**SOP:** BestRAD Illumina Library Prep (for dummies version of Miller/O’Rourke Protocol)

**Last Updated:** 12/6/18 Miller/O’Rourke, with added notes by LMK, then by SMB, then JAS (dec19)

**Name(s):\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

**Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

**Project/Sample Info:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

**General Overview/Notes**

-protocol starts with ~2-4 hours of work that has to be done at the end of the day

-day 2 should be started as close to 12 hours after day is finished (so around 8 am), day two takes 8-9 hours.

-library can be frozen after day 2, day 3 takes ~6 hours

-products can be frozen after day 3, part 4 takes ~3 hours

- Arrange with Ravi to use sonicator at genomics core around 10 am on day 2; day 2 must be a business day.

-It’s nice to prepare the DNA dilutions in advance. This usually takes two hours and a lot of concentration so it’s nice to do in advance, and do step 3-9 of day 1 (~2hrs) at the end of the day.

**Part 1: Digestion, RAD adaptor ligation, and shearing. ~4hrs**

1) Into each 96 plate well, pipet X ul genomic DNA[[1]](#footnote-1) (250 ng in <10 ul).

*X=Calculate how much volume of each sample to add to be 250ng total*

*Can use as little as 50 ng but 250 ng preferred to decrease number of PCR cycles down the line. Want same starting ng for each sample.*

2) Into each 96 plate well, pipet Y ul *(Y = 10 – X)* low TE (10 mM tris (pH 8.0), 0.1 mM EDTA (pH 8.0).

3) Prepare SbfI/PstI digestion master mix into 1.5ml tube(s).

*Notes:*

1. Make sure to add SbfI or PstI **LAST** (and minimize time enzyme spends out of -20C freezer)

2. After SbfI is added, go quickly to next step

3. Always keep SbfI cold and only bring out of freezer for very short periods.

4. To prevent evaporation, keep plate covered/in fridge until right before adding master mix

For one plate (100x): 68 ul water, 120 ul 10X NEBuffer 4, 12 ul SbfI-HF (NEB R3642L) **or** PstI (NEB R0140M)

\_\_\_X: \_\_\_ water, \_\_\_\_\_ NEB 4, \_\_\_\_ SbfI/PstI

4) To each 96 plate well, pipet **2 ul** SbfI/PstI digestion master mix and mix thoroughly.

1. Use a single channel to pipette the volume (+ 1 ul) of MM you need for each row into eight wells of a strip tube, and use a multi channel to add 2ul to each well of the plate (new tips each time). NOTE: use the same pipette for this that you used to make the mastermix to ensure consistency.

2. Pipette mix a few times when add

3. Seal plate (aluminum foil/heat sealer if available), vortex 30 seconds to mix, respin.

5) Incubate plate:

*“Digest” Protocol* = 37°C for 15 minutes followed by heat inactivation at 80°C for 20 minutes on thermocycler. Can store at 10°C (temporarily), or -20°C.

6) Prepare ligation master mix in 1.5ml tube.

*Notes:*

1. Mix all EXCEPT ligase in 1.5ml tube (again, always adding enzyme last)

2. Move to step 7 (Adding adaptors)

3. Then add ligase and move quickly to step 8, 9

For one plate (100x): 128 ul water, 40 ul 10x NEB4, 16 ul rATP (100 mM, Fermentas R0441), 16 ul Ligase (NEB M0202L)

\_\_\_X: \_\_\_ water, \_\_\_\_\_ NEB 4, \_\_\_\_ rATP, \_\_\_ Ligase

7) Add **2 ul** annealed BestRad SbfI/PstI adaptors (50 nM for SbfI digests or 1 uM for PstI digests).

*Notes:*

1. centrifuge before opening. Be VERY careful opening adaptor plate, hold down plate as remove foil lid and go slowly. Splashing, etc risks cross-contaminating adaptors between wells.

2. Use multi-channel to add adaptors to ensure consistency and avoid any mistakes/ cross-contamination.

-these plates are located in -80 freezer, check inventory for location. Use either 1uM working stock plate, or 50nM dilution plate.

8a) add the appropriate amount (\_\_\_ul) of ligase to the ligase MM, vortex and quick spin, keep on ice.

8b) Into each 96 plate well, pipet **2 ul** ligation master mix and mix thoroughly.

-Use a single channel to pipette the volume (+ 1 ul) of MM you need for each row into eight wells of a strip tube, and use a multi channel to add 2ul to each well of the plate (new tips each time)

- pipette mix a few times as add

- cover with aluminum/heat seal lid, vortex and respin.

9) Incubate samples on **thermocycler**:

*“Ligate” Protocol* = 20°C for 12 hours, heat inactivate at 65°C for 20 minutes, infinite hold at 4 degrees. *\*note, this is for less concentrated ligase*

\*Can freeze at -20 after this step if necessary.\*

*Notes:*

- There is an option to use a higher concentrated ligase in reaction above, which then requires just 1hr of incubation. However, it’s ~5x more expensive: <https://www.neb.com/products/m0202-t4-dna-ligase>

- The one listed here is the normal, lower concentrated one *(NEB M0202L)*; the higher one would be *NEB M0202M*.

**Part 2: Pooling and shearing ~ 8 hours**

Before Starting you want to make 80% ethanol and 1x NEB buffer

10) Into a 1.5 ml tube, pipet 3-8 ul each 96 plate well. Store plate for future multiplexing.

- Use a trough/multichannel to combine, then pipette into 1.5ml tube.

*Choosing volume here:*

-Can use 4ul at this step to preserve more DNA to reuse. If you’re going to renormalize (i.e., you want really balanced reads), use 3-4ul so there’s enough left to renormalize after first sequence run

- If not, then can add up to 8ul

11) Add an equal volume of Ampure XP beads *(Agencourt AMPure XP, A63882 Beckman Coulter)*, magnet, wash twice with 80% EtOH, elute in 210 ul low TE.

- add equal volume \_\_\_\_\_\_ul of ampure beads to pooled library: i.e., X ul sample added \*no. samples combined from plate

(e.g. if had 96 samples and added 8ul of each: 96\*8= 768ul to add; if had 96 samples and added 4ul of each=96\*4 =384ul to add)

- After adding Ampure beads, quick vortex, mix and spin, and then leave for 5 minutes (for DNA to bind to beads)

- Put DNA on magnet for 5 min and/or until solution is clear, remove supernatant

- Wash with 80% molecular grade EtOH (volume should be a bit more than original volume of DNA added), vortex, spin, place back on magnet, remove with p1000 or less if have smaller volume; can go quickly at first but then slower at bottom to make sure don’t get beads; check that sup is clear in pipette tip before discarding.

-repeat ethanol wash step, and when washing, after placing back on magnet, turn tube around a few times in magnet to help make sure all gets washed

- after 2nd wash, spin down and go back in with p10 to get out all of EtOH

- let air dry in hood for 10-15 min-until beads dry/cracked before re-suspending. Beads should still appear dark brown and glossy, not light brown (this means too dry). One crack is good, don’t want more than that, but also want all visible solution to be gone. Watch this very closely.

- Elute beads with 210ul low TE regardless of starting volume, mix well, incubate tube at RT for 5 min

- quick spin and place tube on magnet for 5 min and/or until solution clear

-transfer 206 ul of clear supernatant to an Eppendorf lo-bind tube to transport to the genomics core for sonication; discard beads. DO NOT DO MORE VOLUME THAN THIS. The sonicator is only supposed to have 100ul, so don’t want too much or sonication will not be efficient

12) Sonicate with BioRuptor at genomics core (in Morrill).

- Bring 206ul of product from previous step to core in a refrigerated cold block, bring P200 and tips

-Prep sonicator as instructed by Ravi/ core staff

-while sonicator chilling, quick spin 206 ul of product, and pipette 102ul into each of the two sonicator tubes provided by the core (0.2ml), and centrifuge to remove any bubbles

- sonicate for 4 cycles: 30 sec on, 90 sec off

- centrifuge bioruptor tubes (30sec) to remove bubbles, and run an additional 4 cycles

- centrifuge bioruptor tubes (30sec) to remove bubbles, and run 4 more cycles for a total of 12 cycles

-The cycle numbers may need to be adjusted, seems to be based on input DNA quality (e.g., if expect lower input quality, less sonication) e.g., Reid did 8 cycles with varied times, etc. with fin clips (that have been sitting around) vs. fresh fry that require more; Sean notes that is why they run on the fragment analyzer at this step to make sure is what correct size before moving forward because they noticed it was variable, even between the 2 replicate tubes from the same original tube. Having the two split tubes also hedges bets a bit to get what you want here. For libraries where almost all samples are very high quality, HMW, likely need 16 cycles total.

- clean-up sonicator as specified by Ravi/core staff

14) Run 2 ul on a fragment analyzer to assay shearing efficiency; re-shear if required.

-keep remaining sonicated DNA in fridge while FA running

-Looking for peak around 300-400 bp

-can also run gel here, looking for smear with a ‘peak’ about 300-400. Run a 2% agarose gel in 50ml volume, with two 100bp ladders, fresh TBE in rig, and at least 5ul of product with 1 ul orange dye.

**Part 3: RAD tag physical isolation.**

What this step is doing=Removing the DNA without adapters. The streptavidin binds biotin, which is in the adapter. Then

you’re cutting the adapter from the beads with sbfI and freeing the fragments.

Prepare Dynabead M-280 streptavidin magnetic beads

*-2X Binding and Wash (B+W) Buffer: (10 mM Tris-HCl (pH 8.0), 1 mM EDTA pH 8.0, 2 M NaCl).\*see calculations/recipe page*

1) Transfer 20 ul (40 ul for PstI libraries) Dynabeads (Invitrogen 11206D) to a new 1.7 ml tube.

20ul for each library (not per tube split above); e.g., if making 3 libraries, have 6 tubes total, but it’s going to be 20\*3=60ul beads

2) Place tube in magnetic rack for 2 min and discard supernatant.

- make 1X NEB Buffer 4 while waiting for magnet to clear

3) Wash the beads with 100 ul 2X B+W buffer, mix/vortex 30’’, quick spin, magnet 2 min; discard supernatant

- Add 100ul per 20ul beads (so 3 libraries, need 60ul beads and 300ul buffer total, split into the three 1.7ml tubes)

4) Repeat wash once.

5) Re-suspend beads in 200 ul 2X B+W buffer.

- Add 200ul per 20ul beads (so 3 libraries, would need 60ul beads and 600ul buffer total, split into your three 1.7ml tubes-one for each library)

-Using same volume as DNA that was sonicated here, so may need to adjust this volume if deviate from above.

Bead Binding

6) Add washed beads to ~200 ul sonicated DNA. Incubate at RT for 20 min, mix every 2 minutes.

- Add ~100ul of DNA from both of your bioruptor tubes to your 1.7ml tube containing the beads

- Mix is on for 1 min (1400 rpm), off for 1 min, repeat for 20 min; select RADMIX20 program on the **thermomixer**

7) Quick spin, place tube on magnetic rack for 2 min, discard supernatant.

-Place 400 ul of 1x B+W buffer in a 1.5ml tube in the thermomixer set at 56C while waiting for beads to clear on magnet.

8) wash beads in 150 ul room temp **1X** B+W buffer, pipette mix, quick spin, magnet 2min, discard supernatant

- beads can be static-y in solution; if this happens, ‘pipette washing’ can help-basically just pipetting solution up and down and solution should become clearer as beads migrate to magnet

- make sure using 1X B+W buffer here (dilute 2X 1:1 with water)

9) Repeat wash step two additional times with room temp 1x B+W buffer.

10) Repeat wash step 2 additional times with 56°C 150 ul **1X** B+W buffer from the thermomixer. Keep tube of buffer on thermomixer when not in use

Liberate DNA from beads:

10) Resuspend the final washed beads in 100 ul **1X** NEB Buffer 4. Place on magnetic rack 2 min, discard sup.

- Make up how much 1X NEB Buffer 4 you need for all the steps in 1.7ml tube-> e.g., 100ul 10X NEB Buffer 4 + 900ul water

-set thermomixer to 37C!

11) Repeat step 10.

12) Resuspend beads and bound DNA in 40 ul **1X** NEB Buffer 4.

13) Add 2 ul SbfI-HF (NEB R3642L).

- Vortex, quick spin and then flick mix to get beads back in solution

- This cuts off biotin and frees DNA from beads

14) Incubate tube at 37°C for 60 minutes in thermomixer.

15) Quick spin, place tube on magnetic rack 2 min, transfer (~40 ul) supernatant to new tube. **KEEP SUP! This has the DNA you want.**

16) Add an equal volume of Ampure XP beads (40 ul), magnet, wash twice with 150 ul 80% EtOH, resuspend in 52 ul low TE.

*Notes:*

- after adding ~40ul Ampure XP beads, vortex and incubate at RT for ~5 min

- then spin down and magnet for 5 min. discard supernatant

- add 150 ul 80% ethanol, washes don’t need to get beads all back in solution, don’t need to vortex too much, magnet 5 min, discard supernatant

- repeat ethanol wash, turn tubes a few times so beads move toward magnet as in previous steps

- after 2nd EtOH wash, quick spin and then go back in with P10 to get all the EtOH out

- place in hood to dry out completely, until cracks (but not too long)- 12 ish minutes. WATCH CLOSELY THROUGHOUT! Don’t want overcrack because won’t go into solution.

- Then add 52 ul of TE, flick mix and incubate at RT for ~5 min

- when resuspend in TE and mix, beads should go back into solution. If don’t, might have overdried-can heat up briefly (try 37C first, can raise a bit if needed)

17) Transfer 50ul of supernatant into plate wells for library prep

- place on magnet until clear and then **KEEP SUP**

- record library plate -> new well key here and/or create spreadsheet key if have many

-can also aliquot 5 ul if you elute in 57 ul to run on fragment analyzer to check progress.

**Part 3: Library preparation.**

- Use NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB E7370S/L) with no modifications[[2]](#footnote-2). Use 1:10 diluted adaptor and used ampure clean up instead of size-selection for SbfI libraries.

- Also see NEB library prep SOP

*Notes:*

- In this kit, elute in 22ul low TE. Then take 20% of product and run PCR for 15 cycles. Back calculate to figure out how many steps necessary for the other 80% of sample. Keep the 80% of sample

- Once start kit, don’t stop before reaching PCR step. Can pause/freeze sample before running PCR.

**Notes on making buffers & solutions:**

**2X Binding and Washing Buffer**

*(10 mM Tris-HCl (pH 8.0), 1 mM EDTA pH 8.0, 2 M NaCl)*

To make 50ml, Add: (adjust accordingly for different volumes)

500ul Tris-HCl (Bottle is 1M, so want to add 10ul per ml of final solution)

100ul EDTA (Bottle is 0.5M, so want to add 2ul per ml of final solution)

5.844g NaCl (F.W. = 58.44 g/mol -> x 2=116.88 for 2M in 1L -> for 100ml=11.69g, for 50ml=5.844g)

49.4ml Water (50ml-0.6ml from ingredients above; obviously use molecular grade water)

**1X Binding and Washing Buffer**

-Take volume of 2X made above and add equal parts molecular grade water (e.g., 20ml 2X B+W plus 20ml water)

**80% EtOH**

- Add 4 parts EtOH to 1 part water (e.g., 50ml=40ml EtOH + 10ml water)

- This needs to be made fresh…can use for ~1-2days but make new for day 3 of library prep

- Pour unused 80% EtOH into jar for general lab use (cleaning, sample collection, etc.)

1. Genomic DNA quantity should be identical in each plate well. We have used between 50-250 ng per individual. [↑](#footnote-ref-1)
2. We use ~20% of the template to perform a test PCR using 10 cycles and run the product on a fragment analyzer to determine yield. A second PCR is then performed with the remaining template using fewer cycles informed by the fragment analyzer results (typically 6-8, depending on DNA quantity, quality, and restriction enzyme choice). [↑](#footnote-ref-2)