

Project: \_\_\_\_\_ RADseqReady Plate # \_\_\_\_\_ Library # \_\_\_\_\_  
Name: \_\_\_\_\_ Date: \_\_\_\_\_

## **BestRAD Library Preparation for 96-samples**

**Based on protocol of Ali *et al.* 2015 (10.1534/genetics.115.183665)**

**Updated by BVH March 13, 2018**

**This is a 3-day protocol  
(Although it could be completed in 2 long days).  
There are designated stopping points. Please adhere to them!**

### **Section 1: DNA Standardization**

1. Standardize your high quality genomic DNA with 75ng in 15uL (ie. 5ng/uL) in a 96 well plate.
2. All DNA will also have needed to be run out on a gel to survey level (if any) of degradation and shearing. Aim to pool samples of similar quality together.

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## Section 2: Restriction Enzyme Digestion

### Materials Required

- Ice
- 10x Cutsmart Buffer
- Molecular Grade Water
- Sbf*I-HF Restriction Enzyme (NEB R3642L)
- 8-well PCR strips
- 1.5 mL tubes

1. Keep everything on ice as you prepare the master mix and DNA!
2. Prepare restriction enzyme (RE) digestion master mix (MM) as follows:

Item	Total Volume ( $\mu$ L) 96 samples (x110)
Cutsmart Buffer (or NEBBuffer 4)	187
Molecular Grade Water	19.8
<i>Sbf</i> I-HF enzyme (add last & keep cold)	13.2
<b>Total</b>	<b>220 <math>\mu</math>L</b>

3. Aliquot 27ul of the RE digestion MM to each well in the strip tube. If you are preparing two DNA plates, then aliquot 54ul to the strip tube.
4. Add 2 uL of RE digestion MM to each well. Pipette mix. Seal well and quick spin plate.
5. Incubate plate using "*Sbf*I digest" at:
  - a. 37°C for 60 minutes, then
  - b. 80°C for 20 minutes to heat kill RE.

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### Section 3: BestRAD Adapter Ligation

#### Materials Required

- Ice
- Annealed, indexed *SbfI* bestRAD adapters at 75nM
- Molecular grade water
- T4 DNA Ligase (NEB M0202M)
- T4 DNA Ligase Buffer with 10mM rATP (comes with T4 DNA Ligase)
- 1.5 mL tubes
- 8-well PCR strips

1. Keep everything on ice as you prepare the master mix and DNA!
2. Add 2uL of **annealed** indexed *SbfI*-*HF* BestRAD adapters at **75nM** to the digested DNA from Section 2. This is in a 96-well blue plate that contains 96 uniquely barcoded adapters.
3. Prepare adapter ligation (AL) master mix (MM) as follows:

Item	Total Volume (µL) 96 samples (x110)
Molecular Grade Water	140.8
10x T4 DNA Ligase Buffer*	61.6
T4 DNA Ligase	17.6
<b>Total</b>	<b>220</b>

with 10mM rATP

6. Keep adapter ligation (AL-MM) on ice. Aliquot 27ul of the AL-MM into PCR strips to facilitate multi-channel addition to plate. If you are preparing two DNA plates, then aliquot 54ul to the strip tube.
4. Add 2uL of AL-MM to each well and pipette up and down 2x to mix.
5. Seal and quick spin.
6. Incubate **with heated lid off** at:
  - a. 20°C for 60 minutes, then
  - b. 65°C for 15 minutes to inactivate ligase
  - c. Note: “*rad ligation*” program on Sirius/Orion in vonHoldt lab

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#### Section 4: Pool, Magnetic Bead Clean Up

##### *Materials Required*

- 80% ethanol freshly made from 96% Molecular Grade ethanol (ThermoFisher Scientific BP82021)
  - For total 5ml: 4.17mL of 96% ethanol, 833uL molecular grade water
- 8 well PCR strip tubes
- 1.5 mL microcentrifuge tubes
- Agencourt AMPure XP Beads (Beckman Coulter, A63881) **mixed and at room temperature for at least 1 hour**, and Magnetic Rack
- Low TE (TE<sub>0.1</sub>: 10mM Tris-HCl pH 7.5, 0.1 mM EDTA).

1. **Pool** 4uL from each uniquely indexed sample from columns 1-6, and pool separately columns 7-12 into 8 well PCR strips. Then transfer all volume from each strip tube into their respective 1.5mL tube for a total volume of 192uL per pool. This will give you 2 tubes per 96-samples, each tube consists of 48 uniquely barcoded samples. Freeze the rest in case you need to come back to it!
2. **Clean the DNA with 1:1.5 X Agencourt AMPure XP beads** (Beckman Coulter, A63881).
  - a. Add 288 uL Agencourt AMPure Beads to each tube. Gently mix by pipetting and quick spin.
  - b. Incubate the sample at room temperature for 10 minutes to bind DNA to the beads.
  - c. Place the tube on a magnetic rack (**LIDS CLOSED**) and wait until the liquid is clear to capture the beads (10 minutes).
  - d. With tube on rack, carefully open lids, remove and discard the supernatant (in hazardous waste because beads are stored in sodium azide).
  - e. Keep the tube on the rack and add 400-500 uL (cover beads) freshly made 80% ethanol to wash the beads.
  - f. Incubate at room temperature for 30 – 60 seconds.
  - g. Leave tube on rack; carefully remove ethanol & discard.
  - h. Repeat steps e – g again. Remove the residual ethanol with a small pipette tip. Do not disturb beads.
  - i. Dry the beads at room temperature for about 5 minutes. Do not overdry the beads.
  - j. Close lids and remove the tubes from the magnetic rack.
  - k. Add 130 uL Low TE. Mix by pipetting and quick spin.
  - l. Place on magnet for 10 minutes.
  - m. Remove supernatant and place in 1.5 mL tube. **KEEP SUPERNATANT!**

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## Section 5: Covaris Sonication to Selected Size Fragment

### *Materials Required*

- Glass Covaris tubes (available by Covaris LE220 in Princeton Core for use with PN500282 rack)
  1. Transfer over 130ul of DNA to glass tubes
  2. Place the glass tubes in column 1 of the 500282 metal plate
  3. Fill rest of column with blanks filled with water
  4. Screw top metal plate in place tightly.
  5. Test run Covaris by doing a “Load Plate” run with an empty plate loaded (no tubes in it).
  6. Sonicate samples for 400bp on Covaris LE 220 in Princeton Core Facility.
  7. Transfer 130uL back to 1.5 mL tube.

PIP(W)	DF(%)	CPB	Time(s)
450	30	200	60

**Covaris Program:** 20160621\_400bp\_103ul\_500282\_LYR\_300settings.e1proc

**Additional Optional Step:** You can check shearing efficiency using the BioAnalyzer on one of your samples at this point to ensure the correct sample size. Place 3ul in a 1.5mL tube and deposit in the Core Receiving Fridge. Shearing at 400 bp is important for downstream workflow.

# END OF DAY 1

**[Optional: If you want to do a 2-day protocol, continue on to Streptavidin beads, which is a 2-3 hours process]**

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## Section 6: Streptavidin Bead Binding Assay

### *Materials Required*

- Dynabeads M-280 Streptavidin (Invitrogen 11205D); room temperature and resuspended by mixing well (rotate on biorocker).
- 37°C thermomixer
- 56°C incubator
- 2X Binding and Wash Buffer (2X BW Buffer: 10mM Tris-HCl (pH 7.5), 1mM EDTA pH 8.0, 2M NaCl)
- 1X binding and wash buffer (1X BW Buffer: 5mM Tris-HCl (pH 7.5), 0.5mM EDTA pH 8.0, 1M NaCl) – you can dilute 2x BW Buffer 1:1 with Molecular Grade Water
- 1X NEB Buffer 4 (It comes as 10X – dilute 1:9 with Molecular Grade Water)
- Agencourt AMPure XP Beads (warmed and well mixed on biorocker)
- Freshly made 80% ethanol
  - For total 5ml: 4.17mL of 96% ethanol, 833uL molecular grade water
- Low TE (10mM tris-HCl pH 7.5, 0.1 mM EDTA)
- SbfI*-HF restriction enzyme

1. Check 2X and 1X BW buffers! For each tube processed, you will need:
  - a. 300ul **2X BW buffer**
  - b. 300ul **1X BW buffer**
  - c. 300ul **1X BW buffer** (warmed at 56°C in the incubator)
2. Wash Dynabeads
  - a. Transfer 30uL Dynabeads to new 1.5mL tube. One tube for each pool.
  - b. Add 70uL **2X BW buffer** and pipette mix for 5 seconds.
  - c. Quick spin, place on magnet, and let beads aggregate for 1 minute with lids closed.
  - d. Discard supernatant.
  - e. Wash beads with 100uL **2X BW buffer**, pipette mix for 5 seconds. Quick spin, place on magnet, and let beads aggregate for 1 minute with lids closed and discard supernatant.
3. Bind DNA to Dynabeads
  - a. Remove tube from magnet and re-suspend beads in 130uL **2X BW buffer**. Mix well by pipetting.
  - b. Add 130uL of sheared DNA from the previous step to beads. Pipette mix 10x.
  - c. Incubate at room temperature for 20 minutes, with gentle pipetting for 3 seconds every 5 minutes.
  - d. Quick spin (ensure the beads did not clump in bottom of tube), place tube on magnetic rack for 2 minutes. Discard supernatant, move off of magnet, and re-suspend beads in 150uL **1X BW buffer**. Pipette mix 10x.
  - e. Quick spin (ensure the beads did not clump in bottom of tube) and place on magnet. Let beads aggregate for 2 minutes.
  - f. Discard supernatant.

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- g. Re-suspend beads in 150uL **1X BW buffer**. Pipette mix 10x. Quick spin (ensure the beads did not clump in bottom of tube) and place on magnet. Let beads aggregate for 2 minutes. Discard supernatant.
  - h. Re-suspend beads in 150uL **1X BW buffer warmed at 56°C**. Pipette mix 10x. Quick spin (ensure the beads did not clump in bottom of tube) and place on magnet. Let beads aggregate for 2 minutes. Discard supernatant.
  - i. Re-suspend beads in 150uL **1X BW buffer warmed at 56°C**. Pipette mix 10x. Quick spin (ensure the beads did not clump in bottom of tube) and place on magnet. Let beads aggregate for 2 minutes. Discard supernatant.
4. Liberate DNA from Dynabeads
- a. Remove from magnet and re-suspend beads in 100uL of **1X NEB4 Buffer**
  - b. Place tube on magnet for 2 min
  - c. Discard supernatant.
  - d. Remove from magnet and re-suspend beads in 100uL of **1X NEB4 Buffer**. Place tube on magnet for 2 minutes. Discard supernatant.
  - e. Re-suspend beads in 40uL **1X NEBuffer 4**
  - f. Add 2uL of *SbfI*-HF enzyme to re-suspended beads. Pipette mix 10x.
  - g. Incubate at 37°C for 60 minutes in **thermomixer** with gentle pipetting after 30 minutes.
  - h. Quick spin, then place tube on magnetic rack for 5 minutes.
  - i. **TRANSFER supernatant** to a new 1.5 mL tube.
5. AMPure bead cleanup (1:1.5X)
- i. Add 60uL AMPure beads (well mixed and at room temperature) to each sample
  - ii. Gentle pipette mix/spin and incubate at room temperature for 10 minutes.
  - iii. Put on magnet for 5 minutes.
  - iv. Remove and discard supernatant.
  - v. Leave tube on magnet and add 200uL freshly made 80% ethanol.
  - vi. Incubate for 30 seconds.
  - vii. Remove and discard supernatant.
  - viii. Leave tube on magnet and add 200uL freshly made 80% ethanol. Incubate for 30 seconds. Remove and discard supernatant.
  - ix. Let air dry for 5 minutes. Remove all remaining ethanol!
  - x. Remove tubes from rack and add 57uL Low TE.
  - xi. Mix/spin. Let sit for 5 minutes.
  - xii. Put on rack for 5 minutes.
  - xiii. **TRANSFER supernatant** 50uL to a strip tube to prepare for thermocycler work that follows.

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6. Quantify 2uL of the supernatant with HS Qubit. Quantification is usually low (between 0.1 - 0.2 ng/uL).

Library ID	Concentration (ng/uL)

# END OF DAY 2

**Should you proceed, you need to complete the rest of the library prep (there is no good intermediate stopping point).**



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## Section 7: NEBNext Ultra II Library Prep

**Check the NEB website before beginning in case there have been any protocol updates:** <https://www.neb.com/protocols/2015/09/16/protocol-for-use-with-nebnext-ultra-ii-dna-library-prep-kit-for-illumina-and-with-sample-purification-beads-e7645-e7103>

A full manual can be found at: <https://www.neb.com/~ /media/Catalog/All-Products/9CBC60D62F5A4D3AB3970664E099CC73/Datacards%20or%20Manuals/manualE7645.pdf>

### Materials Required

- NEBNext Ultra II DNA Library Prep Kit (E7645S/L)
- PCR tubes
- AMPure XP beads at room temperature and well mixed
- Molecular Grade Water
- Freshly made 80% ethanol
  - o For total 5ml: 4.17mL of 96% ethanol, 833uL molecular grade water
- 1.5 mL tubes

### STEP 1: NEBNext End Prep

1. Add to each tube in the order listed (no master mix is needed):

Item	Volume ( $\mu\text{L}$ )
(green) NEBNext Ultra II End Prep Enzyme Mix	3
(green) NEBNext Ultra II End Prep Reaction Buffer	7
Fragmented DNA	50
<b>Total</b>	<b>60</b>

2. Pipette mix 10x. Quick spin and place on thermocycler program "*NEB\_NEXT\_END\_PREP*"
  - a. 30min at 20°C
  - b. 30min at 65°C
  - c. Hold at 4°C
3. Do not freeze; continue on to next step.

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**STEP 2: Adapter Ligation**

1. Add to each tube in the order listed (no master mix is needed):

Item	Volume (µL)
End Prep reaction mixture from Step 1	60
(red) NEBNext Ultra II Ligation Master Mix (pipette mix prior to adding to sample)	30
(red) NEBNext Ultra II Ligation Enhancer	1
(red) NEBNext Adaptor for Illumina (1uM)	2.5
<b>Total</b>	<b>93.5</b>

2. Pipette mix 10x. Quick spin and place on thermocycler program “NEB\_ADAPT\_LIG1”  
 a. 15min at 20°C with heated lid off
3. Add **3ul of USER Enzyme** to the ligation mixture. Pipette mix and place on thermocycler program “NEB\_ADAPT\_LIG2”  
 a. 15min at 37°C
4. **Side note:** Adapter can be diluted in 10mM Tris HCl by combining 10uL of 1M TrisHCl (ph 7.5) with 990uL molecular grade water. Adapter is at 15uM concentration so you may need to dilute to 1uM by mixing 2uL of 15uM adapter with 28uL of 10mM TrisHCl.

**STEP 3: Size Selection & Bead Cleanup**

1. This step is to select for fragments 300-400bp in size.

Approximate insert size	300-400bp
Approximate final library size (insert+adaptor+primers)	480bp

2. Add **25ul of resuspended beads** to the 96.5ul ligation reaction. Mix well by pipetting 10x.
3. Incubate on bench top for 5 minutes at room temperature.
4. Place the tube on the magnet and incubate for 5 minutes.
5. Carefully **TRANSFER SUPERNATANT** to a new 1.5mL tube. This contains your DNA. Discard beads.
6. Add **10ul of resuspended beads**. Mix well by pipetting 10x.
7. Incubate on bench top for 5 minutes at room temperature.
8. Place the tube on the magnet and incubate for 5 minutes.
9. While on the magnet, carefully **DISCARD THE SUPERNATANT**. Your DNA is bound on the beads. **KEEP BEADS**.
10. While on the magnet, add 200ul of freshly made 80% ethanol. Incubate for 30 seconds then careful remove/discard ethanol.
11. While on the magnet, add another 200ul of freshly made 80% ethanol. Incubate for 30 seconds then careful remove/discard ethanol.
12. While on the magnet, let air dry with lids open for a **maximum of 3 minutes**. Do not over dry!

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13. Remove from magnet and add 18ul of 10mM Tris-HCl or 0.1X TE to the beads.
14. Pipette mix 10x. Incubate for 5 minutes on bench top at room temperature.
15. Place on magnet to incubate for 5 minutes.
16. **Transfer 15ul to strip tubes for PCR amplification.**
17. Use 2ul to Qubit size selected DNA

Library ID	Concentration (ng/uL)

**STEP 4: PCR Amplification (F and R primers are not already combined)**

1. To each tube, add the following items in the order listed. Add all reagents directly to the strip tubes. No master mix is needed.

Item	Volume (µL)
Adapter ligated DNA fragments	15
(blue) NEBNext Ultra II Q5 Master Mix	25
(blue) Index Primer [Look at the sequence tags to ensure that however many indexes you use, that they have a minimum editing distance of 4 mismatches!]	5
(blue) Universal PCR Primer	5
<b>Total</b>	<b>50</b>

Library ID	Index used

2. Pipette mix 10x. Quick spin.
3. Place in thermocycler and perform PCR amplification using the program "*NEB\_UltraII\_PCR*"
  - a. [1 cycle: 98°C for 30sec];[12 cycles: 98°C for 10sec; 65°C for 75sec
  - b. 1 cycle: 65°C for 5min]; Hold at 4°C

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**STEP 5: Cleanup of PCR Amplification**

1. Add **45ul of resuspended beads** to the 96.5ul PCR reaction. Mix well by pipetting 10x.
2. Incubate on bench top for 5 minutes at room temperature.
3. Place the tube on the magnet and incubate for 5 minutes.
4. While on the magnet, carefully **DISCARD THE SUPERNATANT**. Your DNA is bound on the beads. **KEEP BEADS**.
5. While on the magnet, add 200ul of freshly made 80% ethanol. Incubate for 30 seconds then careful remove/discard ethanol.
6. While on the magnet, add another 200ul of freshly made 80% ethanol. Incubate for 30 seconds then careful remove/discard ethanol.
7. While on the magnet, let air dry with lids open for a **maximum of 5 minutes**. Do not over dry!
8. Remove from magnet and add **35uL 0.1x TE** to the beads.
9. Pipette mix 10x. Incubate for 5 minutes on bench top at room temperature.
10. Place on magnet to incubate for 5 minutes.
11. **Transfer 34ul to 1.5uL tubes. This is your final, complete sequencing library to use for preparation of sample submission to sequencing facility.**
12. Use 2ul to Qubit size selected DNA. Expect 5–10 ng/uL, but anything over 3ng/uL is OK. Keep remaining for sequencing.

Library ID	Concentration (ng/uL)

13. Reserve 3uL in another 1.5mL tube for the Bioanalyzer.

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## Section 8: Standardizing Libraries

### *Materials Required*

- Libraries from Section 7
- 0.1% Tween EB buffer (EB Buffer is Qiagen EB Buffer)
- Screw cap low volume tubes.
  1. The two tubes per 96-well plate of DNA now need to be combined into a single tube with equal representation from each half of the plate.
  2. Use the Qubit concentrations from the previous page.
  3. Into a newly labeled 1.5mL tube, add 50ng from each half plate (48 samples). We are combining the half plates to now have a pool of 96 samples. The final volume will be 20ul.
  4. Use  $C_1V_1=C_2V_2$  to adjust with 0.1% Tween EB for a final volume of 20ul and library concentration of 5ng/ul.

## Section 9: Sending for Sequencing at Princeton

See <https://htseq.princeton.edu> for details about sequencing.

1. We send our RADseq libraries to Lewis-Sigler Institute (LSI) at Princeton University. Request 5% PhiX spike in the RAD libraries to ensure maximal reads. On the Illumina Hi-Seq, request 2 lanes (1 flow cell) of sequencing for each library to get the expected 200 Million reads. This is approximately \$2690 per flowcell.
2. Label tube appropriately, and follow sample submission guidelines at on iLab Sequencing Core Service Request. Be sure to charge the correct chart string.
3. Print the sample submission form and place in Ziploc bag with library and deliver to the sequencing core freezer in the "Libraries for Sequencing" box in the mini freezer.
4. Data can be downloaded by logging in and following instructions at: <https://htseq.princeton.edu>.

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## REAGENTS LIST

### **Low TE (TE<sub>0.1</sub>: 10mM Tris-HCl pH 7.5, 0.1 mM EDTA)**

#### ***Materials:***

- Molecular Grade Water
- 1M Tris-HCl (pH 7.5)
- 0.5M EDTA (pH 8.0)

To make 100 mL of Low TE, remove 1.02 mL (1 mL plus 20 uL) from a 100 mL bottle of Molecular Grade Water. Then add 1 mL of 1M Tris-HCl (pH 7.5) and 20 uL 0.5M EDTA. Mix by inversion. It is also *probably* fine to just dilute regular 1X TE in a 1:9 ratio with molecular grade water. But then you end up with 1mM Tris-HCl and 0.1 mM EDTA. It is the EDTA concentration that is the most important here.

### **80% Ethanol**

#### ***Materials:***

- 96% Molecular Grade Ethanol (Fisher BP82021)
- Molecular Grade Water (Fisher BP2484100)
- 5mL tubes or 15 mL conical tubes

Make this fresh each day you are doing a bead clean up or size selection. Mix 4.167 mL of 96% ethanol with 0.833 mL of molecular grade water.

### **2X Binding and Wash Buffer**

Reagent	C1	V1 (uL)	C2 (mM)	V2 (uL)
1M Tris HCl pH 7.5	1000	1000	10	100000
0.5M EDTA pH 7.5	500	200	1	100000
5M NaCl	5000	40	2	100000
Molecular Grade Water		98760*		

\*Remove 1240uL from a 100mL bottle of molecular grade water, then add the other reagents to that bottle. Label and aliquot appropriately.

### **0.1% Tween EB**

#### ***Materials:***

- Tween 20 (Fisher BP337-100)
- EB Buffer (Qiagen 19086)

Tween is really viscous so it is difficult to pipette 1 uL accurately. So, first make a 10% solution with the 100-1000 uL pipette: Eg. mix 1 mL of Tween 20 with 9 mL Buffer EB. Then make a 0.1% Tween EB solution by combining 100 uL of 10% Tween EB with 9900 uL of EB Buffer. This gives you 0.1% Tween EB.