EcoRI/MseI Double Digest RAD Illumina Protocol

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1 Prepare Stocks and Plates

Allow yourself about half of a day to get everything prepared, so that the adapter mixes can be allowed to cool slowly with no rush.

1.1 EcoRI Adapters

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

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 20 ng/μL.) **Adjust the concentrations to be as equal as possible before going on to the next step!**

   To ensure equal DNA concentrations *and* clean DNA, I recommend the following:

- Measure your starting DNA concentrations (I recommend the Qubit), and calculate the total ng of DNA in each sample.
- Perform Ethanol Purification to clean the samples (see separate protocol), and allow the DNA pellet to dry overnight.
- Calculate the amount of water needed to elute the *smallest* sample in a 20ng/μL solution. If this amount is less than 6μL, calculate instead what the concentration of your smallest sample would be in 6μL. If this concentration is below even 10ng/μL, you may need to exclude this sample. Otherwise, you can proceed to the next step.
- Calculate the volume of water needed to elute all of the rest of your samples to a concentration equal to that of your smallest sample, and elute your dried DNA pellet in PCR-grade water.
- Re-check your concentrations on the Qubit, then proceed with add 6μL of each purified and re-concentrated sample to a new 96-well plate.

2 Restriction-Ligation Reactions

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

2. Prepare Master Mix I:

	μL for 1 Sample	μL for 30 samples
10X T4 Ligation Buffer	1	30
1M NaCl	0.5	15
1 mg/mL BSA	0.5	15
MseI Adpater Mix	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 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 (but strongly recommended): 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
M_gCl_2 (50mM)	0.4	24
Mixed PCR Primers (5μM)	1.33	79.8
iProof Taq*	0.2	12
	16 μL 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:

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 μL of product per well with 4 μL of

loading dye. Add 2-5 μL 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.

   - Use UV light and quickly score the gel so that you have cutting guidelines to use without having to leave the UV light on.
   - Weigh an empty microcentrifuge tube and zero the scale; cut gel chunk that is no bigger than 0.3 grams and re-weigh

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

   - Add 300μL of QG buffer for every 0.1g of gel
   - Dissolve at room temp. The protocol instructs you to dissolve at $50°C, but this may reduce quality of DNA fragments. Instead, put in incubator and agitate ($\approx$183 speed) for $\approx$1 hour. Check to make sure there are no gel clumps left when removing from agitator.
   - Add 100μL of Isopropanol per 0.1 g of gel.
   - Transfer mix from previous step to spin columns and spin at max speed (14,000 rpm) for 1 min. Pour off liquid from spin.
   - Add 500 μL of QG buffer and spin at max again. Pour off liquid.
   - Add 750 μL of buffer PE and let solution sit for 2-5 minutes after adding buffer PE. Pour off liquid.
   - Spin column at max again to remove residual wash buffer. Pour off liquid.
   - Place column into labeled 1.5 mL microcentrifuge tube. Add 20 μL of buffer EB and let stand for 1-4 minutes. Spin column for 1 minute. Keep contents of spin in labeled 1.5μL microcentrifuge tube.

5 AMPure Purification

1. Transfer purified products to PCR tubes. You can combine reactions anytime after barcoded adapters have been added, but do not put more than 60μL into 1 tube, or you will not have enough room for the AMPure reagent.

2. Calculate the necessary amount of AMPure reagent (1.8x volume of your sample volume in each tube).

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

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

5. Transfer tubes onto the magnet plate. Let the tubes sit for 2 minutes so that beads can move to side of the wells.

6. 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).

7. While the sample is still on the magnet, add 200 μL of 70% Ethanol into each well and incubate for 30 seconds. Pipette off the Ethanol.

8. Repeat the previous step. Let samples dry for 2 minutes but NO longer as over-drying the beads will reduce the elution efficiency.

9. For final elution step, take the PCR tubes off of plate to facilitate mixing. Use buffer EB instead of water. Pipette up and down $\approx$10 times or until the mixture appears homogeneous. Place the tubes back onto the magnetic plate and let sit for 1 minute.

10. 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 EcoRI Adapter Sequences with Barcodes

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*C
P1-REV-AGGAC	/5Phos/AATTGTCCTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-AGTCA	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTC*A
P1-REV-AGTCA	/5Phos/AATTTGACTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-ATATC	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATAT*C
P1-REV-ATATC	/5Phos/AATTGATATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-ATCGA	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCG*A
P1-REV-ATCGA	/5Phos/AATTTCGATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-ATGCT	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATGC*T
P1-REV-ATGCT	/5Phos/AATTAGCATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-ATTAG	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATTA*G
P1-REV-ATTAG	/5Phos/AATTCTAATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-CAACT	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCAAC*T
P1-REV-CAACT	/5Phos/AATTAGTTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-CACAG	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCACA*G
P1-REV-CACAG	/5Phos/AATTCTGTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-CAGTC	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGT*C
P1-REV-CAGTC	/5Phos/AATTGACTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-CATGA	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCATG*A
P1-REV-CATGA	/5Phos/AATTTCATGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-CCAAC	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCCAA*C
P1-REV-CCAAC	/5Phos/AATTGTTGGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-CCCCA	ATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCCCC*A
P1-REV-CCCCA	/5Phos/AATTTGGGGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-CCGGT	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCCGG*T
P1-REV-CCGGT	/5Phos/AATTACCGGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T
P1-FOR-CCTTG	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTT*G
P1-REV-CCTTG	/5Phos/AATTCAAGGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T

7 MseI Adapters (not Barcoded)

MseI_P2.1_PE	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
MseI_P2.2_PE	CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGC

8 PCR Primers

RAD-FORWARD	AATGATACGGCGACCACCG*A
RAD-REVERSE	CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGC