Version 2

Sep 20, 2022

MAD4HatTeR V.2

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dx.doi.org/10.17504/protocols.io.14egn779mv5d/v2

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ABSTRACT

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

DOI

dx.doi.org/10.17504/protocols.io.14egn779mv5d/v2

PROTOCOL CITATION

Andres Aranda-Diaz, eric.neubauervickers 2022. MAD4HatTeR. **protocols.io** https://protocols.io/view/mad4hatter-b5gaq3se Version created by andres.arandadiaz

CREATED

Feb 22, 2022

LAST MODIFIED

Sep 20, 2022

PROTOCOL INTEGER ID

58594

MATERIALS TEXT

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

Aliquot STOP buffer

For protocol as is:
14 p10 tips
1 p20 tip
19 p200 tips

mPCR

1 

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

Keep Master Mix and thawed primers on ice

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

Aliquot the mix into PCR tubes in a strip so that you can use a multichannel pipette and use smaller volumes.

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**)

Citation: Andres Aranda-Diaz, eric.neubauervickers MAD4HatTeR https://dx.doi.org/10.17504/protocols.io.14egn779mv5d/v2

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
V3/4 (current)	**C1 (≥100)**	**1 in 4**	**0.25 X**
	C2 (<100)	**1 in 4**	**0.25 X**

*: 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
Mix for 8 samples with pools 1A+B, PV3C1
Mix for 8 samples with primer pool 2, PV3C1
Mix for 96 samples, pool 1A+B, PV3
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

2

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

Cover with clear seal for transport to building 100

Take off clear seal and apply heat seal before putting in thermocycler; red line up.

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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)**	**15**
	C2 (<100)	**20**

- 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**
- Bring index primers out of the freezer and thaw **On ice**
- Make mixes (step **6**, step **9** and step **16**) for digestion and indexing PCR
- Make 70 % ethanol **with nuclease-free water** (35 mL ethanol + 15 mL water if using 50 mL conicals)
- Make a plan for sample indexing. Write down what index you will use for each sample

- If using TE at 37 C, move plate or tubes to **37 °C**
- If using a 96-well pipettor, remove the tips corresponding to the empty wells (if any) on your plate. Save tips and put in used box for training purposes later.

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

OR, using the same tips: add **4 µL STOP buffer** to one of the tubes, change the volume in the pipet to 14 uL and transfer all of the volume to the other tube.

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** for 1 primer pool,
31 µL CleanMag Magnetic Beads for 2 pools). Vortex vigorously to mix and incubate for **00:05:00** at **Room temperature**

Do not vortex after this step and treat mixture carefully.

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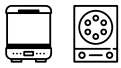
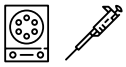
51 µL final volume

7.2 

3m

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 (set to 180). 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

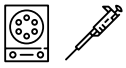
Add **180 µL** **[M]70 % volume ethanol** ethanol

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

You can remove the liquid using the same tips you put it in with

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 (this is only on the second wash, not the first one)

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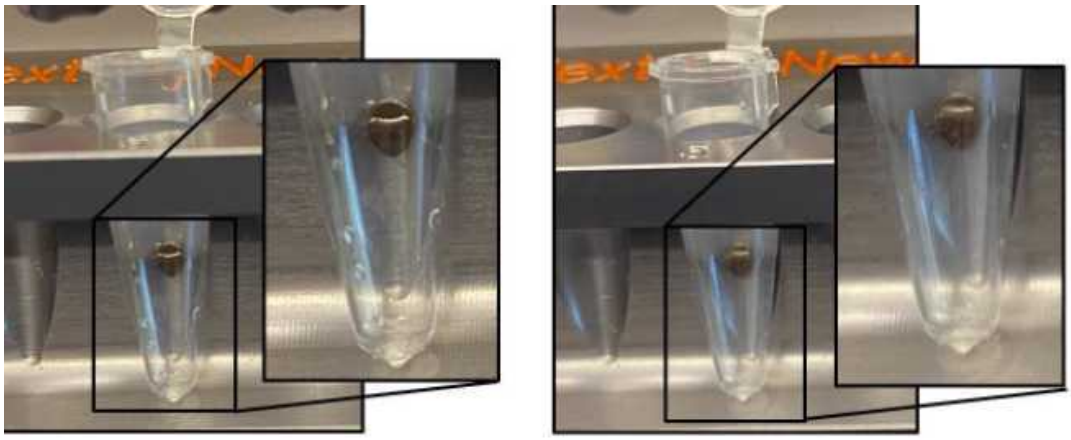
8.4

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 (right in figure), not shiny (left in figure). **Under-drying (carrying ethanol) and over-drying (cracking) can lead to reduced yield**



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

If you have only a few samples, you may want to keep an eye on each sample and close the tubes as they dry so that they all dry to the same extent.

8.5

Add **10 µL** 1X TE taken from the incubator. Close the tubes/wells and vortex vigorously to resuspend the beads. The magnetic beads will not affect the rest of the reactions. Quickly spin down. Return TE to the 37 C.

8.6

This is a safe stopping point

If you want to stop here, store -20 °C

Digestion Reaction

9

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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**

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.

Note: Use a 12 channel pipette with 1-channel reservoir when doing a full plate; there is not enough mix for the a 96-well reservoir

11

10m

Seal plate (do not use hot seal) and incubate at **37 °C** for **00:10:00** , ideally in a thermocycler. Do not incubate for shorter or longer.

Prepare tips for remainder of experiment during at this time

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

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13 ⚠

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

Post-Digestion Purification

14

Perform a 1.3X bead purification **and continue to step 15 right after**

15 ⚠

Proceed **immediately** to indexing PCR reaction

Indexing PCR reaction

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** (Make sure to centrifuge indexing primers before adding)
- Indexing primers MUST only contribute to one well per primer

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40 µL final volume

Spin down before proceeding

Heat seal

Note: If running a plate, use a reservoir to aliquot the mix.

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**

Pooling

18 Pool samples (make sure that indexes are compatible) by mixing them into a single tube.
We recommend skipping using the following volumes if using 15 cycles in mPCR:

- **30 µL for 1 p/µL**
- **20 µL for 10 p/µL**
- **15 µL for 100 p/µL**
- **6 µL for 1,000 p/µL**
- **3 µL for 10,000 p/µL**

19 Perform a **1X bead** purification by adding magnetic beads to the combined sample.
Follow same steps as above but make sure that the volume of beads is the same than the pool volume

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

Elute into **40 µL TE**

Gel purification

20 Run the eluted pool on a 2.5 % agarose gel
We recommend running for 1 h or more, at 140 V using TBE buffer

Use a ladder that allows identification of 200 and 400 bp bands

Cut the 400 bp band and extract using a gel purification kit

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Capillary electrophoresis and quantification

21 (to be filled)

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