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Rapid multiplexed nanopore amplicon sequencing of *Plasmodium falciparum* microhaplotype loci V.2

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We use this protocol and it's working

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Abstract

This protocol outlines a multiplexed nanopore amplicon sequencing (AmpSeq) panel targeting six highly diverse *P. falciparum* microhaplotype loci.

For a detailed explanation of the data analysis, please refer to our **paper** and **github** repository. An example workflow of the bioinformatics pipeline and haplotype inference code used in the pre-print can be found on **zenodo**.

Materials

Reagents

	Item	Supplier	Item code
	Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit	ThermoFisher	Q33231
	Primers (see below)		
	KAPA HiFi HotStart ReadyMix	Roche	KK2601
	Native Barcoding Kit 96 V14	ONT	SQK-NBD114.96
	NEB Blunt/TA Ligase Master Mix	NEB	M0367
	NEBNext Ultra II End repair/dA-tailing Module	NEB	E7546
	NEBNext Quick Ligation Module	NEB	E6056
	AMPure XP beads	Beckman Coulter	A63880
	R10.4.1 flow cell	ONT	FLO-MIN114
	(Optional) Flow Cell Wash Kit	ONT	EXP-WSH004

Plasticware and laboratory basics such as ethanol, nuclease-free water and TE buffer are not included.
Since this protocol uses a higher ratio of magnetic beads than the standard ONT protocol, additional AMPure XP beads are required. Magnetic beads from other manufacturers may also work, but this is at your own risk as we have not tested them.

Primer pool of microhaplotypes

	Primer	Sequence	Quantity	Formulation
	ama1_fw	GAACTCAATATAGACTTCCATCAGG	250 nmole	STD
	ama1_rv	CCTGCATGTCTTGAACATAAAGTC	250 nmole	STD
	celtos_fw	CTGGTACTATTATACCATATGTTGC	250 nmole	STD
	celtos_rv	TCACCAACCTTTTTAGAATCAAGC	250 nmole	STD
	cpmp_fw	GGAAGCTATAGGTATCAGATCC	250 nmole	STD
	cpmp_rv	TAGAATACGTGCTTTATAAACAAAGAG	250 nmole	STD

	Primer	Sequence	Quantity	Formulation
	cpp_fw	AACACAATCTTCCTTAGCCAATTC	250 nmole	STD
	cpp_rv	ATTACTACCTTTCAGCATATCCGA	250 nmole	STD
	csp_fw	GACCCAAACCGAAATGTAGATG	250 nmole	STD
	csp_rv	GAGCCAGGCTTTATTCTAACTTG	250 nmole	STD
	surfin1.1_fw	CACCAAAATATTATATACCACAAGAC	250 nmole	STD
	surfin1.1_rv	GGAAAATCTTTGGTGGGAAAAATAG	250 nmole	STD

Preparation of primer pool

1 Prepare multiplex primer pool (see Materials)

1.1 If you have ordered the primers lyophilised, make them up to [M] 500 micromolar (µM) in TE Buffer (1X Solution pH 8.0, Low EDTA) in a 1.5mL Eppendorf DNA LoBind® Tube.

1.2 Store the resuspended primer stocks at -20 °C .

1.3 Prepare the primer pool by adding the indicated volume (see table below) of *each* forward and reverse [M] 500 micromolar (µM) primer stock to the primer pool. Fill up with nuclease-free H_2O to a total of 500 µL (130 µL fw and rv primers and 370 µL nuclease-free H_2O).

1.4

	Fw Primer	Rv Primer	Amplicon size (bp)	Vol. in Pool (µL)	Final conc. in PCR reaction (µM)
	ama1_fw	ama1_rv	201	7	0.14
	celtos_fw	celtos_rv	123	15	0.3
	cpmp_fw	cpmp_rv	192	10	0.2
	cpp_fw	cpp_rv	184	8	0.16
	csp_fw	csp_rv	143	10	0.2
	surfin1.1_fw	surfin1.1_rv	175	15	0.3

The volume indicated is for *each* of the fw and rv primer to add.

Multiplex PCR

2 Prepare KAPA HiFi HotStart ReadyMix (KK2601) master mix.
Vortex reagents to mix and briefly spin down before opening.

2.1 Generic recipe (without extra % for dead volumes):

	Reagent	Volume (µL)
	2X KAPA HiFi HotStart ReadyMix	12.5
	Multiplex Primer Pool (7-15 µM)	0.5
	Nuclease-Free H2O	8
	TOTAL	21

Note

Instead of the KAPA HiFi HotStart ReadyMix (KK2601) kit, the KAPA HiFi HotStart PCR Kit (KK2501) kit can also be used.

2.2 Aliquot 21 µL PCR mix into PCR tubes/wells (single tubes, strips or plate). Keep tubes On ice .

3 Add DNA

3.1 Add 4 µL DNA sample to each labeled tube/well.

3.2 Close tubes or cover plate with seal. Vortex and spin down before proceeding. 25 µL final volume

4 Run PCR reaction on a thermocycler

	Step	Temp (ºC)	Time	No. cycles
	Initial denaturation	95	3 min	1
	Denaturation	98	15 sec	35
	Annealing	56	15 sec	35
	Extension	72	30 sec	35
	Final Extension	72	2 min	1
	Hold	4	Forever	1

Nanopore library preparation

5 Nanopore library preparation is performed following the "*Native Barcoding Kit 96 V14 (SQK-NBD114.96)*" protocol from Oxford Nanopore Technology (ONT) with some modifications.

> Note
>
> *Alternatively, if only few samples are being processed, the "Native Barcoding Kit 24 V14 (SQK-NBD114.24)" can also be used.*

Make the following changes to the protocol as indicated below:

6 End-prep step

6.1 Omit the DNA Control Sample (DCS) and instead make up each sample to 12.5 µL nuclease-free H_2O. Aim for 500 - 1000 fmol total per sample.

> Note
>
> We recommend including *P. falciparum* positive controls (e.g., mixture of known laboratory strains) in every sequencing run.

6.2 Increase the incubation times for the end-prep to 20 °C for 00:15:00 and 65 °C for 00:15:00 .

6.3 (Optional) Perform a clean-up of the PCR products using AMPure XP beads at a 1.6X ratio (i.e., 24 µL for a 15 µL sample). A clean-up is recommended if possible.

Quantify 1 µL of eluted sample using a Qubit fluorometer.

7 Native barcode ligation

7.1 Take forward an equimolar mass of each sample to be barcoded. Aim for a minimum of >200 fmol total per sample (but this can also be higher).

Make up each end-prepped DNA sample to a maximum of 3.75 µL in nuclease-free H_2O.

> Note
>
> If the DNA concentration varies greatly from sample to sample, it may be necessary to dilute some of the sample. If the amplicon yields are less than 200 fmol, use the entire 3.75 µL for this sample.

7.2 Increase the incubation time to 00:30:00 at room temperature.

7.3 Perform the clean-up step with AMPure XP beads using a 1X ratio instead of 0.4X. Elute the pooled samples in 50 µL instead of 35 µL nuclease-free H_2O.

> Note
>
> The elution volume can be adjusted depending on how much DNA was taken forward. However, the increased volume helps with elution of the DNA as a higher beads ratio is used compared to the standard protocol.
> If overall DNA concentration is low, consider eluting the sample in 35 µL.

8 Adapter ligation

8.1 Increase the incubation time to 00:30:00 at room temperature.

8.2 Perform the clean-up step with AMPure XP beads using a 1X ratio instead of 0.4X (i.e., use 50 µL of beads instead of 20 µL).

8.3 Use short fragment buffer (SFB) for washing the beads to purify all fragments equally.

8.4 Elute the pooled samples in 20-25 µL Elution Buffer (EB).

8.5 Prepare the library to a final volume of 12 µL at 100 - 300 fmol.

Note

The ONT protocol recommends loading 100 fmol for very short (<1 kb) amplicons. We have tried loading up to 300 fmol and did not observe any pore clogging.

Nanopore sequencing

9 Loading SpotON flow cell (R10.4.1)

9.1 Load final library (at 100 - 300 fmol) according to ONT protocol.

Note

Because the amplicons are so short, a large number of reads are generated in a very short time. With a new flow cell (R10.4.1) of good quality and good pore occupancy, nearly 1 million reads can be generated in 1 hour.

We recommend to aim for 60,000 - 120,000 reads per sample, so that ~10,000 - 20,000 reads per marker are obtained. In our protocol, we will remove low quality reads (below Q20), thus removing a portion of the reads prior to haplotype inference.

9.2 (Optional) If the desired number of reads has been obtained and the flow cell has a fair amount of pores remaining (>500), the run can be stopped and the flow cell washed with the Flow Cell Wash Kit (ONT) and reused later.

10 Processing of raw nanopore data after sequencing

For optimal results, we recommend performing basecalling and demultiplexing using ***dorado*** with the following settings:

```
dorado basecaller dna_r10.4.1_e8.2_400bps_sup@v5.0.0 <reads> --
kit-name SQK-NBD114-96 --min-qscore 20 --barcode-both-ends >
calls.bam

dorado demux --output-dir <output-dir> --no-classify --emit-fastq
<input-bam>
```

Resulting fastq files can then be used for haplotype inference (e.g., using our **pipeline**).