Restriction site Associated DNA Sequencing (RAD seq)

Library preparation modified from baddna.uge.edu

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GENOMIC DNA PURIFICATION

- If there is suspicion that the extracted DNA contains residues that may cause problems, it is recommended to get rid of any potential contamination (e.g., phenolic compounds, proteins) by either using magnetic beads (see below), a series of washing steps with ethanol (column based kit), or any other method available to ensure purity.

- Level of contamination can be estimated using a Nanodrop by observing the 260/280 ratio. A ratio from 1.8 to 2.0 can be interpreted as "pure". Less than those values may be indicative of the presence of proteins, phenol or other contaminants.
  (see https://www.nhm.ac.uk/content/dam/nhmwww/our-science/dpts-facilities-staff/Coreresearchlabs/nanodrop.pdf)

DNA clean-up with magnetic beads (CleanNGS)

The following protocol was modified from the following link:
http://www.bulldog-bio.com/GCBiotech/CleanNGS%20User%20Manual%20v3.01.pdf

1. Shake the CleanNGS reagent thoroughly too fully resuspend the magnetic particles prior to usage.
2. Add 1.5x to 1.8x the sample volume of CleanNGS reagent to each well. For Example: if the sample is 50 ul, you should add 75 µl (1.5x) to 90 µl (1.8x) of CleanNGS reagent to the sample.
3. Pipet up and down 5-10 times or vortex for 30 seconds.
4. Incubate at room temperature for 5 minutes.
5. Place the tube in the magnetic separation stand to magnetize the CleanNGS particles. Incubate at room temperature until the CleanNGS particles are completely cleared from solution.
6. Aspirate and discard the cleared supernatant. Do not disturb the CleanNGS particles.
7. Add 500 - 1000 µL 70% ethanol to each well.
8. Incubate at room temperature for 1 minute. It is not necessary to resuspend the CleanNGS particles.
9. Aspirate and discard the cleared supernatant. Do not disturb the CleanNGS particles.
10. Repeat Steps 7-9 for a total of three 70% ethanol wash step.

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11. Leave the tube in the magnetic separation stand for 10-15 minutes to air dry the CleanNGS particles. Remove any residue liquid with a pipet. Note: It is important to dry the CleanNGS particles before elution. Residual ethanol may interfere with downstream applications.
12. Remove the tube from the magnetic separation stand.
13. Add a minimum of 30 µL RNase-free water or Elution Buffer like TE (not provided) to each well.
14. Pipet up and down 20 times or vortex for 30 seconds.
15. Incubate at room temperature for 2-3 minutes.
16. Place the tube in the magnetic separation stand to magnetize the CleanNGS. Incubate at room temperature until the CleanNGS is completely cleared from solution.
17. Transfer the cleared supernatant containing purified DNA and/or RNA to a new (RNase-free) tube or microplate and seal with non-permeable sealing film.
18. Store the plate at 2-8°C if storage is only for a few days. For long-term storage, samples should be kept at -20°C.

DNA quantification with qubit fluorometer

Qubit fluorometer manual
https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017209_Qubit_4_Fluorometer_UG.pdf

- For the DNA quantification use a qubit fluorometer. It requires specific reagents depending the kind of molecule to be measured.

- Store your purified DNA at -20 for further use.

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LIBRARY PREPARATION (3RAD)

The following protocol was modified from:
https://baddna.uga.edu/protocols.html

High-Throughput 3RAD Protocol

1. Normalize all DNA samples to 20 ng/µL (quantified on Qubit), placing ≥ 20 µL in plates or strip tubes. Ensure these (and all other tubes/plates below) are labeled clearly. Protocol will work with much less DNA, but ≥ 5 ng/µL is best.

2. Set up digestion with the following per-sample recipe:
   1.5 µL NEB 10x CutSmart Buffer
   5.0 µL dH_2O
   0.5 µL XbaI (or another Read 1 enzyme)
   0.5 µL EcoRI-HF (or another Read 2 enzyme)
   0.5 µL NheI-HF (or another Read 1 adapter dimer-cutting enzyme)
   1.0 µL ds iTru NheI adapter (5 µM) (or another suitable adapter; see Table 2)
   1.0 µL ds iTru EcoRI adapter (5 µM) (or another suitable adapter; see Table 2)
   5.0 µL genomic DNA (20 ng/µL; if dilute, use a greater volume & reduce water)

3. Incubate samples at 37°C for 1 hr. in a thermal cycler. Proceed immediately to Step 4.

4. Add the ligation reagents. We recommend preparing a master mix shortly before the digestion is completed and adding the following per-reaction mix (i.e., 5 µL) to each tube:

   2.0 µL dH_2O
   1.5 µL ATP (10 mM); note: rATP, NOT dATP
   0.5 µL 10x Ligase Buffer (ensure components are in solution [warm it up!])
   1.0 µL DNA ligase [100 units/µL; make from 400 units/µL stock]

5. Incubate at 22°C for 20 min., 37°C for 10 min., 22°C for 20 min., 37°C for 10 min., 80°C for 20 min., then hold at 10°C.

6. If the multiple ligation products that you have at this step share adapter combinations and therefore pooling is not an option please continue to step 7).

   <u>Pooling ligation products that have unique adapter combinations (from Step 2)</u>
   a. Using the multi-channel pipette and changing tips each time, skloosh, then take 10 µL from each ligation and add it to a strip tube. Thus, when using a 96-well plate, each tube in the strip will have 120 µL. Seal the plate, label it well, and freeze it for potential use later.
   b. Pooling step 2: Into a single 1.5 mL tube, pool 60 µL from each tube of the strip from Step 6a. This should yield 480 µL of ligation product into the 1.5 mL tube. Label the strip tube well and freeze it for potential future use.

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c. Split the pooled ligation products into two 1.5 mL tubes (measured as 2 x 120 µL).

7. Clean and purify ligation products. Either:

   For individual ligation products

   To the 20 µL of ligation product add 30 µL of dH_2O, then add 75 µL of CleanNGS (i.e., 1.5x CleanNGS; see protocol above), purify as normal and resuspend in 20 µL of dH_2O.

OR

   From pooled ligation products:
   To each ligation pool of 240 µL then add 360 µL of CleanNGS (i.e. 1.5x CleanNGS). Purify as normal and resuspend each in 30 µL of dH_2O, then combine into one tube (total of 60 µL).

8. Set up PCR reactions. Either:

PCR recipe to add complete Illumina adapters & indexes in ligation product-pools (three per tube):

10.0 µL 5x Kapa HiFi Buffer (Kapa Biosystems, Wilmington, MA)
1.5 µL dNTP's (10 µM stock from Kapa kit)
7.5 µL dH_2O (to make final total volume 50µL)
1.0 µL Kapa HiFi DNA Polymerase (1 unit/µL from Kapa kit)
5.0 µL i5 Primer (5µM -> 0.5 µM final)
5.0 µL i7 Primer (5µM -> 0.5 µM final)
20.0 µL Linker ligated DNA fragments from step 7 (placed on magnet).

OR

PCR recipe to add complete Illumina adapters & indexes in individual cleaned-ligation products:

5.0 µL 5x Kapa HiFi Buffer
0.75 µL dNTP's (10 µM stock from Kapa kit)
8.75 µL dH_2O (to make final total volume 50µL)
0.5 µL Kapa HiFi DNA Polymerase (1 unit/µL from Kapa kit)
2.5 µL i5 Primer (5µM -> 0.5 µM final)
2.5 µL i7 Primer (5µM -> 0.5 µM final)
5 µL Linker ligated-cleaned DNA fragments from Step 7 (placed on magnet).

Cycling for both methods: 98°C for 1 min.; then, 12 cycles of: 98°C for 20 sec., 60°C for 15 sec., 72°C for 30 sec.; 72°C for 5 min. Hold at 15°C.

9. Purify with CleanNGS by either:

   To each of the pooled-ligations PCR reactions
   Add 50 µL of dH_2O, + 100 µL CleanNGS, skloosh; purify as normal, and resuspend in 20 µL dH_2O. Pool beads from all 3 PCR replicates (60 µL total), then place on magnet and pull off all liquid (~55 µL), leaving the beads behind.

   OR

   To individual PCR reactions
   Add 30 µL of dH_2O, + 60 µL CleanNGS, skloosh; purify as normal, and resuspend in 20 µL dH_2O, incubate few minutes at room temperature, then place on magnet and pull off all liquid (~18 µL), leaving the beads behind.

10. Run 5 µL on agarose gel to ensure each sample/pool worked.

11. Quantify with Qubit, normalize, pool (those that were not pooled after ligation), CleanNGS (1:1.5, dna:CleanNGS), and size select on Pippin (525 bp +/- 10%). [change size as necessary to avoid bright bands; we do *not* know that choosing a narrow size-range is best]

12. Quantify with Qubit, mix in appropriate proportions with other libraries, and send off to HiSeq, NextSeq or MiSeq: Use TruSeq primers & do dual (8nt) index reads!