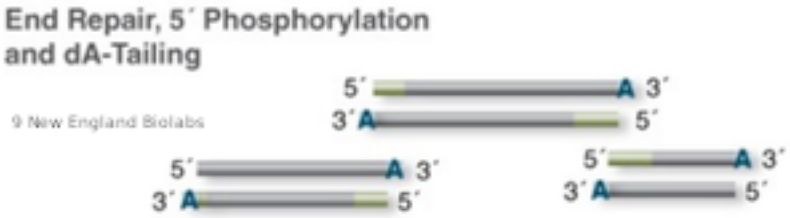
RADtag generation > RADtag isolation > **Illumina library prep**

We use the NEBNext Ultra DNA Library Prep Kit E7370 and Multiplex oligos from E7600.

A. end prep (green tubes)

This prepares the sheared ends of the RADtags for the NEBNext Adaptor to come, A-tailing the 3' ends and phosphorylating the 5'.



Setup

- move library to fridge to thaw
- move (green) End Prep Reaction Buffer to fridge if frozen solid
- move (red) NEBNextAdaptor for Illumina to fridge now if working with a full tube
  - aliquot this to reduce lead time

1. Transfer 55.5 ul of library with cleanup beads to a 0.5 ml PCR tube, add

- 6.5 ul (green) End Prep Reaction Buffer (Ultra kit)
- 3.0 ul of (green) End Prep Enzyme Mix (Ultra kit)

2. Aspirate 55 ul 10X, spin down. A few bubbles are OK.

3. 5-NEB-ENDPREP:

   - Lid 75C, TSP heated lid on, switch off lid at low block temp
   - Temp mode: safe
   - Volume: 65 ul

   1. 20C 30:00
   2. 65C 30:00
   3. 4C ∞

B. adaptor ligation (red tubes)

We ligate circular NEBNext Adaptors to the ends of dsDNA.

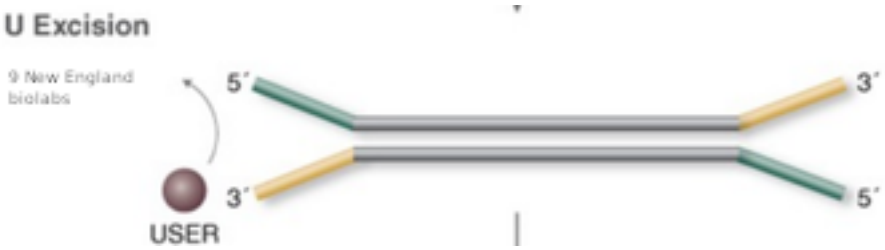
- Take cleanup beads out of fridge.
- Make up adaptor dilution buffer (10 mM Tris-HCl pH 7.5 with 10 mM NaCl). For 1.0 ml of stock: 980 ul H2O, 10 ul 1 M Tris-HCl pH 7.5, 10 ul 1 M NaCl. (equivalent to NEBNext Adaptor Dilution Buffer)

1. Spin end prep reaction down and add directly to the reaction:

- 15 ul (red) Blunt/TA Ligase Master Mix (Ultra). (viscous: take care in aliquoting)
- 1 ul of (red) Ligation Enhancer (Ultra)
- 2.5 ul (red) NEBNextAdaptor for Illumina (Multiplex) **diluted 1:10**
  - 2 ul adaptor, 18 ul adaptor dilution buffer.
  - Use immediately.

2. MM is viscous: mix well. Aspirate 80 ul 10X, spin down. A few bubbles are OK.
3. 6-NEB-LIGA:
   - **Lid off.** Can switch thermocyclers to avoid the hot lid from end prep.
   - temp mode: safe
   - volume: 83.5 ul
   1. 20C ∞
4. Go to the next step after 15 min. have elapsed.

USER enzyme opens up the hairpin loop of the adaptor.



5. Add 3 ul of (red) USER enzyme (Multiplex)
6. Aspirate 80 ul 10X, spin down. A few bubbles are OK.

7. 7-NEB-USER
    - Lid 47C, TSP heated lid on, switch off lid at low block temp
    - temp mode: safe
    - Volume 86.5 ul
    1. 37C 15:00
    2. 4C ∞

Stopping point, store at -20C overnight.

C. size selection of adaptor-ligated DNA

We utilize cleanup beads to restrict the distribution of fragment sizes to a targeted size range.
Large fragment removal *disposes of the largest fragments, preferentially bound to the beads.*

- Make 1.0 ml fresh 80% ethanol: 800 ul 100% EtOH, 200 ul H2O.
- Vortex beads, aliquot for the next 3 bead steps (125 ul + overage). Stage out of the light.
- Bring out (blue) NEBNext Q5 Hot Start HiFi PCR Master Mix to thaw at RT if frozen solid. Invert to mix, watching for precipitates. Put on ice.
- Could move a second library to fridge to thaw now.

1. Transfer ligation rxn to a 1.7 ml tube, place on magnet to separate out prior cleanup beads.
2. **Move eluate** to a new 1.7 ml tube, bringing volume up to 100 ul with H2O.
3. Add 55 ul new cleanup beads, aspirate 150 ul 10X. *Nominal 0.55X PEG:sample ratio*
4. Incubate at RT 5 min.
5. Place on mag rack for $\leq$ 5 min., aspirate beads away from bottom of tube.
6. **Keep eluate** and transfer to a 1.7 ml tube.

Small fragment removal *binds the mid-distribution fragment sizes to the beads.*
Smaller fragment sizes remain behind in solution to be discarded.

7. Add 25 ul (0.8X) of cleanup beads, aspirate 150 ul 10X.
8. Incubate at room temperature for **15** min. [Thaw PCR primers now.]
   Longer incubation gives the DNA more time to "find" the relatively few beads.
9. Move to mag rack for $\leq$ 5 min. separation.
10. **Discard sup.**
11. Add 195 ul 80% ethanol, leave in for 30 s. Remove and discard ethanol.
12. Repeat ethanol wash, remove trace ethanol.
13. Dry on rack with cap open $\leq$ 1.5 min.
14. Resuspend beads with 17 ul 0.1X TE by aspiration.
15. Incubate 5 min.
16. Place on magnet for $\leq$ 5 min.
17. **Transfer 15 ul eluate** to a 0.2 ml PCR tube.
18. Qubit remainder. RFU_____ ng/ul _____

    RFU_____ ng/ul _____

Stopping point, store at -20C overnight.

D. PCR Amplification (blue tubes)

NEBNext i5 and i7 primers incorporate the Illumina indexes that identify the library.

Setup: move primers to fridge. Primers for libraries blended in a lane must be color-balanced.

1. For a low-concentration library, use 2.5 ul of primers. For high, use 5.0. Add to the 15 ul

- 25 ul (blue) NEBNext Q5 Hot Start HiFi PCR MasterMix (Ultra)
- 2.5 | 5.0 ul i5 Index Primer (Multiplex): _____
- 2.5 | 5.0 ul i7 Index Primer (Multiplex): _____
- 5.0 | 0.0 ul H2O

2. Aspirate 40 ul 10X, quick spin.
3. 8-NEB-PCR
    - Lid 105C, TSP heated lid on, switch off lid at low block temp
    - Temp mode: fast
    - Volume 50 ul
    1. 98C, 0:30
    2. 98C, 0:10
    3. 65C 1:15
    - Total # of cycles for steps 2:3 9–12. Default is 12.
    4. 65C 5:00
    5. 4C ∞

Could start end prep on second library now.

E. bead cleanup of PCR reaction

1. Transfer PCR product to 1.7 ml tube, bring up to 50 ul with H20.
2. Transfer 45 ul (0.9X) resuspended cleanup beads, aspirate 75 ul 10X.
3. Incubate at RT 5 min.
4. Place on rack for $\leq$ 5 min., aspirate beads away from bottom of tube.
5. **Remove and discard sup.**
6. Add 195 ul 80% ethanol, incubate 30 s. Remove and discard ethanol.
7. Repeat ethanol wash.
8. Quick spin. Remove trace ethanol.
9. Dry on rack with cap open $\leq$ 2 min.
10. Resuspend beads with 33 ul Qiagen EB (10 mM Tris-HCl). Can include 0.05% Tween 20.
11. Incubate 5 min.
12. Place on rack for $\leq$ 5 min.
13. **Transfer 30 ul eluate** to 1.7 ml tube, refrigerate. At conclusion, store at -20C.
14. Qubit, Nanodrop remainder.
    RFU_____fluor _____ abs _____ 260/230 _____ 260/280 _____
    RFU_____fluor _____ abs _____ 260/230 _____ 260/280 _____
15. Dilute 1 ul sample for bioanalyzer HS-DNA to 500 pg/ul. End with ~75:25 EB:H2O.