



Development of Reference Materials for Detection of Monkeypox

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Introduction

Monkeypox virus (MPXV) is a member of *Poxviridae* family and belongs to the genus of *orthopoxvirus*. MPXV has a double stranded DNA genome of approximately 197 kb with almost 190 nonoverlapping ORFs that encode all of the enzymes needed for transcription and replication process of the genome. Infection in humans can lead to a smallpox-like illness with almost 11% fatality rate in individuals who are not vaccinated. ST-246 (tecovirimat), an FDA approved drug against Smallpox virus, has shown some efficacy against MPXV, making accurate and sensitive diagnostic testing for MPXV critical for patient care. Here we describe the development of AccuPlex™ Monkeypox Reference Material, which is a non-infectious, stable, and reproducible standard to aid nucleic acid testing for MPXV.

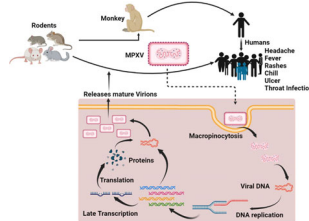


Figure 1: Molecular mechanism of action of MPXV in humans. After entering the human body via macropinocytosis, the viral DNA undergoes replication followed by transcription, translation, and final release of mature virions that again goes through the whole infection cycle.

Materials and Methods

AccuPlex™ Monkeypox Reference Material consists of recombinant Adeno virus bearing four complete MPXV genes: J2L (TNF receptor gene), F8L (DNA polymerase gene), F3L (Double-stranded RNA-binding protein, inhibitor of IFN signaling), and N3R (orthopoxvirus MHC class I-like protein -OMCP). The design of the recombinant virus is based on the sequence of ON585038.1 USA strain from the 2022 outbreak. A digital PCR (dPCR) assay was designed using the E9L Non-variola (NVAR) Orthopox Generic Assay design described in Y. Li et al. /Journal of Clinical Virology 36 (2006) 194-203. The Bio-Rad QX-200 Droplet Digital PCR system was used to quantify the viral concentration (copies/mL) and guide the formulation process. The final product is targeted as a low positive control, approximately 2-3 times the detection limit of commercial PCR testing methods and is formulated in defibrinated human plasma.

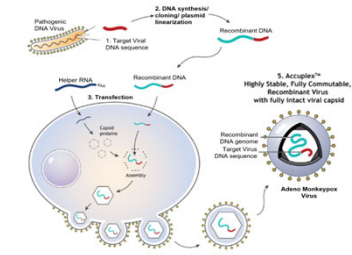


Figure 2: Schematic representation of production of AccuPlex™ Monkeypox Reference Material. A plasmid bearing MPXV sequences was synthesized and transfected into HEK-293 cells. Once the cells reached CPE (viral-induced cytopathic effect) the high titer viral supernatant was collected and purified.

Results

After extraction with the Viral DNA extraction kit, an average concentration of 5.0E+03 copies/mL was measured for the AccuPlex™ Monkeypox Reference Material using digital PCR. The material was tested on multiple RT-PCR platforms externally to verify product performance and compatibility with assay detection limits. Positive detection was observed on all three assays and external sites evaluated (Table 1).

One of the external testing sites, LGC Biosearch Technologies, performed a serial dilution of the original extracted DNA sample in 1X TE buffer and measured each dilution by RT-PCR. The data revealed 100% detection above 12.5 copies per reaction though reduced detection was observed down to ~3 copies per reaction (Table 2, Figure 3).

The concentration of the reference material was also measured by dPCR at dispense, 15 days, 1 month, 3 months, and 4 months using multiple operators and reagent lots. The results indicate that the product has sufficient stability and reproducibility to meet its intended use as a low positive reference material (Figure 4).

Site	RT-PCR Method	Result
Quest Diagnostics	Qualitative	Positive
ARUP Laboratories	Qualitative	Positive
LGC Biosearch Technologies	Quantitative	Positive

Table 1: External test results for the AccuPlex™ Monkeypox Reference Material.

Concentration (copies/mL)	Copies per Reaction	Mean Ct Value	% of detection
5000	100	33.62	100
2500	50	34.70	100
1250	25	35.75	100
625	12.5	36.67	100
313	6.25	37.88	85
156	3.125	38.17	70
0	0	N/A	0
NTC	NTC	N/A	0

Table 2: RT-PCR results for the AccuPlex™ Monkeypox Reference Material serial dilutions of extracted DNA performed by LGC Biosearch Technologies using Kingfisher sample DNA extraction method.

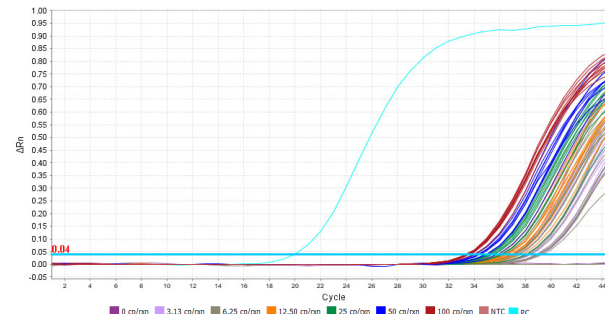


Figure 3: Amplification plot representing the RT-PCR data of AccuPlex™ Monkeypox Reference Material with serial dilutions performed by LGC Biosearch Technologies using Kingfisher Sample DNA extraction method.

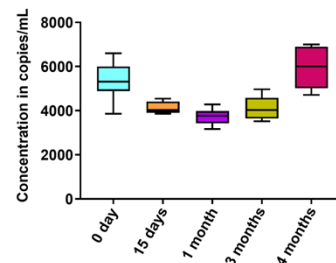


Figure 4: Stability of AccuPlex™ Monkeypox Reference Material over 4 months. Concentration was measured by dPCR and average and standard deviation of 9 replicates is shown. Study data was generated by 3 operators and 3 reagent lots.

Conclusions

- LGC Clinical Diagnostics has developed a stable, non-infectious reference material using recombinant Adenovirus technology for assays that detect MPXV DNA.
- Data presented indicate the compatibility of the reference material with commercially available real time PCR assays.
- The data presented indicate how the material could be used for monitoring the sensitivity of MPXV assays.
- This material will be a useful tool for development, validation, training, and can be used in ongoing Quality Control testing of MPXV detection assays and workflows.



Figure 5: AccuPlex™ Monkeypox Reference Material Kit released by LGC Clinical Diagnostics.