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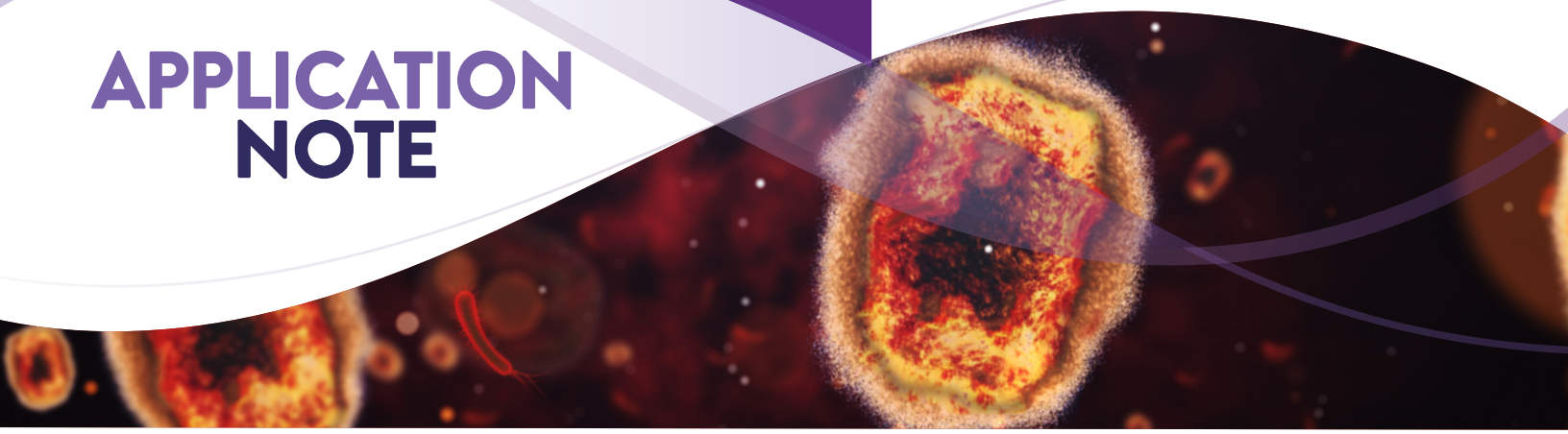
APPLICATION NOTE



Performance Assessment of ATCC® Quantitative Synthetic Analytical Reference Material for Human Monkeypox Virus (hMPXV)

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Abstract

Mpox is a zoonotic viral disease caused by the human monkeypox virus (hMPXV), a double-stranded DNA virus endemic to several countries in Central and Western Africa. In recent years, hMPXV has emerged in regions where it was not previously endemic, posing a growing global health concern. The virus causes a smallpox-like disease in humans. Rapid and accurate detection of this virus during the early stages of infection is essential for containment and timely treatment, making PCR-based methods particularly favorable due to their high sensitivity and relatively rapid turnaround time. However, the development and validation of these methods depend on high-quality reference materials. To address this need, ATCC® designed and developed a BSL-1 quantitative synthetic analytical reference material (ARM) for hMPXV that comprises gene sequences commonly targeted by diagnostic assays. Here, we demonstrate the functionality of the ARM as a positive control for several published quantitative PCR assays.

Introduction

Mpox is a zoonotic viral disease caused by the human monkeypox virus (hMPXV), a double-stranded DNA virus from the genus *Orthopoxvirus* and family *Poxviridae*. Infection typically presents as a smallpox-like disease characterized by fever, muscle aches, swollen lymph nodes, and a characteristic vesiculopustular rash, with potentially serious complications such as secondary bacterial infections, pneumonia, sepsis, or encephalitis.[1] hMPXV infection is endemic in several countries in Central and Western Africa but has gained global attention following a widespread outbreak in previously non-endemic areas in 2022.[2] Two distinct genetic clades are recognized: Clade I and Clade II. Clade I is associated with higher virulence and mortality, historically showing case fatality rates of 5-10%. Clade II generally results in milder disease with case fatality rates typically below 1%. However, certain sublineages like Clade IIB, which drove the 2022 outbreak, have shown greater human-to-human transmissibility despite lower virulence.[3]

Rapid detection during early infection is crucial for containment and treatment. While culture-based detection is time-consuming and requires BSL-3 facilities, PCR-based methods offer a sensitive and rapid alternative. However, this latter methodology relies on high-quality reference materials for development and validation. To address this need, ATCC® developed a quantitative synthetic analytical reference material (ARM) for hMPXV that incorporates diagnostic biomarkers from both virus clades, with gene sequences commonly targeted in detection assays. Furthermore, the construct is synthetically derived and therefore does not contain any viable materials, making it safe to use as a positive control under BSL-1 conditions. In the following study, we showcase the applicability of the synthetic hMPXV ARM as a positive control for several published quantitative PCR assays.

Materials and Methods

When developing the synthetic hMPXV DNA product (ATCC® VR-3270SD™), we applied the same rigorous approach used across our quantitative synthetic nucleic acid portfolio. Extensive research was conducted to identify key target regions within the genome that are compatible with published molecular assays; several published genome sequences were used to inform our design. The resulting construct includes fragments from nine genes: *J2L, D14L, F3L, F8L, A27L, A29L, B6R, B7R,* and *N3R*. The product was authenticated via next-generation sequencing and quantified via digital-based PCR technology, ensuring accuracy and consistency. The concentration specification is between 1×10^5 and 1×10^6 copies/µL, with a lot-specific value reported on the Certificate of Analysis, which aligns with the ATCC® quantitative synthetic nucleic acid product line. Manufactured under ISO 13485 guidance and stabilized in a proprietary storage buffer, this product offers consistent performance and a 5-year-long shelf life.

To verify functionality, the hMPXV synthetic DNA product was tested against 12 published PCR assays, including those from the Centers for Disease Control and Prevention (CDC). Experiments were performed on either the CFX Opus Real-Time PCR System (Bio-Rad) or CFX 96 Real-Time PCR System (Bio-Rad). Assays requiring the use of TaqMan probes were amplified with the Platinum Quantitative PCR SuperMix-UDG (Invitrogen) kit (catalog # 11730017). Cycling parameters and oligonucleotide concentrations emulated those in each source publication. During these experiments, we tested the synthetic DNA at concentrations ranging from 50-50,000 genome copies/reaction (GC/rxn) and genomic DNA at concentrations ranging from 50-5,000 GC/rxn. hMPXV genomic DNA was extracted from Heat-inactivated Monkeypox virus strain hMPX/USA/MA001/2022 (ATCC® VR-3371HK™) via the QIAGEN EZ1 Advanced XL automated extraction system and quantified by digital-based PCR technology. For two of the assays tested, we compare the synthetic DNA with genomic DNA from Clade IA and Clade IB hMPXV (Table 1). A summary of all published assays evaluated, along with compatibility data for ATCC® products, is provided in the Results section (Table 2).

Table 1: Products featured in this study.

Product Number	Product Description	hMPXV Clade
ATCC® VR-3270SD™	Quantitative Synthetic Monkeypox virus DNA	I and II
ATCC® VR-3371HK™	Heat-inactivated Monkeypox virus strain hMPX/USA/MA001/2022	II
BEI Resources NR-2324	Monkeypox Virus Zaire 79 (V79-I-005)	IA
BEI Resources NR-60220	Monkeypox Virus Clade Ib_USA_CA_2024	IB

Results

A unique feature of the synthetic hMPXV DNA product (ATCC® VR-3270SD™) is that it accommodates genetic signatures for both Clades I and II. Therefore, we applied a systematic approach to assess the utility of the hMPXV ARM and its performance relative to hMPXV gDNA across a range of published assays (Table 2).

Table 2: Summary of experimentally verified assay compatibility of quantitative synthetic hMPXV DNA and genomic DNA from various hMPXV strains.

			Experimentally Verified Assay Compatibility			
Assay Specificity	Publication Source	Assay Target	Synthetic DNA (ATCC® VR-3270SD™) Clade I & II compatible	Clade II gDNA (ATCC® VR-3371HK™)	Clade IA gDNA (BEIR NR-2324)	Clade IB gDNA (BEIR NR-60220)
hMPXV Clade I	Negrón, *et al.*, 2023[4]	*C3L*	+	-	+	-
	Li, *et al.*, 2010[5]	*C3L*	+	-	+	-
	Saijo, *et al.*, 2008[6]	ATI/*A27L*	+	-	NT	NT
hMPXV Clade II	Li, *et al.*, 2010[5]	*G2R*	+	+	NT	NT
hMPXV species	CDC, 2022[7]	*G2R*	+	+	NT	NT
	Maksyutov, *et al.*, 2016[8]	*F3L*	+	+	NT	NT
	Kulesh, *et al.*, 2004[9]	*F3L*	+	+	NT	NT
		N3R	+	+	NT	NT
	Shcheklkunov, *et al.*, 2011[10]	*B7R*	+	+	NT	NT
	Neubauer, *et al.*, 1998[11]	ATI ORF	+	+	NT	NT
Orthopoxvirus genus	Dumont, *et al.*, 2014[12]	IMV Surface Protein/*A29L*	+	+	NT	NT
	CDC, 2022[13]	*E9L*	+	+	NT	NT

The "+" symbol indicates amplification of the product (columns) with the assay (rows), whereas the "-" symbol indicates no amplification of the product with the assay. "NT" indicates that the product was not tested with the indicated assay.

We experimentally verified the compatibility of the synthetic hMPXV DNA (ATCC® VR-3270SD™) with clade-specific assays for both Clades I and II and compared it against the performance of the genomic DNA from Clade II hMPXV (ATCC® VR-3371HK™) (Figures 1-2). The synthetic hMPXV DNA was compatible with Clade I assays from Negrón *et al*. 2023,[4] Li *et al*. 2010,[5] and Saijo *et al*. 2008[6] (Figure 1) as well as the Clade II assay from Li *et al*. 2010[5] (Figure 2). As anticipated, the genomic DNA from Clade II hMPXV was not amplified by Clade I–specific assays (Figure 1) but was successfully amplified by the Clade II–specific assay (Figure 2). We also compared the performance of the synthetic hMPXV DNA with genomic DNA from Clade I hMPXV (Figure 3); these experiments were conducted under BSL-3 conditions. Both Clade I assays (Negrón *et al*. 2023[4], Li *et al*. 2010[5]) successfully amplified gDNA from Clade IA hMPXV (BEI Resources NR-2324). The gDNA from Clade IA was not loaded at a known concentration, though it appears more concentrated than the synthetic DNA standard curve. Clade IB is a novel subclade defined in 2024, and it seems that the assays, both published before 2024, could not amplify the gDNA from Clade IB hMPXV (BEI Resources NR-60220).

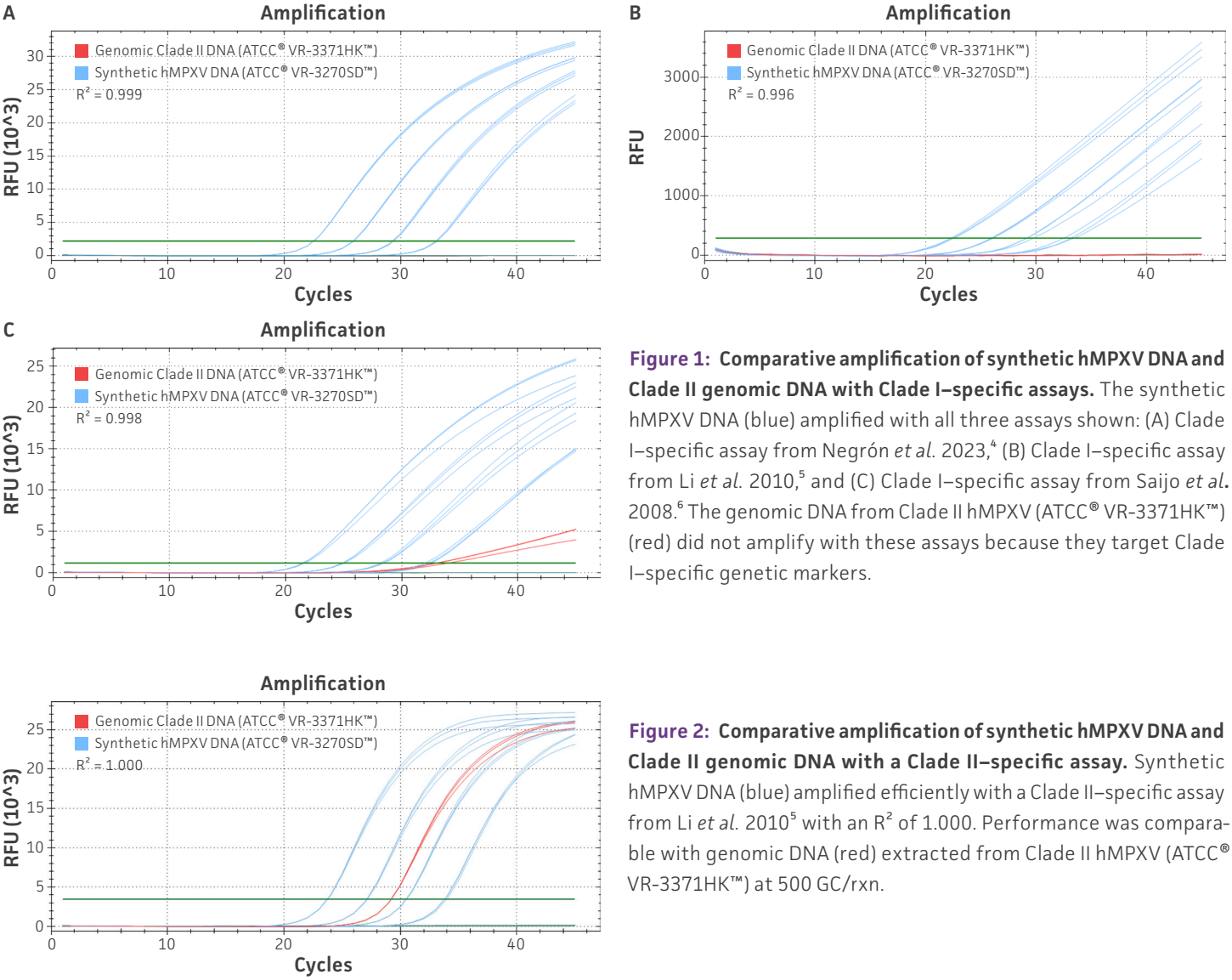

Figure 1: Comparative amplification of synthetic hMPXV DNA and Clade II genomic DNA with Clade I–specific assays. The synthetic hMPXV DNA (blue) amplified with all three assays shown: (A) Clade I–specific assay from Negrón *et al.* 2023,[4] (B) Clade I–specific assay from Li *et al.* 2010,[5] and (C) Clade I–specific assay from Saijo *et al.* 2008.[6] The genomic DNA from Clade II hMPXV (ATCC® VR-3371HK™) (red) did not amplify with these assays because they target Clade I–specific genetic markers.

Figure 2: Comparative amplification of synthetic hMPXV DNA and Clade II genomic DNA with a Clade II–specific assay. Synthetic hMPXV DNA (blue) amplified efficiently with a Clade II–specific assay from Li *et al.* 2010[5] with an R^2 of 1.000. Performance was comparable with genomic DNA (red) extracted from Clade II hMPXV (ATCC® VR-3371HK™) at 500 GC/rxn.

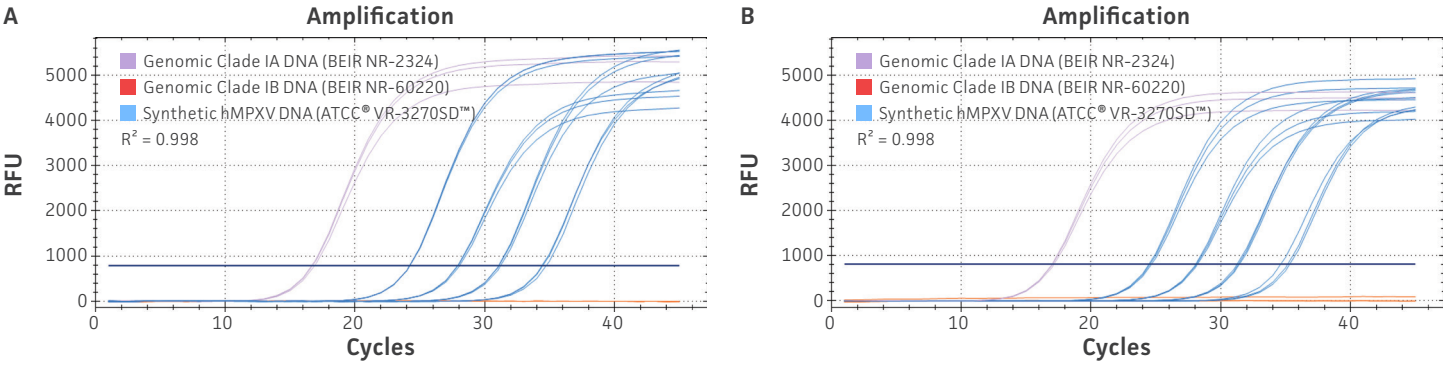

Figure 3: Comparative amplification of synthetic hMPXV DNA and Clade I genomic DNA with Clade I–specific assays. Synthetic hMPXV DNA (blue) and genomic DNA extracted from Clade IA hMPXV (BEI Resources NR-2324) (purple) successfully amplified with both assays: (A) Negrón *et al.* 2023,[4] and (B) Li *et al.* 2010.[5] Neither assay amplified the genomic DNA from Clade IB (novel subclade defined in 2024) hMPXV (BEI Resources NR-60220) (red).

The synthetic hMPXV DNA and the genomic DNA from Clade II hMPXV (ATCC® VR-3371HK™) were both successfully amplified by six general hMPXV assays (Figure 4) from CDC 2022,[7] Maksyutov *et al*. 2016,[8] Kulesh *et al*. 2004,[9] Shcheklkunov *et al*. 2011,[10] and Neubauer *et al*.1998,[11] as well as by two Orthopoxvirus genus assays (Figure 5) from Dumont *et al*. 2014[12] and CDC 2022.[13] In each of these assays, amplification of the synthetic hMPXV DNA was comparable to that of genomic DNA.

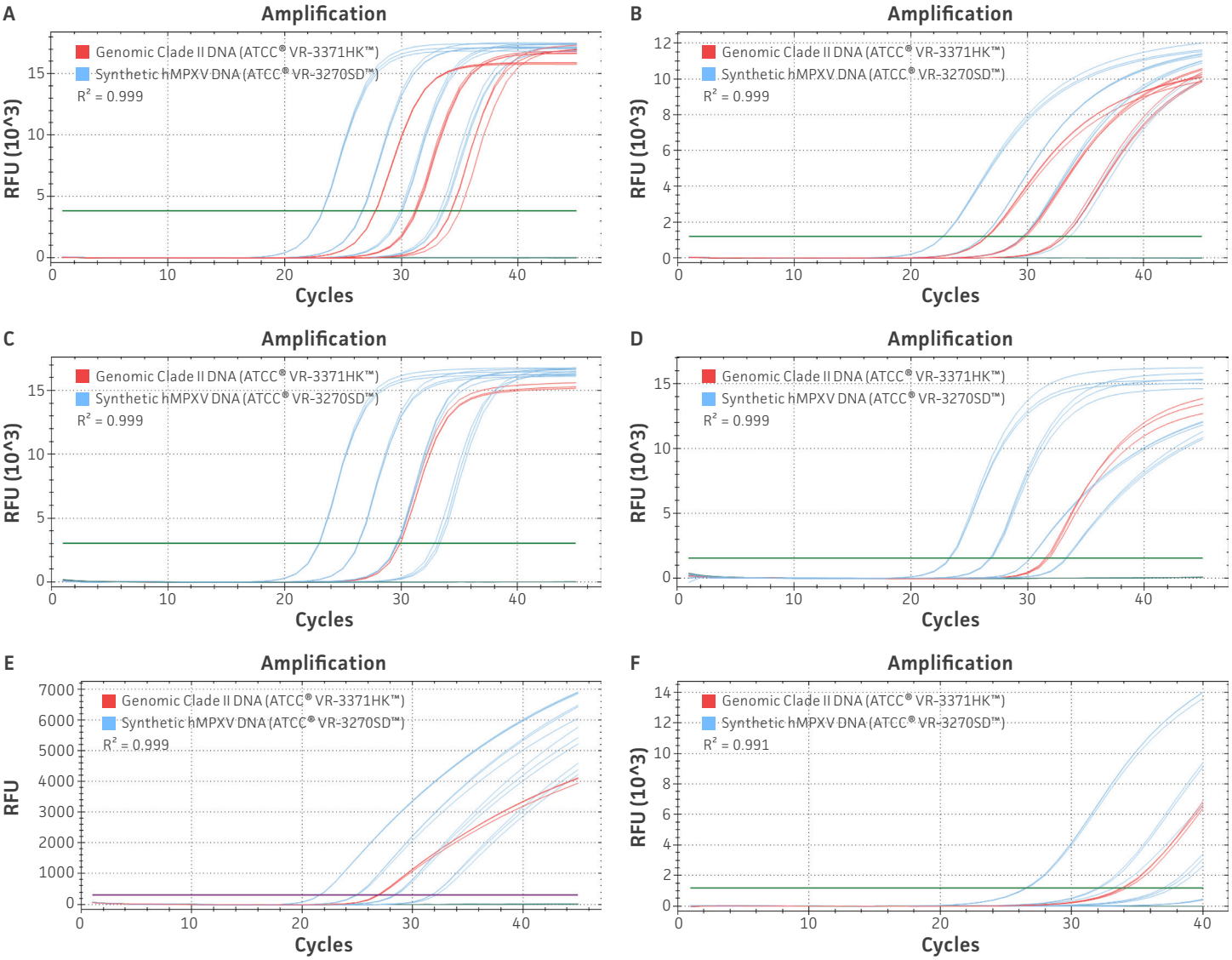

Figure 4: Comparative amplification of synthetic hMPXV DNA and Clade II genomic DNA with hMPXV assays. qPCR amplification of synthetic hMPXV DNA (blue) and gDNA (red) extracted from Clade II MPXV (ATCC® VR-3371HK™) with MPXV assays from (A) CDC 2022,[7] (B) Maksyutov *et al.* 2016,[8] (C, D) Kulesh *et al.* 2004,[9] (E) Shcheklkunov *et al.* 2011,[10] and (F) Neubauer *et al.* 1998.[11] For some assays, (A, B) three ten-fold dilutions of gDNA ranging from 50-5,000 GC/rxn were tested; for others, (C-F) one dilution of gDNA at 500 GC/rxn was tested. In all cases, a dilution series of 50-50,000 GC/rxn synthetic hMPXV DNA (ATCC® VR-3270SD™) was tested. The PCR assay from (F) Neubauer *et al.* 1998 was an endpoint assay designed for agarose gel screening; however, we successfully used SYBR Green qPCR with the primers here, though the amplification was not efficient.

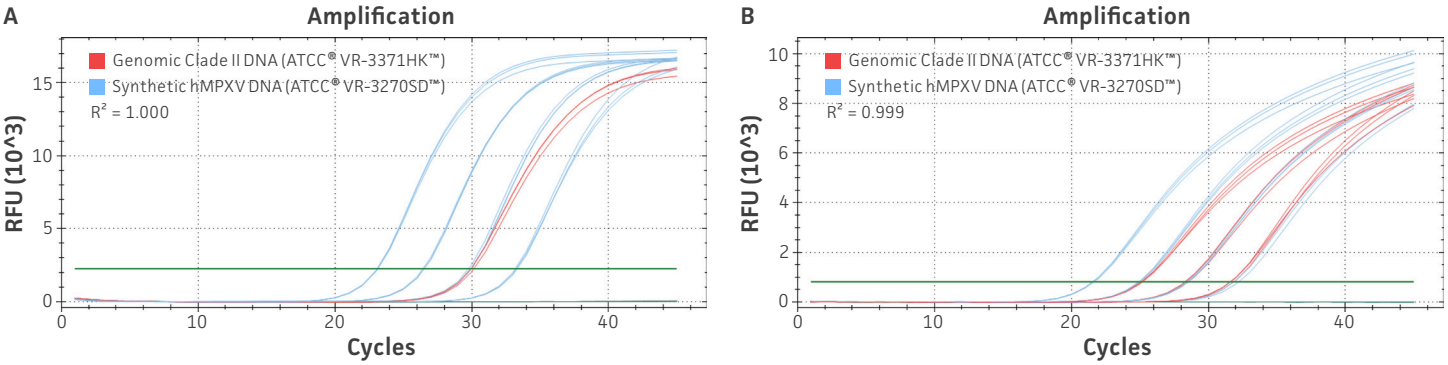

Figure 5: Comparative amplification of synthetic hMPXV DNA and Clade II genomic DNA with assays targeting the *Orthopoxvirus* genus. qPCR amplification of synthetic hMPXV DNA (blue) and gDNA (red) extracted from Clade II MPXV (ATCC® VR-3371HK™) with *Orthopoxvirus* detection assays from (A) Dumont *et al.* 2014[12] and (B) CDC 2022.[13] With the (A) Dumont *et al.* 2014[12] assay, one dilution of gDNA at 500 GC/rxn was tested, and with the (B) CDC 2022[13] assay, three ten-fold dilutions ranging from 50-5,000 GC/rxn were tested. In both cases, a dilution series of 50-50,000 GC/rxn synthetic DNA (ATCC® VR-3270SD™) was tested.

These data collectively indicate that the synthetic hMPXV DNA (ATCC® VR-3270SD™) is effectively designed and suitable for developing and validating molecular-based detection assays. Our findings confirm the compatibility of this ARM with 12 qPCR assays, demonstrating that it can serve as a PCR template control and provide a safe, dependable reference material for molecular assays used in diagnostics and surveillance. Additionally, the synthetic hMPXV DNA achieved impressive R^2 values ranging from 0.991 to 1.000 across all assays, even though we did not perform any in-house assay optimization.

Conclusions

Our data demonstrate that the ATCC® quantitative synthetic hMPXV DNA (ATCC® VR-3270SD™) is a reliable, versatile, and safe ARM for molecular assay development, verification, and validation. It delivers performance comparable to native gDNA while eliminating the biosafety constraints associated with BSL-3 handling. By enabling accurate standard curve generation and compatibility with multiple published qPCR assays for hMPXV detection—including those from the CDC—this synthetic construct provides a critical tool for accelerating diagnostic readiness and surveillance efforts worldwide. Manufactured under ISO 13485 guidance, it ensures regulatory compliance, consistent quality, and enhanced confidence for laboratories seeking reliable positive controls. In short, this ARM bridges the gap between safety and precision, empowering global response efforts against emerging mpox threats.

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