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MAD4HatTeR V.1

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protocol .

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This protocol has been adapted from Paragon Genomics CleanPlex® NGS Panel

Andres Aranda-Diaz 2022. MAD4HatTeR. **protocols.io**
https://protocols.io/view/mad4hatter-b4pjqvkn

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Check expiration date of reagents when you receive them. Make sure to start with reagents that expire first.

- Molecular grade water
- Paragon Kit
- aliquot mag beads

- PCR workstation
-

mPCR

1

In a **PCR Workstation**, prepare mPCR reaction mix

Keep Master Mix and thawed primers on ice

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On ice Prepare a mPCR mix for your samples using the instructions below

Make fresh dilution of primers each time

Vortex reagents before using and spin down to collect liquids

4 µL per reaction :

- **2 µL 5X mPCR Master Mix (green tube)**
- **2 µL diluted mPCR primers (see dilution scheme below)** . You may dilute directly in the mPCR mix (e.g. for a 1:4 dilution, add **0.5 µL primer** and **1.5 µL water**)

Paragon Version	Condition (parasite dentisty [p/µL])	Primer dilution	Final primer concentration
V1 (PGD268)	C1 (≥100)	1 in 2	0.5 X
	C2 (<100)	1 in 4	0.25 X
V2 (PGD375)	C1 (≥100)	1 in 4	0.25 X
	C2 (<100)	1 in 2	0.5 X
V3 (current)	**C1 (≥100)**	**1 in 4**	**0.25 X**
	C2 (<100)	**NA**	**NA**

*: parasite density determined from extraction of DBS (6 mm circle into 150 µL H_2O)

Make at least 10% extra master mix to aliquot into wells. (e.g. for 10 samples, make 11 of the above)

Vortex, briefly spin and **On ice** aliquot **4 µL mPCR mix** into PCR tubes/wells (single tubes, strips or plate)
Step 1 includes a Step case.
Mixing pools
8x PV3C1-1AB
8x PV3C1-2
96x PV3C1-1AB
96x PV3C1-2

step case

Mixing pools

For runs with multiple pools:

1. mPCR-compatible primer pools are mixed in this step equivolume, with each primer at the final primer concentration above. E.g. **For 1A+1B in V3C1** (1 in 4 dilution) add **0.5 µL each primer pool** and **1 µL water** for the primer dilution
2. non-compatible primer pools (e.g. 1A/B and 2) cannot be combined here. 2 independent reactions need to be run for each sample

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Outside the PCR workstation, and ideally in a different area, add **6 µL DNA sample** to each labeled tube/well, independent of parasitemia.

Vortex and spin down before proceeding

10 µL final volume

3

Run PCR reaction on a thermocycler (ideally, in separate room)

- Initial denaturation: **95 °C** **00:10:00**
- Denaturation: **98 °C** **00:00:15** with ramping **3 °C per second**
- Annealing/Extension: **60 °C** **00:05:00** with ramping **2 °C per second**
- Repeat Denaturation and Annealing/Extension for **X total cycles** (see below).

Version	Condition	Total number of cycles
V1 (PGD268)	C1 (≥100)	10
	C2 (<100)	30
V2 (PGD375)	C1 (≥100)	20
	C2 (<100)	20
V3 (current)	**C1 (≥100)**	**10**
	C2 (<100)	**NA**

- Hold at **10 °C**

4 To prepare for following steps, after starting the thermocycler protocol you may want to:

- Bring CleanMag Magnetic Beads and STOP buffer to **Room temperature**
- Start making mixes (step **6**, step **9** and step **16**) for digestion and indexing PCR, as well as 70% ethanol

5

Proceed to next section within **00:30:00**

Post-mPCR bead purification

6

Spin the tubes/plate and add to each tube/well:

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1. **2 µL STOP buffer (red tube)**
2. **10 µL 1X TE**

Vortex briefly and spin down

OPTIONAL:
You may make a mix with 1X TE and STOP buffer (10 volumes 1X TE and 2 volumes STOP buffer) and add **12 µL 1X TE + STOP mix** of that mixture to each tube.

22 µL final volume

Step 6 includes a Step case.
Mixing pools

step case

Mixing pools

If you are mixing 2 mPCR reactions (e.g. mixing pools 1A/B and 2):
Spin the tubes/plate and add to each tube/well **2 µL STOP buffer**
Then transfer the total volume from one of the tubes to the corresponding sample

24 µL final volume

Note that you must adjust CleanMag Magnetic Beads volume in the next section (**31 µL beads**)

7 Incubate PCR products with beads:
CleanMag Magnetic Beads should be at **Room temperature** before adding to the mixture.
If using beads other than CleanMag Magnetic Beads you have to adjust ratios

7.1

Add **1.3 times** the volume of the mixture in CleanMag Magnetic Beads (**29 µL CleanMag Magnetic Beads**). Vortex vigorously to mix and incubate for **00:05:00** at **Room temperature**

Do not vortex after this step and treat mixture carefully.

51 µL final volume

3m

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7.2

Briefly spin down and place on magnetic stand for **00:03:00** or until the beads are collected on the side of the tubes/wells and the liquid is clear.

7.3

Remove all the liquid: first with a P200 pipet. Then briefly spin down and remove the liquid leftovers using a P20 pipet.

PROTIP: Place the tubes in the spinner with the beads so that they are on the outside, further away from the center or axis of rotation.

8

Wash with 70% ethanol (**use only freshly made 70% ethanol**)

8.1 2m

Add **180 µL** **70 % volume ethanol** ethanol

To wash the beads, rotate the tubes/plate so that the beads migrate from one wall to the other. Incubate for **00:02:00** or until all beads have migrated. You may need to **CAREFULLY** flick the tubes/wells.

8.2

Remove the liquid with a P200 pipet and repeat wash **go to step #8.1**

8.3

Spin down and remove all the remaining liquid using a P20 pipet

8.4

Leave tubes/wells open to dry **Room temperature**

Generally, a 5 min dry time is enough, but it will depend on room temperature and humidity. The beads should look matte (left in figure), not shiny (right in figure). **Under-drying (carrying ethanol) and over-drying (cracking) can lead to reduced yield**

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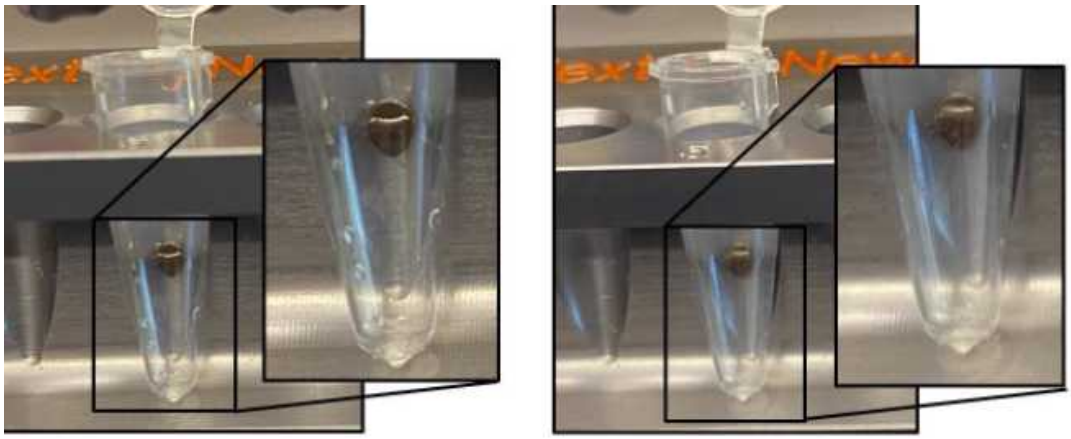

taken from https://www.protocols.io/view/nebnext-artic-sars-cov-2-fs-library-prep-kit-illum-bvrbn52n

8.5

Add **10 µL** 1X TE. Close the tubes/wells and vortex vigorously to resuspend the beads. The magnetic beads will not affect the rest of the reactions.

8.6

This is a safe stopping point
If you want to stop here, store -20 °C

Digestion Reaction

9

Make Digestion Reaction Master Mix
If possible, perform this step in a **PCR Workstation**

On ice

10 µL per reaction :

- **6 µL H2O**
- **2 µL CP Reagent Buffer ***
- **2 µL CP Digestion Reagent**

***Buffer sometimes has a white precipitate after thawing. Make sure that it is completely dissolved before using**

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Make at least 10% extra master mix to aliquot into wells. (e.g. for 10 samples, make 11 of the above)

If you want it is possible to make both the digestion reaction master mix and the Indexing PCR Master Mix (step **16**) at the same time. This is especially useful if the PCR workstation is not physically close to the post-pcr work area.

10 

Add **10 µL Digestion Reaction Mix** to each tube/well. Mix carefully by pipetting up and down at least 5 times or vortex vigorously for ~3 seconds. Avoid prolonged vortexing. Spin briefly to collect the liquid.

11 

10m

Incubate at **37 °C** for **00:10:00** , ideally in a thermocycler. Do not incubate for shorter or longer.

12 

Immediately add **2 µL STOP Buffer** to each tube/well and mix by spinning briefly then vortexing. Spin again briefly to collect the liquid.

22 µL final volume

13 

Proceed to post-digestion bead purification **immediately**.

Post-Digestion Purification

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Perform a 1.3X bead purification **go to step #7**

go to step #8 and continue to step 15 right after

15 

Proceed **immediately** to indexing PCR reaction

Indexing PCR reaction

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16 Make indexing PCR reaction mix in a **PCR Workstation**

16.1

On ice Prepare an indexing PCR mix for your samples using the below

26 µL per reaction :

- **8 µL 5X Second PCR Master Mix**
- **18 µL H2O**

Make at least 10% extra master mix to aliquot into tubes/wells. (e.g. for 10 samples, make 11 of the above)

16.2

Add to the **10 µL** left in the tubes/wells:

- **26 µL indexing PCR Mix**
- **2 µL forward indexing primer** and **2 µL reverse indexing primer** .
  OR **4 µL mixed indexing primers**

40 µL final volume

Spin down before proceeding

17 11m 30s

Run PCR reaction on a thermocycler

- Initial denaturation: **95 °C** **00:10:00**
- Denaturation: **98 °C** **00:00:15** with ramping **3 °C per second**
- Annealing/Extension: **60 °C** **00:01:15** with ramping **2 °C per second**
- Repeat Denaturation and Annealing/Extension for 15 total cycles
- Hold at **10 °C**

Post-indexing PCR purification

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Perform a **1X bead** purification by adding magnetic beads to the combined sample.

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go to step #7 but make sure to modify volumes **go to step #8**

1X ratio should be **40 µL CleanMag Magnetic Beads** into **40 µL indexing PCR**

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After adding **10 µL 1X TE** , vortexing and spinning the solution, the samples can be stored

-20 °C

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