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BCCDC / ARTIC Mpox V2.3.4 2500bp amplicon generation and NGS

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We use this protocol and it's working

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Abstract

This procedure provides instructions on how to generate amplicons and NGS data for the near whole genome of *Mpox*. Illumina, Oxford Nanopore or other NGS sequencing platforms can all be utilized if amplicon specific library preparations and platform specific bioinformatic analysis pipelines are available. We detail specific use of a modified Illumina DNA prep library preparation and the analysis pipelines using the generated data in this protocol. The BCCDC / ARTIC V2.3.4 *Mpox* WGS 2500bp primer scheme was optimized to match and efficiently generate data from the global 2022 West African clade outbreak. The MPV_3000_1_Left to MPV_3000_79_Right primers tile the near whole genome of the *Mpox* virus.

Since the first and last ~6500bp of the *Mpox* genome (ITR) are usually identical, the last ~6500bp of the genome is masked out on the reference genome to simplify analysis using short read Illumina data. The resulting ITR regions thus represent a consensus ITR combining the amplification products from the dual ITR primer binding sites. For specific ITR region sequence information the use of unique external primer binding sites is required along with removal of all but one common internal ITR binding site to generate specific region spanning PCR products. These products can then be sequenced using Nanopore long read sequencing, see **here** for some candidate primer combinations (MPV_3000_4_RIGHT, MPV_3000_80_LEFT, and MPV_3000_1_LEFT), to provide specific ITR region sequences. This approach was used to generate the BCCDCmpx2 sequence (available on GISAID, EPI_ISL_13351002) which used both Oxford Nanopore and Illumina data to fully sequence the individual ITR regions and repeat regions refractory to short read technologies alone.

The sample PCRs are performed in two pools before being combined for library preparation in order to minimize primer interactions and allow near complete coverage tiling of the *Mpox* genome. Sequences for the primers and locations on reference genomes can be found in the Materials section tables as well as linked file locations and **here**.

Effort were made to increase sequencing efficiency / genome coverage for a given amount of generated sequence data by performing a number of rounds of primer pair concentration re-balancing in the PCR primer pools (Figure 1.). The primer amounts for V2.3.4 are detailed in this protocol and Materials section.

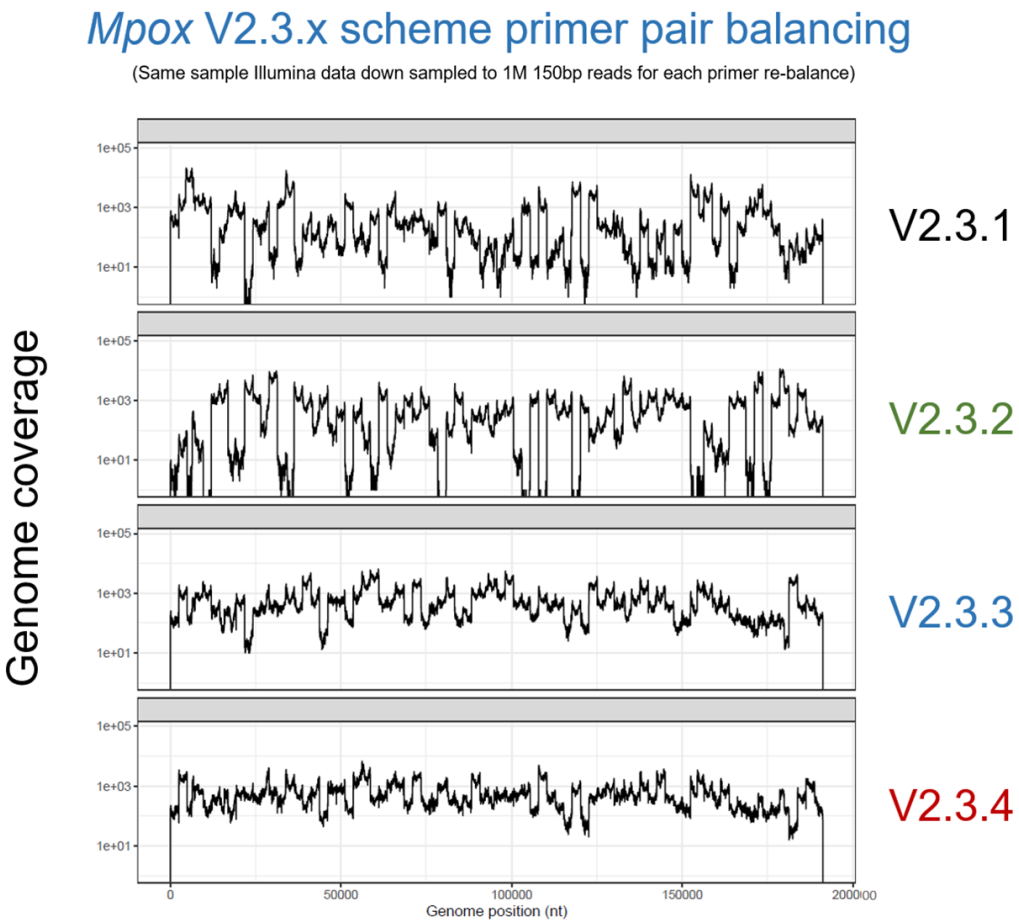

Figure 1. Iterative experimental amplicon balancing to increase sequencing efficiency for a given sample

Materials

Samples: Nucleic acid extracts previously found to be positive for Mpox virus by a validated laboratory method.

Reagents	Equipment	Supplies
Modified custom primers, see sequences below	Post-PCR thermalcycler	96-well PCR plate
Q5 Hot Start High Fidelity DNA Polymerase (New England BioLabs, M0493L)	Appropriate volume pipettes (single and multi-channel)	Plate adhesive covers
10mM dNTPs (SuperScript IV First Strand Synthesis Kit ThermoFisher #18091200	Vortex Mixer	Filtered pipet tips; various sizes
Amplirun MPXV DNA control (Somagen Diagnostics, MBC146-R)	Plate centrifuge	1.7mL microcentrifuge tubes
Ultrapure water	Biological safety cabinet	
	Repeater pipet	

Quality Control:

Negative control: PCR Grade Water

Positive control: Amplirun MPXV DNA control (Somagen Diagnostics, MBC146-R) [2] is purified DNA of the complete viral genome of Mpox virus.

BCCDC/ARTIC Mpox V2.3.4 Primer Scheme sequences

Mapping locations and reference specific bed files etc. for analysis pipeline can be found **here**.

Table 1. BCCDC/ARTIC Mpox V2.3.4 primer Pool 1 sequences and 10x stock pool amounts

A	B	C
Pool 1 Primer	Sequence	Vol 100uM (uL)
MPV_3000_1_LEFT	AACTCTAGAGGGTAAGAAAAATCAATCG	4

	A	B	C
	MPV_3000_1_RIGHTmod	CGGGAACTTACGCTTTCAGATTATG	4
	MPV_3000_3_LEFT	TTAACGATGCGCACAATCTCGTC	2
	MPV_3000_3_RIGHT	AGCCACTAATAAAAACAGTTGGTTAATTACTTC	2
	MPV_3000_5_LEFTmod	CCATGTTCGAGCATACTAGCCATG	2
	MPV_3000_5_RIGHT	GACAGGATCGAATTCCATATCCGC	2
	MPV_3000_7_LEFT	GATATCGCCGAATGTCATATACTCAATTAG	4
	MPV_3000_7_RIGHT	GGATAAACTATTATCTGCCCATGATACRTTT	4
	MPV_3000_9_LEFT	ACAGTGAGTTTATCTTTCTTTGAAGTGATA	8
	MPV_3000_9_RIGHT	CGTGATATTTTCTATCAATGGGGAAATTATTACG	8
	MPV_3000_11_LEFT	TGCGCTGGGCTCAATGTCTTA	6
	MPV_3000_11_RIGHTmod	AGCCATATATCTACTAATCAGATCTATTAGAGA	6
	MPV_3000_13_LEFT	CCGCCTTTGAATAAATAATAGGAATAAAGTTCA	6
	MPV_3000_13_RIGHT	TGAAGTTAATACTATACTAATGAACAACAAGGG	6
	MPV_3000_15_LEFT	GTATTCACCCACACGTTTTTCGAAAAA	4
	MPV_3000_15_RIGHT	TAATCCTCCACCAAAATAGAGTATATCATCTCA	4
	MPV_3000_17_LEFT	CATTCAATAGGGTAGTGATATTTGTATGTATGA	8
	MPV_3000_17_RIGHT	TCCATTGATCGATATTATACGAAAGCG	8
	MPV_3000_19_LEFT	TAGCCCCGCAATACTCCTCAT	3
	MPV_3000_19_RIGHT	TCAGAGTTTTAAATCCTCAAAATGAATAGG	3
	MPV_3000_21_LEFT	GATTTTCCATCTGCCTTATCGAATACTCTT	3
	MPV_3000_21_RIGHT	TTCCCCTCCGTGAAATAATGGATTAAG	3
	MPV_3000_23_LEFT	ATTCAGATACGTCTATACAGATAATACCAAACA	4
	MPV_3000_23_RIGHT	TAACAGGAGAGAAGATTATCTTTAGATCTCCA	4
	MPV_3000_25_LEFT	TCTCRACATCTTCAATAGATACCTTGCTA	4
	MPV_3000_25_RIGHT	TCAATAGTATCAGTGCCCGTGC	4
	MPV_3000_27_LEFT	ACCACTCTCCRTCATCCTTCAC	4
	MPV_3000_27_RIGHT	TGGACCAAAAAGTCAATTAATGTGATATGTG	4
	MPV_3000_29_LEFT	GTTGCACAGTGTCACGAATATAAATCATATTTA	6
	MPV_3000_29_RIGHT	GATATCTACTTGGGAATTTCTAATTTCGGATTC	6
	MPV_3000_31_LEFT	GTTACCCTGCCAATTAATGTACGCA	3
	MPV_3000_31_RIGHT	CTAGATGAATGACGGTTCTACCACAAC	3
	MPV_3000_33_LEFT	TGGCGCTAGTCATCACATTAACTATTTTT	3
	MPV_3000_33_RIGHT	TTTGAATCGCATCAAACTAATCACAAAGTC	3
	MPV_3000_35_LEFTmod	GCTACTCGTTTGGAATCACAGACATTAT	6
	MPV_3000_35_RIGHT	ATTAAGAATATAACCTCTTCCTTCTGGATCCT	6
	MPV_3000_37_LEFT	ATCCAGTCTTTAATGAATTAACGAGATATATGC	6

	A	B	C
	MPV_3000_37_RIGHT	CAGCGCTTTATCTTTAATATTATCATGTCT	6
	MPV_3000_39_LEFT	GCGGTTTTTATGTTAATGGAAACTATGTTT	6
	MPV_3000_39_RIGHT	CTATAAACTTAATAGGTACAACAACGGACTTAA	6
	MPV_3000_41_LEFT	TGTGTGTGGTATAGATCCGTATCCAAA	6
	MPV_3000_41_RIGHT	TTTGTACCATTTAACCAACAAGTATAGAAATGC	6
	MPV_3000_43_LEFT	CCGTATATAAGGCGTATCTCCATAGAG	8
	MPV_3000_43_RIGHT	CAATAATTTGATCACTCGTTAGAGATATTCKTC	8
	MPV_3000_45_LEFT	TTAGTGACGGACTTAACATGAGACATAAATAAA	4
	MPV_3000_45_RIGHT	GGTTAAGTCTCACGTAGCTCTTAGAGAA	4
	MPV_3000_47_LEFT	GCAGGAAGTTTAATACCGAATAGTGC	4
	MPV_3000_47_RIGHT	CCTAAAATCATACTGTTTCAGAATAACGACATC	4
	MPV_3000_49_LEFT	GTAATGTCCAAATCTAGTTGTGGAAATACTTC	4
	MPV_3000_49_RIGHT	TCGGAACCGATAAGTATCTTGGATAGAAT	4
	MPV_3000_51_LEFT	GTGGTTAATCTTAGAAATCAACGATCTAATACA	4
	MPV_3000_51_RIGHT	GCATTATACGTCTTTTGTAACGACCATAAAG	4
	MPV_3000_53_LEFT	CCAGATATGGTCAAATCRATTAAGGAACTAA	12
	MPV_3000_53_RIGHT	TTTTCGATCTCTATATGAATTACCAGACATGA	12
	MPV_3000_55_LEFT	GTCATCGCATCCTAACGTACGG	3
	MPV_3000_55_RIGHT	ACAGATTTCCGAATATCAGGAGCATAAC	3
	MPV_3000_57_LEFT	GCATCATCTTTATCATTATTAGGTGGGGG	6
	MPV_3000_57_RIGHT	GCAAATACATGCATCAGTAGAAAACATAGAAA	6
	MPV_3000_59_LEFT	GTTGTATTGGGGAAGGTAACAATTAATGATC	4
	MPV_3000_59_RIGHT	AYATCTAACATGATATCATAGTCGCATGC	4
	MPV_3000_61_LEFT	CATGGCAATAACGCAACCAAACA	4
	MPV_3000_61_RIGHT	GARCTGTATGTCAATACCGGCATTA	4
	MPV_3000_63_LEFT	AAATGCATTAGTTTACTTTTCTACTCAGCA	4
	MPV_3000_63_RIGHT	ATTATCGTCTAATGAATTAATCAACTCGTTCTG	4
	MPV_3000_65_LEFT	TCTACTCATCTAAACGATTTAGTAAACTTGACT	6
	MPV_3000_65_RIGHT	AACTTAAACCACCATCAAAAATCCATGT	6
	MPV_3000_67_LEFT	CGATAAAGAAAAAGATTGTTGGACATTGGATAA	8
	MPV_3000_67_RIGHT	CGTCTAGATACAACGTATCCTTTATGC	8
	MPV_3000_69_LEFT	ACATAATCTCGGGTTATCATCCATTAATAT	4
	MPV_3000_69_RIGHT	GCATCGTAGATATCAACATCCACTGAAG	4
	MPV_3000_71_LEFTmod	TATTCCAAAATGTTCCAGAACTCTCTGTATT	4
	MPV_3000_71_RIGHTmod	CCATTAGCTCCATAATACAGTATTGTCAATAC	4
	MPV_3000_73_LEFTmod	GGAGACGGTAGAATAAGTGTAGCAAATAAAA	4

	A	B	C
	MPV_3000_73_RIGHT	CTGATCTATCATAACCATAGAATTCATCAACAA	4
	MPV_3000_75_LEFT	ATGTTACCCGATATACCCATAGCATTATCTA	3.5
	MPV_3000_75_RIGHT	TCTCCTCGATAATAAGCCATATCTGG	3.5
	MPV_3000_77_LEFT	CTACAAATGATGGTCTACAGAGTCCAA	4
	MPV_3000_77_RIGHT	AATGATGAAAACAAGCACGTGGTTTATC	4
	MPV_3000_79_LEFT	CTGGATAGACATAAATATCCTCCTCGTAATAATG	12
	MPV_3000_79_RIGHT	GCTATCGAACCATTTTTGTAGTCTAAAGC	12

Table 2. BCCDC/ARTIC Mpox V2.3.4 primer Pool 2 sequences and 10x stock pool amounts

	A	B	C
	Pool 2 Primer	Sequence	Vol 100uM (uL)
	MPV_3000_2_LEFT	CACACGGYGTACATTGTGTATTAGT	4
	MPV_3000_2_RIGHT	TGACAATCTCCTATCAAAACTTCCATTAGAAAT	4
	MPV_3000_4_LEFT	GATCTGAGATAAATTATACAATCTTCGCTATCGA	3
	MPV_3000_4_RIGHT	ATTCTATTGATCTAACGCTGTAYGACG	3
	MPV_3000_6_LEFT	AAAGTTGTGGTAGTATGATCTCCATATTTA	3
	MPV_3000_6_RIGHT	TTGAAAACAATGAACTCGGATGTCCT	3
	MPV_3000_8_LEFT	GTACACTGTATGATAAGATACATATTCTGACAA	4
	MPV_3000_8_RIGHT	GCTAAAGATATAAATACGATGTCATTCGAC	4
	MPV_3000_10_LEFT	CTTTAGATGGTGATCCTGAATGTGTTTAAAAA	4
	MPV_3000_10_RIGHTmod	AAACAAAACAAATTTGGAGATAGTCCTCTTA	4
	MPV_3000_12_LEFT	ATGTCTGAAACGAGACGCTAATTAGTG	2
	MPV_3000_12_RIGHT	CCGCTTTATTGTCATGTATCGGAAATG	2
	MPV_3000_14_LEFT	GGTAATGAATGAAATTGTCGCCAATCT	3
	MPV_3000_14_RIGHT	AATGTTACCAATCCGTTTGATTTCATGG	3
	MPV_3000_16_LEFT	AGTAAAAAGTTCACATTGAAACTATGTCAGTAG	6
	MPV_3000_16_RIGHT	TTCTGCATTATTGTAATTCGTATGCTGATAC	6
	MPV_3000_18_LEFT	CCGTATCGTTCTAAAGCCAGTGC	4
	MPV_3000_18_RIGHT	GATGAACAAAATGGGYATTCTAGACAAAAA	4
	MPV_3000_20_LEFT	CTCTGCCTAAATAGGTTTCTATAATCTTTAATG	2
	MPV_3000_20_RIGHT	AACTCTCCTACATTTTATGCCTGTGTAG	2
	MPV_3000_22_LEFT	GACGAATTAGGAGAACTTATAGGCGTAAATTA	3
	MPV_3000_22_RIGHT	TTATAGTCTCCTGTTTGATGTATATACCTAGC	3
	MPV_3000_24_LEFT	GATTTCTCTCATAAATACAGAGATGTACAGCTT	6
	MPV_3000_24_RIGHT	TCTCTAGAGGATATTCTCTCACATATAGATAATG	6

	A	B	C
	MPV_3000_26_LEFT	CATCTACCATTTCATTGATTTTAGCAGTGAA	4
	MPV_3000_26_RIGHT	ATCTATTTATAGCTCTTTGGATTCCTGATCT	4
	MPV_3000_28_LEFT	AACAAAGTTATTCATCGTCGTCTACTATTCTAT	8
	MPV_3000_28_RIGHT	CCTTACCATGAATAATATACATATCATACGGTA	8
	MPV_3000_30_LEFT	AAGTGAGCAATTCCCAAGATTTCATCTA	4
	MPV_3000_30_RIGHT	AGTTTTAACTTCTGAAACATCAATCTCCTTAC	4
	MPV_3000_32_LEFT	CCTATTAGGACAGATACTACTATTTGTYTAAGT	8
	MPV_3000_32_RIGHT	GTTTGTTAGAAGTCAGTCTAGTACTATTATAGT	8
	MPV_3000_34_LEFT	TTACAGATGGAAGGGTCAAACTTAATAAAGG	4
	MPV_3000_34_RIGHT	ATGTATCTGATTGTACCATTTCATTGGGAA	4
	MPV_3000_36_LEFT	GAGTATTGGGTAGAGTCTTACCTTACTATCTT	4
	MPV_3000_36_RIGHT	CTAATTTTGTTTTGCTATCCGTTAATGATTTCC	4
	MPV_3000_38_LEFT	TGTATTTCATTACCCAAGTTTGAGATTTCA	6
	MPV_3000_38_RIGHT	GTGTTAATAACCCTACTGTTTCATTCTCCT	6
	MPV_3000_40_LEFT	GATTGTGTTATATAACGGCGTTAATTATCT	3
	MPV_3000_40_RIGHT	TTTGGTGATTCGAACGGTACACC	3
	MPV_3000_42_LEFT	ACGAGGGATTAGATGGTAAAATACAGAATAATAG	3
	MPV_3000_42_RIGHT	GTCTTCACGAATTTCTATTTTCTTGGCC	3
	MPV_3000_44_LEFT	GACAATTCTTTTGTATTCATAGACACTCGTTTT	4
	MPV_3000_44_RIGHT	CGTAAGCGATAGTTGGTTGAAAGATAG	4
	MPV_3000_46_LEFT	CCTTATCTTCATAGAATACTAAAGGCAATAAAG	8
	MPV_3000_46_RIGHT	TAGAAAACCAGAAACTAATTACTACATTCATCC	8
	MPV_3000_48_LEFT	CGACGTCAATTGTCTATAAATCAGAGTATAAAT	6
	MPV_3000_48_RIGHT	ATTTGGATCGTACGAAGATGTAGATAATAA	6
	MPV_3000_50_LEFT	CCATCATTTCATTAATAGTATACGCAGAAAAGA	6
	MPV_3000_50_RIGHT	CTTATTAACTTCKGTTGATTTATACGGAGTCT	6
	MPV_3000_52_LEFT	TTGGATAATTCGCGCACCAACAA	1.5
	MPV_3000_52_RIGHT	AGTGCCGGAATAAAATAAGGTGGATAC	1.5
	MPV_3000_54_LEFT	CATACATCTGCTGCCAAAGTTATTTGC	6
	MPV_3000_54_RIGHT	CTCTAATTCATCATGTGCYAACGTCT	6
	MPV_3000_56_LEFT	ATATTATCGGATTCGGTATTGTTACTCGAG	3
	MPV_3000_56_RIGHT	AGGAGATAAGGAGGACCAACCAATTAA	3
	MPV_3000_58_LEFT	TAGGATATGCTTTCATAAAGTCCCTAATAACTT	6
	MPV_3000_58_RIGHT	ACTCCATGATATGATAAATCCATGTAAAATAGC	6
	MPV_3000_60_LEFTmod	CAACATTTATAGTATTTGTACAGCATACAGATC	6
	MPV_3000_60_RIGHT	GCTTATACATCGATATCAGTTGTATTTTCTACT	6

	A	B	C
	MPV_3000_62_LEFT	GTGTACGTGCAACTGGTAATTAAAATAAAAAG	4
	MPV_3000_62_RIGHT	GTAGTGTATATCATTAACTTCATTACGTATGAC	4
	MPV_3000_64_LEFT	CTTTCCTAGATACTGCATATACTATCATAGATCA	6
	MPV_3000_64_RIGHT	AGAAATCAAACAGGAGAATAATCAATGATGC	6
	MPV_3000_66_LEFT	GATGGATGAGATATACAGCTATTAATTTCGAAA	8
	MPV_3000_66_RIGHT	CTATGTTTAAMCGGTTCGCATTTATACATT	8
	MPV_3000_68_LEFT	ATAAAATGGATAACGGTACTCTAGAATTTACAC	4
	MPV_3000_68_RIGHT	CGTCCATTTTCAAGCATTAGTCTTATACTATT	4
	MPV_3000_70_LEFT	CATAAATTGCTACCGTGAATATAAATCCGTTA	2
	MPV_3000_70_RIGHT	ATGTCTCCCGCCTCTTGATCAC	2
	MPV_3000_72_LEFT	GAAAGAATGTATTCATTTCTCCAGCGTC	3
	MPV_3000_72_RIGHT	AGGATTAATGTTTAACCAGGATGTAATAACAC	3
	MPV_3000_74_LEFT	CTAGTAGATTACGACATTAAACTCAAAACCAAT	2
	MPV_3000_74_RIGHTmod	GTGTTGGGTACGACCGCTTATA	2
	MPV_3000_76_LEFT	TTAACCGTGCGTAAAATGACTCGAG	2
	MPV_3000_76_RIGHT	ATAATCATCATGTTGAGGAAGTGGATTAC	2
	MPV_3000_78_LEFT	GGTTGGTTAAAATCTAGTGGYTATGGG	6
	MPV_3000_78_RIGHT	GTTTAGGACGGAACMCAAAAGGG	6

Preparation of primer pools from individual primers

1

> **Note**
>
> See ***Materials*** for primer sequences.

1.1 Combine separately the respective primer volumes listed in Table 1 and Table 2 using 100µM stocks to create the **10x Pool 1** and **10x Pool 2** primer pool stocks.

1.2 **10x Pool 1:** Combine the volumes listed in Table 1 for each of the 100µM 80 primers into a 1.7mL tube
10x Pool 2: Combine the volumes listed in Table 2 for each of the 100µM 78 primers into a 1.7mL tube

Pool 1 primer	Vol (µL)
MPV_3000_1_LEFT	4
MPV_3000_1_RIGHTmod	4
MPV_3000_3_LEFT	2
MPV_3000_3_RIGHT	2
MPV_3000_5_LEFTmod	2
MPV_3000_5_RIGHT	2
MPV_3000_7_LEFT	4
MPV_3000_7_RIGHT	4
MPV_3000_9_LEFT	8
MPV_3000_9_RIGHT	8
MPV_3000_11_LEFT	6
MPV_3000_11_RIGHTmod	6

	Pool 1 primer	Vol (µL)
	MPV_3000_13_LEFT	6
	MPV_3000_13_RIGHT	6
	MPV_3000_15_LEFT	4
	MPV_3000_15_RIGHT	4
	MPV_3000_17_LEFT	8
	MPV_3000_17_RIGHT	8
	MPV_3000_19_LEFT	3
	MPV_3000_19_RIGHT	3
	MPV_3000_21_LEFT	3
	MPV_3000_21_RIGHT	3
	MPV_3000_23_LEFT	4
	MPV_3000_23_RIGHT	4
	MPV_3000_25_LEFT	4
	MPV_3000_25_RIGHT	4
	MPV_3000_27_LEFT	4
	MPV_3000_27_RIGHT	4
	MPV_3000_29_LEFT	6
	MPV_3000_29_RIGHT	6
	MPV_3000_31_LEFT	3
	MPV_3000_31_RIGHT	3
	MPV_3000_33_LEFT	3
	MPV_3000_33_RIGHT	3
	MPV_3000_35_LEFTmod	6

	Pool 1 primer	Vol (µL)
	MPV_3000_35_RIGHT	6
	MPV_3000_37_LEFT	6
	MPV_3000_37_RIGHT	6
	MPV_3000_39_LEFT	6
	MPV_3000_39_RIGHT	6
	MPV_3000_41_LEFT	6
	MPV_3000_41_RIGHT	6
	MPV_3000_43_LEFT	8
	MPV_3000_43_RIGHT	8
	MPV_3000_45_LEFT	4
	MPV_3000_45_RIGHT	4
	MPV_3000_47_LEFT	4
	MPV_3000_47_RIGHT	4
	MPV_3000_49_LEFT	4
	MPV_3000_49_RIGHT	4
	MPV_3000_51_LEFT	4
	MPV_3000_51_RIGHT	4
	MPV_3000_53_LEFT	12
	MPV_3000_53_RIGHT	12
	MPV_3000_55_LEFT	3
	MPV_3000_55_RIGHT	3
	MPV_3000_57_LEFT	6
	MPV_3000_57_RIGHT	6

	Pool 1 primer	Vol (µL)
	MPV_3000_59_LEFT	4
	MPV_3000_59_RIGHT	4
	MPV_3000_61_LEFT	4
	MPV_3000_61_RIGHT	4
	MPV_3000_63_LEFT	4
	MPV_3000_63_RIGHT	4
	MPV_3000_65_LEFT	6
	MPV_3000_65_RIGHT	6
	MPV_3000_67_LEFT	8
	MPV_3000_67_RIGHT	8
	MPV_3000_69_LEFT	4
	MPV_3000_69_RIGHT	4
	MPV_3000_71_LEFTmod	4
	MPV_3000_71_RIGHTmod	4
	MPV_3000_73_LEFTmod	4
	MPV_3000_73_RIGHT	4
	MPV_3000_75_LEFT	3.5
	MPV_3000_75_RIGHT	3.5
	MPV_3000_77_LEFT	4
	MPV_3000_77_RIGHT	4
	MPV_3000_79_LEFT	12
	MPV_3000_79_RIGHT	12

Table 1 - 10x Pool 1 of 2500bp V2.3.4 amplicon primer scheme

	Pool 2 primer	Vol (µL)
	MPV_3000_2_LEFT	4
	MPV_3000_2_RIGHT	4
	MPV_3000_4_LEFT	3
	MPV_3000_4_RIGHT	3
	MPV_3000_6_LEFT	3
	MPV_3000_6_RIGHT	3
	MPV_3000_8_LEFT	4
	MPV_3000_8_RIGHT	4
	MPV_3000_10_LEFT	4
	MPV_3000_10_RIGHTmod	4
	MPV_3000_12_LEFT	2
	MPV_3000_12_RIGHT	2
	MPV_3000_14_LEFT	3
	MPV_3000_14_RIGHT	3
	MPV_3000_16_LEFT	6
	MPV_3000_16_RIGHT	6
	MPV_3000_18_LEFT	4
	MPV_3000_18_RIGHT	4
	MPV_3000_20_LEFT	2
	MPV_3000_20_RIGHT	2
	MPV_3000_22_LEFT	3

	Pool 2 primer	Vol (µL)
	MPV_3000_22_RIGHT	3
	MPV_3000_24_LEFT	6
	MPV_3000_24_RIGHT	6
	MPV_3000_26_LEFT	4
	MPV_3000_26_RIGHT	4
	MPV_3000_28_LEFT	8
	MPV_3000_28_RIGHT	8
	MPV_3000_30_LEFT	4
	MPV_3000_30_RIGHT	4
	MPV_3000_32_LEFT	8
	MPV_3000_32_RIGHT	8
	MPV_3000_34_LEFT	4
	MPV_3000_34_RIGHT	4
	MPV_3000_36_LEFT	4
	MPV_3000_36_RIGHT	4
	MPV_3000_38_LEFT	6
	MPV_3000_38_RIGHT	6
	MPV_3000_40_LEFT	3
	MPV_3000_40_RIGHT	3
	MPV_3000_42_LEFT	3
	MPV_3000_42_RIGHT	3
	MPV_3000_44_LEFT	4
	MPV_3000_44_RIGHT	4

	Pool 2 primer	Vol (µL)
	MPV_3000_46_LEFT	8
	MPV_3000_46_RIGHT	8
	MPV_3000_48_LEFT	6
	MPV_3000_48_RIGHT	6
	MPV_3000_50_LEFT	6
	MPV_3000_50_RIGHT	6
	MPV_3000_52_LEFT	1.5
	MPV_3000_52_RIGHT	1.5
	MPV_3000_54_LEFT	6
	MPV_3000_54_RIGHT	6
	MPV_3000_56_LEFT	3
	MPV_3000_56_RIGHT	3
	MPV_3000_58_LEFT	6
	MPV_3000_58_RIGHT	6
	MPV_3000_60_LEFTmod	6
	MPV_3000_60_RIGHT	6
	MPV_3000_62_LEFT	4
	MPV_3000_62_RIGHT	4
	MPV_3000_64_LEFT	6
	MPV_3000_64_RIGHT	6
	MPV_3000_66_LEFT	8
	MPV_3000_66_RIGHT	8
	MPV_3000_68_LEFT	4

Pool 2 primer	Vol (µL)
MPV_3000_68_RIGHT	4
MPV_3000_70_LEFT	2
MPV_3000_70_RIGHT	2
MPV_3000_72_LEFT	3
MPV_3000_72_RIGHT	3
MPV_3000_74_LEFT	2
MPV_3000_74_RIGHTmod	2
MPV_3000_76_LEFT	2
MPV_3000_76_RIGHT	2
MPV_3000_78_LEFT	6
MPV_3000_78_RIGHT	6

Table 2 - 10x Pool 2 of 2500bp V2.3.4 amplicon primer scheme

1.3 Create 10µM (1x) stock solutions of **pool 1** and **pool 2** of an appropriate volume in individual 1.7mL tubes from the 100µM stocks created above by dilution in Ultrapure water. Primers at this concentration should only be freeze/thawed a maximum of three times.

Preparation of PCR Reagents

2 Prepare both PCR Master Mix 1 (**MM1** containing Primer Pool 1) and 2 (**MM2** containing Primer Pool 2) separately in a PCR Clean Room as follows:

Reagent	Volume (Rxn)	Volume (24 Rxns)
Ultrapure water	15.65µl	375.6µl
5X Q5 Reaction Buffer	5µl	120µl
10mM dNTPs	0.5µl	12µl
Q5 Hot Start DNA Polymerase	0.25µl	6µl

Reagent	Volume (Rxn)	Volume (24 Rxns)
Primer Pool **1 or 2**	1.1µl	26.4µl
Total	**22.5µl**	**540µl**

Label two PCR Plates with the experiment code followed by “PCR Pool 1” or “PCR Pool 2.”
Using a repeater pipet, pipet 22.5µl of PCR **MM1** into “PCR Pool 1” and 22.5µl of PCR **MM2** into “PCR Pool 2.”

3 Seal and move all the plates containing your master mixes from the Clean Reagent Preparation Room
into a Genomic Level Room.

Generate PCR amplicons

4

Note

Sample template material
Sample nucleic acid extracts previously found to be positive for Mpox virus by a validated laboratory method are used. At the BCCDC PHL this Mpox DNA template material is generated using the 5X MagMax - 96 Viral Isolation kit from Applied Biosystems from preferred higher yielding clinical sample site swabs, such as lesions and mucosal sources, using a Copan swab with Universal Transport Media.

5 Pipet 2.5µl of template into their respective wells in PCR Pool 1 plate. Pipet to mix.
Pipet 2.5µl of template into their respective wells in PCR Pool 2 plate. Pipet to mix.
Seal the two PCR plates with an adhesive seal and spin down.

6 Place the two PCR plates on thermal cyclers and run at the following conditions:

A	B	C
Temperature	**Time**	**Cycles**
98°C	30 seconds	1
98°C	15 seconds	35
65°C	5 minutes	
4°C	∞	1

Thermal cycling program for Mpox amplicon generation. This program takes 3.5 hours. PCR amplicons are stable for several days at 4°C and months at -20°C

Amplicons from primer pools 1 and 2 are combined post-PCR for NGS library preparation.

Sequencing

7 Proceed with library preparation and sequencing of Mpox amplicons. A condensed library preparation protocol for the Illumina NextSeq may be used for Mpox amplicons as per Hickman et al (2024). Protocols.io link **here**. For other NGS platforms proceed with appropriate methods and analysis.

CITATION

Hickman R, Nguyen J, Lee TD, Tyson JR, Azana R, Tsang F, Hoang L, Prystajecky NA (2024). Rapid, high-throughput, cost-effective whole-genome sequencing of SARS-CoV-2 using a condensed library preparation of the Illumina DNA Prep kit..

LINK

https://doi.org/10.1128/jcm.00103-22

Bioinformatics

8 For consensus building and variant calling from Illumina DNA Prep generated data see the BCCDC Nextflow pipeline **here** that uses a modified ARTIC network fieldbioinformatics tool for Mpxv created by the Simpson lab **here**.

For primer scheme .bed, primer_pair.tsv and associated reference sequences files see **here**

We use the below files for compatibility and integration with the publicly available **Mpox Nextstrain** instances using the same reference sequence **NC_063383.1** :-

NC_063383.1_masked_right_itr.fasta (Mpox reference sequences with right ITR region masked)

NC_063383.1_masked_right_itr.fasta.fai (Index file for above mpox reference fasta file)

BCCDC-PHL_mpxv_v2.3_primer_pairs.tsv (File containing bracketing primer pairs for the generated amplicon regions. This is specific to the Illumina analysis pipeline we use and is for short read quality filtering when using library preps that fragment amplicons)

BCCDC-PHL_mpxv_v2.3_NC_063383.1.scheme.bed (.bed file containing primer binding site information for a specific reference for use in analysis pipelines)

BCCDC-PHL_mpxv_v2.3_NC_063383.1.primer.bed (.bed file containing individual primer reference location, name, pool number, orientation and sequence information ****not compatible with analysis pipeline but useful to have information all together****)

9 For QC analysis see the **companion pipeline for quality control analysis**.

Protocol references

Hickman R, Nguyen J, Lee TD, et al. Rapid, high-throughput, cost-effective whole-genome sequencing of SARS-CoV-2 using a condensed library preparation of the Illumina DNA Prep kit. *J Clin Microbiol*. 2024;62(3):e0010322. doi:10.1128/jcm.00103-22

GitHub - BCCDC-PHL/mpxv-artic-nf

Primer .bed, primer_pair.tsv and references sequences used

GitHub - BCCDC-PHL/mpxv-qc: Companion pipeline to perform quality control for BCCDC-PHL/mpxv-artic-nf