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MAD4HatTeR V.4

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Andres Aranda-Diaz[1,2], eric.neubauervickers[1,2]

[1]University of California, San Francisco; [2]Chan Zuckerberg Biohub

EPPIcenter

Andres Aranda-Diaz
UCSF

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Protocol status: Working
We use this protocol and it's working

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Abstract

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

Information about the primer pools can be found **here**

Safety warnings

A version of this protocol made an SOP in pdf format, including a didactic version, can be found at eppicenter.ucsf.edu/resources. That version may be more up to date.

Before start

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mPCR

1 Prepare mPCR mix and

1.1 Thaw primer pools at Room temperature. Keep On ice after thawing

1.2 If expecting to run full protocol in one day, thaw CP Reagent Buffer (white tube)at Room temperature and keep On ice after thawing

1.3 Bring 5X mPCR Master Mix (green tube) into PCR Workstation in a cold rack

1.4 Combine the following volumes to prepare the mPCR reaction mix. Keep mix On ice or cold rack.
Vortex reagents to mix and briefly spin down before opening.

Generic recipe (without extra % for dead volumes):

	Reagent	Volume (µL)
	5X mPCR Master Mix (green tube)	2
	Each primer pool	0.5
	Nuclease-free H2O If you are using less than 6 µL of input DNA, increase water volume (e.g. if using 3 µL DNA, add water up to 7 µL)	Up to 4

Note: pools 1B and 2 are incompatible. Pools 3 and 4 are subsets of 1A and 5 is a subset of 1B. Incompatibilities also hold for subsets (5 and 2 are not compatible)
Pools are used at 0.25X for high parasitemia samples. When using more cycles (low parasitemia samples), halve primer concentration.

1.5 Vortex mixes, briefly spin and keep on ice

1.6 Aliquot 4 µL (or 7 µL if using 3 µL input DNA) mPCR mix into PCR tubes/wells (single tubes, strips or plate). Keep tubes On ice

1.7 Put primers and 5X mPCR Master Mix back in freezer

1.8 Transfer tubes outside of the PCR Workstation, to area where to add DNA

2 Add DNA

2.1 Add 6 µL DNA sample to each labeled tube/well, independent of parasitemia.

2.2 Close tubes or cover plate with seal. Vortex and spin down before proceeding

10 µL final volume

3 Run PCR reaction on a thermocycler (in a 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).

	Sample parasitemia	Total number of cycles
	≥100 p/µL	15
	<100 p/µL	20

- Hold at 10 °C

Reagent preparation

4 Prepare for next steps

4.1 Bring CleanMag Magnetic Beads and STOP buffer to Room temperature

4.2 Bring STOP buffer to room temperature and aliquot into PCR tube strip (~200 µL per tube) so that you can use a multichannel

4.3 Make 70% ethanol with nuclease-free water (you will need 800 µL per sample)

5 If you are not stopping at safe stopping point after first bead clean-up, also do this:

- 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.

5.1 Bring index primers out of the freezer and thaw On ice

5.2 Make a plan for sample indexing. Write down what index you will use for each sample

5.3 Make Digestion Mix (If you are splitting the protocol in 2 days, make this mix at the beginning of the second day):
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 for dead volume (e.g. for 10 samples, make 11 of the above)

5.4 Make Indexing PCR Mix (If you are splitting the protocol in 2 days, make this mix at the beginning of the second day):
Perform this step in a PCR Workstation

On ice
26 µL per reaction :

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

Make at least 10% extra for dead volume (e.g. for 10 samples, make 11 of the above)

5.5 If using warm TE, move plate or tubes to 37 °C

6 Proceed to next section within 00:30:00 of finishing mPCR

Post-mPCR bead purification

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

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

22 µL final volume

8 If you haven't, prepare [M] 70 % (v/v) ethanol with nuclease-free water

Make sure that CleanMag Magnetic Beads are at Room temperature and are well mixed

STEP CASE

Mixing pools 70 steps

If you are mixing 2 mPCR reactions (e.g. mixing pools 1A/B and 2):
Spin the tubes/plate and 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

9 Incubate PCR products with beads

9.1 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)

9.2 Vortex vigorously to mix and incubate for 00:05:00 at Room temperature 5m

After this step, and until resuspension in TE, do not vortex and treat mixture carefully.

9.3 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. 3m

9.4 Remove all the liquid with a pipet set to >60 µL

9.5 Briefly spin down and remove the liquid leftovers using pipet set to 10-20 µL

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. so that centrifuge force doesn't push them towards the opposite wall

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

10.1 Add 180 µL [M] 70 % volume ethanol ethanol 2m

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

10.2 Remove the liquid with a P200 pipet

10.3 Repeat wash: 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

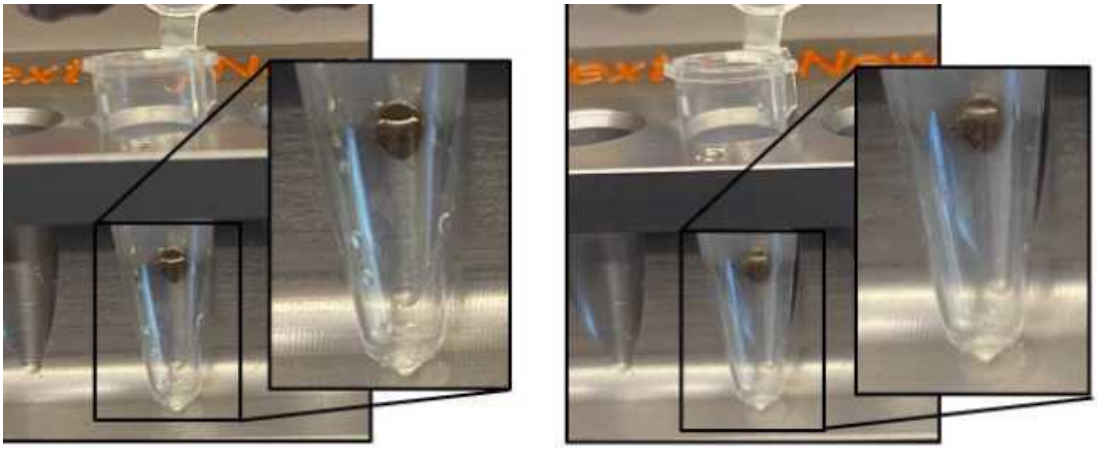
10.4 Remove the liquid with a P200 pipet

10.5 Briefly spin down and remove the liquid leftovers using pipet set to 10-20 µL

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. so that centrifuge force doesn't push them towards the opposite wall

10.6 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.

10.7 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, there is no need to remove them.
Quickly spin down.

10.8 Return TE to 37 °C

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

Digestion Reaction

11 **If you stopped in the previous step and left samples at -20 °C**

12 Bring thermocycler block to 37 °C

13 Perform Digestion reaction

13.1 Vortex Digestion reaction mix and briefly spin down to collect liquids

13.2 Add 10 µL Digestion Reaction Mix to each tube/well.

13.3 Close tubes or seal plate (do not use a hot seal). Vortex and quickly spin down to collect liquids

13.4 Incubate at 37 °C for 00:10:00 ,. 10m

13.5 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

Post-Digestion Purification

14 Perform a 1.3X bead purification
Proceed **immediately** to indexing PCR reaction

Indexing PCR reaction

15 Mindexing PCR

15.1 Vortex Indexing PCR mix and indexing primer plate and briefly spin down to collect liquids

15.2 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

40 µL final volume

15.3 Close tubes or seal plate
Vortex and briefly spin

16 Run PCR reaction on a thermocycler 11m 30s

- 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

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

Capillary electrophoresis check

18 Briefly spin down tubes and place on magnetic stand to separate beads, which cannot be loaded into capillary electrophoresis systems

19 Select a random subset (8-16 depending on experiment design) of samples to run on capillary electrophoresis. Include negative and positive controls when available

20 Follow the instructions corresponding to the system you are using

Pooling

21 Before starting make 70% ethanol with nuclease-free water and bring CleanMag Magnetic Beads to room temperature

22 Create a sample sheet and double check that indexes are compatible

23 Briefly spin down tubes and place on magnetic stand to separate beads.

Pool samples by mixing them into a single 1.5 mL microcentrifuge 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

If you have capillary electrophoresis data for each of the samples, pool with volumes inversely proportional to the concentration of the 300-500 bp region

24 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

24.1 Add **1X** the volume of the pool in CleanMag Magnetic Beads.

You may need to split in more than 1 tube if the total volume is > 1.5 mL

24.2 Vortex vigorously to mix and incubate for 00:05:00 at Room temperature 5m

After this step, and until resuspension in TE, do not vortex and treat mixture carefully.

24.3 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. 3m

24.4 Remove all the liquid

24.5 Briefly spin down and remove the liquid leftovers using pipet set to 10-20 µL

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. so that centrifuge force doesn't push them towards the opposite wall

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

24.7 Add 1 mL [M] 70 % volume ethanol ethanol 2m

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

24.8 Remove all the liquid

24.9 Repeat wash: 2m
Add 1 mL [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

24.10 Remove all the liquid

24.11 Briefly spin down and remove the liquid leftovers using pipet set to 10-20 µL

PROTIP: Place the tubes in the spinner with the beads so that they are on the outside, fur

24.12 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.

24.13 Add 43 µL 1X TE taken from the incubator. Close the tubes/wells and vortex vigorously to resuspend the beads.
If using multiple tubes, resuspend in one tube and use that resuspension to resuspend the rest of the tubes.

24.14 Quickly spin down and incubate at Room temperature for 00:02:00 2m

24.15 Place tube in magnetic stand and incubate at room temperature for 00:03:00 or until liquid is clear 3m

24.16 Transfer 40 µL to a clean, labeled tube

Capillary electrophoresis

25 Follow the instructions corresponding to the system you are using

26 If there is no 150-250 bp peak or it is <5% proceed to loading the pool into sequencer

Gel purification

27 Cast a 2.5% agarose gel in TBE buffer with 1X SYBRsafe

28 Place the gel in an electrophoresis system and fill up with TBE buffer

29 Load 5-10 µL DNA ladder to the first lane. Use a ladder that allows to distinguish 200 bp from 400 bp peaks.

30 Add 8 µL 6X loading buffer to the 40 µL pool tube. Vortex and spin to collect liquids

31 Load the pool into 1 or more lanes (depending on comb size you may not be able to fit in one lane). Leave an unused lane between the ladder and the pool

32 Run with constant voltage at 140 V for 1 h

33 After 1 h quickly image the gel. If there is a clear separation between primer dimers (~200 bp) and amplicons (~400 bp), you may continue to excise. Otherwise, run for longer

34 Once the 2 bands are well separated, excise the 400 bp band

35 Using a DNA gel extraction kit, dissolve the excised gel and run through a column following manufacturer's instructions

36 Elute with 15 µL elution buffer

Capillary electrophoresis check

37 Follow the instructions corresponding to the system you are using

38 If there is no 150-250 peak or it is <5% proceed to loading the pool into sequencer. Otherwise, you may need to run another gel extraction

Sequencing

39 You are ready to sequence on an Illumina platform!