Apr 23, 2024

PCR based amplicon sequencing of P. vivax antigens

DOI
dx.doi.org/10.17504/protocols.io.261gedwddv47/v1

Paolo Bareng[1]

[1]Deakin University

Paolo Bareng
Deakin University

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.261gedwddv47/v1

Protocol Citation: Paolo Bareng 2024. PCR based amplicon sequencing of P. vivax antigens. **protocols.io** https://dx.doi.org/10.17504/protocols.io.261gedwddv47/v1

Protocol status: In development
We are still developing and optimizing this protocol

Created: October 05, 2023

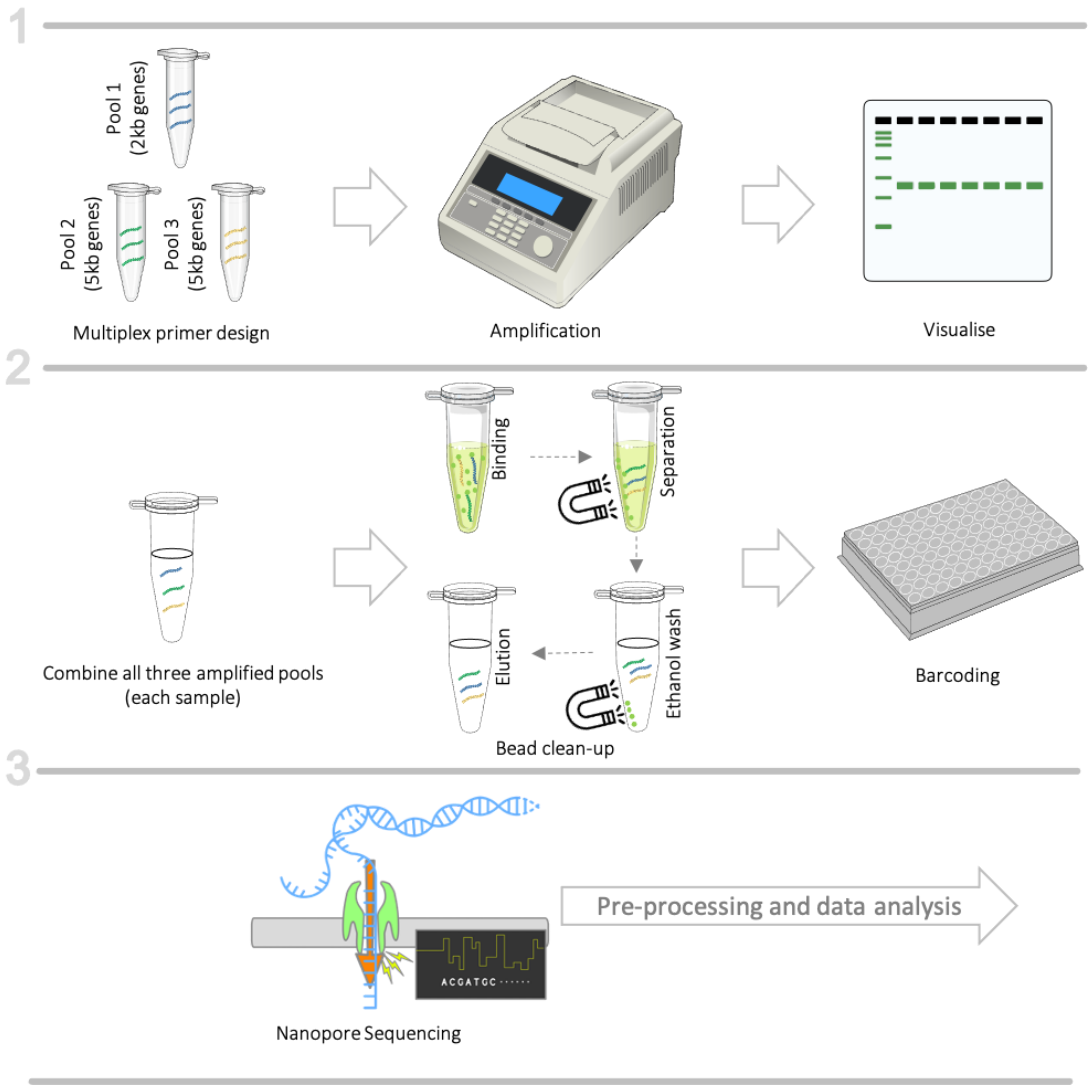
Last Modified: April 23, 2024

Protocol Integer ID: 88854

Disclaimer

PCR based amplicon sequencing of P. vivax antigens

Abstract


1
Pool 1 (2kb genes)
Pool 2 (5kb genes)
Pool 3 (5kb genes)
Multiplex primer design
Amplification
Visualise
2
Combine all three amplified pools (each sample)
Binding
Separation
Elution
Ethanol wash
Bead clean-up
Barcoding
3
ACGATGC
Nanopore Sequencing
Pre-processing and data analysis

Primer pool(s) preparation

1 Prepare the primer pools 1, 2, and 3 by reconstituting lyophilized primers to a concentration of 100µM using nuclease-free water

1.1 The tables below show the volume of each 100µM forward and reverse primers stock to be added in the respective pools. The total volume provided in the table is 100µL with a concentration of 5µM for each pool. However, it is recommended to make larger volume pool then split into aliquots.

Pool 1 genes	Size (bp)	Volume for each primer (µL)
AMA-1	1720	5
CSP	1712	5
CyRPA	1723	5
MSP8	1704	5
P41	1700	5
s12	1743	5
s28	1708	5
TRAMP	1747	5
TRAP	1731	5
Water	--	10
Total	--	100

Note

Genes in Pool 1 were grouped together according to their sequence length, ranging from approximately 700bp to 1600bp. To ensure uniformity in size, extra base pairs were incorporated around the target genes, resulting in ~1700bp in length. Primers were designed within these flanking regions.

Pool 2 genes	Size (bp)	Volume for each primer (µL)
DBP	4300	5
MSP9	4333	5
MSP1_1	4338	5
RBP1a_1	4318	5
RBP2a_1	4314	5

	Pool 2 genes	Size (bp)	Volume for each primer (µL)
	RBP2b_1	4357	5
	RON2_1	4350	5
	Water	--	30
	Total	--	100

Note

Genes like msp1, rbp1a, rbp2a, rbp2b, and ron2 have sequence lengths from 5kb to 8kb bps. These longer fragment genes were each split into two segments in order to standardize the size to be similar with DBP (~3.7kb) and MSP9 (~2.6kb). The split genes are now referred to as "gene name_1" for first gene segment and "gene name_2" for the second gene segment. Extra base pairs flanking the target region were added to reach ~4300 bp in total length. Primers were designed within these flanking regions.

	Pool 3 genes	Size (bp)	Volume for each primer (µL)
	MSP1_2	4381	5
	RBP1a_2	4387	5
	RBP2a_2	4326	5
	RBP2b_2	4303	5
	RON_2	4310	5
	Water	--	50
	Total	--	100

Note

Initially, Pools 2 and 3 were grouped together, however during our preliminary experiments, we did not observe any amplified products in these genes. We believe that the issue may be due to the overlap between the forward primer of the second gene segment and the reverse primer of the first gene segment, potentially causing interference during PCR amplification. As a result, we made a decision to separate the second gene segment into a different pool.

Multiplex PCR reaction

2 Combine PCR components as follows. The reaction is for 1x sample. Volume and concentration are the same for all three pools.

	PCR components	1x volume (µL)
	2x KAPA HotStart HiFi RM	6.25
	0.5µM primer pool (s)	1.5
	Nuclease-free water	3.75
	Template DNA (1-100ng)	1
	Total	12.5

3 Incubate the reaction into a thermal cycler following the conditions below. Pools 1 and Pools 2/3 have different PCR conditions

	Step	Temperature (C)	Time	Number of cycle
	Initial denaturation	95	3 mins.	1
	Denaturation	98	20 secs.	35
	Annealing	63	15 secs.	
	Extension	72	4 mins.	
	Final extension	72	5 mins.	1
	Hold	4	∞	1

PCR conditions for Pool 1

	Step	Temperature (C)	Time	Number of cycle
	Initial denaturation	95	3 mins.	1
	Denaturation	98	20 secs.	35
	Annealing	63	15 secs.	
	Extension	72	6 mins, 30 seconds	
	Final extension	72	10 mins.	1
	Hold	4	∞	1

PCR conditions for Pools 2 and 3

Note

The PCR conditions for pools 2 and 3 PCR conditions have been adjusted to accommodate the longer gene sequences in these pools hence, the longer extension times.

Note

Safe stopping point. Products can now be stored at -20 or -4C

4 Run 2-3 µL of the amplified product on a 1% agarose gel. Strong bands in the expected sizes indicate that the target genes have been amplified in the samples.

5 Transfer 5µL from each of the amplified pools into a new tube, then continue with the bead clean-up process.

Bead clean up

6 Resuspend AMPure beads by vortexing. Then, add one volume of AMPure beads to the combined PCR product; mix by gentle flicking.

7 Incubate for 5 mins at room temperature, either on rotator, or gently flick every ~1 min.

8 Spin down the sample and pellet on a magnet until the eluate is clear and colourless

9 Leaving the tube on the magnet, carefully pipette off and discard the supernatant

10 Keep the tube on magnet, and wash beads with 200 µl of freshly prepared 75% ethanol (without disturbing the pellet; leave ~15 secs). Remove the 75% ethanol using a pipette and discard.

11 Repeat ethanol wash (step 10)

12 After the second ethanol wash, spin down the sample briefly then put the sample back on the magnet. Pipette off residual wash. Allow to dry (~1-2 min)

13 Remove the tube from the magnetic rack and resuspend pellet in 13 µl nuclease-free water by gentle flicking. Incubate for 5-10 minutes at 37 °C (heat block).

14 Pellet beads on magnet until the eluate is clear and colourless.

15 Transfer eluate to fresh DNA LoBind tube. Retain 1 µl of DNA for quantification (yield should be >10ng/µL).

16 You can now discard the tube with beads.

Indexing

17 Combine PCR components as follows. The reaction is for 1x sample.

	PCR components	1x volume (µl)
	LongAmp mastermix	12.5
	PCR Barcode (Nanopore)	1
	Purified DNA	5-10
	Nuclease-free water	1.5-6.5
	Total	25

Note

Volume of nuclease-free water is adjusted according to the volume of DNA template added. DNA concentration should be 50ng to 90ng in total.

18 Incubate the reaction into a thermal cycler following the conditions below.

	Step	Temperature (C)	Time	Number of cycles
	Initial denaturation	95	3 mins.	1
	Denaturation	95	15 secs.	18
	Annealing	62	15 secs.	
	Extension	65	5 mins., 30 secs.	

	Step	Temperature (C)	Time	Number of cycles
	Final extension	72	10 mins.	1
	Hold	4	∞	1

Note

Safe stopping point. Products can now be stored at -20 or -4C

19 Bead clean up. Refer to Bead clean up step.

Pooling samples

20 Ensure that the concentration of each sample is equal when pooling multiple samples, targeting a total of 70-80 ng per sample. Calculate the necessary volume for each sample individually and then pool them together.

21 Total volume of pooled sample will be >100 µl, depending on the number of samples and volume added. Proceed to final bead clean-up before library sequencing preparation.

22 Refer to Bead clean up step.

Note

Instead of adding one volume of AMPure beads into the tube, include beads at a 0.4x ratio. This will ensure that shorter gene sequences (~>1kb) will be removed.

Note

Add 50 µl nuclease free-water instead of 13 µl for reconstituting the pellet.

23 Sample is now ready for library preparation and sequencing