# Extraction free direct detection of Monkeypox virus (MPXV) using a field portable thermocycler and real time–Polymerase Chain Reaction (RT-PCR) assay

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## BACKGROUND

Monkeypox virus (MPXV) is an enveloped DNA virus belonging to family *Poxviridae*, genus orthopoxvirus (OPV). Other closely related members are variola virus (VARV) and zoonotic vaccinia (VACV), cowpox (CPXV), and camelpox viruses. These viruses cause human febrile disease with a rash, which may range from benign lesions to a severe fatal systemic infection such as smallpox. It was first detected in humans in 1970. The first outbreak in humans outside of Africa was reported in 2003 in the USA. Since then, there have been many small outbreaks in the western hemisphere. The recent outbreak has spread to at least 75 countries, with about 16000 cases worldwide. World health organization (WHO) declared monkeypox a public health emergency on July 23rd, 2022. We have evaluated a real-time polymerase chain reaction (RT-PCR) test developed to detect orthopox DNA for detection of monkeypox virus DNA directly without nucleic acid extraction.

**Figure 1.** Portable Thermocycler T-COR 8™, with a portable mixer, cartridge, T-COR 8 tubes, and simple sample collection device.

**Table 1.** The monkey pox virus strains and quantitative synthetic DNA used for verification and validation of the assay parameters

| # | Catalog # | Product description | Comments | Material provided | Titer |
|---|---|---|---|---|---|
| 1 | NR-58622 | Virus Classification: Poxviridae, Orthopoxvirus Species: Monkeypox virus Strain/Isolate: hMPXV/USA/MA001/2022 Original Source: Monkeypox virus, hMPXV/USA/MA001/2022 was isolated from a human in Massachusetts, USA in May of 2022, during an outbreak of monkeypox. | Monkeypox virus, hMPXV/USA/MA001/2022 belongs to Clade IIb (previously west African clade) and lineage B.1.2 The complete genome of monkeypox virus, hMPXV/USA/MA001/2022 has been sequenced (GenBank: ON563414.3 and GISAID: EPI_ISL_13052289). | Each vial contains approximately 0.5 mL of cell lysate and supernatant from Cercopithecus aethiops kidney epithelial cells (BSC-40; ATCC® CRL-2761™) infected with monkeypox virus, hMPXV/USA/MA001/2022. | $1.8x10^6$ TCID50 per mL |
| 2 | NR-2500 | Virus Classification: Poxviridae, Orthopoxvirus Species: Monkeypox virus Strain/Isolate: USA-2003 Original Source: 2003 monkeypox outbreak in Wisconsin. | The complete genomic sequences of monkeypox virus, USA-2003-039 (GenBank: DQ011157) and USA-2003-044 (GenBank: DQ011153) have been determined. | Each vial contains approximately 1 mL of cell lysate and supernatant from African green monkey cells (MA-104 Clone 1; ATCC® CRL-2378.1™; also available as BEI Resources NR-595) infected with monkeypox virus, USA-2003. | $3.2x10^7$ pfu/mL |
| 3 | NR-58627 | NR-58627 was prepared from synthetic DNA from monkeypox virus (ATCC® VR-3270SD™). The product can be used for assay development, verification, validation, monitoring of day-to-day test variation and lot-to-lot performance of molecular-based assays. | The quantitative format allows for the generation of a standard curve for quantitative PCR (qPCR) to determine viral load. The preparation includes fragments from J2L, D14L, F3L, F8L, A27L, A29L, B6R, B7R and N3R regions. | Each vial of synthetic DNA from monkeypox virus contains approximately 100 µL of a proprietary stabilization matrix. The vial should be centrifuged prior to opening. | $1.05x10^8$ Genome copies/mL |

The Tetracore's assay reagents for the detection of MPXV include three forward and three reverse primers and two FAM-labeled probes. This assay has one primer-probe set designed by CDC. The other two primer sets and probe were designed at Tetracore. We performed *in-silico* characterization of all primers and probes by screening them against all publicly available MPXV sequences downloaded from GISAID and NCBI on October 10, 2022. After removing incomplete and low-quality sequences, we performed a BLAST analysis of Tetracore's assay. As shown in the tables below, the Tetracore primer and probe set had a higher *in-silico* specificity for the MPXV targets. It should be noted that the depositors label a significant number of the MPXV sequences present in the dataset as partial or unverified. The MPXV target sequences for the CDC and Tetracore assays are ~18,000 bases apart.

**Table 2.** *In-silico* characterization of primer and probe sequences

| | Specificity of Primer and Probe sequences to MPXV dataset | | | | | |
|---|---|---|---|---|---|---|
| F = Forward primer<br>P= Probe<br>R = Reverse primer | Blastable MPXV sequences with Perfect Matches | % Blastable MPXV sequences with Perfect Matches | Blastable MPXV sequences with Mismatch | % of Blastable MPXV sequences with Mismatch | Minor Mis-matches | % of Total sequences (N=3244) |
| CDC MPXV-F | 67 | 2.3 | 2823 | 87.0 | 2890 | 89.1 |
| CDC MPXV-P | 2888 | 99.9 | 2 | 0.1 | 2890 | 89.1 |
| CDC MPXV-R | 174 | 6.0 | 2549 | 83.7 | 2890 | 89.1 |
| Tetracore MPXV-F1 | 3017 | 99.9 | 5 | 0.2 | 3021 | 93.1 |
| Tetracore MPXV-F2 | 3012 | 99.9 | 13 | 0.4 | 3015 | 92.9 |
| Tetracore MPXV-P | 3023 | 99.9 | 4 | 0.1 | 3025 | 93.2 |
| Tetracore MPXV-R1 | 2935 | 99.9 | 69 | 2.1 | 3000 | 92.5 |
| Tetracore MPXV-R2 | 67 | 2.2 | 2937 | 90.5 | 3004 | 92.6 |

We also determined the selectivity of these primers and probes to a collection of other members of the *Poxviridae* family, including vaccinia and variola virus targets.

**Table 3.** This table indicates the identities between each primer and probe to the *Poxviridae* family. Pink highlighted boxes indicate if the primer would be able to participate in PCR assays based on the primer binding.

| Viral Target | Identities for Primer and Probe Sequences compared to *Poxviridae* family | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CDC MPXV-F | CDC MPXV-P | CDC MPXV-R | Tetracore MPXV-F1 | Tetracore MPXV-F2 | Tetracore MPXV-P | Tetracore MPXV-R1 | Tetracore MPXV-R2 |
| Monkeypox virus isolate hMPXV/Austria/MUW1525179/2022 | 25/26 | 30/30 | 23/24 | 31/31 | 32/32 | 24/24 | 22/22 | 21/22 |
| Camelpox virus M-96 from Kazakhstan, complete genome | 24/26 | 22/30 | 23/24 | 24/31 | 21/32 | 12/24 | 13/22 | 17/22 |
| VACCG Vaccinia virus Copenhagen, complete genome | 24/26 | 11/30 | 23/24 | 12/31 | 14/31 | 12/24 | 13/22 | 11/22 |
| Variola virus, complete genome | 23/26 | 22/30 | 24/24 | 24/31 | 21/32 | 12/24 | 13/22 | 17/22 |

**RESULTS:**

- We heat inactivated the virus and then tested using both lab based Quant studio 5 and the portable T-COR 8 thermocycler. The detection was successfully performed using these samples directly.
- Sensitivity of detection on T-COR 8 matches the lab based QuantStudio (Tables 4 and 5).

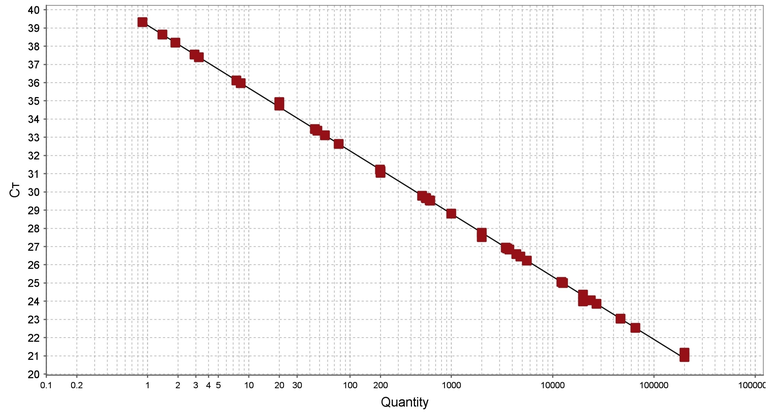


**Figure 2.** To determine analytical sensitivity of the assay we used synthetic DNA from BEI resources (described in Table 1). The assay sensitivity was determined to be 20 copies per reaction. $R^2$ = 0.998: Slope= -3.447; Efficiency = 95.02%.

**Table 4.** Two monkey pox virus strains were tested on QuantStudio™ 5

| | | NR-58622 | | NR-2500 | |
|---|---|---|---|---|---|
| Well | Sample dilution | Average Ct MPXV | Average Ct IC | Average Ct MPXV | Average Ct IC |
| 1 | 10 | 23.96 | 35.95 | 25.02 | 37.21 |
| 2 | 100 | 26.71 | 32.11 | 26.92 | 32.30 |
| 3 | 1000 | 29.66 | 31.94 | 29.67 | 31.53 |
| 4 | 10000 | 33.28 | 31.82 | 33.00 | 31.57 |
| 5 | 100000 | 37.16 | 31.45 | 37.47 | 31.84 |
| 6 | 1000000 | | 32.05 | | 32.05 |
| 7 | Negative control | | 32. 35 | | 31.96 |

**Table 5.** Two monkey pox virus strains were tested on portable thermocycler T-COR 8

| | | NR-58622 | | NR-2500 | |
|---|---|---|---|---|---|
| Well | Sample dilution | Average Ct MPXV | Average Ct IC | Average Ct MPXV | Average Ct IC |
| 1 | 10 | 25.85 | 34.60 | 26.20 | 34.35 |
| 2 | 100 | 28.15 | 31.25 | 28.25 | 31.40 |
| 3 | 1000 | 31.50 | 31.35 | 31.40 | 31.50 |
| 4 | 10000 | 34.80 | 31.65 | 34.60 | 31.65 |
| 5 | 100000 | 38.60 | 31.45 | 37.10 | 31.65 |
| 6 | 1000000 | | 31.45 | | 31.50 |
| 7 | Positive control | 24.40 | 30.90 | 24.15 | 30.85 |
| 8 | Negative control | | 31.55 | | 31.35 |

**CONCLUSIONS:**

The integrated system of the portable thermocycler T-COR 8 and the Tetracore's assay reagents provides a sensitive tool for detection of MPXV. We continue to seek partnerships to obtain clinical samples for further assay characterization.

**Acknowledgements**

The following reagent was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH: Monkeypox Virus, hMPXV/USA/MA001/2022 (Lineage B.1, Clade IIb), NR-58622; Monkeypox Virus, USA-2003, NR-2500; Quantitative Synthetic DNA from Monkeypox Virus, NR-58627 (Table 1)