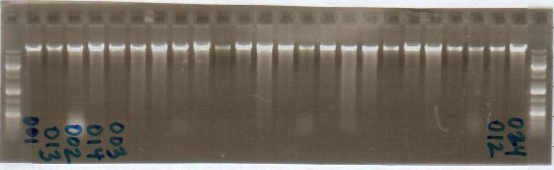
**BestRAD Illumina Library Preparation for 96 Multiplexed Samples**

**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 50 ng of DNA in 10 μl volume. (EpMotion)
2. Into 1.5 ml tube, pipet SbfI digestion master mix: (Start later in the day)

1X 110X

H2O 0.68 μl 74.8 μl

NEBuffer 4 1.2 μl 132 μl

SbfI-HF (NEB R3642L) 0.12 μl 13.2 μl

\*Don’t vortex SbfI-HF

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

Thermocycler: TONY

RAPTUR>RAPDig

OR

Thermocycler: SORK

BESTRA>BEDig

1. Incubate plate at 37° C for 60 minutes.
2. Incubate plate at 80° C for 20 minutes.

**Part 3: BestRad SbfI Adaptor Ligation**

1. Into each plate well, pipet 2 μl annealed BestRad SbfI adaptors (50 nM).
2. Make ligation master mix (make in 1.5 mL tube and then pour into tray):

1X 110X

H2O 1.28 μl 140.8 μl

NEBuffer 4 0.4 μl 44.0 μl

rATP (100mM, Fermentas Ro441) 0.16 μl 17.6 μl

T4 DNA Ligase (NEB M0202M) 0.16 μl 17.6 μl

1. Into each 96 plate well, pipet 2 μl ligation master mix.
2. Incubate plate at 20° C overnight.

Thermocycler: TONY

RAPTUR>RAPLig

Or

SORK> BestLig

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

\*can stop and freeze here\*

**Part 4: Clean up**

1. Pipet 8 μl from each of the 96 plate wells in trough, mix and then transfer into 1.5ml tube. Split total volume into two tubes and perform bead clean up separately. 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 ~350 μl AMPure beads to each 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 tubes from the magnetic stand and add 106 μl of LowTE to each tube. 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 105ul of the supernatant from each tube 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 500 bp using BioRuptor NGS. 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 10 cycles of 15 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:

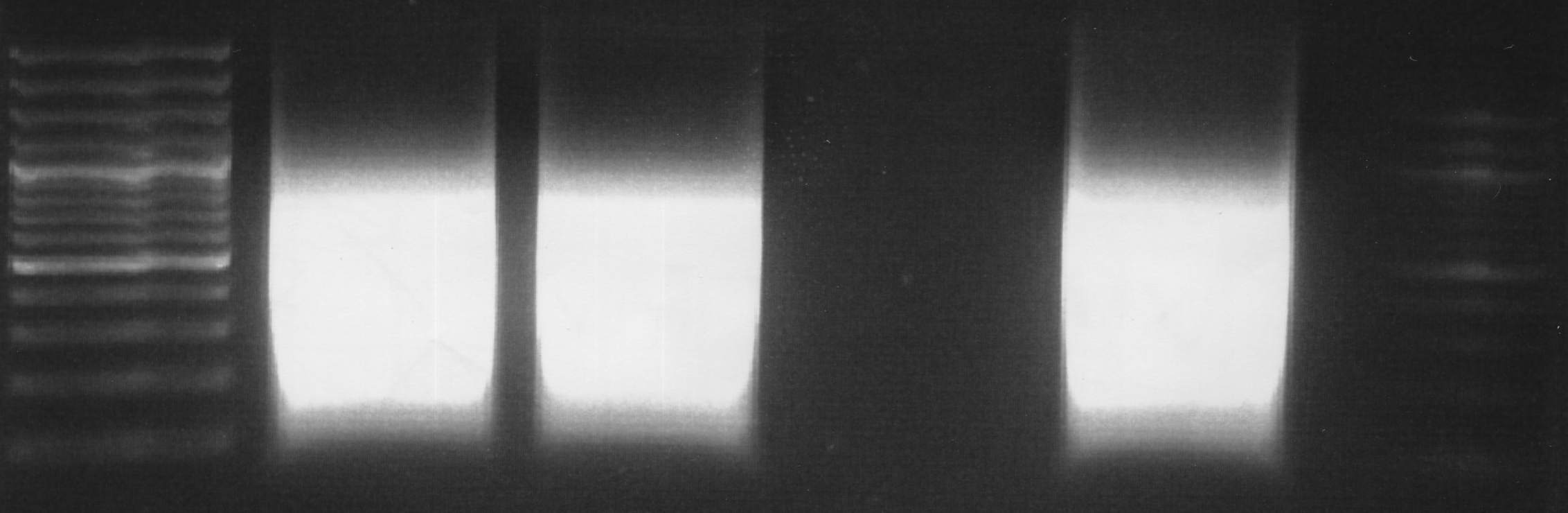
>1% surface tension gel

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. 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. Combine both bioruptor tubes, ~100ul each into a new 1.7 ml tube to have a total of ~200 ul.

\*can stop and freeze here\*

**Part 6: Prepare Dynabead M280 streptavidin magnetic beads**

1. Transfer 20 ul Dynabeads to a new 1.7 ml tube
2. Place tube in magnetic rack for 2 min. or when the solution clears and remove supernatant.
3. Wash the beads with 100 ul 2X B+W buffer, mix 30 sec., quick spin, place on mag. rack for 2 min. and remove supernatant.
4. Repeat wash once.
5. Resuspend beads in 200 ul 2X B+W buffer.

**Part 7: Bind Ligated fragments to Dynabeads**

\*Place 300ul of 1X B+W buffer in incubator at 56°C

1. Add resuspended dynabeads to the tube of ~200 ul sonicated DNA. Incubate at RT for 20 min while mixing every 2 minutes.
2. Quick spin, place tube on magnetic rack for 2-3 min., remove supernatant, resuspend beads in 150 ul 1X B+W Buffer.
3. Repeat wash step two additional times. Each time place on mag. for 1 min.
4. Repeat wash step two additional times with 56°C 150 ul 1X B+W buffer

**Part 8: Liberate DNA from Dynabeads**

1. Resuspend the final washed beads in 100 ul 1X NEB Buffer 4.
2. Place on magnetic rack for 1 min., remove supernatant.
3. Repeat NEB 4 wash once.
4. Resuspend beads and bound DNA in 40 ul 1X NEB Buffer 4.
5. Transfer solution to a PCR tube.
6. Add 2 ul SbfI-HF (NEB R3642L).

Thermocycler: JOHN

BESTRA>LIBDNA

1. Incubate tube at 37°C for 60 minutes.
2. Quick spin, place tube on magnetic rack for 2-3 min., **keep supernatant**

**Part 9: 2nd Clean-up**

1. Add ~45 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.
2. Quickly spin down the tube and place it on a magnetic stand for 5 minutes or until solution is clear.
3. Carefully remove and discard supernatant, making sure not to disturb the beads. \*\*keep beads\*\*
4. While the tube is still on the magnetic stand, add 200 μl freshly prepared 80% ethanol. Incubate at room temperature for 30 seconds, then carefully remove and discard the supernatant.

EtOH 400 ul

H2O 100 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 5 minutes. Do not over-dry.
3. Remove the tube from the magnetic stand and add 56μl 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 new PCR tube, making sure not to disturb the beads. \*\*discard beads\*\* 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 and associated reagents for Illumina *without* modification)**

**Part 10: 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 11: NEBNext Adaptor Ligation -** This step ligates the NEBNext Adaptor 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

NEBNext Adaptor for Illumina (1.5μM) 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.

Thermocycler: JOHN

RAD>NEBLigat

1. Incubate at 20°C for 15 minutes in a thermal cycler.
2. Add 3 ul of USER enzyme to the ligation mixture from step 3.

Thermocycler: JOHN

BESTRA>USERENZ

1. Mix well and incubate at 37°C for 15 minutes.

\*can stop and freeze here\*

**Part 12: Size Selection of Adapter-ligated DNA**

This step of the protocol removes free un-ligated or concatomerized BestRAD 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.

\*Transfer to 1.5 ml tube

1. Add 13.5 l dH20 to the ligation reaction for a 100 l volume.
2. **Remove large fragments**: Add 45 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.
3. Incubate for 5 minutes at room temperature.
4. 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.
5. **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.

3X:

EtOH 560 ul

H2O 140 ul

6X:

EtOH 1040 ul

H2O 260 ul

1. 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!**)
2. 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.
3. Repeat Step 8 2x for a total of three washes.
4. Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open.
5. 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 13: Library Amplification**

**A. Test PCR** - The first PCR is a test run to see if there is amplification. This step only uses 20% of the library template with 15 PCR cycles.

1. Mix the following components in sterile PCR tube:

Adapter Ligated DNA Fragments 5 l

NEBNext Q5 Hot Start HiFi PCR Master Mix 25 l

H2O 10 l

Index 1 Primer (10 M) 5 l

Universal PCR Primer (10 M) 5 l

Total Volume 50 l

PCR cycling conditions:

|  |  |  |  |
| --- | --- | --- | --- |
| **Thermocyler: JOHN 🡪 BESTRA🡪 NEBTESTP** | | | |
| **Cycle Step** | **Temp** | **Time** | **Cycles** |
| Initial Denaturation | 98°C | 30 seconds | 1 |
| Denaturation | 98°C | 10 seconds | 15 |
| Annealing/Extension | 65°C | 75 seconds |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ |  |

1. **Recommended:** Run a test gel with 5 ul PCR product on a 1% gel to determine whether or not the PCR worked. If there is amplification, we carry on with the final library PCR in the next step. If the band is super bright, adjust final PCR to fewer cycles.

**B. Final PCR Enrichment -** The final PCR amplification uses the leftover 80% of the library template with only 12 PCR cycles. We reduce the number of PCR cycles to reduce the chance of PCR duplicates and maintain library complexity. Stronger libraries can get away with even fewer cycles (9 – 12).

1. Mix the following components in sterile PCR tube:

Adapter Ligated DNA Fragments 15 l

NEBNext Q5 Hot Start HiFi PCR Master Mix 25 l

Index 1 Primer (10 M) 5 l

Universal PCR Primer (10 M) 5 l

Total Volume 50 l

PCR cycling conditions:

|  |  |  |  |
| --- | --- | --- | --- |
| **Thermocyler: JOHN 🡪 BESTRA🡪 NEBFINPC** | | | |
| **Cycle Step** | **Temp** | **Time** | **Cycles** |
| Initial Denaturation | 98°C | 30 seconds | 1 |
| Denaturation | 98°C | 10 seconds | 12 |
| Annealing/Extension | 65°C | 75 seconds |
| Final Extension | 65°C | 5 minutes | 1 |
| Hold | 4°C | ∞ |  |

1. **Recommended:** Run 5 ul PCR product on a 1% gel. If there is amplification, proceed to clean up.

\*can stop and freeze here\*

**Part 14: Cleanup of PCR Amplification:** This size selectin step is to remove adaptor and PCR dimers.

1. Vortex AMPure XP beads to re-suspend.

\*Transfer to 1.5 ml tube

1. Add 55 l dH20 to the ligation reaction for a 100 l volume.
2. **Remove large fragments**: Add 45 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.
3. Incubate for 5 minutes at room temperature.
4. 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.
5. **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.

3X:

EtOH 560 ul

H2O 140 ul

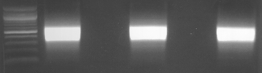
6X:

EtOH 1040 ul

H2O 260 ul

1. 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!**)
2. 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.
3. Repeat Step 8 2x for a total of three washes.
4. Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open.
5. 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. Leave at room temperature for 5 minutes. 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 supernatant to a new PCR tube. Store libraries at – 20°C.

A strong library should look something like:



**Fig. 4.** PCR product from the three libraries. The libraries are 300-600 bp in size.

1 2 3 4 5 6

**Part 15: Prepare for shipment to sequencing facility**

1. Quantify library concentration using PicoGreen or Qubit. Dilute library to <3 ng/l with water and run on Agilent BioAnalyzer DNA High Sensitivity chip to check the size distribution.
2. Dilute library to required concentration and volume (for Berkeley: 10 nM in 10 l) for sequencing.

**Materials and Supplies**

***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

Dynabeads (Life Technologies, 11205D) https://www.thermofisher.com/order/catalog/product/11205D?ICID=search-11205d

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 Singleplex Oligos (NEB EE7350)

2X Binding and Wash (B+W) Buffer: (10mM Tris-HCL (pH 7.5), 1mM EDTA (pH 8.0), 2M NaCl)

1X Binding and Wash (B+W) Buffer: (5mM Tris-HCL (pH 7.5), 0.5mM EDTA (pH 8.0), 1M NaCl)

***Equipment:***

* Qubit® Fluorometer or plate reader (<http://products.invitrogen.com/ivgn/product/Q32871>)
* Bioruptor NGS – for sonicating samples <http://www.diagenode.com/en/catalog/sonication-54/bioruptor--55/product/bioruptor-standard-1>
* 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>)