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Monkeypox virus multiplexed PCR amplicon sequencing (PrimalSeq) V.1.

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In Development Share

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DISCLAIMER

ABSTRACT

Background: The current global outbreak of Monkeypox virus (MPXV) concurrent with an ongoing SARS-CoV-2 pandemic has further highlighted the need for genomic surveillance and pathogen whole genome sequencing. While metagenomic and hybrid capture sequencing approaches were used to sequence many of the early MPXV cases, the viability of these methods is dependent on samples with high viral DNA concentrations. Given the atypical clinical presentation of cases associated with the current outbreak and uncertainty regarding viral load across both the course of infection and anatomical body sites, there is a strong need for a more sensitive and broadly applicable sequencing approach. Amplicon-based sequencing (PrimalSeq) was initially developed for sequencing of Zika virus, and later adapted as the main sequencing approach for SARS-CoV-2. Here, we used PrimalScheme to design a primer scheme for

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MPXV and we validated it with widely used SARS-CoV-2 sequencing protocols. Based on initial validation, our approach shows notably higher depth and breadth of coverage across the genome, particularly with higher PCR cycle threshold (Ct) samples, as compared to metagenomic sequencing. While further testing is needed, the early success of this approach has significant implications for sequencing efforts of the current MPXV outbreak and serves as a proof of concept of amplicon-based sequencing for use with other large-genome DNA viruses and potentially bacterial genomes.

Overview of Design: We used PrimalScheme (https://primalscheme.com/) to generate an MPXV primer scheme using a pre-outbreak A.1 clade reference genome (GenBank accession: MT903345). The primer scheme comprises a total of 163 primer pairs with an amplicon length ranging between 1597 and 2497 bp (average length of 1977 bp). The primer scheme is compatible with current ARTIC and COVIDSeq SARS-CoV-2 sequencing protocols, and while validated only with Illumina library prep methods and sequencing platforms, it would likely see a high degree of success with other sequencing platforms such as the Oxford Nanopore Technologies MinION.

Initial Validation: We validated our MPXV primer scheme with clinical specimens at the Massachusetts Department of Public Health, Massachusetts State Public Health Laboratory under the IRB Project Titled "Rash Illness: Alternate Specimen Types and Sequencing" (protocol number 1917413). A total of 10 clinical specimens were included in this initial validation comprised of both throat and swabs of fluid from lesions from two individuals, belonging to the current outbreak-associated hMPXV B.1 clade as determined by prior characterization at the Centers of Disease Control and Prevention. Ct values were determined with the non-variola orthopox diagnostic qPCR assay developed for use by the Laboratory Response Network (Rapid Diagnostic Testing for Response to the Monkeypox Outbreak — Laboratory Response Network, United States, May 17–June 30, 2022 | MMWR (cdc.gov)). Clinical samples ranged in cycle threshold (Ct) values from 15.03 (high viral concentration) to 34.63 (low viral concentration), and each sample was sequenced in parallel using a metagenomics approach and the provided amplicon-based approach. Extractions and sequencing analysis were performed in accordance with current biosafety guidance including extraction in a BSL-3 setting. An evaluation of site-specific biosafety practices should be developed in consultation with your organization's biosafety officer. Libraries were prepared with the Illumina DNA prep kit and sequenced on the MiSeq (v2 kit running 2x150 nt reads). Consensus genomes were generated at 10X coverage using the TheiaCoV_Illumina_PE Workflow Series on Terra.bio. A MPXV "fork" was developed for us by Curtis Kapsak from Theiagen, which included the MPXV reference sequence, primer scheme, and consensus genome length, and can currently be accessed at: https://github.com/theiagen/public_health_viral_genomics/tree/cjk-MPXV-theiacov. A non-MPXV specific TheiaCov workflow can also be found at: https://public-health-viral-genomics-theiagen.readthedocs.io/en/latest/theiacov_workflows.html with an accompanying protocols.io at: https://www.protocols.io/private/0E095FC2AC5211EC82F60A58A9FEAC02. We found comparable genome coverage between amplicon and metagenomic sequencing with low Ct (<18) samples, yet a significant increase in genome coverage with amplicon sequencing in higher Ct samples (>18; **Figure 1.**) Our findings highlight how amplicon-based approaches can significantly expand MPXV sequencing to a wider variety of samples.

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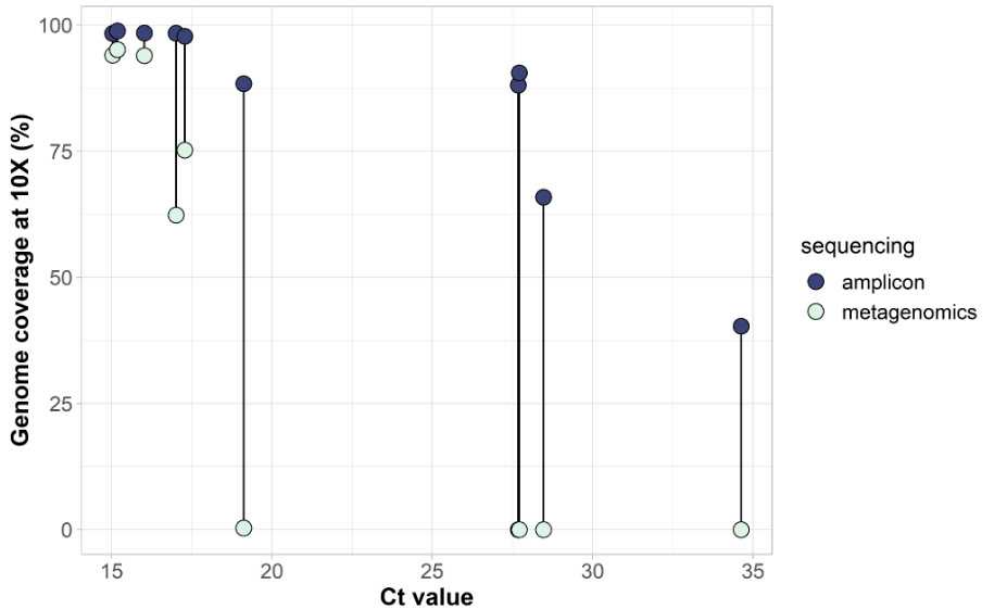

Figure 1. Percent genome coverage at 10X for clinical specimens sequenced with metagenomic and amplicon-based approaches.

Amongst samples with a low Ct (<18), genome coverage via amplicon sequencing was consistently >97%, with minimal amplicon drop-outs (**Figure 2**). Amplicons 11, 75, and 118 showed consistent drop-out across the sequenced samples, while none of the primers had mismatches, except for a single nucleotide mismatch in the 11_RIGHT primer. We did obtain coverage for these amplicons when sequencing clade 2 DNA from cultured virus (strain USA-2003; NR-4928) obtained from BEI Resources (NIAID, NIH). This suggests that the dropouts of these primers may be a specific issue related to the current hMPXV B.1 clade genomes. As this protocol is still in development, we will further investigate performance as we continue to sequence additional samples.

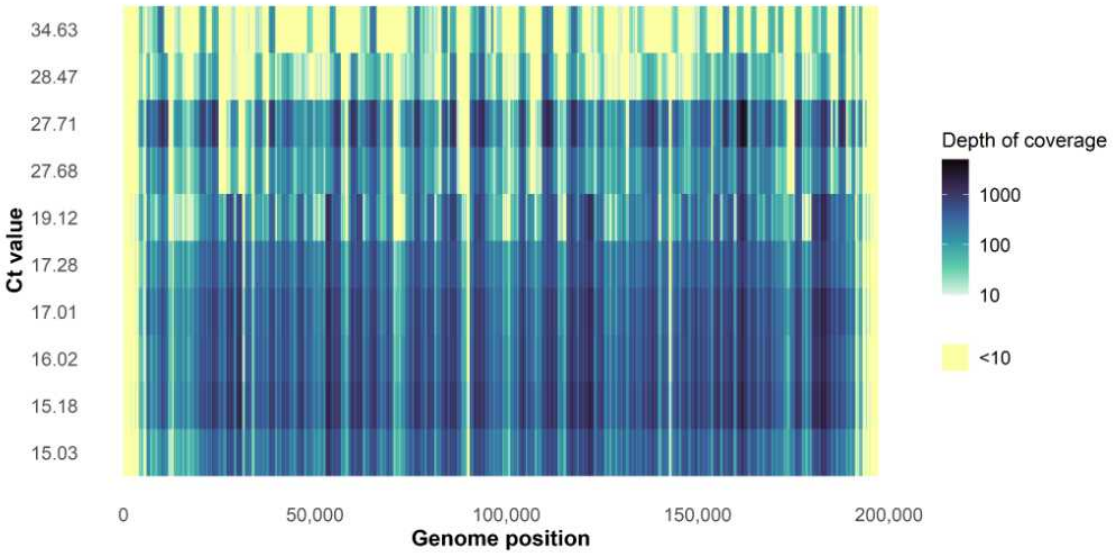

Figure 2. Depth of coverage by genome position and Ct value for 10 clinical specimens sequenced using the amplicon-based sequencing approach.

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Conclusion: We developed an amplicon-based sequencing (PrimalSeq) approach for MPXV that improved the depth and breadth of genome coverage with low viral concentration specimens as compared to metagenomic sequencing.

This protocol represents the first iteration of development. Further versions will be uploaded to protocols.io with an accompanying description of changes as appropriate.

DOI

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PROTOCOL CITATION

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KEYWORDS

Monkeypox, MPXV, Amplicon sequencing, PrimalSeq

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Jul 15, 2022

PROTOCOL INTEGER ID

65116

MATERIALS TEXT

MPXV Primer Scheme

MPXV-primer_genome-positions.tsv

For instructions on how to prepare MPXV Primer Pool 1 and 2 (10 uM) see step 1 of the protocol.

Library Preparation Method: Illumina DNA Prep

Reagents: https://www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/nextera-dna-flex.html

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https://www.neb.com/products/m0494-q5-hot-start-high-fidelity-2x-master-mix#Product%20Information

Library Preparation Method: CovidSeq

Reagents: https://www.illumina.com/products/by-type/ivd-products/covidseq.html

A	B	C
Illumina COVIDSeq Test Box 1 – 3072 Samples, Part # 20044408		
Reagent	**Description**	**Storage**
ITB	Illumina Tune Beads	Room Temperature
ST2 HT	Stop Tagment Buffer 2 HT	Room temperature, post-amp environment
Illumina COVIDSeqTest Box 2 – 3072 Samples, Part # 20044409		
EBLTS HT	Enrichment BLT HT	2°C to 8°C post-amp environment
TWB HT	Tagmentation Wash Buffer HT	2°C to 8°C post-amp environment
RSB HT	Resuspension Buffer HT	2°C to 8°C, post-amp environment
Illumina COVIDSeq Test Box 3 – 3072 Samples, Part # 20044410		
IPM HT	Enhanced PCR Mix HT	-25°C to -15°C, pre-amp environment
TB1 HT	Tagmentation Buffer 1 HT	-25°C to -15°C, post-amp environment
EPM HT	Enhanced PCR Mix HT	-25°C to -15°C, pre-amp environment
Index Adapater Part Numbers : 20043132, 20043133, 20043134, 20043135		
Index Adapters	IDT for Illumina- PCR Indexes Set 1-4	Room Temperature

Additional Materials

A	B	C
Reagent	**Description**	**Storage**
80% EtOH	80% Ethanol	Room Temperature
Nuclease-free water		Room Temperature

SAFETY WARNINGS

Processing of any sample type which could potentially be positive for MPXV should be

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conducted in BSL2+ settings. Before starting work with these samples, please contact your local EHS (environment, health and safety) or biosafety office for proper guidance on how to work with these samples in your laboratory.

DISCLAIMER:

BEFORE STARTING

This protocol is currently in the developmental phase. It is for research purposes only and should not be used in a diagnostic capacity.

If using clinical samples, DNA will need to be extracted and purified prior to beginning library preparation.

Primer diluting and pooling requires several hours and may be conducted well in advance so long as the pooled primers are stored at -20°C.
Following primer dilution and pooling, this workflow can be completed in one day, however, it is recommended to be conducted across two, with the amplicon generation step on the first day and all subsequent wet-lab steps on the second day.

Dilute and Pool Primers

1 **Reagents:**

Primer Preparation

Reagent	Storage	Instructions
82 Odd Numbered Primer Pairs (100 µM)	-20°C	Thaw at RT
81 Even Numbered Primer Pairs (100 µM)	-20°C	Thaw at RT
Nuclease-free water	RT	

Primers should be ordered lyophilized or resuspended (100 uM; recommended). Lyophilized primers should be resuspended to 100 uM in nuclease-free water.

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Primer Scheme: MPXV-primer_genome-positions.tsv

1.1

If not already done, separate odd and even numbered primer pairs into two separate boxes. These will constitute the two pools

For example:
Primer Pool 1: *1 left, 1 right, 3 left, 3 right, etc.*
Primer Pool 2: *2 left, 2 right, 4 left, 4 right, etc.*

1.2 Label 164, 8-strip tubes with the corresponding odd-numbered primer name (e.g. 3 left)

1.3 To each tube add **90 µL** of nuclease-free water

This will comprise the 10X dilution to arrive at a final primer concentration of 10uM

1.4

For each odd-numbered primer tube:

- Spin down
- Pipette 10 times to mix
- Add **10 µL** to the corresponding labeled tube
- Pipette 10 times to mix

1.5 After all 164 primers have been aliquoted, combine 10uL from each tube into a 2mL tube. This will be the odd-numbered primer pool

To most efficiently pool all 164 primers, use a multi-channel pipette to remove

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10uL from each 8-strip and pool into a new 8-strip.
Then combine each of the pooled 8 strip tubes into a single 2mL tube

1.6 Repeat steps 1.2-1.5 with the even -numbered primers (**NOTE:** There will be two fewer primers in the even set than the odd)

1.7

Safe Stopping Point: Pooled Primers Can Be Stored at -20°C

2 Library Preparation Method

Step 2 includes a Step case.

Illumina CovidSeq Test (RUO)

Illumina DNA Prep

Amplicon Generation

step case

Illumina CovidSeq Test (RUO)

3 Reagents:

Reagent	Storage	Instructions
MPXV Primer Pool 1 (10 uM)	-20°C	Thaw at RT
MPXV Primer Pool 2 (10 uM)	-20°C	Thaw at RT
IPM HT	-20°C	Thaw at RT

3.1 In two separate tubes, prepare the following master mixes:

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Pool 1

Reagent	Volume in 20 µL master mix
IPM HT	12.5 µL
MPXV Primer Pool 1 (10 uM)	3.6 µL
Nuclease-free water	3.9 µL

Pool 2

Reagent	Volume in 20 µL master mix
IPM HT	12.5 µL
MPXV Primer Pool 2 (10 uM)	3.6 µL
Nuclease-free water	3.9 µL

Master mix volumes are for one reaction and do not account for lost volume due to pipetting. Multiply volumes by reaction number accordingly.

3.2 Label two sets of PCR tubes/plates for Pool 1 and Pool 2

3.3 Add the following:

- **20 µL Pool 1 master mix** to each Pool 1 tube/well
- **20 µL Pool 2 master mix** to each Pool 2 tube/well

3.4 Add **5 µL DNA** to each tube in **both** sets

- Mix by pipetting up and down 10 times
- Briefly centrifuge tubes/plates

Be sure to include a negative PCR control (NTC; nuclease-free water) for **each pool**

3.5

Place on thermocycler and run the following program (choose preheat lid option):

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PCR Program			
Step(s)	**Temperature**	**Time**	**Cycles**
Initial denaturation	98°C	3min	1
Denaturation	98°C	15sec	35 times
Anneal and extension	63°C	5min	
Hold	4°C	Hold	Hold
Volume Amount: 25 µL		*Lid: 105°C*	

3.6

Safe Stopping Point: Amplified DNA may remain in the thermocycler at 4°C or stored at -20°C until ready to use

Amplicon Tagmentation and Clean Up

4 **Reagents:**

Reagent	Storage	Instructions
EBLTS HT	4°C	Thaw at RT; vortex to mix
TB1 HT	-20°C	Thaw at RT; vortex to mix
ST2 HT	RT	Vortex before use
TWB HT	4°C	Vortex before use

4.1 Spin down PCR tubes/plates

4.2 Prepare the following master mix:

Reagent	Volume in 30 µL master mix
TB1	10 µL
EBLTS	3.3 µL
Nuclease-free water	16.7 µL

4.3 In **each** PCR tube add:

- **30 µL Master mix**
- **10 µL Pool 1 amplicons**

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- **10 µL** **Pool 2 amplicons**

For a total reaction volume of **50 µL** per PCR tube/well

Pooling of amplicons should be conducted on a dedicated post-PCR bench to prevent contamination

4.4 Mix by pipetting up and down and briefly centrifuge

4.5

Place on thermocycler and run the following program (choose preheat lid option):

Temp	Time
55°C	3 minutes
10°C	hold

4.6 Once the thermocycler reaches **10 °C** , remove tubes/plates and spin down

4.7 Add **10 µL** **ST2** to each tube/well, mix by pipetting up and down, and briefly centrifuge

4.8 Incubate at **Room temperature** for **00:05:00** 5m

4.9 Place on magnetic stand and wait until liquid is clear (a few minutes)

4.10 Remove and discard all supernatant

4.11 Remove tubes/plates from magnetic stand and add **100 µL** **TWB** to each

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tube/well

- Mix by pipetting up and down 10 times and spin down. **Be careful to not introduce bubbles**.

4.12 Repeat steps 4.9 - 4.11. **Leave the supernatant after the second wash so that the beads don't dry out**

Amplify Tagmented Amplicons

5 **Reagents:**

Reagent	Storage	Instructions
EPM HT	-20°C	Invert to mix
Index adapters	-20°C	Thaw at RT; vortex to mix

5.1 Prepare the following master mix:

Reagent	Volume in 40 µL master mix
EPM	20 µL
Nuclease-free water	20 µL

5.2 Place the tubes/plates with tagmented amplicons on the magnetic stand and remove the supernatant once the liquid is clear

5.3 Use a **20µL pipette** to remove any residual TWB from tubes/wells

5.4 Remove the tubes/plates from the magnetic stand

5.5 Add **40 µL master mix** to each tube/well

5.6 Add **10 µL dual-barcoded index adapters** to each tube/well

Note which set of indexes are used for each tube for bioinformatic processing

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5.7 Pipette up and down to mix and spin down

5.8

Place on thermocycler and run the following program (choose preheat lid option):

Temp	Time	Repeat
72°C	3 minutes	
98°C	3 minutes	
98°C	20 seconds	7 times
60°C	30 seconds	
72°C	1 minute	
72°C	3 minutes	
10°C	hold	

Pool and Clean Up

6 **Reagents:**

Reagent	Storage	Instructions
ITB	RT	Vortex thoroughly to mix
RSB HT	4°C	Bring to RT; vortex to mix
80% EtOH	RT	Prepare immediately before use

6.1 Briefly centrifuge tubes/plates

6.2 Place on magnetic stand and wait until liquid is clear (a few minutes)

6.3 Pool libraries by equal volume:

Number of individual samples	Volume to pool per sample
1-24	40 µL
25-48	20 µL
49-72	10 µL
72-96	5 µL

6.4 Pipette up and down to mix pooled libraries and briefly spin down

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6.5 Calculate the volume of **ITB** to reach a **0.6X** beads:total pool volume ratio

For example: Total pooled volume is 400 µL (10 samples x 40 µL/sample) then add 0.6 x 400 µL = 240 µL ITB

6.6 Mix by pipetting up and down, briefly centrifuge, and incubate at 5m
Room temperature for **00:05:00**

6.7 Place on magnetic stand and wait until liquid is clear (a few minutes)

6.8 Transfer supernatant to a new tube **(do not discard, this is your final library)**. This volume will be slightly lower than the total combined volume

For example: 400µL+ 240µL = total 640µL > transfer 630µL

6.9 ⚠

Calculate the volume of beads to add to the supernatant to attain a second clean-up beads:sample ratio of **0.9X:**

$$\frac{\text{beads from 1}^{st}\text{ step} + \text{beads added in 2}^{nd}\text{ step} \times \frac{Vo}{Vt}}{\text{Volume of DNA sample from right-side clean-up}}$$

Vo = total volume of sample + beads from step 5.5
Vt = transferred volume of supernatant

***For example**: 630µL of supernatant is transferred to a new tube*

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$$240 + \mathbf{118} * \frac{640}{630} = 360$$

$$\frac{360}{400} = \mathbf{0.9}\boldsymbol{X}$$

*Add **118µL** of ITB to **630µL** of transferred supernatant*

***Calculation source**:* https://support.illumina.com/bulletins/2020/07/library-size-selection-using-sample-purification-beads.html

6.10 Add **beads** to supernatant, mix by pipetting up and down, briefly centrifuge, and incubate at **Room temperature** for **00:05:00** 5m

6.11 Place on magnetic stand and wait until liquid is clear (a few minutes)

6.12 Carefully discard supernatant

6.13 Add **1000 µL** **80% EtOH**

6.14 Wait **00:00:30** 30s

6.15 Repeat steps 6.12 - 6.14

6.16 Remove supernatant using a **20µL pipette** to remove all residual EtOH

6.17 Add **55 µL** of **RSB** to the tube, mix by pipetting up and down, and briefly centrifuge

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6.18 Incubate at **Room temperature** for **00:02:00** 2m

6.19 Place on magnetic stand and wait until liquid is clear (a few minutes)

6.20 Transfer **50 µL** of final pooled library to a new 1.5mL tube

6.21

Quantify library on a Qubit and obtain fragment distribution using a Bioanalyzer/Tape Station

Qubit SOP: **Agilent High Sensitivity DNA Kit Guide.pdf**
Bioanalyzer SOP: **Agilent High Sensitivity DNA Kit Guide.pdf**

Sequencing

7 Protocol validated on the MiSeq (v2 kit running 2x150 nt reads)

Sequencing may be performed on Illumina (and potentially Oxford Nanopore Technologies) sequencing platforms following standard protocols

Bioinformatics/Analysis

8 Sequencing results may be analyzed utilizing a standard amplicon sequencing bioinformatics pipeline, including those employed for SARS-CoV-2 sequencing.

If utilizing a SARS-CoV-2 based bioinformatics pipeline, adjustments may be required to account for the consensus genome length

Optional bioinformatics pipeline: **Terra.bio_TheiaCov_Illumina_PE_workflow**
Reference Sequence: **MPXV.reference.fasta**

Citation: Nicholas F.G. Chen*, Luc Gagne*, Matthew Doucette, Sandra Smole, Erika Buzby, Joshua Hall, Stephanie Ash, Rachel Harrington, Seana Cofsky, Selina Clancy, Curtis J Kapsak, Joel Sevinsky, Kevin Libuit, Chrispin Chaguza, Nathan D. Grubaugh, Daniel J. Park, Glen R. Gallagher#, Chantal B.F. Vogels# Monkeypox virus multiplexed PCR amplicon sequencing (PrimalSeq) V.1.ÃÂ
https://dx.doi.org/10.17504/protocols.io.5qpvob1nbl4o/v1

Bed file: MPXV.primer.bed

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