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Modified Andolfatto- reduced rep sequencing

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1 Works for me dx.doi.org/10.17504/protocols.io.bbjbikin

Mimulus

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Before You Start

1 Before you start:
·To order enough restriction enzyme for all of your samples. Calculate the total amount needed for all the samples that you plan to do, and order the appropriate amount of restriction enzyme. Ben Blackman did an *in silico* digest of the *Mimulus* genome and determined that Csp6I (G^TAC, not methylation sensitive)is a good enzyme to use, less frequent cutter, less cutting in repetitive regions that are difficult to map reads to. Order Csp6I, Fisher ER0211, 1,500 Units, 10U/ µL, ~300 digestion reactions per tube (or ~3 plates worth of samples).
·T4 DNA ligase may also need to be ordered. It goes bad quickly, especially if it is not kept consistently frozen, so buy more to be safe. NEB M0202M, 2,000,000Units/mL, 100,000 units. (50 µL per tube, calculate the number of tubes you need, 2 plates = 40 µL T4 ligase vol.)
·Carefully extracted genomic DNA that is not too sheared or degraded, these samples should also be quantified (using the Pico Green protocol if you have several plates or the Qbit if you are just doing a few samples.
·Phusion PCR mix may need to be ordered, check to make sure there is enough. You'll need 25 µL per pool of samples.
·Check the amount of Agencourt AMPure PCR purification beads we have, order more if there isn't enough. You'll need ~225 µL of beads per pool of samples. (96 samples = 2 pools)
·You will need access to a UV table; a refrigerated centrifuge is also highly recommended

Anneal Adaptors

2 1. Prepare annealing buffer:
To a 50 mL conical tube add:
- 39.12 mL ultrapure water
- 400 uL 1M Tris pH 7.5
- 80 uL 0.5 EDTA
- 400 uL 5M NaCl

3 Pipette 95 uL of annealing buffer to 48 wells (half a plate or 8 strip tubes)

4 Pipette 5uL of FC 1-1 and 5uL of FC 1-2 into the first well; repeat for remaining barcodes (FC 2-1/2-2 through 48-1/48-2

5 Anneal in thermocycler - hold the reaction at 95°C for two minutes, then cool to 30°C by lowering the temp 1°C per minute, then hold at 4°C.

Dilute DNA

6 Dilute your genomic DNA samples to a standard concentration. 5ng/ µL is ideal for this protocol, but any concentration between 2 – 5ng/µL will work.

DNA restriction enzyme digest

7 Master Mix:

per sample:	Total (for 2 plates)
2.0 µL Buffer B (10X)	400 µL Buffer B (10X)
0.5 µL Csp6I (5U)	100 µL Csp6I (5U)
12.5 µL water	2500 µL water
15.0 µLTOTAL	3000 µLTOTAL

8 Transfer 15ul of the master mix into each well of a new 96-well PCR plate.

9 Pipette 5 µL (=10-25ng) of your diluted genomic DNA into each well of the new 96-well PCR plate that now has master mix in it.

10 Digest the DNA in a thermocycler for 3 hours at 37°C with a 20-minute incubation at 65°C at the end to inactivate the restriction enzyme

Ligate Bar-Coded Adapters

11 See *Annealing Adapters Section* to make adapters stock

Master Mix:

Amounts **per sample**	Master Mix for **2 plates**:
5.0 µL T4 DNA Ligase Buffer (10X)	1000 µL T4 DNA Ligase Buffer (10X)
0.2 µL NEB T4 DNA ligase, LC	40 µL NEB T4 DNA ligase, LC
1.0 µL Unique Barcoded Adapter pair (5 µM stock)	1.0 µL Unique Barcoded Adapter pair (5 µM stock)
23.8 µL water	4760 µL water
30.0 µLTOTAL	5800 µLTOTAL

12 Transfer 29 µL of the master mix into each well of the digested DNA plate

13 ·Add 1 µL of the unique adaptor pair into each digested DNA sample.

14 Ligate in a thermocycler for 3 hours at 16°C.

Isopropanol Precipitation and Pooling

15 This is the point in the protocol where you can pool sets of 48 samples, where each sample has a unique Barcoded Adapter pair.

Stop the ligation reaction by adding the following to each well:
1/10 volume (5.0 µL) of 3M Sodium Acetate, pH 5.2; **for 2 plates = 1,000 µL**
1 volume (50 µL) of isopropanol; **for 2 plates = 10, 000 µL**

16 Ligation reactions with unique Barcoded Adapters should be **pooled** into 15 mL Falcon tubes together here. We have 48 unique barcodes; each plate of samples could condense to 2 pools.

17 Incubate overnight at 4°C (this step should precipitate the DNA out of solution)

18 Centrifuge for 30 minutes at 4°C

19 As soon as the spin is done, carefully pour off the supernatant

20 Wash the sides of the Falcon tube with cold 70% Ethanol (stored at -20°C). Use enough Ethanol to fully submerge the DNA pellet.

21 Centrifuge for 5 minutes at 4°C at the same speed as before

22 As soon as the spin is done, carefully pour off the supernatant, remove as much of the Ethanol as possible, can use Kimwipes to dry the insides of the Falcon tube, as well as re-spin briefly and pipette the remaining Ethanol out.

23 Air dry the pellet. You must ensure that the pellet is completely dry (clear, not opaque!) before re-suspending.

24 Re-suspend the pellet in 100 µL TE pH 8 at 65°C for 30 minutes with intermittent vortexing. (The pellet will be difficult to re-suspend! May need to incubate at RT or 4C overnight, or use a pipette to disrupt the pellet)

25 Once the pellet is re-suspended, move the entire volume to a 1.5mL Eppie tube.

Bead Purify DNA using Agencourt AMPure PCR purification kit:

26 **Always** bring AMPure beads and your sample to room temperature before using (~45min).

Gently shake the AMPure bead bottle to re-suspend the beads (they settle out of solution quickly) make sure that no beads remain settled when you go to pipette them

27 Estimate the average volume of DNA and add 1.5 volumes of beads (~150 µL). Mix by pipetting up and down 10 times. Use a pipette to estimate the volume by pipetting the solution

28 Incubate at Room Temp for 5 minutes

29 Place the tubes in the magnetic rack for 10 minutes, keep the tubes in the magnetic rack until step 6 below. The roman numeral steps are to wash off anything not tightly bound to the beads/magnet

i. Aspirate and discard the supernatant
ii. Add 200 µL of 70% EtOH
iii. Incubate at RT for 1 minute
iv. Aspirate and discard the supernatant
v. Add another 200 µL of 70% EtOH
vi. Incubate at RT for 1 minute
vii. Aspirate and discard the supernatant. Remove as much ethanol as possible without disturbing the beads/pellet
viii. Leave the tube cap open and let the samples air dry

30 Take the tubes off the magnet, add 22 µL of TE to elute the barcoded DNA off the beads

31 Incubate at RT for 1 minute

32 Place tubes back on the magnet for 5 minutes

33 Transfer the supernatant (20 µL) to a new Eppie tube

Agarose Gel Size Selection

34 The beads do some size selection, but cutting a gel band is a much more precise size selection method, and a narrow fragment size distribution means increased coverage per sequenced site. You may not see your DNA band on the gel, but this does not mean there is no DNA there, cut the gel based on the ladder position anyway.

35 Add 6 µL loading dye to each sample.

36 Prepare a 2% agarose gel (the higher percent gel is better for separating the small fragments that you are looking for).

For a small gel rig- Mix 70 mL TAE buffer with 1.4 grams of Bio-Rad low range ultra-agarose + 7 µL Cyber Safe. Use a comb with thick/ wide teeth

37 Put the solidified gel into the small rig, pour TAE buffer into the rig, but do not submerge the gel. Stop pouring when the TAE is just above the bottom of the gel

38 Load the samples into the dry wells. Skip a lane between each sample. Into the skipped lanes load ladder (Fermentas GeneRuler 50bp ladder) ~20ul

39 Using a pipette top off each well with TAE running buffer, but do not exceed the capacity of the well. I.e. Do not let the buffer spill out of the well. If you do not add buffer to top off the wells the DNA bands will run distorted, and you will not get a clean cut.

40 Run the gel rig at 70V for 10 minutes to embed the DNA in the gel.

41 Pour enough TAE into the rig to fully submerge the gel

42 Continue to run the gel at 70V for about 2 hours. (check that you have good separation between ladder bands)

43 Pre-weigh empty 1.5 or 5mL Eppie tubes, one for each gel band. Write the weight on the side of the tube.

44 Using a UV light table to cut a band out of the gel that is between 250bp and 300bp, using the ladder as a guide. (Use a new blade for each sample, so you don't get cross contamination). Once the band is cut, place each band in its own labeled/weighed Eppie tube.

45 Follow the QIAquick Gel Extraction kit protocol to recover the DNA from the gel band. (each sample should go in a separate tube / column)

46 Quantify 2 µL of the column cleaned DNA on the Qubit.

Amplify the barcoded DNA fragments

47 Using the Phusion PCR kit, this will amplify the targeted fragments and add indices to each sample using index labeled primers.

Set up a PCR reaction using the following formula:

25.0 µLPhusion mix
2.5 µLFC1 (10 µM) INDEXED primer
2.5 µLFC2 (10 µM) common primer
?? µL 2ng of template DNA
?? µL Water to bring the reaction to a total of 50 µL

48 Run the PCR using the following program

i. 98°Cfor 30 sec
ii. 98°Cfor 10 sec
iii. 60°C for 15 sec
iv. 72°C for 15 sec
v. Repeat steps ii. to iv. For *n** cycles
vi. 72°C for 7 minutes
vii. 4°C hold

* the ideal cycle number may be different for each library. A good starting point is 14 cycles, and should not exceed 18 cycles. Therefore, it is recommended to optimize the number of cycles by trying 14, 16, and 18. The success of each PCR reaction can be estimated by cleaning the test-PCR reactions using the procedure in part 8 below, then Qubit quantifying, and then calculating the molecular (Molar) concentration of the library.

Bead purify the PCR products using the Agencourt AMPure beads

49 This part removes any leftover PCR reagents, enzymes, buffer salts, etc. The result from this step will be submitted for sequencing, so make sure to follow these steps carefully.

Gently shake the bottle of AMPure beads to re-suspend them, make sure that no beads remain settled at the bottom. Shake them up just prior to pipetting because they will settle out quickly.

50 Add 1.5 volumes of beads (~75 µL), mix by pipetting up and down 10 times

51 Incubate at RT for 5 minutes

52 Place the tubes in the magnetic rack for 10 minutes, keep the tubes in the magnetic rack until step 5 below. The roman numeral steps are to wash off anything not tightly bound to the beads/magnet, so the tubes must stay on the magnet until the washes are complete.

i. Aspirate and discard the supernatant
ii. Add 200 µL of 70% EtOH
iii. Incubate at RT for 1 minute
iv. Aspirate and discard the supernatant
v. Add another 200 µL of 70% EtOH
vi. Incubate at RT for 1 minute
vii. Aspirate and discard the supernatant. Remove as much ethanol as possible without disturbing the beads/pellet
viii. Leave the tube cap open and let the samples air dry for 5 minutes

53 Take the tubes off the magnet, add 22 µL of **Qiagen Buffer EB.**

54 Incubate at RT for 1 minute

55 Place the tubes back in the magnetic rack for 5 minutes

56 Transfer the supernatant (~20 µL) to a new labeled tube + quibit to quantify yield.