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NOMADS-MVP: Rapid Genomic Surveillance of Malaria with Nanopore V.1

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We use this protocol and it's working

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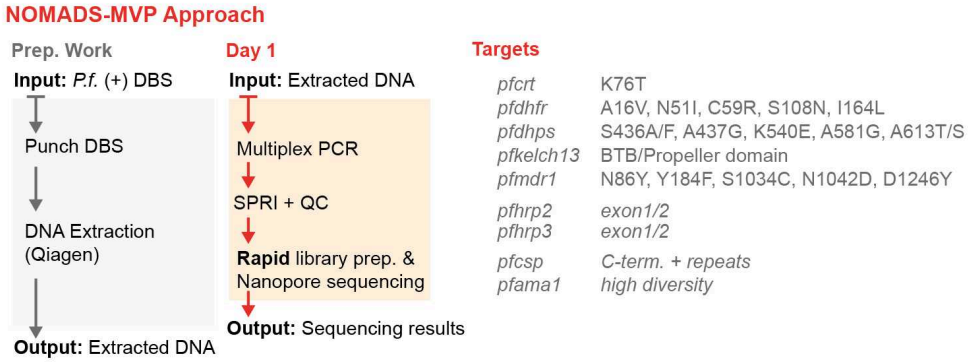
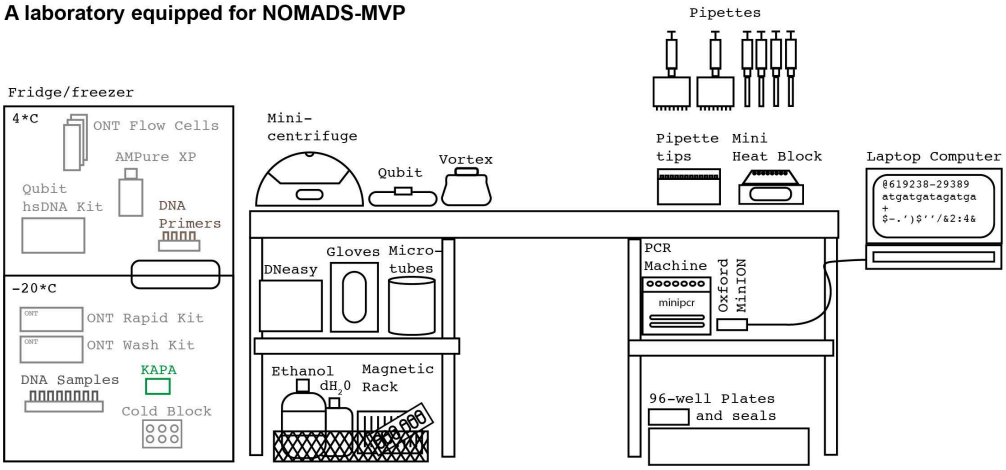
Abstract

The NOMADS Minimum Viable Panel (MVP) approach enables rapid genomic surveillance of *P. falciparum* malaria using targeted nanopore sequencing. It includes amplicons targeting key antimalarial drug resistance genes (*pfcrt, pfdhfr, pfdhps, pfkelch13, pfmdr1*), rapid diagnostic test (RDT) antigens (*pfhrp2, pfhrp3*), a high diversity gene (*pfama1*), and the RTS'S vaccine target (*pfcsp*). From extracted DNA to data takes under one day and results can be visualised real-time using the *Nomadic* dashboard (https://jasonahendry.github.io/nomadic).



The protocol uses a single multiplex PCR from extracted DNA without preamplification, combined with the Rapid Barcoding Kit (SQK-RBK114.96) from Oxford Nanopore Technologies (ONT) to accelerate and simplify library preparation. The median read length is typically betweeen 400 - 600bp, and >80 samples can be multiplexed in a run. Across a variety of field dried-blood spot (DBS) sample sets, pass rate is moderate (>70%) for samples with >100p/uL, and good (>90%) for samples with >1000p/uL. The protocol has been validated for SNP detection in clonal samples and hrp2/3 deletion detection.



Materials

MVP Primers

	Name	Sequence	Quantity to order (nmole)	Formulation
	ama1-d2-18-ck_v43_F	CAACACGCATATCCAATAGACCA	25nm	STD
	ama1-d2-18-ck_v43_R	TGATCCGAAGCACTCAATTCAA	25nm	STD
	crt-k76_v27_F	AGCAAAAATGACGAGCGTTATAGA	25nm	STD
	crt-k76_v27_R	AGCTTCGGTGTCGTTCCTAAA	25nm	STD
	csp-rtss-repeat_v4_F	TCGCAAACGTAATTAAATATTCACAAA	25nm	STD
	csp-rtss-repeat_v4_R	CCTTATTCCAGGAATACCAGTGC	25nm	STD
	dhfr-p51-p164_v19_F	CCATTTTTGTATTCCCAAATAGCTAGT	25nm	STD
	dhfr-p51-p164_v19_R	TCCCTAGTACCATTAGCTTCCCA	25nm	STD
	dhps-p436-p613_v55_F	TCCATTCCTCATGTGTATACAACA	25nm	STD
	dhps-p436-p613_v55_R	TGTTTAATCACATGTTTGCACTTTCC	25nm	STD
	hrp2-exon2-complete_v26_F	TCGCTATCCCATAAATTACAAAACA	25nm	STD
	hrp2-exon2-complete_v26_R	CCGTTTTTGCCTCCGTACTT	25nm	STD
	hrp3-exon2-complete_v21_F	AGACAGTAGAAAAATCGCTATCCT	25nm	STD
	hrp3-exon2-complete_v21_R	GCCGTTTTTGCTTCCGTACTT	25nm	STD
	kelch13-cterm_v5_F	AAGGGAAAATCATAAACAATCAAGT	25nm	STD
	kelch13-cterm_v5_R	GGAAGACATCATGTAACCAGAGA	25nm	STD
	mdr1-p1034-p1246_v19_F	GCGGAGTTTTTGCATTTAGTTCAG	25nm	STD
	mdr1-p1034-p1246_v19_R	CCAATGTTGCATCTTCTCTTCCA	25nm	STD
	mdr1-p86-p184_v26_F	CGTTTAAATGTTTACCTGCACAACA	25nm	STD
	mdr1-p86-p184_v26_R	ACTTGCAACAGTTCTTATTCCCA	25nm	STD

MVP Multiplex PCR Reagents

	Product	Order number	Vendor
	KAPA HiFi HS RM (6.25ml)	07958935001 (KK2602)	Roche
	Qubit 1X dsDNA HS Assay Kit	Q33231	Thermo Fisher Scientific
	AMPure XP Beads	A63881	Beckman Coulter

Rapid Sequencing

	Product	Order number	Vendor
	Rapid sequencing DNA V14	SQK-RBK114.96	Oxford Nanopore
	Flow Cell R.10	FLO-MIN114	Oxford Nanopore
	Flow Cell Wash Kit	EXP-WSH004	Oxford Nanopore

	Abbr.	Definition
	MVP	Minimum Viable Panel
	DNA	Deoxyribonucleic acid
	PCR	Polymerase chain reaction

	Abbr.	Definition
	DBS	Dried blood spot
	EB	Elution buffer
	bp	Base pair
	QC	Quality control
	AMPure XP beads	Magnetic beads used for purification
	RFU	Relative fluorescence units
	ng	Nanogram
	ul	microliter
	min	Minutes
	sec	Seconds

Plasticware

	Plastics	Comment
	1.5 ml Low Bind Tubes	Eppendorf
	100 ml Reservoir	Needed in purification step
	15ml or 50ml Falcon Tube	For ethanol preparation
	96-well Plates	Ensure they fit your thermalcycler
	96-well Plate Seals	
	Strip Tubes	For aliquoting master mix
	Qubit Tubes	

Important Information

1 **Including controls**

Always include at least one positive control (lab strain) and one negative control (water only) in each sequencing run.

2 **Avoiding contamination**

DNA sequencing is very sensitive to contamination.
Please take the following steps to avoid contamination during your work:

- Before starting, clean the surfaces with 10% bleach and then wash with 70% ethanol (alternatively, you can use 70% ethanol and then a solution like DNA exitus).
- Aliquot large volume reagents into smaller volumes so they are not used across multiple experiments. For example, we **strongly recommend aliquoting the KAPA HiFi ReadyMix** (aliquots of 845uL are adequate for batches of 48 samples).
- If available, do all pre-PCR work in a separate clean space.
- Always change tips between samples.
- Always spin tubes or plates before opening.
- Never touch reagents or pipettes without gloves.

3 **Selecting suitable samples for sequencing**

Parasitemia: We have processed over 500 field dried-blood spots (DBS) with this protocol from several countries and studies across Sub-Saharan Africa. In general, we observe very good pass rates (>90%) for samples with >1000p/uL; and moderate pass rates (>70%) for samples with >100p/uL.
DNA extraction: We have observed better performance for samples extracted with Qiagen kits, rather than Chelex.
Quality: It is important to use Whatman 3 filter paper or equivalent for dried-blood spots (DBS). Extracting DNA promptly following DBS collection and storing at -20°C is the best way to maintain DNA quality. Samples that spend a long time as DBS (>6-12 months) may perform worse.

4 **If you are working in a 96-well plate:**

Each addition of a reagent (e.g. master mix, or water in the elution step) can be first added to a 8-well strip tube, so that you can use a multichannel pipette to add reagents column-wise. Always check the uptake and release of the reagent in each tip when using a multichannel pipette.

MVP Multiplex PCR

5 **MVP PCR**

Prepare single primers:

The primers will come lyophilised and need to be reconstituted to 100uM in nuclease free water or low TE-buffer as follows:

- Spin down all of the lyophilised primers before opening.

- On each tube you can find a **molarity information e.g. 24.8 nmol**. You would need to add 248uL of nuclease-free water or low TE-Buffer to get a **100 uM** stock concentration.

Attention: the volume required for the individual primers will be different!

Example:

Primer **csp-rtss-repeat_v4_F**

26.7 nmol noted on the tube

Add **267 uL** water

- Make all primers up to 100uM and vortex them well and spin down.

Prepare the primer pool:

- To prepare the primer pool **combine the volumes of each primer (100uM) mentioned in the following table** in a new tube. Your total volume will be 135uL.

	Name	Sequence	Volume into Pool (ul)
	ama1-d2-18-ck_v43_F	CAACACGCATATCCAATAGACCA	5
	ama1-d2-18-ck_v43_R	TGATCCGAAGCACTCAATTCAA	5
	crt-k76_v27_F	AGCAAAAATGACGAGCGTTATAGA	5
	crt-k76_v27_R	AGCTTCGGTGTCGTTCCTAAA	5
	hrp2-exon2-complete_v26_F	TCGCTATCCCATAAATTACAAAACA	5
	hrp2-exon2-complete_v26_R	CCGTTTTTGCCTCCGTACTT	5
	hrp3-exon2-complete_v21_F	AGACAGTAGAAAAATCGCTATCCT	5
	hrp3-exon2-complete_v21_R	GCCGTTTTTGCTTCCGTACTT	5
	mdr1-p1034-p1246_v19_F	GCGGAGTTTTTGCATTTAGTTCAG	5
	mdr1-p1034-p1246_v19_R	CCAATGTTGCATCTTCTCTTCCA	5
	mdr1-p86-p184_v26_F	CGTTTAAATGTTTACCTGCACAACA	5
	mdr1-p86-p184_v26_R	ACTTGCAACAGTTCTTATTCCCA	5
	csp-rtss-repeat_v4_F	TCGCAAACGTAATTAAATATTCACAAA	10
	csp-rtss-repeat_v4_R	CCTTATTCCAGGAATACCAGTGC	10
	dhfr-p51-p164_v19_F	CCATTTTTGTATTCCCAAATAGCTAGT	10
	dhfr-p51-p164_v19_R	TCCCTAGTACCATTAGCTTCCC	10
	dhps-p436-p613_v55_F	TCCATTCCTCATGTGTATACAACA	10
	dhps-p436-p613_v55_R	TGTTTAATCACATGTTTGCACTTTCC	10
	kelch13-cterm_v5_F	AAGGGAAAATCATAAACAATCAAGT	7.5
	kelch13-cterm_v5_R	GGAAGACATCATGTAACCAGAGA	7.5

MVP Primer Pool preparation

- **Take 100uL of the primer pool and add 900uL** nuclease-free water or low TE-Buffer to make a **10 uM working dilution** and vortex well.
- Make 4 aliquots of the primer pool with 250uL each.
- Store 3 aliquots at -20°C and keep the working aliquot at 4°C.

Prepare the PCR program

	Step	Temp (°C)	Time	No. of Cycles
	Prepare Block	95	Forever	1
	Initial Denaturation	95	3 min	1
	Denaturation	98	20 sec	35
	Extension	60	3 min	
	Final Extension	60	10 min	1
	Hold	8	Forever	1

Total runtime is approximately 2hrs and 28mins.

Prepare samples

- Transfer 8uL of extracted DNA from each sample to a unique well in a 96-well plate.
- Cover the plate while preparing the master mix.

Prepare master mix

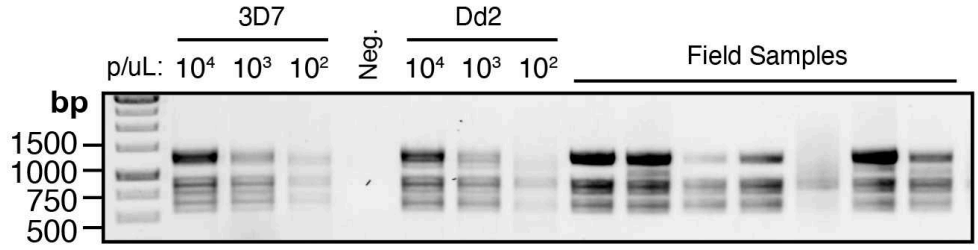
- Before preparing the master mix, start the PCR program to preheat the block to 95°C.
- Prepare the master mix **on ice:**

	Reagent	Vol. per sample (ul)	Vol. per 48 sample (ul) [+10% excess]
	KAPA ReadyMix 2X	15.5	818
	MVP primer pool (10uM)	1.5	79
	Total	17	897

- Mix the master mix by pipetting.
- Transfer 17uL of master mix to each sample. When preparing many samples, aliquot your master mix into a 8-well strip tube, and then deliver the master mix to the samples by multichannel pipette to improve consistency.
- **Mix well by pipetting**, seal the plate and spin down.
- Place the plate in the preheated thermal cycler, and press "Resume" or "Skip Step" to begin the PCR.

Quality control by electrophoresis

- After PCR, run 1uL of each PCR product on a 0.66% agarose gel with an 1kb ladder (140 Volt for 40 minutes).
- Your results should look similar to:



Example agarose gel of NOMADS-MVP. Controls are shown at three parasitemia levels. Negative control is pure water. Seven field samples are shown; one has failed.

Post-PCR DNA Clean Up

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

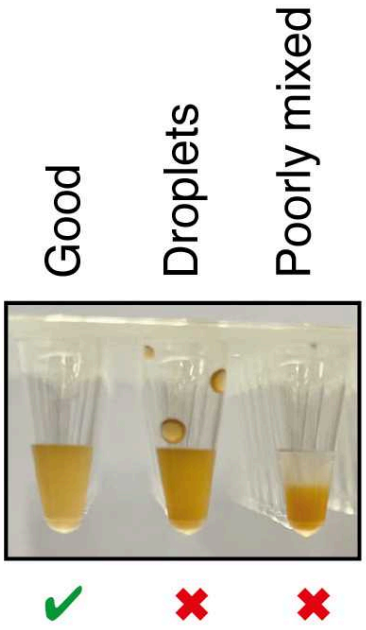
	# of samples	Vol. Ethanol	Vol. Water	Total vol.
	24	12 ml	3 ml	15 ml
	48	20 ml	5 ml	25 ml
	96	36 ml	9 ml	45 ml

Guidelines for preparation of 80% ethanol. Some excess is included for use with reservoir.

Post-PCR DNA Clean Up

7
- Aliquot mixed AMPure XP beads to a strip tube and then deliver by multichannel pipette to improve consistency.
- Add 12uL of AMPure XP beads to the PCR products (0.5X ratio)
- **Mix thoroughly by pipetting**. Visually check to ensure the beads are mixed throughout the solution **(see image below)**.
- Incubate for 5 mins at room temperature (not on the magnet!).
- Incubate on the magnet for 5 mins.
- Remove 30uL of the supernatant, leaving approximately 5uL behind. Take care not to transfer any beads.
- While keeping the plate on the magnet, wash the beads by adding 175uL of 80% ethanol.
- Leave for 30 secs. (for 96-well plate, add the ethanol to a reservoir, deliver with multichannel pipette).
- Remove and discard the supernatant.
- Repeat the ethanol wash by adding another 175uL of 80% ethanol, leaving for 30s, and removing the supernatant.
- Spin down the samples, place back on the magnet and remove any residual ethanol. Ensure all ethanol is removed.

- Air dry the beads until they look dry, e.g. 30s to 1min.
- Resuspend the beads in 15uL of nuclease-free water by pipetting. Aliquot water to a strip tube, deliver by multichannel pipette, **make sure you resuspended all the beads**.
- Wait 3 mins while DNA is being released from the beads.
- Return samples to the magnet for 2 mins or until beads have pelleted and the solution is clear.
- Transfer 14uL of supernatant to a clean 96-well skirted plate.
- QC using Qubit DNA assay with 1uL of the purified DNA. A good concentration is >30 ng/uL or higher.
- Safe stopping point. Store at -20°C or 4°C.



DNA Clean-up Bead Example. The left well the beads are properly mixed. The middle well has droplets; the plate should be spin down. The right well needs more pipetting mixing.

DNA Quantification with Qubit

8 Bring all the reagents (an aliquot of the Qubit buffer and the Qubit standards) to room temperature.

Always keep the Qubit reagent protected from light.

- Prepare one Qubit tube per sample and two Qubit tubes for the standards and label the tube lids. Always use Qubit assay tubes (Q32856) for measurement.
- For each of the two standards aliquot 190uL of the Qubit reagent into labeled tubes.
- For sample tubes aliquot 199uL of the Qubit reagent per sample.
- The final volume in each tube has to be 200uL (after adding samples or standards).
- Add 10uL of each standard into the prepared tubes. Ensure to add 10uL or your readings will be incorrect!

- Add 1uL of your sample into each tube.
- Shortly vortex all tubes and incubate for 2 mins at room temperature (protected from light).
- Make sure you have no air bubbles within the reagent.
- After incubation measure the two standards (following the device instructions)
- Approximate standard values could be:

  Standard I : ~ 100 RFU

  Standard II: ~ 45000 RFU
- Measure samples and note the concentrations.
- QC using Qubit DNA assay with 1uL of the purified DNA. A good concentration is >30 ng/uL or higher.

Rapid Sample Barcoding

9 This is a **shortened version of the original rapid sequencing protocol**. It is strongly recommended to refer to the original protocol on the website for more detailed instructions, especially on flow cell checking and loading.
Note: be very careful when taking of the seal off the rapid barcode plate! You must avoid cross-contamination of the barcodes.
Take out all the reagents from the freezer, thaw at room temperature and store on ice until you need it.

10
- Transfer approximately 50-600 ng per sample into a new plate, in no more than 10uL total volume.
- **If possible, use equal volumes for all samples to avoid contamination**. Fill up to 10uL with nuclease-free water.
- Spin down the rapid barcodes plate and place on ice. Carefully take off the seal.
- Add 1uL of the rapid barcode to each sample. Use an multichannel pipette and add the barcodes column-wise, so that every sample has a different barcode. Double check each pipette tip to ensure barcodes are being added! **Very critical!**
- Mix thoroughly by pipetting and spin down briefly.
- Incubate the plate in thermal cycler:

	Step	Temp. (°C)	Time
	Prepare block	30	Forever
	Tagmentation	30	2 min
	Inactivation	80	2 min
	Hold	8	Forever

11 **Pool barcoded samples and purify the pool**

- Spin down the plate to collect the liquid at the bottom.
- Pool 5uL of each barcoded sample into one 1.5 ml Low Bind Tube:
- With a multichannel pipette first take 5ul of each sample (from each column of the plate) into an PCR strip tube.

- Finally transfer from the PCR strip tube into a clean 1.5 ml tube. **Remeasuring the total volume with an single channel pipette of your pool is strongly recommended** to add the right volume of AMPure XP beads in the next step.
- Store the remaining barcoded samples as a backup at 4°C.

12 **Post-pooling Clean Up**
Ensure your AMPure XP beads are at room temperature!
Vortex your AMPure XP beads for 30s or more to ensure they are in suspension before pipetting!
Prepare a fresh stock of 80% ethanol each time.

	# of samples	Vol. Ethanol	Vol. Water	Total vol.
	24	0.8 ml	0.2 ml	1 ml
	48	0.8 ml	0.2 ml	1 ml
	96	2.4 ml	0.6 ml	3 ml

Guidelines for preparation of 80% ethanol.

13
- Perform a 0.5X ratio AMPure XP bead clean-up.
- For a 150uL pool, add 75uL vortexed AMPure XP beads. **(check the volume of your pool with an single channel pipette, to add the right volume of AMPure XP beads).**
- Incubate 5 mins at RT.
- Prepare fresh 80% ethanol according to the table above.
- Place the pool on a magnet for 5 mins or until the solution is clear. Pipette off the supernatant.
- Keep the tube on the magnet and wash the beads by adding freshly prepared 80% ethanol until the **beads are completely covered with ethanol**, without disturbing the pellet. Usually 500uL of ethanol will be enough.
- Remove the supernatant.
- Repeat previous step.
- Briefly spin down the tube and place back on the magnet. Pipette off any residual ethanol. Allow to air dry for 1 min.
- Remove the tube from the magnet and resuspend the pellet in 15uL Elution Buffer or water. If you have more than 48 samples pooled, you can increase the elution volume e.g. to 30uL.
- Incubate for 8 min at RT.
- Pellet the beads for one minute on a magnet until the eluate is clear.
- Remove and retain 14uL of eluate into a new tube. For higher sample numbers >48 elution can be done in 30uL.
- Use 1uL to make a 1:10 dilution in water. Quantify 2uL of the 1:10 dilution with Qubit. The concentration of your undiluted pool should be >50 ng/uL.
- Transfer around 800ng of the pool into a new tube, in no more than 11uL. If necessary fill up to 11uL with EB.

14 **Rapid Adapter ligation**

Always use a freshly prepared Rapid Adapter dilution. Do not use the diluted Adapter over several days.

- Mix the Rapid Adapter (RA) by pipetting, and the Adapter Buffer (ADB) by vortexing.
- In a new 1.5 ml Eppendorf DNA LoBind tube, dilute the Rapid Adapter (RA) as follows and pipette mix:

	Reagent
	1 uL Rapid Adapter (RA)
	2.3 uL Adapter Buffer (ADB)

- Add 1uL of the diluted Rapid Adapter to the barcoded DNA.
- Mix gently by flicking the tube, and spin down.
- Incubate the reaction for 5 mins at RT.

Priming and loading the SpotON flow cell

15

- Prepare the priming mix by combining the following reagents in a fresh tube. Make 1170uL aliquots of the Flow Cell Flush when thawing it the first time. Take out one aliquot per sequencing run and add the Flow Cell Tether:

	Volume	Reagent
	1170 uL	Flow Cell Flush (FCF)
	30 uL	Flow Cell Tether (FCT)

- Mix by inverting the tube and pipette mix at RT.
- Open the priming port, set a 1000uL pipette to 200uL, scroll up until you can see a small volume of buffer entering the pipette tip. (To get rid of the air)
- Load 800uL of the priming mix into the flow cell via the priming port. **Avoid the introduction of air bubbles!**
- Wait for 5 minutes.
- During this time, prepare the library for loading:

	Volume	Reagent
	37.5 ul	Sequencing Buffer (SB)
	25.5 ul	Library Beads (LIB) mixed immediately before use
	12 ul	DNA Library (adapter ligated pool)

- Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
- Load 200uL of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.
- Add 75uL of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.
- Gently replace the SpotON sample port cover and close the priming port.
- Start the run.