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P. vivax microhaplotype deep sequencing assays

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Angela Rumaseb[1], Trent Peter[2], Ashley Osborne[1], Hidayat Trimarsanto[1], Mariana Kleinecke[1], David Hawkes[2], Sarah Auburn[1]

[1]Menzies School of Health Research; [2]Australian Genome Research Facility

Malaria_NGS

Angela Rumaseb
Menzies School of Health Research

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We use this protocol and it's working

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Abstract

This SOP is adapted from a high throughput rhAmpSeq library preparation protocol by integrated DNA technologies (IDT) and has been optimized at the Australian Genomic Research Facility. The SOP describes the laboratory procedures for amplifying a panel of 98 *Plasmodium vivax* markers, using high-throughput multiplex PCR, for downstream genotyping by sequencing on the Illumina platform. The 98 markers include a panel of 93 microhaplotype loci (loci comprising of 3 or more globally high diversity SNPs) distributed across the *P. vivax* genome, as well as one *Plasmodium* species confirmation marker and 4 putative drug resistance markers.
Further information on potential use cases for *P. vivax* microhaplotype genotyping, including recurrence classification, characterization of infection diversity, and spatiotemporal monitoring, can be found in Siegel et al [1].

Materials

	Item	Supplier	Size	Catalog#
	Reagents			
	IDTE, pH 7.5	IDT	10 × 2 mL	11-01-02-02
	IDTE pH 8.0	IDT	10 × 2 mL	11-01-02-05
	Nuclease-Free Water	IDT	10 × 2 mL	11-04-02-01
	rhAmpSeq Library Kit	IDT	16 rxn 100 rxn 500 rxn 5000 rxn	10000064 10000065 10000066 10000067
	rhAmpSeq *pv* custom PCR panel	IDT	0.4nmole (1.6mL)	Sales Order# 9413072 WO#3363760
	xGen 10nt UDI Index Primer Plates	IDT	Plate 1-4 Plate 1-8 Plate 1-16	10008052 10008053 10008054
	SPRIselect	Beckman Coulter	5 mL 60 mL 450 mL	B23317 B23318 B23319
	Ethanol (200 proof)			
	High Sensivity D1000 ScreenTape	Agilent	7 ScreenTapes	AG 5067-5584
	High Sensitivity D1000 Reagents	Agilent	112 reactions	AG 5067-5585
	Collibri™ Library Quantification Kit	Invitrogen		A38524500
	Consumables			

	Item	Supplier	Size	Catalog#
	DNA LoBind Tubes	Eppendorf	250 pcs (1.5 mL) 250 pcs (2 mL)	022431021 022431048
	Eppendorf twin.tec‰ microbiology PCR Plate 96, semi-skirted, 250 µL	Eppendorf	10 pcs	0030129326
	Axygen™ 8-Strip PCR Tubes, 0.2 mL	Thermo Fisher Scientific	125 pcs	14-222-251
	Microseal 'B' PCR Plate Sealing Film	Bio-Rad	100 pcs	MSB1001
	Agilent Loading Tips	Agilent	112 pcs	AG 5067-5598
	Agilent Strip caps	Agilent	120 pcs	AG 401425
	Agilent Optical tube strip	Agilent	120 pcs	AG 401428
	Equipment			
	DynaMag™2 Magnet or equivalent	Thermo Fisher Scientific		12321D
	Agilent 4150 TapeStation	Agilent		
	96-well thermocycler			
	Microcentrifuge			
	Vortex			
	Centrifuge with plate adapter			

Before start

The only safe stopping point in the procedure is **after** clean-up (Step 48).

Sample Preparation

1 Input DNA does not require prior quantification or normalization. Using a *cox1 P. vivax* qPCR assay (Gruenberg et al., 2018), input DNA with a CT value of <30 (roughly ≥100 parasites/µL) yielded a read depth of ≥100 reads/marker. If DNA amount is limited (11µL of DNA is required for this protocol), gDNA can be diluted using IDTE, pH 8.0.

Rebalanced Working Primer Preparation

2 Thaw an aliquot of Rebalanced rhAmpSeq PCR Panel (Forward and Reverse Stock Primer Pool from -20°C storage.

3 After thawing the primers, briefly vortex, then centrifuge.

4 Prepare working primer pools using a 1 in 5 dilution.
To sequence 96-samples, add 42µL of Forward/Reverse **Stock** Pool to 168µL of IDTE, pH 7.5.
Tip: Prepare multiple aliquots of rhAmpSeq forward/reverse primer pools and store at -20°C to avoid multiple freeze-thaw cycles.

Perform Targeted rhAmpSeq PCR_1

5 Thaw 4X rhAmpSeq Library Mix 1 and Rebalanced rhAmpSeq Working PCR Panel (Forward and Reverse **Working** Pool) completely to room temperature (15-20°C).

6 After thawing the reagents, briefly vortex and then centrifuge.

7 Prepare the following **PCR_1** master mix in a Lo-bind Eppendorf tube for the number of samples being processed:

	Component	Per reaction
	4X rhAmpSeq Library Mix 1	5µL

	Component	Per reaction
	rhAmpSeq pv custom PCR Panel – Forward Working Pool	2µL
	rhAmpSeq pv custom PCR Panel – Reverse Working Pool	9µL
	Total	**9µL**

Targeted rhAmp PCR_1 master mix

8 Briefly vortex and centrifuge down the mastermix.

9 In a new 96-well plate, add 9µL of PCR_1 master mix to each reaction well.

10 Add 11µL of gDNA to each reaction well.

11 Seal the plate, then briefly vortex and centrifuge.

12 Place the plate in a thermocycler and run the following program (Lid set to 105°C):

	Step	Cycle	Temperature	Duration
	Activate enzyme	1	95°C	10 min
	Amplify	14	95°C	15 sec
			61°C	8 min
	Deactivate enzyme	1	99.5°C	15 min
			4°C	Hold

Targeted rhAmp PCR_1 Program

13 Once the program is complete, remove the plate from the thermocycler and proceed immediately to **Dilute PCR_1 product**.

Dilute PCR_1 product

14 Briefly vortex the PCR_1 product, then centrifuge.

15 In a new 96-well plate, add 95µL of Nuclease-Free Water to each well.

16 Transfer 5µL of the PCR_1 product to each well (To prepare a final dilution of 1 in 20 dilution). Pipette up and down to ensure the entire volume is dispensed.

17 Seal the plate and thoroughly vortex to mix, then centrifuge before proceeding immediately to Perform Indexing PCR_2.

Perform Indexing PCR

18 Thaw 4X rhAmpSeq Library Mix 2 and xGen 10nt UDI Index Primer **Working** Plate completely to room temperature (15-20°C).

19 Briefly vortex, then centrifuge the reagents.

20 Prepare the following **PCR_2** master mix in a Lo-bind Eppendorf tube for the number of samples being processed:

	Component	Per reaction
	4x rhAmpSeq Library Mix 2	5µL
	Nuclease Free Water	2µL
	Total	**7µL**

21 In a new 96-well plate, add 7 µL of PCR_2 master mix to each reaction well.

22 Add 2 µL of xGen 10nt UDI Index Primer Working Concentration to each corresponding well using a multichannel pipette.

23 Add 11µL of diluted rhAmpSeq PCR_1 product to each reaction well in a 96-well plate using a multichannel pipette.

24 Important! Note down the index plate and well position used for each sample.

25 Seal the plate, then briefly vortex and centrifuge.

26 Place the plate in a thermocycler and run the following program (lid set to 105°C):

	Step	Cycle	Temperature	Duration
	Activate enzyme	1	95°C	3 min
	Amplify	24	95°C	15 sec
			60°C	30 sec
			72°C	30 sec
	Final extension	1	72°C	1 min
			4°C	Hold

Indexing PCR Cycling Condition

27 Once the program is complete, remove the plate from the thermocycler and proceed immediately to **Pool libraries**.

Pool libraries

28 Using a multichannel pipette, pool each row by transferring 5µL of each indexed library into an 8-well strip tube (use 10µL if the total number of individual libraries processed is less than 20 samples).

29 Cap the strip tube, briefly vortex, then centrifuge down.

30 Combine all libraries from the strip tube into a single 2mL LoBind tube.

31 Important! Pool the complete volume from each well for an even sample coverage.

32 Properly seal and store the plate with remaining individual indexed libraries at -20°C then proceed immediately to clean up the pooled libraries.

Clean up library

33 Prepare 2mL of fresh 80% ethanol solution by combining 400µL of molecular-grade water and 1,600µL of molecular-grade ethanol (Absolute ethanol).

34 Transfer 100µL of the rhAmpSeq library pool to a new 1.5mL tube.

35 Vortex the SPRI beads thoroughly before use.

36 Add 70µL of SPRI (0.7X) to the library pool.

37 Thoroughly mix the contents of the tube by pipetting up and down.

38 Incubate for 10 minutes at room temperature.

39 Briefly pulse spin down the tube then place it on a magnetic rack for 5 minutes, or until the solution is clear.

40 Keeping the tube on the magnet, do the following:

40.1 Aspirate with the pipette, then discard the supernatant.

40.2 Add 1000µL of 80% ethanol to the tube.

40.3 Incubate at room temperature for 30 seconds.

40.4 Aspirate, then discard the supernatant.

40.5 Repeat the 80% ethanol wash one more time (steps 40.1 - 40.4).

40.6 Using a new P200 pipette tip, remove the remaining ethanol from the tube.

40.7 Allow the beads to dry for 3 minutes at room temperature.

41 Remove the tube from the magnet.

42 Add 22µL of IDTE, pH 8.0, to elute the library pool.

43 Thoroughly vortex to fully resuspend the beads, then briefly pulse spin down the tube.

44 Incubate at room temperature for 3 minutes.

45 Place the tube on the magnet to collect the beads for 1 minute, or until the solution is clear.

46 While keeping the tube on the magnet, transfer 20µL of the final library pool elution into a new 1.5mL LoBind tube, ensuring no beads are carried over.

47 Proceed to quantify and check the size of the library.

48 Store any remaining library pool at -20°C for **up to 3 weeks**.

Library QC

49 Quantify the library concentration using Illumina NGS library quantification kit (such as The Invitrogen Collibri Library Quantification Kit)

50 Using capillary electrophoresis, check the fragment size of the library before and after beads clean-up to ensure that adapter dimers have been successfully removed.

Sequencing

51 Follow Illumina's recommended protocol for a 2 × 150bp paired-end sequencing run on your Illumina platform

Protocol references

[1] Siegel, S.V., Trimarsanto, H., Amato, R. *et al.* Lineage-informative microhaplotypes for recurrence classification and spatio-temporal surveillance of *Plasmodium vivax* malaria parasites. *Nat Commun* **15**, 6757 (2024). https://doi.org/10.1038/s41467-024-51015-3

[2] Gruenberg et al:
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