**EcoRI/MseI Double Digestion RAD Illumina Protocol**

**September 4, 2012**

**1. Prepare Stocks and Plates (~ Half day to prepare and perform this step)**

1.1 EcoRI Adpaters (ERA – referenced below)

1. Mix 1μL of each oligo in a pair (from 100μM stock) with 98μL water to make 100μL of 1pmol/μL (1μM) of double-stranded stock. (in a 96-well plate).
2. Heat to 95°C for 5 minutes and cool slowly to room temperature.

1.2 MseI Adapters (MIA – referenced below)

1. Mix 10μL of each MseI oligo (from 100μM stock) with 80μL water to make 100μL of 10pmol/μL (10μM) stock (in a 500μL tube).
2. Heat to 95°C for 5 minutes and cool slowly to room temperature.

1.3 PCR Primers

1. Mix 5 μL of each (Forward and Reverse) Illumina PCR oligo (from 100μM stock) with 90μL of water to make a working solution of 5μM (2.5μM each oligo).

1.4 DNA Plate

1. Prepare a 96-well plate with at least 6 μL of each DNA sample (at a concentration close to 20ng/μL). Adjust concentrations to be as equal as possible BEFORE starting digestion reactions.

**2. Restriction-Ligation Reactions (RL ~ Half day to prepare and perform this step; best to set up in the afternoon and allow digestion to run overnight)**

1. Place 6uL of sample DNA in each well of a new plate and keep on ice.

2. Prepare Master Mix I:

|  |  |  |
| --- | --- | --- |
|  | μL for 1 Sample | Amt. for 30 samples |
| 10X T4 Ligation Buffer | 1 | 30 |
| 1M NaCL | 0.5 | 15 |
| 1 mg/mL BSA | 0.5 | 15 |
| MseI Adapter Mix (MIA; 10uM) | 1 | 30 |

3. Prepare Master Mix II:

|  |  |  |
| --- | --- | --- |
|  | μL for 1 Sample | μL for 30 samples |
| Water | 0.2825 | 8.475 |
| 10X T4 Ligation Buffer | 0.1 | 3 |
| 1M NaCl | 0.05 | 1.5 |
| 1 mg/mL BSA | 0.05 | 1.5 |
| MseI (enzyme) | 0.1 | 3 |
| EcoRI (enzyme) | 0.25 | 7.5 |
| T4 DNA Ligase | 0.1675 | 5.025 |

4. Combine Master Mix I and Master Mix II together; vortex and centrifuge. Add 4μL of combined mix to each sample, keeping everything on ice.

5. Add 1μL of EcoRI adapters (ERA) to each well on the plate.

6. Total reaction volume should be 11μL. Cover and seal the plate, vortex, centrifuge (for centrifuge, spin samples until centrifuge reaches max speed and then stop the spin), and incubate at 37°C for 18 hours on a thermal cycler with a heated lid.

7. Optional: Perform AMPure bead purification at this step to remove un-ligated adapters and dimers prior to PCR amplification. Details of AMPure protocol are given below.

\* To store the restriction-ligation reaction long-term (i.e. frozen), dilute with 189μL of 0.1X TE.

**3. PCR Amplification**

1. Take **2** aliquots of 4μL each from each Restriction-Ligation (R/L) reaction and put into separate PCR tubes or a new plate.

2. Prepare a PCR Master Mix:

|  |  |  |
| --- | --- | --- |
|  | μL per Sample | μL for 60 samples |
| Water | 9.67 | 580.2 |
| 5X iProof HF buffer | 4 | 240 |
| dNTP Mix (10mM) | 0.4 | 24 |
| MgCl2 (50mM) | 0.4 | 24 |
| Mixed PCR Primers (5μM) | 1.33 | 79.8 |
| iProof Taq | 0.2 | 12 |

16 uL Total

\*Using iProof because it is a high-fidelity polymerase.

3. Add 16μL of PCR Mix to each R/L aliquot, and place on a thermal cycler with the following conditions (Known as RAD-Gompert on Uy Biorad thermal cyclers):

98°C for 30 seconds

**30 cycles of:**

98°C for 20s

56°C for 30s

72°C for 40s

Final extension: 72°C for 10 minutes

**4. Gel Purification**

1. Pool all PCR products (for a total volume of 960μL for 24 starting DNA samples). Prepare a gel (1% agarose) that can accommodate all of the DNA (probably about 40 wells in a single gel – two 20 lane combs = 40 total wells on big gel rig-you will probably need to run 2 big gels to get through all of the samples). Add 20 uL of product per well with 4 uL of loading dye. Add 2-5 uL of 100 bp ladder (from Promega) to the first and last wells of each row (the 4 extreme ends).

2. Run the PCR product on the gel for as long as needed (100 volts for 2-3 hours if doing 40 lanes in 1 gel), and be sure to include 100bp ladder in multiple locations.

3. Cut the desired region from the gel (between 300-500bp seems best) using a sterile pipette tip or razor blade.

3a. Use UV light to score the gel so that you have cutting guidelines when cutting outside of the UV light.

3b. Weigh the microcentrifuge tube and zero the scale; cut gel chunk that is no bigger than 0.3 grams and reweigh

4. Purify the excised gel regions using QiaQuick Gel Extraction Kits.

4a. Add 300uL of QG buffer for every 0.1g of gel

4b. Dissolve at room temp. The protocol instructs to dissolve at 50°C, but this may reduce quality of DNA fragments. Instead, put in incubator and agitate (~183 speed) for ~ 1 hour. Check to make sure there are no gel clumps left when removing from agitator.

4c. Add 100uL of Isopropanol per 0.1 g of gel.

4d. Transfer mix from 4d to spin columns and spin at max speed (14,000 rpm) of small centrifuge for 1 min. Pour off liquid from spin.

4e. Add 500 uL of QG buffer and spin at max again. Pour off liquid.

4f. Add 750 uL of buffer PE and let solution sit for 2-5 minutes after adding buffer PE. Pour off liquid.

4g. Spin column at max again to remove residual wash buffer. Pour off liquid.

4h. Place column into labeled 1.5 mL microcentrifuge tube. Add 20 uL of buffer EB and let stand for 1-4 minutes. Spin column for 1 minute. Keep contents of spin in labeled 1.5uL microcentrifuge tube.

5. To further remove adapter dimers, use the AMPure beads kit at this stage to remove excess small fragments.

5a. Transfer purified products from Step 4 to PCR tubes. You can combine reactions at this stage, but do not put more than 60uL into 1 tube, or you will not have enough room for the AMPure reagent.

5c. Calculate necessary amount of AMPure reagent using table (1.8x volume of PCR tube volume)

5d. AMPure reagent tends to separate so vortex until thoroughly mixed. When drawing up AMPure reagent, pipette up and down several times to confirm that it is mixed.

5e. Add mixed AMPure reagent to PCR tube and pipette up and down several times (about 10 times) to mix DNA strands with AMPure beads. Let this mixture sit for 5 minutes so that DNA can bind to beads.

5f. Transfer mixture from step 5e onto the magnet plate. Let the mixture sit for 2 minutes so that beads can move to side of the wells.

5g. Pipette off the cleared solution WITHOUT disturbing the ring of beads on the side. Dispose of aspirated solution (I prefer to use Gel Loading Pipet Tips for this, as the thin capillary sections fit more easily into the PCR tubes without disturbing the sides).

5h. While the sample is still on the magnet, place 200 uL of 70% Ethanol into each well and incubate for 30 seconds. Pipette off the Ethanol.

5i. Repeate step 5h. Let samples dry for 2 minutes but NO longer as overdrying the beads will reduce the elution efficiency.

5j. For final elution step, take PCR tube off of plate to facilitate mixing. Use buffer EB instead of water. Pipette up and down ~10 times or until the mixture is homogeneous. Place the mixture back onto expensive magnetic plate and let sit for 1 minute.

5k. Remove the liquid and transfer to a new PCR tube. Do not include any beads in final solution so as to avoid damaging the sequencing machine.

6. Use the Qubit to measure the final concentration of the library.

6a. Requires special Qubit tubes.

6b. Need two standards for each set of measurements. Use DNA-HS (high sensitivity) reagent for RAD-tag DNA. For working solution, need 200 uL per sample tubes (including standards). Use a 1:200 (Fluorescent reagent: Buffer) dilution.

6c. Make standards using 10:190 solution. First solution is a blank standard and the second has a DNA concentration.

6d. Use 1 uL of sample and 199 uL of solution for DNA samples.

6e. Vortex samples and then spin them down quickly.

6f. Turn on Qubit  DNA Double-strand high sensitivity Click on yes for

new standards Put in blank standard Insert second standard Then it will say insert assay tube if ready to go then put in samples and hit read.

**ADAPTER SEQUENCES**

**I. ERA adapters (indexed)**

|  |  |  |  |
| --- | --- | --- | --- |
| P1-FOR-AAAAA | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAAAA\*A | P1-REV-AAAAA | /5Phos/AATTTTTTTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-AACCC | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAACC\*C | P1-REV-AACCC | /5Phos/AATTGGGTTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-AAGGG | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGG\*G | P1-REV-AAGGG | /5Phos/AATTCCCTTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-AATTT | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAATT\*T | P1-REV-AATTT | /5Phos/AATTAAATTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-ACACG | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACAC\*G | P1-REV-ACACG | /5Phos/AATTCGTGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-ACCAT | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACCA\*T | P1-REV-ACCAT | /5Phos/AATTATGGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-ACGTA | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACGT\*A | P1-REV-ACGTA | /5Phos/AATTTACGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-ACTGC | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACTG\*C | P1-REV-ACTGC | /5Phos/AATTGCAGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-AGAGT | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAGAG\*T | P1-REV-AGAGT | /5Phos/AATTACTCTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-AGCTG | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCT\*G | P1-REV-AGCTG | /5Phos/AATTCAGCTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-AGGAC | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAGGA\*A | P1-REV-AGGAC | /5Phos/AATTGTCCTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-AGTCA | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTC\*C | P1-REV-AGTCA | /5Phos/AATTTGACTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-ATATC | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATAT\*T | P1-REV-ATATC | /5Phos/AATTGATATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-ATCGA | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCG\*G | P1-REV-ATCGA | /5Phos/AATTTCGATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-ATGCT | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATGC\*C | P1-REV-ATGCT | /5Phos/AATTAGCATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-ATTAG | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATTA\*A | P1-REV-ATTAG | /5Phos/AATTCTAATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-CAACT | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCAAC\*C | P1-REV-CAACT | /5Phos/AATTAGTTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-CACAG | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCACA\*A | P1-REV-CACAG | /5Phos/AATTCTGTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-CAGTC | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGT\*T | P1-REV-CAGTC | /5Phos/AATTGACTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-CATGA | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCATG\*G | P1-REV-CATGA | /5Phos/AATTTCATGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-CCAAC | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCCAA\*A | P1-REV-CCAAC | /5Phos/AATTGTTGGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-CCCCA | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCCCC\*C | P1-REV-CCCCA | /5Phos/AATTTGGGGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-CCGGT | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCCGG\*G | P1-REV-CCGGT | /5Phos/AATTACCGGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |
| P1-FOR-CCTTG | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTT\*T | P1-REV-CCTTG | /5Phos/AATTCAAGGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT\*T |

5’ Phosphorylation as indicated

Phosphorothioate linkage between bases marked with \*

**II. MIA Adapters (Universal)**

|  |  |  |  |
| --- | --- | --- | --- |
| MseI\_P2.1\_PE | GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT | MseI\_P2.2\_PE | CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGC |

**III. PCR Primers (Universal)**

|  |  |
| --- | --- |
| RAD-FORWARD | AATGATACGGCGACCACCG\*A |
| RAD-REVERSE | CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGC |