Jul 12, 2022

Monkeypox virus whole genome sequencing using combination of NextGenPCR and Oxford Nanopore

Matthijs Welkers[1], M. Jonges[1], Anton van den Ouden[2]

[1]AmsterdamUMC location AMC, Dept. of Medical Microbiology & Infection Prevention, Amsterdam, The Netherlands;

[2]Molecular Biology Systems B.V., Goes, The Netherlands

In Development

Share

This protocol is published without a DOI.

Anton van den Ouden

DISCLAIMER

ABSTRACT

Rapid genomic surveillance of monkeypox virus (MPXV) can provide valuable insights in order to guide public health interventions. Current sequencing protocols make use of direct Oxford Nanopore Sequencing. However, the obtained depth is a limiting factor which prevents multiplexing samples on a flowcell making sequencing very costly. Here, we provide the protocol for a PCR-based amplicon tiling approach (inspired by SARS-CoV-2 Midnight Protocol by Nikki Freed et. al. and the ARTIC network) for MPXV consisting of a total of 88 primer sets divided over 2 amplicon pools. The amplicon size is ~2,5kB. Our approach will increase the coverage (depth) significantly and allow for multiplexing up to 20 samples on a single Nanopore flowcell.

In our experience clinical samples can be successfully sequenced with CT-values <25. Homopolymer regions will remain an issue in our approach, requiring manual curation of obtained consensus sequence genomes.

PROTOCOL CITATION

Matthijs Welkers, M. Jonges, Anton van den Ouden 2022. Monkeypox virus whole genome sequencing using combination of NextGenPCR and Oxford Nanopore. **protocols.io** https://protocols.io/view/monkeypox-virus-whole-genome-sequencing-using-comb-ccc7sszn

KEYWORDS

Monkeypox virus, amplicon-based tiling PCR, Nanopore sequencing, Oxford Nanopore sequencing, Monkeypox

CREATED

Jun 30, 2022

LAST MODIFIED

Jul 12, 2022

PROTOCOL INTEGER ID

65663

Primer pool preparation

1 If required, resuspend lyophilised primers to a concentration of 100µM each

Primers for this protocol were designed by Martin Schou Pedersen (Department of Clinical Microbiology, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark) using Primal Scheme to generate overlapping 2500bp amplicons.

Primers are available as two pre-mixed pools from Monkeypox - MBS - Ultra Fast NEXTGENPCR®

1.1 Primers used to generate 2500 bp amplicons are here:

name	pool	seq	size	%gc	tm
monkeypox-2500_1_LEFT	1	AAAAATGTGTGACCCACGACCG	22	50	62,07
monkeypox-2500_1_RIGHT	1	CCGGGAACTTACGCTTTCAGAT	22	50	60,86
monkeypox-2500_3_LEFT	1	GTTAACGATGCGCACAATCTCG	22	50	61,01
monkeypox-2500_3_RIGHT	1	TAGTGAGAGCGAGAGTGACAGT	22	50	60,47
monkeypox-2500_5_LEFT	1	AATAGTCTGTAGACCTTTATCGTCGT	26	38,46	59,9
monkeypox-2500_5_RIGHT	1	ACTGCTAGAATCCGGTTCAGATG	23	47,83	60,43
monkeypox-2500_7_LEFT	1	CACTGTAAGCATGTCCGTACCA	22	50	60,53
monkeypox-2500_7_RIGHT	1	TGAGAACGAGCTCTTCAAACACT	23	43,48	60,43
monkeypox-2500_9_LEFT	1	AGGCTATGTTTCGCCCATCATC	22	50	60,99
monkeypox-2500_9_RIGHT	1	GTCCTTTACGATGAGCTCAAATGT	24	41,67	59,68
monkeypox-2500_11_LEFT	1	TGTCACTCCATAACTACCACGC	22	50	60,27
monkeypox-2500_11_RIGHT	1	AGTTTCGTCGATAGTACTGTGTGT	24	41,67	60,1
monkeypox-2500_13_LEFT	1	TCCTTATGAAGATGATGTTTGGCG	24	41,67	59,74
monkeypox-2500_13_RIGHT	1	CCCTCCTGGAGAACGACAGTTA	22	54,55	61,33
monkeypox-2500_15_LEFT	1	TGGAAGCGAATGATCCGGAAAA	22	45,45	60,8

monkeypox-2500_15_RIGHT	1	TCCGTGGTTTCTAGTGGGTGTA	22	50	60,94
monkeypox-2500_17_LEFT	1	ACCTTGGCTGTCTCATTCAATAGG	24	45,83	60,95
monkeypox-2500_17_RIGHT	1	TGAATGGCTGTCGTCAAAAGGT	22	45,45	60,93
monkeypox-2500_19_LEFT	1	AGGCTTCCAAAAATTTTTCATCCGT	25	36	60,84
monkeypox-2500_19_RIGHT	1	ACGTCGCTGTAATAGACAAGGC	22	50	60,91
monkeypox-2500_21_LEFT	1	CCCTAGGACGAACTACTGCCAT	22	54,55	61,46
monkeypox-2500_21_RIGHT	1	TTGTGCTGCTCTTATCGTCTGA	22	45,45	59,95
monkeypox-2500_23_LEFT	1	AAAAACCCTAGTATTCTTCCATCGC	25	40	59,79
monkeypox-2500_23_RIGHT	1	AACGGTATGTTACGGTTTGCCA	22	45,45	60,99
monkeypox-2500_25_LEFT	1	CTCGCCATTTCGACATCTGGAT	22	50	60,98
monkeypox-2500_25_RIGHT	1	CGGGACCAAATGTAGTCAAGCT	22	50	60,8
monkeypox-2500_27_LEFT	1	ACGCGTTCACTATCTCCAGAGA	22	50	61,12
monkeypox-2500_27_RIGHT	1	TACGCACGCTTCTCCTACCTTA	22	50	61,12
monkeypox-2500_29_LEFT	1	TTGACTTTTTGGTCCACTTTTCCA	24	37,5	59,8
monkeypox-2500_29_RIGHT	1	ATATTCGTGACACTGTGCAACG	22	45,45	59,76
monkeypox-2500_31_LEFT	1	TCCGGACATGATGGTAAAGACC	22	50	60,01
monkeypox-2500_31_RIGHT	1	AACGAATTCTGCGTCTCGTTCA	22	45,45	61,04
monkeypox-2500_33_LEFT	1	TAGGCTCACCGATGATCATTGG	22	50	60,14
monkeypox-2500_33_RIGHT	1	AACACAGCATCCAACTGAGCAT	22	45,45	61
monkeypox-2500_35_LEFT	1	ACAGGGGCAATGTTTACCACAA	22	45,45	60,88
monkeypox-2500_35_RIGHT	1	CTAGACGCCACGGGGTTTAAAA	22	50	61,05
monkeypox-2500_37_LEFT	1	TTGTTTCGTCAACAAGTTGGATGA	24	37,5	59,92

monkeypox-2500_37_RIGHT	1	CGGATACCAGAGTGATAATTTCGGT	25	44	60,89
monkeypox-2500_39_LEFT	1	CCGCATTGGTGTTCCGATCTTA	22	50	61,18
monkeypox-2500_39_RIGHT	1	TGAACCTGAGGCATGGAAAAGG	22	50	61
monkeypox-2500_41_LEFT	1	CCACAGATTCCAATTATCAGTTGGC	25	44	60,83
monkeypox-2500_41_RIGHT	1	AGACGACTCTCCAAAGATAATTGGT	25	40	60,2
monkeypox-2500_43_LEFT	1	TGTACAGGTACCTCCATCATTAGGA	25	44	60,73
monkeypox-2500_43_RIGHT	1	TTGGTTGTCGACTTCCCAGTTG	22	50	61,18
monkeypox-2500_45_LEFT	1	TCCTGAAAACGATGATGGCAATC	23	43,48	59,87
monkeypox-2500_45_RIGHT	1	AACTCTTCGAAGTGAGGATCGAT	23	43,48	59,56
monkeypox-2500_47_LEFT	1	CTCCCGGATCACGATTTTGTCT	22	50	60,6
monkeypox-2500_47_RIGHT	1	GAACATATAGCGACGCCACCAA	22	50	61,23
monkeypox-2500_49_LEFT	1	TTGCATCTACATCATCCGTGGA	22	45,45	59,75
monkeypox-2500_49_RIGHT	1	AATGGAAGCCGTGGTCAATAGC	22	50	61,45
monkeypox-2500_51_LEFT	1	TCTCTGTAGTCGACGCTCTCAA	22	50	60,79
monkeypox-2500_51_RIGHT	1	ACGGCCGGAAATAGTTAAGAGAC	23	47,83	60,68
monkeypox-2500_53_LEFT	1	GTTGTATGGCATTGCGCAGAAA	22	45,45	60,85
monkeypox-2500_53_RIGHT	1	CAAGGATGGTGTTTGTGTTGGC	22	50	60,98
monkeypox-2500_55_LEFT	1	CTGACAATGTACTGGGCCATGT	22	50	60,8
monkeypox-2500_55_RIGHT	1	ACATCATCGGAGGATAATACGCTAA	25	40	59,96
monkeypox-2500_57_LEFT	1	TTGGGAGAACTTAAGCGGCAAG	22	50	61,31
monkeypox-2500_57_RIGHT	1	AAACGATAAGAGTGGCCGCTTG	22	50	61,68
monkeypox-2500_59_LEFT	1	AAGATTGCGGCTAATTGCTTCG	22	45,45	60,4

monkeypox-2500_59_RIGHT	1	GAGGGAATTGACTCGCGAAAGA	22	50	60,85
monkeypox-2500_61_LEFT	1	ACAGAACAATTAGAGCGGCAGG	22	50	61,12
monkeypox-2500_61_RIGHT	1	ACACGATGCGACAATGTATAGACT	24	41,67	60,52
monkeypox-2500_63_LEFT	1	GACGATGATGATTGATCACTATTACACA	28	35,71	60,19
monkeypox-2500_63_RIGHT	1	AATCCATCCATTGCCGTCTGAT	22	45,45	60,34
monkeypox-2500_65_LEFT	1	TCAATCCCAAACCCAAAACCGT	22	45,45	61,14
monkeypox-2500_65_RIGHT	1	CCCAGTAAGCAACTCCATAGCA	22	50	60,28
monkeypox-2500_67_LEFT	1	ACTTTCGAGGTTATTGGTTGTGGA	24	41,67	60,77
monkeypox-2500_67_RIGHT	1	GCATACGCTACTCCAGAGAACG	22	54,55	61,03
monkeypox-2500_69_LEFT	1	TGATGCACTAACGAGAAAATTAGAAGG	27	37,04	60,42
monkeypox-2500_69_RIGHT	1	ACTTAAACCACCATCAAAAATCCATGT	27	33,33	60,7
monkeypox-2500_71_LEFT	1	GGTGGAGTCGTTAAAGGTGACA	22	50	60,4
monkeypox-2500_71_RIGHT	1	TGCCTTGCATGTGATAAGACCT	22	45,45	60,21
monkeypox-2500_73_LEFT	1	ATTGGATTCACGGTGGGTCATG	22	50	61,13
monkeypox-2500_73_RIGHT	1	TCACAGACAGCATTTGGATCCA	22	45,45	60,14
monkeypox-2500_75_LEFT	1	ATTCGATCGTCATGGGCATAGT	22	45,45	59,88
monkeypox-2500_75_RIGHT	1	TGTATCTGAATCCATGTTAGTAGTAAGCA	29	34,48	60,78
monkeypox-2500_77_LEFT	1	GTTGGGACTGACAGATGTGTTCT	23	47,83	60,75
monkeypox-2500_77_RIGHT	1	TGTATCGCATTCCACCCTTTCC	22	50	60,86
monkeypox-2500_79_LEFT	1	GATAGATCAGTGGGTGTCCATGAT	24	45,83	60,04
monkeypox-2500_79_RIGHT	1	GTGTTGGGTACGACCGCTTATA	22	50	60,34
monkeypox-2500_81_LEFT	1	CACCTGATGGTCTGGACATACC	22	54,55	60,34

monkeypox-2500_81_RIGHT	1	ACTACGTCCTTTTGCCATTGCA	22	45,45	61,26
monkeypox-2500_83_LEFT	1	CCACATTGGCTAGAGGAATGCC	22	54,55	61,51
monkeypox-2500_83_RIGHT	1	TGATAAGCGACGCCATTCATGT	22	45,45	60,92
monkeypox-2500_85_LEFT	1	ACTAAATCTCCTTCATGCTCTCTCAC	26	42,31	60,85
monkeypox-2500_85_RIGHT	1	ACCTGCTCGGTTACTTCTGTGT	22	50	61,79
monkeypox-2500_87_LEFT	1	CCAAGCTAAGCGACTACCATCT	22	50	60,08
monkeypox-2500_87_RIGHT	1	TGATGCAATTGTCTGACAACCTAGA	25	40	60,9

Primers for Pool 1

name	pool	seq	size	%gc	tm
monkeypox-2500_2_LEFT	2	TGTTCTACACCCTGATGCTCCT	22	50	61,01
monkeypox-2500_2_RIGHT	2	TCCACCCACCTTTCTTGAAATGA	23	43,48	60,38
monkeypox-2500_4_LEFT	2	GTAGCAGTAGTTGGTGCATGGT	22	50	60,8
monkeypox-2500_4_RIGHT	2	TGTGTCCTCTCCTCTTATAACATCG	25	44	60,08
monkeypox-2500_6_LEFT	2	AGCGTTGACTTATGGACTCTGG	22	50	60,27
monkeypox-2500_6_RIGHT	2	TACCTATCCAACGACAGGCACT	22	50	61,07
monkeypox-2500_8_LEFT	2	TTGCGGACATGTTACACTCCTT	22	45,45	60,41
monkeypox-2500_8_RIGHT	2	ACTATGGATCCCCACCACTTGA	22	50	60,75
monkeypox-2500_10_LEFT	2	TCGCCGTCATTTCTCCAAAGAA	22	45,45	60,73
monkeypox-2500_10_RIGHT	2	TCTGTTGTTTACCACTCAGCGG	22	50	60,99
monkeypox-2500_12_LEFT	2	GGAACCGTTTTCGTACCGTACT	22	50	60,78
monkeypox-2500_12_RIGHT	2	AGTCAGGTCTTGAAGGCTACCA	22	50	60,95

monkeypox-2500_14_LEFT	2	TGATCCAAACCCTTGATCTCCTC	23	47,83	60,06
monkeypox-2500_14_RIGHT	2	ACGGATTTCAGATGGCCATTGA	22	45,45	60,54
monkeypox-2500_16_LEFT	2	GGCTGCTCCTGTTCTTGTAGTC	22	54,55	61,11
monkeypox-2500_16_RIGHT	2	GATAACGCCAAAATCGCTGCTC	22	50	61,03
monkeypox-2500_18_LEFT	2	AAATTCGCGCCCACAATTCATC	22	45,45	60,91
monkeypox-2500_18_RIGHT	2	TCGCCGTTTCATTTTCAACAGC	22	45,45	61,03
monkeypox-2500_20_LEFT	2	AGAAATGCCAAATCTATAAGAAAAGTCCT	29	31,03	60,27
monkeypox-2500_20_RIGHT	2	CCTTTATCAACAAGGAAAGCGTGT	24	41,67	60,34
monkeypox-2500_22_LEFT	2	TCGTATTGTGGTTATATGGCTACAATT	27	33,33	59,56
monkeypox-2500_22_RIGHT	2	TGAATTGTTGCAACGGTTTCCA	22	40,91	60,01
monkeypox-2500_24_LEFT	2	TCAGTCGTTCTAACTCCTTTGCT	23	43,48	59,93
monkeypox-2500_24_RIGHT	2	CACGCTTCTATGTTGCCGTCTA	22	50	60,91
monkeypox-2500_26_LEFT	2	AGACAGAATATCGTGAACAGGTGG	24	45,83	60,7
monkeypox-2500_26_RIGHT	2	TGTTTCGACTGGAGAATCATCCA	23	43,48	59,99
monkeypox-2500_28_LEFT	2	TAACTCCAGGCCGTTTGTTTCC	22	50	61,25
monkeypox-2500_28_RIGHT	2	TTGTGTACCAGAACTCCACCTAAA	24	41,67	59,92
monkeypox-2500_30_LEFT	2	CTGCCACGTTAGAGGATGACAG	22	54,55	60,92
monkeypox-2500_30_RIGHT	2	ACTAACGTTTCTTAGCGGAGGC	22	50	60,85
monkeypox-2500_32_LEFT	2	CAAGACGTTAGAGACAAGAGACGT	24	45,83	60,63
monkeypox-2500_32_RIGHT	2	CAACGCCACAGATTTCTGGAGA	22	50	61,05
monkeypox-2500_34_LEFT	2	GCTATTTAAATGGGTGCCGCAG	22	50	60,72
monkeypox-2500_34_RIGHT	2	GGTGATGATCCTTGACGGAAGA	22	50	60,01

monkeypox-2500_36_LEFT	2	GGCCGCCATCATGATCCTATTC	22	54,55	61,44
monkeypox-2500_36_RIGHT	2	TTACCGCCTTCTGGATAACCTG	22	50	60,01
monkeypox-2500_38_LEFT	2	AGGTGGTGGAACTCCTATTGGA	22	50	60,68
monkeypox-2500_38_RIGHT	2	CACCGCTTCGAAACCATGAAAC	22	50	61,09
monkeypox-2500_40_LEFT	2	TCACGTCAGCGGCATCTAAATT	22	45,45	60,86
monkeypox-2500_40_RIGHT	2	TTCATGTGAAACTTTGTCCTTTCCT	25	36	59,73
monkeypox-2500_42_LEFT	2	AGCCCGTAAATGCAATCAGTGA	22	45,45	60,8
monkeypox-2500_42_RIGHT	2	GCCGTTAAACCAAGCGAATACA	22	45,45	60,02
monkeypox-2500_44_LEFT	2	ACGTGTACTGTATCGACCGGAT	22	50	61,18
monkeypox-2500_44_RIGHT	2	ACGGGTTCAGAAATATCGACGT	22	45,45	60,01
monkeypox-2500_46_LEFT	2	CCAAGATCAAAAGACACGCACG	22	50	60,84
monkeypox-2500_46_RIGHT	2	TTGATGATGTGGAAGGGTCTGC	22	50	60,8
monkeypox-2500_48_LEFT	2	AGATGGGCCCGTTCTCTGAATA	22	50	61,15
monkeypox-2500_48_RIGHT	2	TGTAGCTGTTGTAGACATAACGGTA	25	40	59,97
monkeypox-2500_50_LEFT	2	GCTACTTCGTCGATGGAAACCA	22	50	60,85
monkeypox-2500_50_RIGHT	2	TCCTTAAATCTGGTGCCGTTGT	22	45,45	60,41
monkeypox-2500_52_LEFT	2	AACCAAAAAGTCACACGCTCCA	22	45,45	61,38
monkeypox-2500_52_RIGHT	2	TTCTATGCAGGATCTCCCGAAG	22	50	59,55
monkeypox-2500_54_LEFT	2	GAGAACATAATGCCGCCGTAGT	22	50	60,98
monkeypox-2500_54_RIGHT	2	TGACGTACATCCAGGAGAACCT	22	50	60,74
monkeypox-2500_56_LEFT	2	CACACACGGCAGAAAAACCATC	22	50	61,03
monkeypox-2500_56_RIGHT	2	GTTCCGTTCCCATCATAGTCGT	22	50	60,34

monkeypox-2500_58_LEFT	2	GAAACGGAATCGGTAGATCGTCT	23	47,83	60,49
monkeypox-2500_58_RIGHT	2	CATAGCGTCTCCGGATTCCAAG	22	54,55	61,04
monkeypox-2500_60_LEFT	2	ACTCGACGAGCTCACGTTTAAG	22	50	60,84
monkeypox-2500_60_RIGHT	2	GTTCGACGATTAACGGAGAGCA	22	50	60,91
monkeypox-2500_62_LEFT	2	GCTTCGCGTTTAGTCTCTGGAT	22	50	60,91
monkeypox-2500_62_RIGHT	2	TCGATGCCTGTAAAGGGGAAAC	22	50	60,8
monkeypox-2500_64_LEFT	2	ACCATCATCATAGCATGCGACT	22	45,45	60,14
monkeypox-2500_64_RIGHT	2	GTGTTTGGTTGCGTTATTGCCA	22	45,45	60,98
monkeypox-2500_66_LEFT	2	TAATAAGTTCGAGGATGCCGCC	22	50	60,73
monkeypox-2500_66_RIGHT	2	TTTTCCATGGACTTGTTCAACGT	23	39,13	59,56
monkeypox-2500_68_LEFT	2	ATGTCTCGTGGGGCATTAATCG	22	50	60,99
monkeypox-2500_68_RIGHT	2	ACCGGATTCATCGTCGTAACAA	22	45,45	60,27
monkeypox-2500_70_LEFT	2	AGACTAGTGTATGTGGAAATGTCATAGA	28	35,71	60,24
monkeypox-2500_70_RIGHT	2	TCGGATTATAGCTAAGGACTAGATTCG	27	40,74	60,21
monkeypox-2500_72_LEFT	2	GCAAAAATCAATGGGTCGTTGGAC	24	45,83	61,93
monkeypox-2500_72_RIGHT	2	GTGACACCCATTCATCTGGAGA	22	50	59,95
monkeypox-2500_74_LEFT	2	TCCTTTTAGTGCTCGACAGTGT	22	45,45	59,82
monkeypox-2500_74_RIGHT	2	ACATTGTTTGCCACGTCTTGAT	22	40,91	59,56
monkeypox-2500_76_LEFT	2	TCTTCCGATATCTACAAGGATATTCCA	27	37,04	59,61
monkeypox-2500_76_RIGHT	2	ACGGATGATCTGCACAGAACTC	22	50	60,6
monkeypox-2500_78_LEFT	2	CTCATGTTCTTGTGTAATCGCAGT	24	41,67	59,92
monkeypox-2500_78_RIGHT	2	TGTTCTGCGTCATCTACATCTGA	23	43,48	59,81

monkeypox-2500_80_LEFT	2	AGCGAGAGATCTAGCAACTAGAGT	24	45,83	60,77
monkeypox-2500_80_RIGHT	2	TCGAGTCATTTTACGCACGGTT	22	45,45	61,04
monkeypox-2500_82_LEFT	2	GCTCAATCTGCCAGGATCAAGT	22	50	60,87
monkeypox-2500_82_RIGHT	2	TCAATGGAGCAGGAAAATGGGT	22	45,45	60,41
monkeypox-2500_84_LEFT	2	GACCTCACAACACAGTGCAAGA	22	50	61,18
monkeypox-2500_84_RIGHT	2	CCAGCTAACATAAGAGCCAATCTCA	25	44	61,07
monkeypox-2500_86_LEFT	2	AAAACCATGATGTGATAAAGCTCTGT	26	34,62	60,01
monkeypox-2500_86_RIGHT	2	CCATTGGATGGTGCATGTGGT	21	52,38	61,34
monkeypox-2500_88_LEFT	2	CCGGGAACTTACGCTTTCAGAT	22	50	60,86
monkeypox-2500_88_RIGHT	2	AAAAATGTGTGACCCACGACCG	22	50	62,07

Primers pool 2

2 If you have ordered each primer independently and need to generate primer pool stocks: add **5 µL** of each primer from Pool 1 to a **1.5 mL** Eppendorf labeled “Pool 1 (100µM)” and each primer from Pool 2 to a **1.5 mL** Eppendorf labelled “Pool 2 (100µM)”. These are your 100µM stocks of each primer pool.

Primers should be diluted and pooled in the **mastermix cabinet** which should be cleaned with decontamination wipes and UV sterilised before and after use.

Multiplex PCR 42m

3 In the mastermix hood set up the multiplex PCR mastermix as follows in an **1.5 mL** Eppendorf PCR tube:

Components	volume (pool 1)	Volume (pool 2)
Nuclease Free Water	**3.94 µL**	**3.94 µL**
Primer Pool 1 (100µM)	**1.06 µL**	

Primer Pool 2 (100µM)		1.06 µL
NextGenPCR™ Arctic Fox HF Chemistry-2x	10 µL	10 µL
Total	15 µL	15 µL

4 Add 5 µL of each DNA sample to the NextGenPCR microplate containing 15 µL **Pool 1.** Mix well by pipetting

Add 5 µL of each DNA sample to the NextGenPCR microplate containing 15 µL **Pool 2.** Mix well by pipetting

The **extraction and sample addition cabinet** should be cleaned with decontamination wipes and UV sterilized before and after use.

5 Set-up the following program on the NextGenPCR™ Instrument #10001, (Molecular Biology Systems B.V., The Netherlands): 42m

Step	Temperature	Time	Cycles
Heat Activation	98 °C	00:01:00	1
Denaturation	98 °C	00:00:10	35
Annealing and Extension	65 °C	00:01:00	35

Total PCR time is **42** minutes

Pooling

6 Label a 0.2 mL PCR tube for each sample and combine the two pools from the individual PCR reaction as follows:

Component	Volume
Pool 1 PCR reaction	10 µL
Pool 2 PCR reaction	10 µL

Total **20 µL**

individual sample Bead cleaning (Optional)

7 Ampure XP Bead Cleanup. Add a total of **30 µL** of beads to **20 µL** of pooled samples.

7.1 Vortex or resuspend beads thoroughly to ensure they are well resuspended, the solution should be a homogenous brown colour.

7.2 Incubate for 5 minutes at room temperature

7.3 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear. 2m

7.4 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

7.5 Add **150 µL** of freshly prepared room-temperature **80 % volume** volume ethanol to the pellet.

7.6 Keeping the magnetic rack on the benchtop, rotate the bead-containing tube by 180°. Wait for the beads to migrate towards the magnet and re-form a pellet. Remove the ethanol using a pipette and discard.

7.7 Repeat step 7.5 and 7.6

7.8 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette

7.9 With the tube lid open incubate for **00:01:00** or until the pellet loses it's 1m

shine (if the pellet dries completely it will crack and become difficult to resuspend)

7.10 Remove the tube from the magnetic rack. Resuspend Pellet in **20 µL** Nuclease free water mix gently by flicking and incubate at room temperature for **00:02:00** 2m

7.11 Place on magnet for beads to collect on the magnetic side of the holder and the supernatant to become clear and then transfer **18 µL** sample to a clean 96 wells Microplate ensuring no beads are transferred into this tube.

Quantification

8 Prepare a mastermix of Qubit™ working solution for the required number of samples and standards. The Qubit dsDNA kit requires 2 standards for calibration.

8.1

Per sample:

Qubit® dsDNA HS Reagent **1 µL** µL

Qubit® dsDNA HS Buffer **199 µL** µL

8.2 Aliquot Qubit™ working solution to each tube:

- standard tubes requires 190µL of Qubit™ working solution
- sample tubes require anywhere from **180 µL** to **199 µL** (depending how much sample you wish to add)

The final volume in each tube must be 200µL once sample/standard has been added.

8.3 Add 10µL of standard to the appropriate tube.

8.4 Add 1–20µL of each user sample to the appropriate tube.

8.5 Mix each tube vigorously by vortexing for 3–5 seconds.

8.6 Allow all tubes to incubate at room temperature for 2 minutes, then proceed to “Read standards and samples”.

8.7 On the Home screen of the Qubit™ 3 Fluorometer, press DNA, then select 1X dsDNA HS as the assay type. The Read standards screen is displayed. Press Read Standards to proceed.

8.8 Insert the tube containing Standard #1 into the sample chamber, close the lid, then press Read standard. When the reading is complete (~3 seconds), remove Standard #1.

8.9 Insert the tube containing Standard #2 into the sample chamber, close the lid, then press Read standard. When the reading is complete, remove Standard #2.

8.10 The instrument displays the results on the Read standard screen. For information on interpreting the calibration results, refer to the Qubit™ Fluorometer User Guide, available for download at thermofisher.com/qubit.

8.11 Press Run samples.

8.12 On the assay screen, select the sample volume and units:
- Press the + or – buttons on the wheel, or anywhere on the wheel itself, to select the sample volume added to the assay tube (from 1–20µL).
- From the unit dropdown menu, select the units for the output sample concentration (in this case choose ng/µL).

8.13 Insert a sample tube into the sample chamber, close the lid, then press Read tube. When the reading is complete (~3 seconds), remove the sample tube.

8.14 The top value (in large font) is the concentration of the original sample and the bottom value is the dilution concentration. For information on interpreting the sample results, refer to the Qubit™ Fluorometer User Guide.

8.15 Carefully record all results and store run file from the Qubit on a memory stick.

8.16 All negative controls should ideally be 'too low' to read on the Qubit machine, but MUST be < 1ng per ul. If your negative controls >1ng per ul, considerable contamination has occurred and you must redo previous steps.

Normalisation

9 Adjust the amount of DNA in the tube to be 100 ng total per sample in 7.5 µL molecular grade water. For example if your PCR reaction is at 100ng/ul, add 1ul of the PCR reaction to 6.5ul of molecular grade water. Input to the Rapid Barcoding kit will vary depending on the amplicon length but we have determined 50-200 ng works for efficient barcoding of this amplicon length. If there is under 100ng or you do not know the concentration, simply use all 7.5 µL of the pooled PCR reaction. Use the full 7.5 µLof the negative control, even if there is no detectable DNA in the PCR reaction.

Rapid barcoding 9m 30s

10 Add **7.5 µL** of each diluted PCR reaction to the labeled PCR tube.
Set up the following reaction from each sample:

Component	Volume
DNA amplicons	**7.5 µL**
Fragmentation Mix RB01-12	**2.5 µL**
Total	**10 µL**

10.1 Mix gently by flicking the tube, and spin down.

10.2 Incubate the reaction in a PCR machine: 2m 30s
30 °C for **00:01:00**

80 °C for **00:01:00**

4 °C for **00:00:30**

10.3 Pool all barcoded samples, noting the total volume

11 Bead Cleanup. Use a 1:1 ratio of sample to beads.

11.1 Vortex SPRI beads thoroughly to ensure they are well resuspended, the solution should be a homogenous brown colour.

11.2 Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. For example add **50 µL** room temperature SPRI beads to a **50 µL** reaction.

11.3 Pulse centrifuge to collect all liquid at the bottom of the tube.

11.4 Incubate for **00:05:00** at room temperature. 5m

11.5 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear. 2m

11.6 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

11.7 Add **200 µL** of freshly prepared room-temperature **80 % volume** volume ethanol to the pellet.

11.8 Keeping the magnetic rack on the benchtop, rotate the bead-containing tube by 180°. Wait for the beads to migrate towards the magnet and re-form a pellet. Remove the ethanol using a pipette and discard.

11.9 And repeat ethanol wash (steps 11.7-11.8)

11.10 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette

11.11 With the tube lid open incubate for **00:01:00** or until the pellet loses it's shine (if the pellet dries completely it will crack and become difficult to resuspend) 1m

11.12 Remove the tube from the magnetic rack. Resuspend Pellet in **10 µL** 10 mM Tris-HCl pH 8.0 with 50 mM NaCl, mix gently by flicking and incubate at room temperature for **00:02:00** 2m

11.13 Place on magnet and transfer sample to a clean **1.5 mL** Eppendorf tube ensuring no beads are transferred into this tube.

11.14 Add **1 µL** of RAP (from the SQK-RBK110.96 kit) to **10 µL** cleaned, barcoded DNA, mix gently by flicking the tube, and spin down.

11.15 Incubate the reaction for **00:05:00** at room temperature. 5m

11.16 The prepared library is used for loading into the MinION flow cell according to Oxford Nanopore Rapid Barcoding (SQK-RBK110.96) protocol. Please refer to the Oxford Nanopore Rapid Barcoding SQK-RBK110.96 protocol at this stage. Store the library on ice until ready to load.

MinION sequencing

12 Start the sequencing run using MinKNOW.