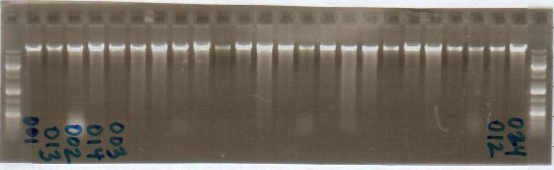
**RAD-SE Illumina Library Preparation for 48 Multiplexed Samples**

**Procedure**

**Part 1: Prepare DNA**

Use PicoGreen to quantify the amount of dsDNA and run DNA out on a check gel. Genomic DNA should consist of fairly tight high molecular weight bands without any visible degradation products or smears.

**Example:**



\*can stop and freeze here\*

**Part 2: Digestion**

1. Into each 96 plate well, pipet X μl of genomic DNA and Y μl of water so that your final concentration is equal to 100 ng of DNA in 20 μl volume.
2. Into 1.5 ml tube, pipet SbfI digestion master mix:

1X 53X

106X:

H2O 144.2

Buffer 254.4

SbfI 25.4

H2O 1.36 μl 72.10 μl

CutSmart Buffer 2.4 μl 127.20 μl

SbfI-HF (NEB R3642L) 0.24 μl 12.70 μl

\*Don’t vortex SbfI-HF

1. To each 96 plate well containing the DNA, pipet 4 μl of SbfI digestion mix. Pipet up and down to mix or place on a plate mixer (if available). Spin down plate. \*use fresh tube of SbfI for each prep, unless done on the same day\*
2. Incubate plate at 37° C for 60 minutes.

Thermocycler: TONY

RAD>RADigest

1. Incubate plate at 65° C for 20 minutes.

**Part 3: P1 Adapter Ligation**

1. Into each plate well, pipet 4 μl indexed Sbf1 P1 RAD adapter (10nM).
2. Make ligation master mix (make in 1.5 mL tube and then pour into tray):

1X 53X

106X:

H2O 271.4

Buffer 84.8

ATP 33.9

Ligase 33.9

H2O 2.56 μl 135.7 μl

CutSmart Buffer 0.8 μl 42.4 μl

rATP (100mM, Fermentas Ro441) 0.32 μl 17 μl

T4 DNA Ligase (NEB M0202M) 0.32 μl 17 μl

\*Use fresh T4 DNA Ligase and don’t vortex Ligase

1. Into each 96 plate well, pipet 4 μl ligation master mix.
2. Incubate plate at 20° C for 60 min.

Thermocycler: TONY

RAD>RADLig

1. Incubate plate at 65° C for 20 min.

\*can stop and freeze here\*

**Part 4: Clean up**

1. Pipet 10 μl from each of the 48 plate wells in trough, mix and then transfer into 1.5ml tube. Store plate for additional multiplexing in future.
2. Clean the sample using AMPure beads, making sure the beads are well mixed and at room temperature and ethanol is freshly prepared:
3. Add ~480 μl AMPure beads to the tube (1:1 ratio) and mix thoroughly. Volume of beads may vary depending on actual volume of DNA. Incubate on a rocker for 5 minutes at room temperature.
4. Quickly spin down the tube and place it on a magnetic stand for 5 minutes or until solution is clear.
5. Carefully remove and discard supernatant, making sure not to disturb the beads. \*\*keep beads\*\*
6. While the tube is still on the magnetic stand, add 800 μl freshly prepared 80% ethanol. Incubate at room temperature for 30 seconds, then carefully remove and discard the supernatant.

2X:

EtOH 1360 ul

H2O 340 ul

4X:

EtOH 2640 ul

H2O 660 ul

1. Repeat step d once.
2. Leave the cap of the tube open to air dry the beads at room temperature for up to 10 minutes. Do not over-dry.
3. Remove the tube from the magnetic stand and add 100 μl of LowTE. Mix thoroughly. Incubate on a rocker for 5 minutes at room temperature.
4. Quickly spin down the tube and place it on a magnetic stand for 5 minutes or until solution is clear.
5. Carefully transfer the supernatant to a new bioruptor tube, making sure not to disturb the beads. \*\*discard beads\*\*

\*can stop and freeze here\*

\*Turn on Bioruptor 1 hour before starting, wait until bottom unit cools down to 4° C.

**Part 5: Sonication**

1. Shear sample to an average size of 300 bp using BioRuptor NGS and specialized tubes. The goal is to create sheared product that is predominantly smaller than 1 kb in size.
   1. Make sure that the water level in sonicator is correct and set machine to 12 cycles of 30 seconds on and 90 seconds off, high power. This is a fairly standard starting point, but you can do more if needed. If you don’t sonicate well then when you go to size select you will lose a lot of your DNA. **This is a critical step!!**
   2. Run the sheared DNA on a check gel to see if the size is correct. Here is an example of what you should expect to see if things are working well:

\*Check gel:

>1% surface tension gel

Make ~40 ml

2 ul DNA + 1 ul dye

2 ul 100 bp ladder

Run gel for ~15 min @ 120 V

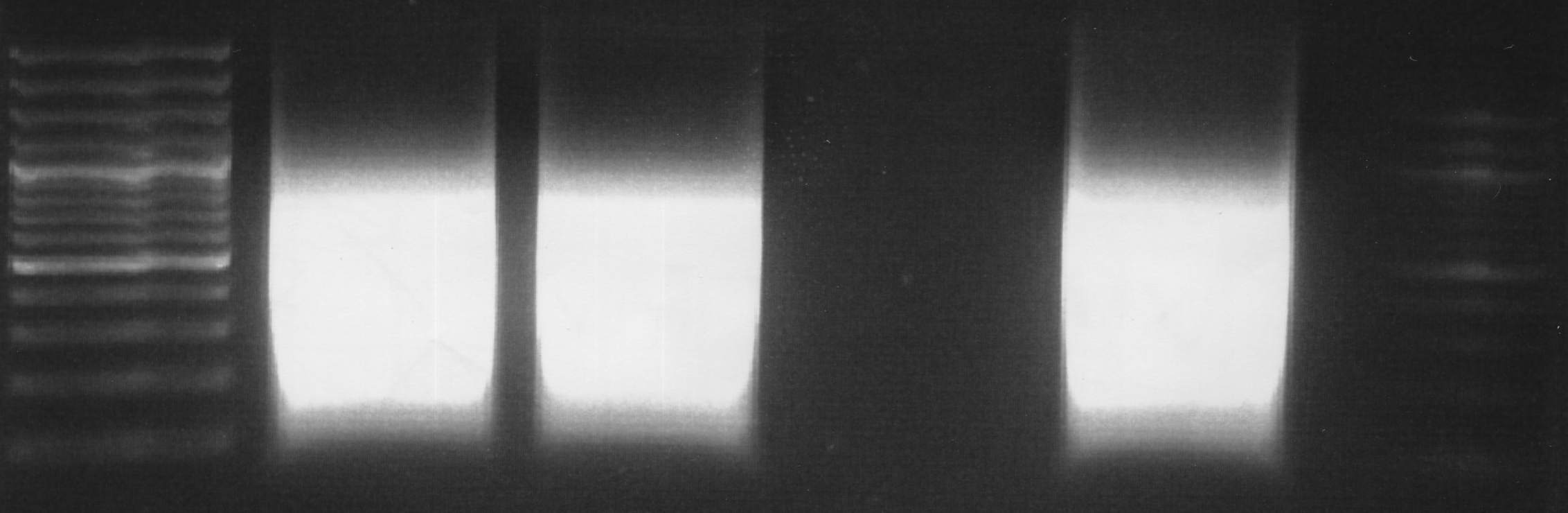


Fig. 2. Three barcoded and multiplexed RAD tag libraries. **2**, **3** & **5** each contain two DNA samples that were restriction digested, ligated to barcoded P1 adapters, combined, sheared, purified and then loaded on an agarose gel. **2** - *F0*parental DNA samples cut with *Sbf*I. **3** - *F2* pools cut with *Sbf*I. **4** - blank. **5** - *F0*parental DNA samples cut with *EcoR*I. Libraries contain 2 μg total combined genomic DNA each. **1** & **6** - 2.0 μl GeneRuler 100 bp DNA Ladder Plus.

1000 bp

500 bp

1 2 3 4 5 6

1. Use half of the sheared DNA (50 μl sample + 5.5 μl water or TE) for the next step. The next step – NEBNext End Prep – calls for 55.5 μl Fragmented DNA

\*can stop and freeze here\*

**The following steps use the NEBNext Ultra DNA Library Prep Kit for Illumina.**

**Part 6: Blunt End Repair**

1. Mix the following components in a sterile nuclease-free tube:

End Prep Enzyme Mix 3.0 μl

End Repair Reaction Buffer (10X) 6.5 μl

Fragmented DNA 55.5 μl

Total Volume 65 μl

1. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.
2. Place in a thermocycler, with the heated lid on, and run the following program:

30 minutes @ 20°C

Thermocycler: JOHN

RAD>NEBENDRP

30 minutes @ 65°C

Hold at 4°C

**Part 7: P2 Adapter Ligation**

This step ligates the P2 adapter onto the ends of blunt DNA fragments.

1. Add the following components directly to the End Prep reaction mixture and mix well:

Blunt/TA Ligase Master Mix 15 l

P2 RAD Adapter (5μM) 2.5 l

Ligation Enhancer 1.0 l

Total Volume 83.5 l

1. Mix by pipetting followed by a quick spin to collect all liquid from the slides of the tube.
2. Incubate at 20°C for 15 minutes in a thermal cycler.

Thermocycler: JOHN

RAD>NEBLigat

\*can stop and freeze here\*

**Part 8: Size Selection of Adapter-ligated DNA**

This step of the protocol removes free un-ligated or concatomerized P1 adapters and restricts the size range of tags to be sequenced. The target size range varies (from 300- 500bp (Etter) to 400 - 600bp (Miller) to 200- 700pb (for RAD-PE).



1. Vortex AMPure XP beads to re-suspend.
2. Add 16.5 l dH20 to the ligation reaction for a 100 l volume.

\*Transfer to 1.5 ml tube

1. **Remove large fragments**: Add 55 l of re-suspended AMPure XP beads (amount will vary depending on size selection range) to the 100l ligation reaction. Mix well by pipetting up and down at least 10 times.
2. Incubate for 5 minutes at room temperature.
3. Quickly spin the tube and place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully transfer the supernatant containing your DNA to a new tube (**Caution: do not discard the supernatant!**). Discard the beads that contain the unwanted large fragments.
4. **Remove small fragments:** Add 25 l re-suspended AMPure XP beads (amount will vary depending on size selection range) to the supernatant, mix well and incubate for 5 minutes at room temperature.
5. Quickly spin the tube and place it on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA targets (**Caution: do not discard the beads!**)
6. Add 200 l of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

3X:

EtOH 560 ul

H2O 140 ul

6X:

EtOH 1040 ul

H2O 260 ul

1. Repeat Step 8 2x for a total of three washes.
2. Air dry the beads for 10 minutes while the tube is on the magnetic stand with the lid open.
3. Elute the DNA target from the beads into 20 l of 10 mM Tris-HCL or LowTE. Mix well on a vortex mixer or by pipetting up and down. Incubate for 5 minutes at room temperature. Quickly spin the tube and place it on a magnetic stand. After the solution is clear (about 5 minutes), transfer supernatant to new tube. Transfer 5 l to a new PCR tube for amplification.

\*can stop and freeze here\*

**Part 9: PCR Amplification**

1. Mix the following components in sterile strip tubes:

\*If 1st PCR doesn’t work, use 2X template:

Fragments 10 ul

MM 25 ul

H2O 13 ul

P1 Primer 1 ul

P2 Primer 1 ul

Adapter Ligated DNA Fragments 5 l

NEBNext High Fidelity 2X PCR Master Mix 25 l

H2O 18 l

P1 Adapter Primer (25 M) 1 l

P2 Adapter Primer (25 M) 1 l

Total Volume 50 l

1. PCR cycling conditions:

Thermocycler: JOHN

RAD>NEBPCR

*\*\* This protocol recommends 15 cycles, more cycles may skew the representation of the library.*

1. **Recommended:** Run a test gel with 1 ul RAD library template (the size selected DNA before amplification) and 5 ul PCR product on a 1% gel to determine the quality of the library relative to the unamplified fragments (as in Figure 3). If the amplified version looks twice as bright as the unamplified version, then proceed to clean up. If the libraries look poor, then increase the amount of template and try to amplify again. \*If the library looks less bright than the template, you should reprep the library from the plate and restart at step 2\*

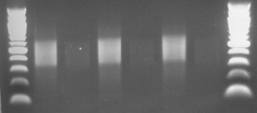


Fig. 3. Test amplification PCR product from the three libraries shown in **Fig. 2**. **2**, **4** & **6** contain 5.0 μl amplified PCR product. **2** - *F0**Sbf*I library. **4** - *F2* *Sbf*I library. **6** - *F0* *EcoR*I library. **3**, **5** & **7** contain 1.0 μl template used for amplification in the lane to the left. Template was loaded at 5X the amount used in the equivalent volume loaded for amplified reactions. **1** & **8** - 2.0 μl GeneRuler 100 bp DNA Ladder Plus. Libraries are 3-600 bp in size.

1 2 3 4 5 6 7 8

\*Test gel:

Pour thinner gel and use smallest combs

1 ul Template + 1 ul dye

5 ul PCR product + 1 ul dye

Run gel for ~15 min @ 120 V

\*can stop and freeze here\*

**Part 10: Cleanup of PCR Amplification (Post PCR bench)**

1. Vortex AMPure XP beads to resuspend.
2. Add 50 l of resuspended AMPure XP beads to the PCR reactions (~50 l). Mix well by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube and place it on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets (**Caution do not discard beads!)**
5. Add 200 l of fresh 80% ethanol to the PCR plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

EtOH 400 ul

H2O 100 ul

1. Repeat Step 5 once.
2. Air dry the beads for 10 minutes while the PCR plate is on the magnetic stand with the lid open.
3. Elute DNA target from beads into 30 l 10 mM Tris-HCL, pH 8.0 or 0.1X TE. Mix well by pipetting up and down at least 10 times. Quickly spin the tube and place it on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully transfer 28 l pr more of supernatant to a new PCR tube. Store libraries at – 20°C.

A strong library should look something like:

\*For cleaner library, repeat clean-up for a total of 2 cleanups. Just make sure that you mix DNA with equal amount of beads—1:1 ratio.

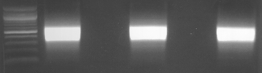


Fig. 4. PCR product from the three libraries shown in **Figs. 2 & 3** after the final large volume amplification and purification. **2**, **4** & **6** each contain 20 μl purified PCR product from 100 μl amplifications. **2** - *F0**Sbf*I library. **4** - *F2* *Sbf*I library. **6** - *F0* *EcoR*I library. **1** - 2.0 μl GeneRuler 100 bp DNA Ladder Plus. **3**, **5** & **7** are blank. Libraries are 3-600 bp in size.

1 2 3 4 5 6

Quantify library concentration using PicoGreen or Qubit. Take 1 l, dilute to <3 ng/l with water and run on Agilent BioAnalyzer DNA High Sensitivity chip to check the size distribution.

Dilute library to required concentration and volume (for Berkeley: 10 nM in 10 l) for sequencing.

**Materials and Supplies used by the Miller lab**

***Reagents:***

PicoGreen dsDNA assay kit (<http://products.invitrogen.com/ivgn/product/P11496>)

Fisher Scientific:

Water, Sterile BP2470-1

Tris Hydrocloride 1M BP1758-100ml

rATP (100mM, Fermentas R0441)

EDTA BP2482 – 100

Sbf1-HF (NEB R3642S) <https://www.neb.com/products/r3642-sbfi-hf>

NEB Buffer 4 (<https://www.neb.com/products/b7004-nebuffer-4>)

T4 DNA Ligase NEB (M0202M; <https://www.neb.com/products/m0202-t4-dna-ligase>)

Agencourt AMPure XP, 60 mL (A63881)

Bioruptor tubes

NEBNext Ultra DNA Library Prep Kit for Illumina (<https://www.neb.com/products/e7370-nebnext-ultra-dna-library-prep-kit-for-illumina>)

NEBNext Multiplex Oligos (NEB EE7335) – only if multiplexing P2 adapter

**New P1 adapter primer:**

5’ AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC\*G 3’

**P2 adapter primer (from Mike’s protocol online):**

5' CAAGCAGAAGACGGCATACGA 3'

**P2 RAD adapter information (from Mike’s protocol) – need to anneal before use:**

Top oligo sequence:

5' /5Phos/GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCAGAACA\*A 3'

Bottom oligo sequence:

5'CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC\*T 3'

***Equipment:***

* Qubit® Fluorometer or plate reader (<http://products.invitrogen.com/ivgn/product/Q32871>)
* Bioruptor NGS – for sonicating samples, runs about 14K <http://www.diagenode.com/en/catalog/sonication-54/bioruptor--55/product/bioruptor-standard-1>
* Advanced Analytical Fragment Analyzer (<http://www.aati-us.com/instruments/fragment_analyzer/>), serves as a gel box replacement, but more sophisticated. Runs about 29K.
* EP Motion – used for sample normalization, serial dilution transfer etc. It is most useful in this context for sample normalization. (<http://www.eppendorf.com/int//index.php?page=1&action=epmotion&contentid=3&page=2>)