosen as a control in this study since it does not contain resistance-associated mutations (Table 1). This synthetic reference material consists of two synthetic RNA transcripts designed to include diagnostically relevant regions from the HA, NP, NA, M1/M2, and NEP/NS1 genes, collectively representing ~50% of the influenza genome. Both transcripts are quantified by digital-based PCR and fall within the range of 1×10^5 and 1×10^6 genome copies/µL.

We assessed the performance of these analytical reference materials (ARMs) using published qRT-PCR assays from the WHO[7] and literature sources[8] that would detect all three items, and with oseltamivir-resistance biomarker detection assays[9,10] (Table 2). All oligonucleotides, except for the oseltamivir-resistance biomarker assay TaqMan probes, were ordered from Integrated DNA Technologies, and TaqMan probes were tagged with FAM and double-quenched. The oseltamivir-resistance biomarker detection probes were obtained from Thermo Fisher Scientific with FAM and VIC labels and were quenched by a minor-groove binding (MGB) quencher. The qRT-PCR data were generated on the CFX Opus Real-Time PCR System (Bio-Rad). Amplification was achieved using the Invitrogen SuperScript III Platinum One-Step qRT-PCR Kit (Thermo Fisher Scientific). We compared three ten-fold dilutions of Influenza A virus H1N1pdm09 strains A/Washington/29/2009 (ATCC® VR-1988™) and A/Connecticut/11/2023 (ATCC® VR-3441™) ranging from 50-5,000 genome copies (GC) per reaction (rxn) with four ten-fold dilutions of the synthetic ARM ranging from 50-5×10^4 GC/rxn.

Table 1: Influenza A virus and nucleic acid products featured in this study

ATCC® Item	Strain	Serotype	Antiviral Drug Resistance and Corresponding Mutation Profile
VR-3388SD™	A/Netherlands/2629/2009	H1N1pdm09	None
VR-1988™	A/Washington/29/2009	H1N1pdm09	Amantadine, rimantadine, M2: S31N; Oseltamivir, NA: H275Y
VR-3441™	A/Connecticut/11/2023	H1N1pdm09	Oseltamivir, NA: I223V and NA: S247N

Table 2: Published assays used in this study

Publication Source	Assay Target	Application
WHO, Molecular Detection of Influenza viruses, 2021[7]	HA	H1N1pdm09 detection
WHO, Molecular Detection of Influenza viruses, 2021[7]	NA	H1N1pdm09 detection
Hoffmann *et al.*, 2016[8]	M	Influenza A detection
Nakauchi *et al.*, 2011[9]	NA	Oseltamivir resistance mutation H275Y detection
Takayama *et al.*, 2013[10]	NA	Oseltamivir resistance mutation S247N detection

Results

In this publication, we present the utility of two oseltamivir-resistant influenza H1N1pdm09 strains (Table 1), by demonstrating compatibility with published qRT-PCR assays, including mutation-specific detection assays (Table 2). In addition, we demonstrate the utility of synthetic influenza A/H1N1pdm09 RNA (ATCC® VR-3388SD™) as an analytical reference material, here serving as a control without oseltamivir-resistance biomarkers. First, we tested the products using assays we believed would successfully amplify all three H1N1pdm09 influenza variants; assay selection was guided by in silico analysis. Next, we evaluated assays designed to detect specific oseltamivir resistance-associated mutations known to be present in Influenza A virus H1N1pdm09 strains A/Washington/29/2009 (ATCC® VR-1988™) and A/Connecticut/11/2023 (ATCC® VR-3441™).

We observed comparable amplification between native gRNA derived from oseltamivir-resistant strains and the synthetic RNA control across three different assays (Figure 1). These assays targeted HA (Figure 1A) and NA (Figure 1B), as described in the WHO Information for the Molecular Detection of Influenza Viruses (2021),[7] and M (Figure 1C), as described in Hoffmann *et al.*, 2016.[8] The R^2 results for the synthetic RNA product (ATCC® VR-3388SD™) with the two WHO assays[7] (Figure 1A, 1B) varied between 0.996-0.999, and those of the native gRNA were between 0.992-0.999, indicating high linearity. In the case of the M assay from Hoffmann *et al.*, 2016[8] (Figure 1C), we do not observe linearity in the amplification curves; however, the amplification patterns between gRNA and synthetic RNA across all three ATCC® items were similar, with approximately equal Cq values at equivalent concentrations, indicating comparable performance.

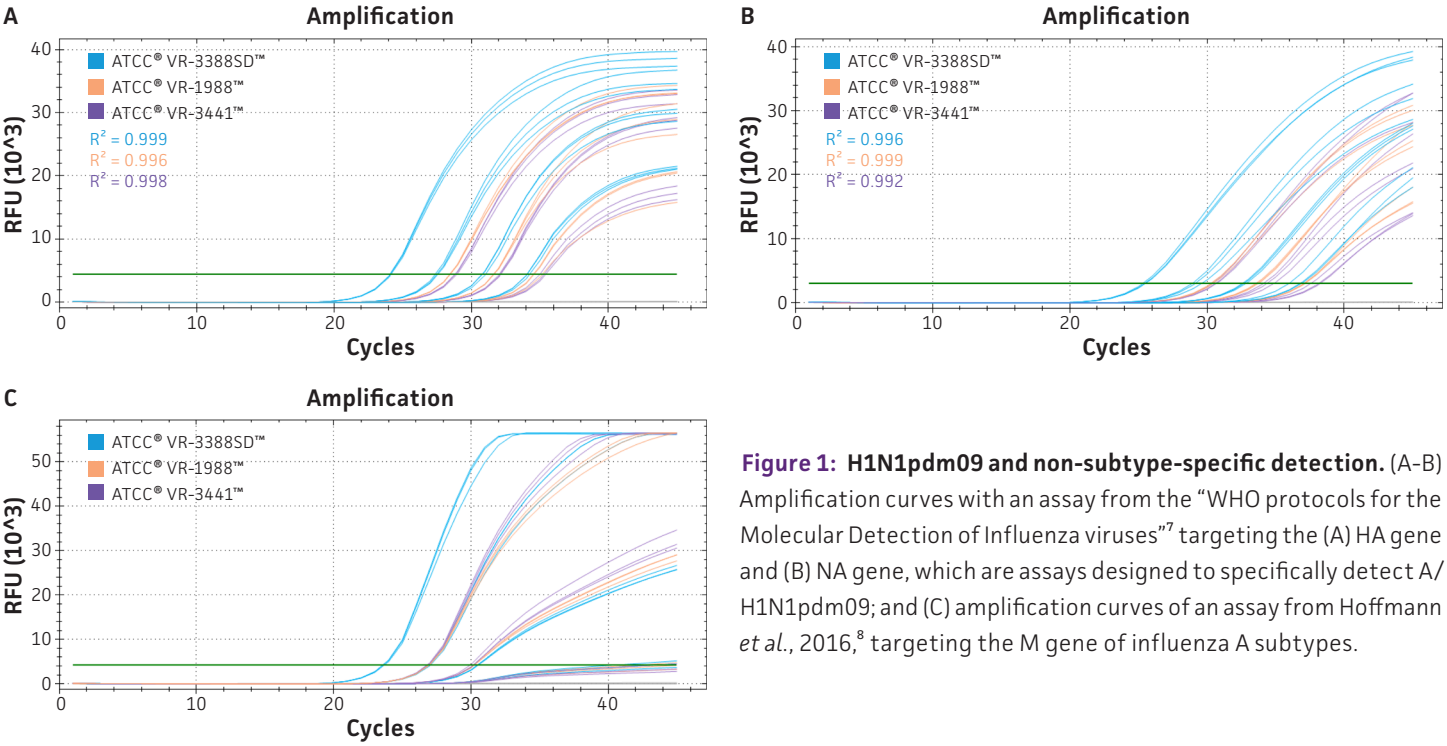


Figure 1: H1N1pdm09 and non-subtype-specific detection. (A-B) Amplification curves with an assay from the "WHO protocols for the Molecular Detection of Influenza viruses"[7] targeting the (A) HA gene and (B) NA gene, which are assays designed to specifically detect A/H1N1pdm09; and (C) amplification curves of an assay from Hoffmann *et al.*, 2016,[8] targeting the M gene of influenza A subtypes.

Next, we tested the assays that detect specific mutations associated with oseltamivir resistance that are present in Influenza A virus H1N1pdm09 strains A/Washington/29/2009 (ATCC® VR-1988™) and A/Connecticut/11/2023 (ATCC® VR-3441™). The assay from Nakauchi *et al.*, 2011,[9] detects the H275Y mutation present in A/Washington/29/2009 (ATCC® VR-1988™). In this assay, there are two TaqMan probes that differ by a single nucleotide (nt) at the 5' end; these probes distinguish strains with H275 (linked to oseltamivir sensitivity), which amplify in the VIC channel, from strains with Y275 (linked to oseltamivir resistance), which amplify in the FAM channel. Because this difference in amplification is achieved by detecting a single nucleotide polymorphism (SNP) at the 5' end of the Taqman probe, there is some cross-amplification in both channels (Figure 2A, 2B). Therefore, it is more appropriate to visualize the data using an end-fluorescence plot (Figure 2C), as proposed by Nakauchi *et al.*[9] In this plot, we observe more clearly that the predominantly VIC amplification of the synthetic RNA control (ATCC® VR-3388SD™) indicates H275 detection (associated with oseltamivir sensitivity) and the predominantly FAM amplification of A/Washington/29/2009 (ATCC® VR-1988™) indicates Y275 detection (associated with oseltamivir resistance). Regarding the performance of A/Connecticut/11/2023 (ATCC® VR-3441™) with this assay, we initially expected to observe VIC amplification associated with H275; however, the result was a lack of amplification with either probe. Following these results, we performed in silico mismatch analysis of the oligonucleotides with the A/Connecticut/11/2023 (ATCC® VR-3441™) genome sequence available from the ATCC® Genome Portal[11] and determined that a SNP in the probe binding site at the fifth nt from the 3' end of the probe may be preventing successful binding to the sequence, thus preventing the expected amplification pattern.

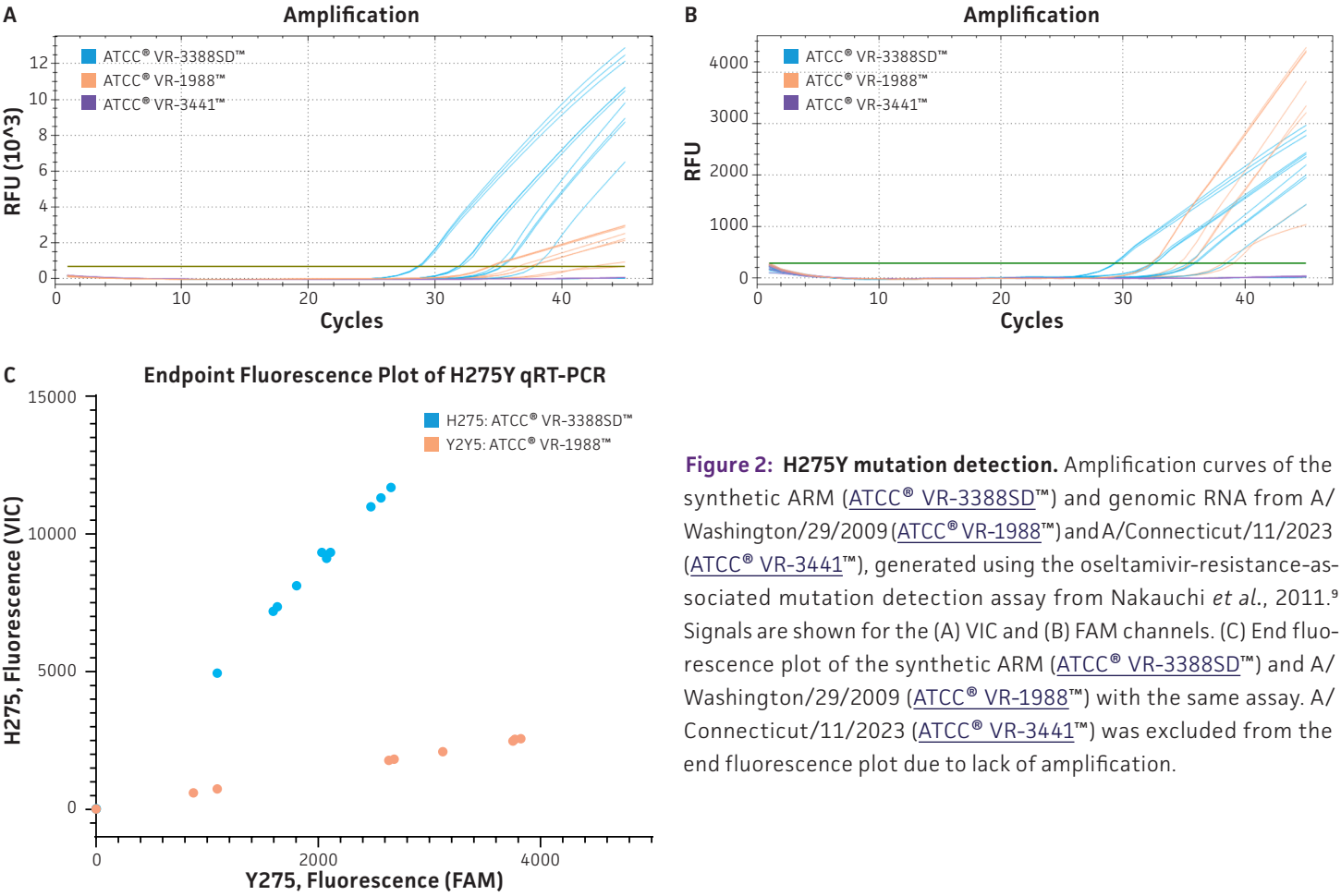


Figure 2: H275Y mutation detection. Amplification curves of the synthetic ARM (ATCC® VR-3388SD™) and genomic RNA from A/Washington/29/2009 (ATCC® VR-1988™) and A/Connecticut/11/2023 (ATCC® VR-3441™), generated using the oseltamivir-resistance-associated mutation detection assay from Nakauchi *et al.*, 2011.[9] Signals are shown for the (A) VIC and (B) FAM channels. (C) End fluorescence plot of the synthetic ARM (ATCC® VR-3388SD™) and A/Washington/29/2009 (ATCC® VR-1988™) with the same assay. A/Connecticut/11/2023 (ATCC® VR-3441™) was excluded from the end fluorescence plot due to lack of amplification.

The assay from Takayama *et al.*, 2013,[10] detects the S247N mutation present in A/Connecticut/11/2023 (ATCC® VR-3441™). In this assay, as in the prior assay, there are two TaqMan probes that differ by one nt; although, in this case the SNP lies in the middle of the probe sequence. The probes differentiate between strains containing S247 (associated with oseltamivir sensitivity) and those containing N247 (associated with oseltamivir resistance), with S247 strains amplifying in the VIC channel and N247 strains amplifying in the FAM channel. With this assay, we observed minimal cross-amplification across the channels. In the VIC channel (Figure 3A), the synthetic ARM (ATCC® VR-3388SD™) and A/Washington/29/2009 (ATCC® VR-1988™) amplified, and in the FAM channel (Figure 3B), A/Connecticut/11/2023 (ATCC® VR-3441™) amplified. The end-fluorescence plot (Figure 3C) further illustrates the successful detection of S247 (associated with oseltamivir sensitivity) in the synthetic ARM (ATCC® VR-3388SD™) and N247 (associated with oseltamivir resistance) in A/Connecticut/11/2023 (ATCC® VR-3441™). The expectations and results for all five assays tested with each of the three ATCC® items are summarized in Table 3.

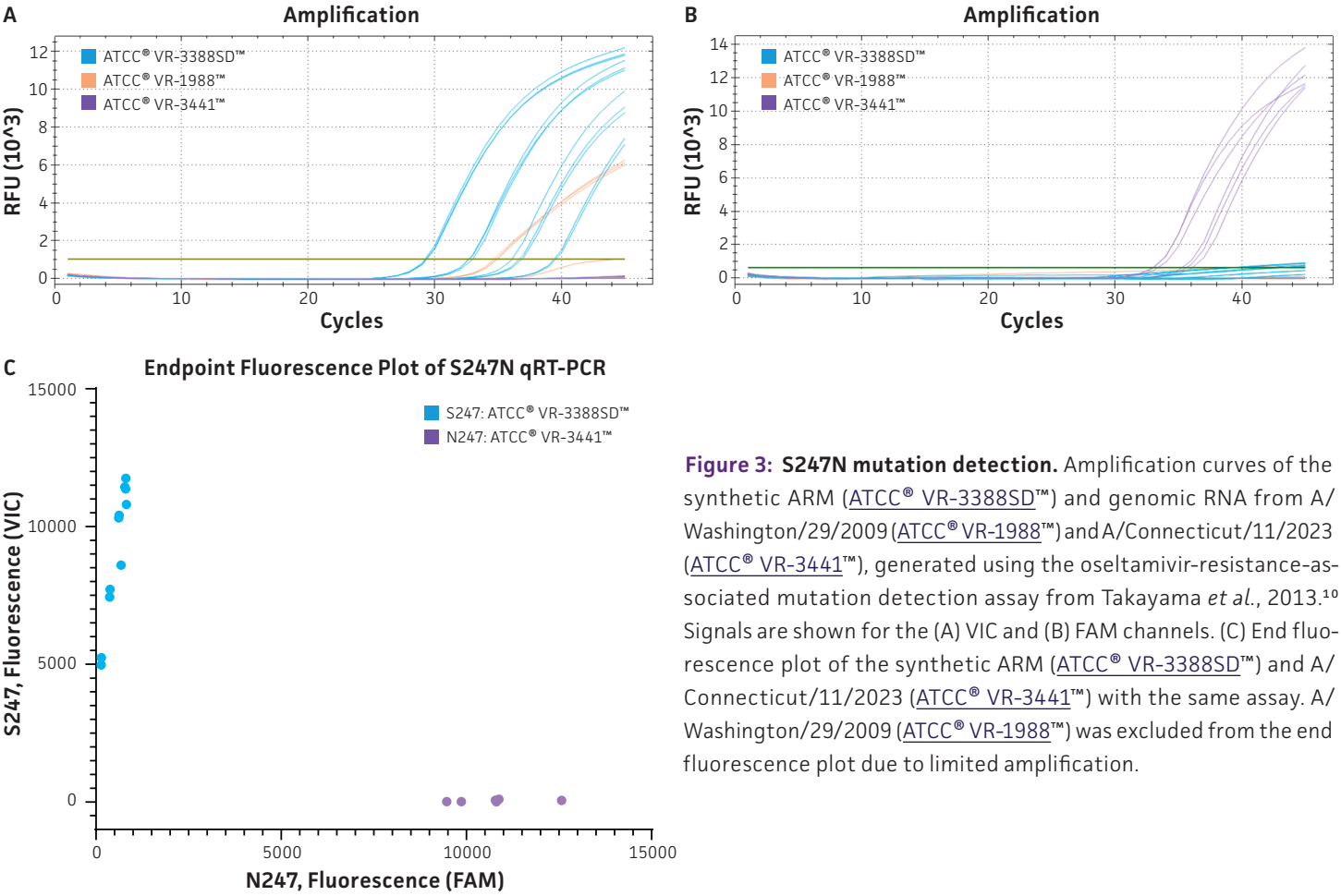


Figure 3: S247N mutation detection. Amplification curves of the synthetic ARM (ATCC® VR-3388SD™) and genomic RNA from A/Washington/29/2009 (ATCC® VR-1988™) and A/Connecticut/11/2023 (ATCC® VR-3441™), generated using the oseltamivir-resistance-associated mutation detection assay from Takayama *et al.*, 2013.[10] Signals are shown for the (A) VIC and (B) FAM channels. (C) End fluorescence plot of the synthetic ARM (ATCC® VR-3388SD™) and A/Connecticut/11/2023 (ATCC® VR-3441™) with the same assay. A/Washington/29/2009 (ATCC® VR-1988™) was excluded from the end fluorescence plot due to limited amplification.

Table 3: Summary of results of ATCC® items with each assay in the study

Publication Source	Assay Target	Synthetic A/H1N1pdm09 RNA, ATCC® VR-3388SD™		gRNA from A/Connecticut/11/2023, ATCC® VR-3441™		gRNA from A/Washington/29/2009, ATCC® VR-1988™	
		Expected	Actual	Expected	Actual	Expected	Actual
WHO, Molecular Detection of Influenza viruses, 2021[7]	HA	+	+	+	+	+	+
	M	+	+	+	+	+	+
Hoffmann *et al.*, 2016[8]	NA	+	+	+	+	+	+
Nakauchi *et al.*, 2011[9]	NA, H275	+	+	+**	-**	-	-
	NA, Y275*	-	-	-	-	+	+
Takayama *et al.*, 2013[10]	NA, S247	+	+	-	-	+	+
	NA, N247*	-	-	+	+	-	-

*Mutation associated with oseltamivir resistance
**Based on the current genome sequence analysis, A/Connecticut/11/2023 (ATCC® VR-3441™) contains an additional SNP in the probe binding site at the fifth nt from the 3' end of the Nakauchi 2011 probe, which may prevent binding to the sequence. Thus, this gRNA may not be a reliable control for the Nakauchi 2011 assay.

Conclusions

Our results demonstrate that Influenza A virus H1N1pdm09 strains A/Washington/29/2009 (ATCC® VR-1988™) and A/Connecticut/11/2023 (ATCC® VR-3441™) can serve as valuable analytical reference materials for molecular detection of mutations associated with oseltamivir resistance. The gRNA extracted from these strains showed robust and assay-appropriate performance across multiple published qRT-PCR assays, including mutation-specific detection of H275Y and S247N, supporting their utility as positive controls for diagnostic assay development and antiviral resistance surveillance.

In parallel, the quantitative synthetic Influenza A virus H1N1pdm09 RNA (ATCC® VR-3388SD™) demonstrated consistent amplification and linearity across subtype-specific and non-subtype-specific assays, confirming its suitability as an analytical reference material for influenza A virus detection. Together, these findings highlight the complementary value of well-characterized native viral strains and synthetic RNA reference materials in supporting accurate, reliable, and adaptable molecular surveillance of influenza antiviral resistance.

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10801 University Boulevard
Manassas, Virginia 20110-2209

703.365.2700 703.365.2701 sales@atcc.org www.atcc.org

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