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Cost-effective targeted nanopore sequencing of P. falciparum malaria

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ABSTRACT

This protocol outlines a cost-effective approach for amplicon sequencing of *P. falciparum* malaria from dried blood spots (DBS). The protocol can be conducted in ~2-3 days and costs ~USD $25/sample. We currently have two target panels, NOMADS8 and NOMADS16, which were developed using multiply (https://github.com/JasonAHendry/multiply).

Here, we describe how to generate the primer pools and prepare the library for sequencing. The protocol starts from dried-blood spot (DBS) extracted DNA. To date, most of our tests have been with samples extracted using Qiagen QIAamp kits (see Materials) and we observe good performance for samples with parasitemia > 1000p/uL. The assay performs better on fresher samples compared to those that have spent a long time as DBS. It is advisable to include positive controls in your experiment (for example, lab strains mixed with human DNA to mimic DBS-extracted DNA) as well as non-template controls.

We welcome your questions and/or feedback on the protocol.

MATERIALS

Major reagents

	Step	Item	Supplier	Item Code
	General	Qubit™ dsDNA HS Assay Kit (500)	ThermoFisher	Q32854
	DNA Extraction	QIAamp DNA Micro Kit (50)	Qiagen	56304
	sWGA	phi29 DNA Polymerase	NEB	M0269L
	sWGA	1000uM sWGA Primers		
	sWGA	NEB 10mM dNTP Solution Mix	NEB	N0447L
	Multiplex PCR	NOMADS16 Primers		
	Multiplex PCR	KAPA HIFI + dNTPS (100U)	Roche	KK2101

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Protocol status: Working
We use this protocol and it's working

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	Step	Item	Supplier	Item Code
	Barcoding	Native Barcoding Expansion 96	ONT	EXP-NBD196
	Barcoding	NEBNext Ultra II DNA Library Prep Kit with Sample Purification Beads*	NEB	E7103L
	Adapter Ligation	Ligation Sequencing Kit	ONT	SQK-LSK109
	Adapter Ligation	NEBNext Quick Ligation Module	NEB	E6056L
	Sequencing	R9.4.1 Flow Cell	ONT	FLO-MIN106D

Note that plasticware and laboratory basics like ethanol and nuclease-free water are not included here.
*This kit contains enough material perform sample barcoding for 480 samples, as well as SPRI beads.

sWGA Primer Pool

	Primer	Sequence	Quantity	Formulation
	Pf1	ATATATATAT*A	250 nmole	STD
	Pf2	TATATATATAT*T	250 nmole	STD
	Pf3	TATATATATA*A	250 nmole	STD
	Pf4	TAATATATA*T	250 nmole	STD
	Pf5	TATATATATT*T	250 nmole	STD
	Pf6	ATTATTATTA*T	250 nmole	STD
	Pf7	TAATAATAAT*A	250 nmole	STD
	Pf8	AAAAAAAAAAA*A	250 nmole	STD
	Pf9	AATAATAATA*A	250 nmole	STD
	Pf10	TATTATATA*T	250 nmole	STD

The asteriks (*) indicates that the 3’ nucleotide is joined thorugh a phosphorothioate bond to inhibit the exonucleotide acitivity of phi29; note there is an extra charge per primer for the modification.

NOMADS8 Primer Pool

Primer	Sequence	Quantity	Formulation
CRT1_s8_F	TTTCATTGTCTTCCACATATATGACA	25 nmole	STD
CRT1_s8_R	TGCTCCTTTGACACAGTATGA	25 nmole	STD
DHFR_s0_F	AGCCATTTTTGTATTCCCAAATAGC	25 nmole	STD
DHFR_s0_R	ACCTCCTTAACAGAACTAGCCG	25 nmole	STD
DHPS_s14_F	AGAACAGCTATCGTATGAGGTACA	25 nmole	STD
DHPS_s14_R	TCACATGTTTGCACTTTCCTTT	25 nmole	STD
K13_s14_F	ATCCAAGCCTAGAACCTAATCC	25 nmole	STD
K13_s14_R	GGCGTAAATATTCGTGTTATAATTTCT	25 nmole	STD
MDR1part_s6_F	AGAGTTGAACAAAAAGAGTACCGC	25 nmole	STD
MDR1part_s6_R	CCAATGTTGCATCTTCTCTTCCA	25 nmole	STD
MSP2_s6_F	CACATGGACAACCTGCATCAC	25 nmole	STD
MSP2_s6_R	GTGCCACATTATAGCGCCCT	25 nmole	STD
PMI_s9_F	AAACAGACAATACCCGTCTACA	25 nmole	STD
PMI_s9_R	ACAAACTAGCCATTTGTATGCCT	25 nmole	STD
PMIII_s6_F	AAAAACGGGTCTGCCAAAAT	25 nmole	STD
PMIII_s6_R	TCAACCCAGCATGTGTTTCT	25 nmole	STD

NOMADS16 Primer Pool

Primer	Sequence	Quantity	Formulation
AMA1_S11_F	CACCTTGTTTTGAAACCTTTACACA	25 nmole	STD
AMA1_S11_R	TGTTGTACACATTTTCCCATAACA	25 nmole	STD
CSP_S3_F	CTTTTCCGGGGTCCCTTTCA	25 nmole	STD
CSP_S3_R	CGTGTAAAAATAAGTAGAAACCACGT	25 nmole	STD
HRP2_S12_F	TCGCTATCCCATAAATTACAAAACA	25 nmole	STD
HRP2_S12_R	ACCATAATAAATTACTTTCCAGTCACA	25 nmole	STD
HRP2dwn_S5_F	GCAGTAACATCATGGTTTTACATTACA	25 nmole	STD
HRP2dwn_S5_R	GGCTAATTTTTCAGGAGCTTCTACC	25 nmole	STD
HRP2up_S11_F	TGTGGGAATTTATAAGTTTCCAAGT	25 nmole	STD
HRP2up_S11_R	ACCACACCTACGTCAGTAACA	25 nmole	STD

	Primer	Sequence	Quantity	Formulation
	HRP3_S12_F	AGCATATTATCTTCACTCACCCC	25 nmole	STD
	HRP3_S12_R	GTGGTATTCAGAACATTCGGATTTATT	25 nmole	STD
	HRP3dwn_S1_F	GCTAGGTGTATACGCACAGA	25 nmole	STD
	HRP3dwn_S1_R	ATTCCCATGTTAAGCCTCTATTTTT	25 nmole	STD
	HRP3up_S6_F	TCAATCTGAGGGATTAGTTGATGA	25 nmole	STD
	HRP3up_S6_R	ACACCGTCAAATCCTCGATA	25 nmole	STD

NB: These primers are combined **in addition to the NOMADS8 primers** to create the full NOMADS16 panel.

Preparation of primer pools.

1 Prepare the sWGA primer pool (see Materials).

1.1 If you have ordered the primers lyophilised, make them up to 1000uM in nuclease-free water.

1.2 To make the 1000uM sWGA primer pool, combine equal volumes of each primer (e.g. Pf1-Pf10) in a 1.5mL LoBind tube.

1.3 Store the primers at 4C.

2 Prepare the NOMADS8 or NOMADS16 primer pools (see Materials).

2.1 If you have ordered the primers lyophilised, make them up to 100uM in nuclease-free water.

2.2 If you want to run the **NOMADS8 panel**, use the table below to add the indicated volume of each 100uM primer stock to the primer pool. Afterwards dilute the primer pool to 10uM in nuclease-free water.

	F primer	R primer	Amplicon Size (bp)	Vol. into pool (uL)*
	CRT1_s8_F	CRT1_s8_R	3874	6
	DHFR_s0_F	DHFR_s0_R	3463	3
	DHPS_s14_F	DHPS_s14_R	3656	3
	K13_s14_F	K13_s14_R	3826	1.5
	MDR1part_s6_F	MDR1part_s6_R	3773	1.5
	MSP2_s6_F	MSP2_s6_R	3720	3
	PMI_s9_F	PMI_s9_R	3101	6
	PMIII_s6_F	PMIII_s6_R	3468	3
			TOTAL (uL)	54

*This is the volume of *each of the F and R primer to add.* So for CRT1_s8; you will be adding 6uL of CRT1_s8_F *and* 6uL of CRT1_s8_R.

Note

For reasons of consistency, it is often advisable to make a larger volume pool than indicated above and split into aliquots.

Note

Why aren't all the volumes the same? In preliminary experiments we found that *mdr1* and *kelch13* amplicons were very abundant, whereas *crt1* amplicons were relatively few. Thus the primer volumes were adjusted to mitigate against this imbalance.

2.3 If you want to run the **NOMADS16 panel**, use the table below to add the indicated volume of each 100uM primer stock to the primer pool. Afterwards dilute the primer pool to 10uM in nuclease-free water.

	F primer	R primer	Amplicon Size (bp)	Vol. into pool (uL)*
	CRT1_s8_F	CRT1_s8_R	3874	6
	DHFR_s0_F	DHFR_s0_R	3463	6
	DHPS_s14_F	DHPS_s14_R	3656	3
	K13_s14_F	K13_s14_R	3826	3
	MDR1part_s6_F	MDR1part_s6_R	3773	1.5
	MSP2_s6_F	MSP2_s6_R	3720	3
	PMI_s9_F	PMI_s9_R	3101	3
	PMIII_s6_F	PMIII_s6_R	3468	3
	AMA1_S11_F	AMA1_S11_R	3138	1.5
	CSP_S3_F	CSP_S3_R	3107	3
	HRP2_S12_F	HRP2_S12_R	3097	3
	HRP2dwn_S5_F	HRP2dwn_S5_R	3333	3
	HRP2up_S11_F	HRP2up_S11_R	3366	3
	HRP3_S12_F	HRP3_S12_R	3444	3
	HRP3dwn_S1_F	HRP3dwn_S1_R	3304	3
	HRP3up_S6_F	HRP3up_S6_R	3066	6
			TOTAL (uL)	108

*This is the volume of *each of the F and R primer to add.* So for CRT1_s8; you will be adding 6uL of CRT1_s8_F *and* 6uL of CRT1_s8_R.

Note

Treat these volumes as suggestions. For the NOMADS16 panel, we did one round of amplicon balance optimisation but the balance can still be improved. If you find a combination of primer volumes that produces more balanced coverage across targets, please let us know! :)

3 Prepare the sample DNA.

3.1 Place a 96-well skirted PCR plate on ice.

3.2 Add 10ng to 40ng of extracted DNA per well and bring to 30uL with nuclease-free water, or low TE.

> **Note**
>
> The 10ng to 40ng recommendation is a rough guideline. We have had successful experiments using less than 10ng; and also had reactions fail using 40ng. Probably more important than input DNA mass is sample quality and parasitemia.

3.3 Seal the plate and leave on ice while preparing the master mix.

> **Note**
>
> If you are using a cool block (e.g. Eppendorf 3881000031), be careful not to freeze the DNA while preparing the master mix.

4 Prepare the sWGA master mix.

	Reagent	Vol. per sample (uL)	Vol. per 96-well plate (uL) [+10% excess]
	Nuclease-Free H2O	8.625	910.8
	10x Phi29 Buffer	5	528
	20mg/mL BSA	0.25	26.4
	1000uM sWGA Primer Mix	0.125	13.2
	10mM dNTP	5	528
	phi29 (10U)	1	105.6
	TOTAL (uL)	20	2112

Note

If working from a plate, aliquot master mix into a PCR strip tube and deliver by multichannel pipette to improve consistency.

5 Add 20uL of the master mix to each sample, mix by pipetting and incubate the plate in a thermal cycler with the following program:

	Step	Temp. (*C)	Time
	Amplify	35	5 min
		34	10 min
		33	15 min
		32	20 min
		31	30 min
		30	16 hr
	Heat inactivation	65	15 min
	Hold	10	Forever

Note

The total reaction time is 17h35min. If you set the reaction up late afternoon (e.g. 3-4pm), it will be done for 9am the following morning.

5.1 (Optional) QC the sWGA product using a Qubit DNA assay. Good reaction performance will yield >20ng/uL; if your product is only at ~5ng/uL, it probably failed.

NOMADS Multiplex PCR

6 Transfer 2uL of sWGA product into a new skirted 96-well PCR plate.

7 Prepare KAPA HiFi polymerase (KK2101) master mix:

	Reagent	Vol. per sample (uL)	Vol. per 96-well plate (uL) [+10% excess]
	5X Buffer with Mg2+	5	528
	10mM dNTPs	0.75	79.2
	Multiplex Primer Pool (10uM)*	1.5	158.4
	Nuclease-Free H20	15.25	1610.4
	KAPA Pol. (1U/uL)	0.5	52.8
	TOTAL	23	2428

*either NOMADS8 or NOMADS16 primers, as prepared in Section 1.

Note

If working from a plate, aliquot master mix into a PCR strip tube and deliver by multichannel pipette to improve consistency.

Note

We have also successfully amplified the NOMADS8 panel using the KAPA HiFi HotStart ReadyMix (KK2602). We haven't tried yet with the NOMADS16 panel; if you do and it works, let us know.

7.1 Mix by pipetting; or flick mix and spin down.

8 Add 23uL of master mix to each sample, and incubate the plate in a thermal cycler with the following program:

	Step	Temp. (*C)	Time	No. cycles
	Initial Denaturation	95	3 mins	1
	Denaturation	98	20 secs	30
	Annealing	50	15 secs	

	Step	Temp. (*C)	Time	No. cycles
	Extension	60	6 mins	
	Final Extension	60	10 mins	1
	Hold	8	Forever	1

Note

Why such a long extension time? According to the technical data sheet, KAPA HiFi extends at 15-60s/kbp. Our amplicons are 3-4kbp, so if we followed these guidelines a 4min extension should be adequate.

However, inspired by *Xin-zhuan et al. (1996). NAR.*, we run with a reduced extension temperature (60*C rather than 72*C), which will decrease the rate of catalysis. With 6min we are trying to take this into account, and ensuring our longer and more difficult amplicons (e.g. *crt1*) are amplified.

Note

This is a safe stopping point. Store the PCR products at -20*C or 4*C.

9 (Optional) Run 2uL of PCR product on a 0.66% agarose gel. If you see a strong bands in the 3-4kbp range, this is a very good predictor that sequencing will be successful. If there are no bands visible in the 3-4kbp range, the multiplex PCR or sWGA has failed, and it is worth troubleshooting rather than continuing with sequencing.

SPRI bead clean-up of multiplex PCR products

10 Perform a 0.5X ratio SPRI bead clean-up of your multiplex PCR products.

Note

Ensure your SPRI beads are **at room temperature**!
Vortex your SPRI beads **for 30s or more** to ensure they are in suspension before pipetting!
Prepare a *fresh stock of 80% ethanol* each time.

10.1 To the PCR product, add 12uL of SPRI beads.

10.2 Mix **thoroughly** by pipetting or vortexing. *Visually check to ensure the beads are mixed throughout the solution*. If vortexing, spin down but do not pellet the beads.

> Note
>
> Especially when working in a 96-well plate, it is easy to inadvertently pipette the SPRI beads to the bottom of each well, pipette mix them at the bottom of the well, and therefore never mix them properly with the PCR product.

10.3 Incubate for 5mins at room temperature (RT).

10.4 Incubate on the magnet for 8mins.

10.5 Remove the supernatant. Do not transfer any beads; if this is a risk leave ~5uL of supernatant behind with the beads.

10.6 Wash the beads in 175uL of 80% ethanol. Leave for 30secs.

10.7 Remove and discard the supernatant. Repeat ethanol wash.

10.8 Spin down the samples, place back on the magnet and remove ay residual ethanol. *Ensure all ethanol is removed.*

10.9 Air dry the beads until the look dry, e.g. ~30secs.

10.10 Resuspend the beads in 15uL of nuclease-free water by pipetting.

10.11 Wait 2 to 5mins while DNA is being released from the beads. Waiting longer helps recovered longer DNA fragments.

10.12 Return samples to the magnet for 2mins or until beads have pelleted and the solution is clear.

10.13 Transfer 14uL of supernatant to a clean 96-well skirted plate.

11 QC using Qubit DNA assay. A good range is between 30-100ng/uL.

> **Note**
>
> We have observed a positive correlation between samples' ng/uL in this QC step and their *P.falciparum* mapping percentages in the sequencing data.

> **Note**
>
> This is a safe stopping point. Store at -20*C or 4*C.

One-pot barcoding by ligation (modified from *Josh Quick's* o..

12 Prepare the one-pot program on your thermal cycler, and start the program to bring the block to 20*C.

	Step	Temp. (*C)	Time
	Prepare block	20	Forever
	End prep.	20	15 min

	Step	Temp. (*C)	Time
	Heat inactivate	65	15 min
	Hold	8	Forever
	Prepare block	20	Forever
	Barcode ligation	20	20 min
	Heat inactivate	65 or 70	10 min
	Hold	8	Forever

Note

If possible it is better to have the heated lid off for all steps that are not heat inactivation. If this is not possible on your thermal cycler, set the lid temperature lower (e.g 75*C) instead.

13 Transfer between 100-600ng of each sample's PCR product into a new plate, in no more than 10uL volume.

Note

You can attempt some sort of sample normalisation at this point, however in our experience ng of PCR product into the barcoding step only weakly influences final number of reads observed; so at present we feel it is preferable to transfer a consistent volume across the plate to minimise time and risk of cross-contamination.

14 Perform end preparation.

14.1 Prepare the end preparation master mix as follows:

	Reagent	Vol. (uL) per sample	Vol. (uL) per 96-well plate [includes 20% excess]
	NEBNext 10X End Prep Buffer	1.4	161.3
	NEBNext End Prep Enzyme Mix	0.6	69.1

14.2 Add 2uL of the master mix to each sample. Mix by pipetting or flicking, and spin down.

14.3 Place the plate in the thermal cycler and press "Enter" to move the program forward; through the 20°C for 15 mins -> 65°C for 15 mins -> 8°C forever, steps.

15 Perform barcode ligation.

15.1 While the end preparation is incubating, thaw the native barcodes in the fridge. There may be condensation on the side of the barcode tubes requiring they are spun down.

15.2 Also while incubating, prepare the barcode ligation master mix:

	Reagent	Vol. (uL) per sample	Vol. (uL) per 96-well plate [includes 20% excess]
	Ultra II Ligase Master Mix	6	691.2
	Ligation Enhancer	0.2	23

15.3 Once the thermal cycler block reaches 8*C, take out the plate and press "Enter" to move the program to the next 20*C hold.

15.4 Place the plate on ice for 1 min.

> **Note**
>
> The volume here is very small, if you are using a cool block (e.g. Eppendorf 3881000031), be careful not to freeze.

15.5 To each sample, add 0.5uL of the assigned native barcode.

Note

This step requires attention; double check your tips to ensure that 0.5uL of barcode is being delivered to every sample.

If you cannot consistently aspirate 0.5uL with your pipettes, you can transfer a larger volume (e.g. 0.75uL), although this increases assay costs.

Consider using long-reach thin-tipped pipette tips for this step. Inconsistencies in pipetting here directly influence sample balance!

15.6 Add 6.2uL of the barcode ligation master mix to each sample, mix well by pipetting and spin down.

15.7 Return the plate to the thermal cycler and press “Enter” to move the program through: 20°C for 20 mins -> 65°C for 10mins -> 8°C forever.

Sample pooling and SPRI bead clean-up.

16 Pool equal volumes of each sample to a LoBind 1.5mL Eppendorf Tube.

17 Perform a 0.4X ratio SPRI bead clean-up.

Note

Ensure your SPRI beads are **at room temperature**!
Vortex your SPRI beads **for 30s or more** to ensure they are in suspension before pipetting!
Prepare a *fresh stock of 80% ethanol* each time.

17.1 To the pooled DNA, add SPRI beads at a 0.4X ratio.

Note

For example, if your pool is 100uL add 40uL of SPRI beads.

17.2 Mix thoroughly by pipetting or vortexing. *Ensure the beads are mixed visually*. If vortexing, spin down but do not pellet beads.

17.3 Incubate for 5 mins at RT.

17.4 Incubate on the magnet for 8 mins.

17.5 Remove the supernatant. Do not transfer any beads, if this is a risk leave ~5uL of supernatant behnid with the beads.

17.6 Wash the beads with 80% ethanol. Leave for 30 secs. Ensure the ethanol complete covers the pelleted beads – use an amount greater than the amount of sample + beads.

17.7 Remove and discard supernatant. Repeat ethanol wash.

17.8 Spin down the samples, place back on the magent and remove any residual ethanol. *Ensure all ethanol is removed.*

17.9 Air dry the beads until they look dry, e.g. ~30s.

17.10 Resuspend the beads in 70uL nuclease-free water by pipetting.

17.11 Wait 2 to 5 mins while DNA is released from the beads. Wait longer for longer DNA fragments.

17.12 Return samples to the magnet for 2 mins or until beads have pelleted and the solution is clear.

17.13 Transfer 67uL of supernatant to a clean plate.

18 QC 2uL of the cleaned pool using the Qubit.

18.1 Aim to pass 1 to 2ug (1000 to 2000ng) of pooled DNA into the adapter ligation reaction, making up to 65uL in nuclease-free water.

Library preparation and sequencing

19 From this point forward, follow the protocol from Oxford Nanopore Technology (ONT)...

"1D Native barcoding genomic DNA (with EXP-NBD104, EXP-NBD114, and SQK-LSK109)"

...starting from the *'Adapter ligation and clean-up'* step.

Make the following changes:

- Use LFB for the clean up
- Transfer 200-300fmol (~400-600ng) of DNA to the Flow Cell.