



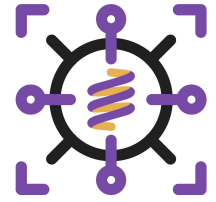
Apr 08, 2025

Version 5

Monkeypox virus multiplexed PCR amplicon sequencing (PrimalSeq) V.5

DOI

<https://dx.doi.org/10.17504/protocols.io.5qpvob1nbl4o/v5>



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Protocol 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, Mallery Breban, Chrispin Chaguzza, Nathan D. Grubaugh, Daniel J. Park, Jiusheng Deng, Crystal Gigante, Glen R. Gallagher#, Chantal B.F. Vogels# 2025. Monkeypox virus multiplexed PCR amplicon sequencing (PrimalSeq) . **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.5qpvob1nbl4o/v5>Version created by **Chantal Vogels**

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Protocol status: Working

We use this protocol and it's working

Created: April 08, 2025

Last Modified: April 08, 2025

Protocol Integer ID: 126391

Keywords: Monkeypox, MPXV, Amplicon sequencing, PrimalSeq, hMPXV, current global outbreak of human monkeypox virus, pathogen whole genome sequencing, human monkeypox virus, hmpxv primer scheme with clinical specimen, efforts of the current hmpxv outbreak, monkeypox virus, current hmpxv outbreak, metagenomic sequencing, monkeypox outbreak, hmpxv reference sequence, zika virus, sequencing analysis, whole genome sequencing, genome dna virus, clade iia dna from cultured virus, sequencing clade, covidseq sar, primer scheme for hmpxv, sequencing approach, sequencing clade iia dna, comparable genome coverage, comparable genome coverage between amplicon, high viral dna concentration, hmpxv primer scheme, genome coverage via amplicon, sequencing effort, improved genome coverage, metagenomics approach, sequencing, sequencing platform, sequencing protocol, genomic surveillance, pathogen, current global outbreak, need for genomic surveillance, metagenomic, genome coverage, significant increase in genome coverage, consensus genome leng

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Abstract

Version 5 Updates:

- *Release of updated primer scheme (v1.1) that includes 11 additional primers to be spiked into the original primer scheme to improve coverage when sequencing clade I viruses. See materials tab for primer information and step 8 for new clade I reference and BED files.*

Complete validation study: <https://doi.org/10.1101/2022.10.14.22280783>

Background: The current global outbreak of human Monkeypox virus (hMPXV) 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 mpox 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 hMPXV 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 hMPXV outbreak and serves as a proof of concept of amplicon-based sequencing for use with other large-genome DNA viruses and potentially bacterial genomes.

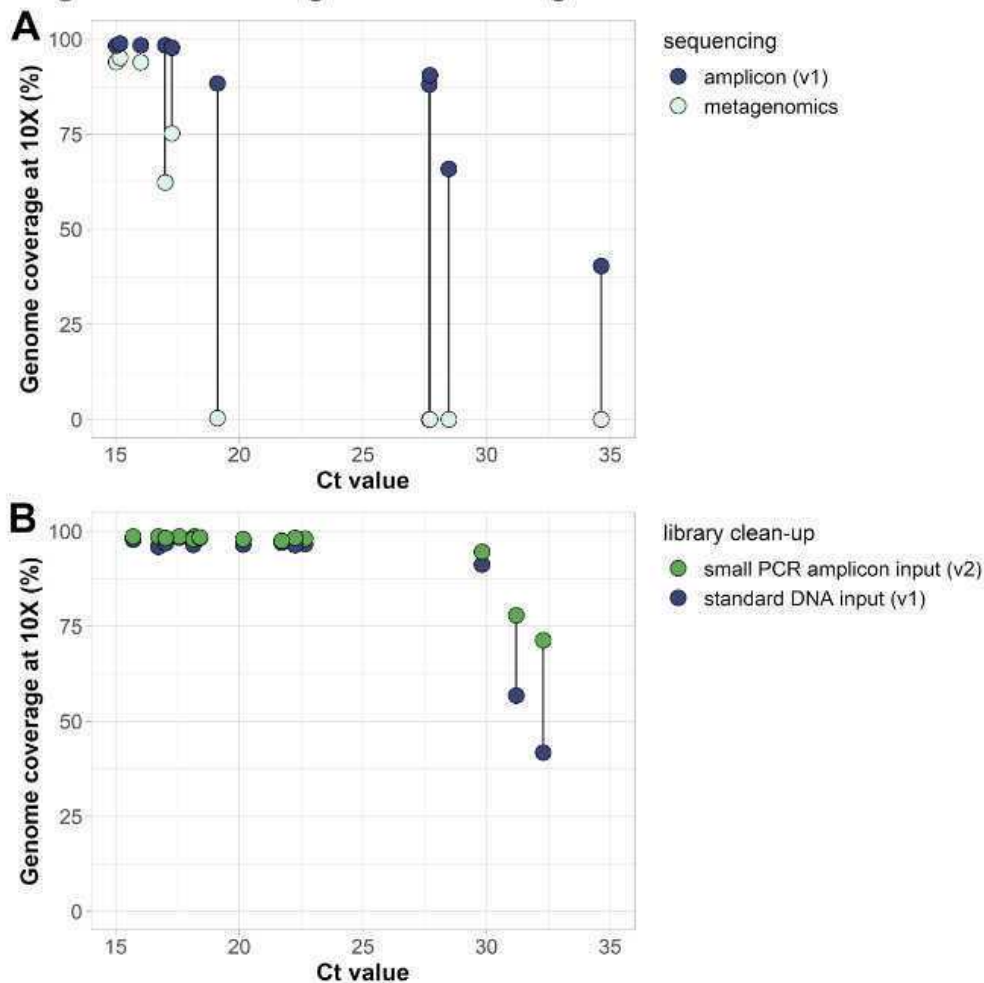
Overview of Design: We used PrimalScheme (<https://primalscheme.com/>) to generate an hMPXV primer scheme using a pre-outbreak A.1 lineage reference genome (GenBank accession: MT903345). The primer scheme comprises a total of 163 primer pairs with an amplicon length ranging between 1597 and 2497 bp (average 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 hMPXV 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 25 clinical specimens were included in this initial validation comprised of both throat and swabs of fluid from lesions of 8 individuals, belonging to the current outbreak-associated hMPXV B.1 lineage 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 2×150 nt reads). Consensus genomes were generated at 10X coverage using the TheiaCoV_Illumina_PE Workflow Series on Terra.bio. An hMPXV "fork" was developed for us by Curtis Kapsak from Theiagen, which included the hMPXV 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. 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 1A**.) The library clean up stage of the amplicon based Illumina DNA prep protocol was conducted using the 'standard DNA input' option for comparison to metagenomics. Following optimization with 15 additional paired samples unrelated to the initial 10 samples, we found improved genome coverage with higher Ct samples (>25) using the 'small PCR amplicon input' option of the Illumina DNA prep protocol (**Figure 1B**.) Our findings highlight how amplicon-based approaches can significantly expand hMPXV sequencing to a wider variety of samples.

Figure 1: Percent genome coverage at 10X



A. Comparison in genome coverage for 10 paired clinical samples sequenced with metagenomics and amplicon approaches using the v1 standard DNA input library clean up method. **B.** Comparison between the v2 Illumina DNA prep small PCR amplicon input and the v1 standard DNA input library clean up methods for 15 paired clinical samples

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 IIa 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 lineage genomes. As this protocol is still in development, we will further investigate performance as we continue to sequence additional samples.

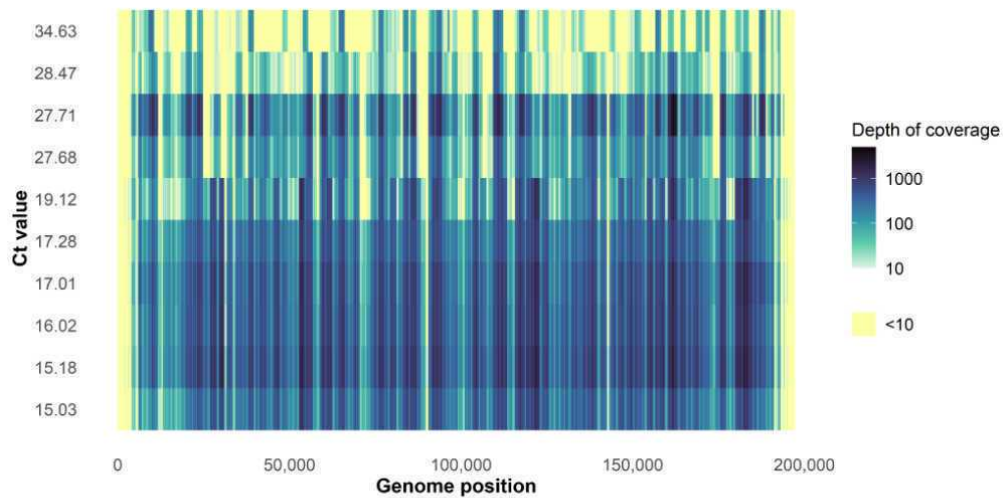


Figure 2. Depth of coverage by genome position and Ct value for 10 clinical specimens sequenced using the amplicon-based sequencing approach.

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

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

Materials

MPXV Primer Scheme

V.1

Primers may be ordered from any oligonucleotide company using the following file as a reference. For instructions on how to prepare hMPXV Primer Pool 1 and 2 (10 uM) see the "Unpooled Primers" fork of step 1 in the protocol.

 MPXV-primer_genome-positions.tsv

Our pre-pooled primer scheme (v.1) can also be ordered through Integrated DNA Technologies (IDT) with the following link. For preparation instructions, follow the "IDT Pre-Pooled Primers" fork of step 1 in the protocol.

<https://go.idtdna.com/Yale-Mpox-Amplicon-Panel-Request-Consult.html>

V1.1

An additional 11 primers were designed to improve coverage when sequencing clade I viruses. This improved scheme can be used to sequence both clade I and II viruses. If you already have V1 primers, you can order the 11 primers and spike into existing primer pools (5 odd numbered primers to be added to pool 1 and 6 even numbered primers to be added to pool 2) at equal primer concentrations.

 MPXV_spike-in_v1.1.xlsx 10.3KB (new primers only)

 MPXV_primers_v1.1.fasta 13.2KB (all V1.1 primers combined)

Library Preparation Method: Illumina DNA Prep

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



A	B	C
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 Adapter 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 hMPXV should be 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.

Before start

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.

Protocol Recommendations:

To maximize sequencing coverage, we recommend the following best practices based on our [multi-site validation study](#):

1. Prioritizing samples with cycle threshold (Ct) values <29 if resources are limited
2. Generating at least 1 million sequencing reads per sample

1 Primer Source

STEP CASE

IDT Pre-Pooled Primers From 56 to 81 steps

Integrated DNA Technologies (IDT) Pre-Pooled Yale hMPXV Amplicon Panel:
<https://go.idtdna.com/Yale-Mpox-Amplicon-Panel-Request-Consult.html>

1.1 Dilute primers to a working concentration of 10uM with nuclease-free water (i.e. 1:10 dilution)



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



2 Library Preparation Method

STEP CASE

Illumina CovidSeq Test (RUO) 53 steps

Amplicon Generation

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:

**Pool 1**

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

Pool 2

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

Note

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

3.4 Add  5 μ L **DNA** to each tube in **both** sets

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

Note

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):





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**
-  10 μL **Pool 2 amplicons**

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

Note

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

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 μ L
25-48	20 μ L
49-72	10 μ L
72-96	5 μ L

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

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

Note

*For example: Total pooled volume is 400 μ L (10 samples x 40 μ L/sample)
then add $0.6 \times 400 \mu\text{L} = 240 \mu\text{L}$ ITB*

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

5m

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

Note

For example: $400\mu\text{L} + 240\mu\text{L} = \text{total } 640\mu\text{L} > \text{transfer } 630\mu\text{L}$

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





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

V_o = total volume of sample + beads from step 5.5

V_t = transferred volume of supernatant

Note

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

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

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

Add **118 μL** of ITB to **630 μL** 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
- 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 

Note

Qubit SOP:  Agilent High Sensitivity DNA Kit Gui...

Bioanalyzer SOP:  Agilent High Sensitivity DNA Kit Gui...

Sequencing

- 7 Protocol validated on the MiSeq (v2 kit running 2×150 nt reads)

Note

Note: For sequencing we recommend generating at least 1 million reads per sample for optimal sequencing coverage

Sequencing may be performed on Illumina and 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.

Note

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](#)

V.1 files:

Reference Sequence (MT903345):  MPXV.reference.fasta

Bed file:  MPXV.primer.bed

New V1.1 files:

Reference Sequence (NC_003310):  NC_003310.fasta

Bed file:  MPXV_primers_v1.1.bed