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Development and Validation of a Quantitative Synthetic Molecular Standard for Monkeypox virus

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Background and Introduction

Mpox is a zoonotic viral disease caused by the Monkeypox virus, which is endemic to central and west Africa and has been increasingly appearing in urban areas. Belonging to the genus *Orthopoxvirus* and the family Poxviridae, Monkeypox virus causes a small-pox like disease in humans. Symptoms typically lasts between 2 to 4 weeks and include a fever, intense headache, muscle aches, back pain, low energy, swollen lymph nodes, and a skin rash or lesions. Therefore, the rapid and accurate diagnosis of this disease during the early stages of infection is essential for timely and proper treatment.

While culture-based approaches can be used to detect Monkeypox virus, they are typically time consuming, labor intensive, and require BSL-3 facilities. PCR-based methods provide a highly sensitive and rapid alternative screening approach; however, the development and validation of these assays are dependent on the availability of high-quality reference materials. To address this need, ATCC designed and developed quantitative synthetic molecular standards for Monkeypox virus. Here, we used a proprietary strategy to incorporate genes typically targeted in various assays for viral detection and identification. As proof-of-concept, the Monkeypox virus standard was quantified using Droplet Digital™ PCR (ddPCR™; Bio-Rad), validated via published qPCR assays, and further tested with the CDC non-variola *Orthopoxvirus* and Monkeypox virus generic assays.[1-4]

ATCC Synthetic Molecular Standards

BSL-1	Quantitative	Stabilized

ATCC Catalog Number	Product Description
ATCC® VR-3270SD™	Quantitative Synthetic Monkeypox virus DNA

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

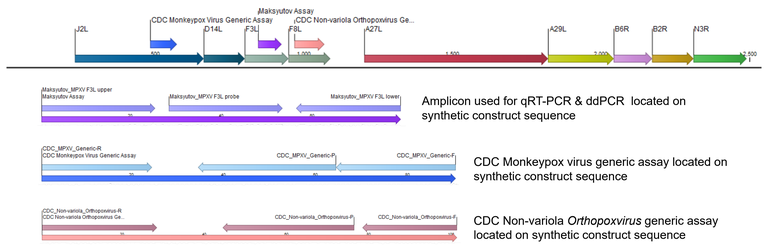
Materials and Methods

Quantitative Synthetic DNA
Using a proprietary method, we designed a synthetic DNA construct for Monkeypox virus (ATCC VR-3270SD). This standard comprises fragments from the following genomic regions: J2L, D14L, F3L, F8L, A27L, A29L, B6R, B7R, and N3R. Following construction, the standard was authenticated via next-generation sequencing and then quantified via droplet digital PCR.

qPCR Assay
qPCR assays were performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad) according to the manufacturer's instructions with slight modifications.

Droplet digital PCR Assay
Droplet digital PCR assays were performed according to the manufacturer's instructions using the QX200™ droplet reader and QuantaSoft™ software 1.7.4.0917 (Bio-Rad) for droplet generation and data analysis.

Synthetic Design



Amplicon used for qRT-PCR & ddPCR located on synthetic construct sequence

CDC Monkeypox virus generic assay located on synthetic construct sequence

CDC Non-variola *Orthopoxvirus* generic assay located on synthetic construct sequence

Results

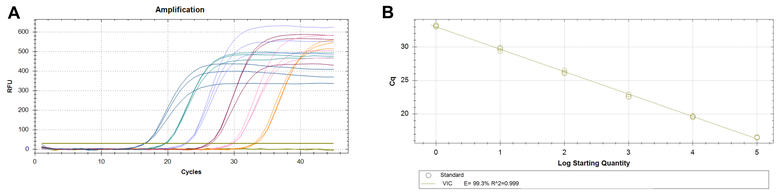


Figure 1: qPCR assay to verify the functionality of the synthetic molecular standards. (A) An amplification plot and (B) standard curve were generated with the Monkeypox virus standard. The qPCR assay was performed as previously described by Maksyutov et al.[1] Cycling conditions were 50°C for 2 min and 95°C for 2 min, followed by 45 cycles of 95°C for 15 sec and 63°C for 1 min. Standard curves were generated by using serial 10-fold dilutions that ranged from 5 copies/µL to 5×10^5 copies/µL. The DNA standard was tested in triplicate.

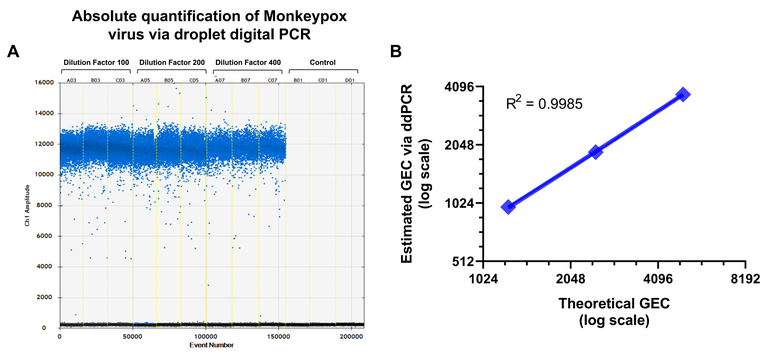


Figure 2: Absolute quantification of Monkeypox virus via droplet digital PCR. (A) One-dimensional (1-D) amplitude scatter plots of positive and negative digital PCR droplet reactions for three dilutions (dilution factors 100, 200, and 400) in triplicate were quantified by ddPCR by using a modified protocol of the published qPCR assay.[1] Digital PCR was performed as followed: initial denaturation at 95°C for 10 min, amplified 40× at 94°C for 30 sec and 60°C for 1 min, and enzyme deactivation at 98°C for 10 min. Droplets were analyzed in the QX200 droplet reader. Data were analyzed with QuantaSoft software (Bio-Rad). (B) Average calculated genome equivalent copy numbers/µL per dilution of stock material.

Results (continued)

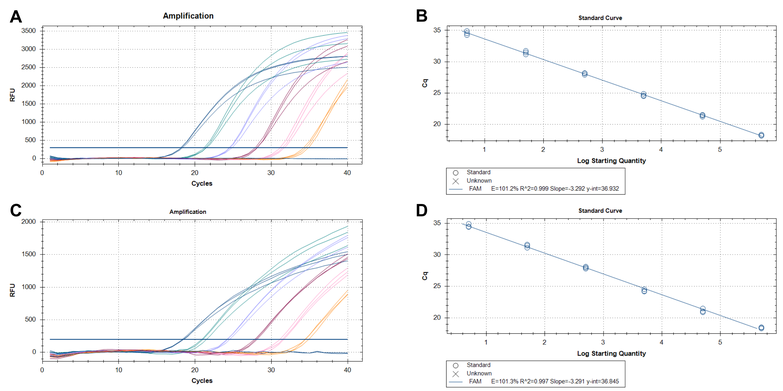


Figure 3: CDC qPCR assays. (A, C) Amplification plots and (B, D) standard curves were generated with the Monkeypox virus standard using the primers and probe from the (C, D) CDC non-variola *Orthopoxvirus* and (A, B) monkeypox generic assays. The qPCR assay was performed as previously described by CDC.[2,3] Cycling conditions for the orthopox assay were 50°C for 2 min and 95°C for 20 sec, followed by 40 cycles of 95°C for 3 sec and 63°C for 30 sec. Cycling conditions for the Monkeypox virus assay were 50°C for 2 min and 95°C for 20 sec, followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec. Standard curves were generated by using serial 10-fold dilutions that ranged from 5 copies/µL to 5×10^5 copies/µL. The synthetic DNA standard was tested in triplicate.

Conclusions

- Our proof-of-concept data demonstrates that the ATCC quantitative synthetic molecular standard for Monkeypox virus can be used as a control for assay development, verification, and validation.
- The standard was manufactured under ISO 13485 guidance and can be used to determine the viral load of unknown mpox samples through the generation of a standard curve.
- The standards are compatible with numerous published assays[1-4] and exhibited minimal variability, as evident from the slope and R^2 values.
- Taken together, this standard provides well-characterized controls for viral detection and quantification.

Order the Synthetic Monkeypox virus DNA

References

1. Maksyutov RA, Gavrilova EV, and Shchelkunov SN. Species-specific differentiation of variola, monkeypox, and varicella-zoster viruses by multiplex real-time PCR assay. J Virol Methods 236: 215-220, 2016. PubMed: 27477914
2. Centers for Disease Control & Prevention Poxvirus & Rabies Branch (PRB). Test Procedure: Non-variola Orthopoxvirus Generic Real-Time PCR Test. Rev. No. 02, 2022.
3. Centers for Disease Control & Prevention Poxvirus & Rabies Branch (PRB). Test Procedure: Monkeypox virus Generic Real-Time PCR Test. Rev. No. 01, 2022.
4. Li Y, Zhao H, Wilkins K, Hughes C, Damon IK. Real-time PCR assays for the specific detection of monkeypox virus West African and Congo Basin strain DNA. J Virol Methods 169: 223–227, 2010. PubMed: 20643162

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