**Paired-end Restriction Associated DNA (RAD) Library**

This protocol describes the modifications necessary for Paired-end (PE) high- throughput sequencing based on the method “Amplified restriction fragments for genomic enrichment” by Parchman, Gompert, and Buerkle (version 2.3 August 2011). The RAD library is purified with three successive rounds of AMpure XP Bead to improve DNA migration during Blue Pippin Size Selection. We first process multiple volumes in parallel and then collapse the recovered sample into a single volume for the remainder of the protocol.

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# RESTRICTION DIGEST AND LIGATION

Restriction digest with EcoRI and MseI Prepare a 96-well plate with each well containing DNA from a different, unique source. Briefly centrifuge the plate to bring its contents to the bottom of the wells. Seal it with PCR adhesive film and store the prepared DNA plate at -20°C to -80°C until ready to use. \*\* Different concentrations.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Required genomic DNA Amount | | |
|  | | **Low Input** | High Input |
| DNA Amount | | 100 – 400 ng | 400 – 1200 ng |
| Final Volume | | 12.0 ul | 6.0 ul |

Thaw the restriction digest reagents on ice. Keep enzymes in freezer until ready to use. Remove the prepared DNA plate from the freezer. Briefly centrifuge the plate. Place on ice.

|  |  |  |
| --- | --- | --- |
| Low Input DNA  Restriction Digest Master Mix (RDMM) | | |
| Component | **Amount per rxn (µl)** | Amount 96 rxn  plus 20% extra (µl) |
| 10X T4 Buffer | 1.767 | 203.6 |
| 2M NaCl \*\* | 0.46 | 53.0 |
| 2mg/mL BSA \*\* | 0.46 | 53.0 |
| Water | 0.163 | 18.8 |
| MseI enzyme, 10,000U/mL | 0.10 | 11.5 |
| EcoRI enzyme, 20,000U/mL | 0.25 | 28.8 |
| Final Volume | 3.20 | 368.6 |
| \* Transfer 45 µl to each well of 8-well PCR strip tube | | |
|  |  |  |
| Master Mix per rxn | 3.2 µl |  |
| DNA volume | 12.0 µl |  |

|  |  |  |
| --- | --- | --- |
| High Input DNA  Restriction Digest Master Mix (RDMM) | | |
| Component | **Amount per rxn (µl)** | Amount 96 rxn  plus 20% extra (µl) |
| 10X T4 Buffer | 1.0 | 115.2 |
| 1M NaCl \*\* | 0.52 | 59.9 |
| 1mg/mL BSA \*\* | 0.52 | 59.9 |
| Water | 0.2125 | 24.5 |
| MseI enzyme, 10,000U/mL | 0.10 | 11.5 |
| EcoRI enzyme, 20,000U/mL | 0.25 | 28.8 |
| Final Volume | 2.6025 | 299.8 |
| \* Transfer 35 µl to each well of 8-well PCR strip tube | | |
|  |  |  |
| Master Mix per rxn | 2.6 µl |  |
| DNA volume | 6.0 µl |  |

1. On ice, prepare the Restriction Digest Master Mix (RDMM) in a microcentrifuge tube (1.5mL) according to the table. Pipette gently 5 times to mix. Then, transfer 45µl Low Input or 35µl High Input Restriction Digest Master Mix (RDMM) into each well of an 8-well PCR strip tube. Centrifuge briefly and place on ice.
2. Using an 8-channel P10 pipette, add Restriction Digest Master Mix (RDMM) to each sample. Pipette gently 3 times to mix.
   1. 3.2 µl for Low Input
   2. 2.6µl for High Input
3. Seal the DNA plate with PCR adhesive film and centrifuge briefly.
4. In a thermal cycler, incubate the DNA plate at 37°C for 2 hours, then at 65°C for 20 minutes with heated lid at 105°C, followed by a 4°C hold.
5. Remove the DNA plate from the thermal cycler. Briefly centrifuge the plate to bring its contents to the bottom of the wells. Place on ice, and proceed to the next step. This is now your restriction digested DNA plate.

Ligation of EcoRI Adapter and MseI Adapter to DNA fragments Thaw the barcoded EcoRI Adapter plate, MseI Adapter, and ligation reagents on ice. Be sure to minimize warming of the T4 DNA Ligase. Centrifuge briefly all components and keep on ice. **\*\* Different concentrations.**

|  |  |  |  |
| --- | --- | --- | --- |
| Low Input DNA  Adapter Ligation Master Mix (ALMM) | | | |
| Component | **Amount per rxn (µl)** | Amount 96 rxn  plus 20% extra (µl) | |
| 10 µM MseI Adapter | 2.010 | 231.6 | |
| Water | 0.043 | 5.0 | |
| 10X T4 Buffer | 0.444 | 51.1 | |
| 2M NaCl \*\* | 0.113 | 13.0 | |
| 2mg/mL BSA \*\* | 0.113 | 13.0 | |
| T4 DNA Ligase, 400,000U/mL | 0.1675 | 19.3 | |
| Final Volume | 2.9 | 333.0 | |
| \* Transfer 40 µl to each well of 8-well PCR strip tube | | | |
|  |  |  | |
| Master Mix per rxn | 2.9 µl |  |
| 1µM EcoRI Adapter | 2.0 µl |  |

|  |  |  |
| --- | --- | --- |
| High Input DNA  Adapter Ligation Master Mix (ALMM) | | |
| Component | **Amount per rxn (µl)** | Amount 96 rxn  plus 20% extra (µl) |
| 10 µM MseI Adapter | 1.0 | 115.2 |
| Water | 0.072 | 8.3 |
| 10X T4 Buffer | 0.1 | 11.5 |
| 1M NaCl \*\* | 0.05 | 5.8 |
| 1mg/mL BSA \*\* | 0.05 | 5.8 |
| T4 DNA Ligase, 400,000U/mL | 0.1675 | 19.3 |
| Final Volume | 1.4395 | 165.8 |
| \* Transfer 20 µl to each well of 8-well PCR strip tube | | |
|  |  |  |
| Master Mix per rxn | 1.4 µl |  |
| 1µM EcoRI Adapter | 1.0 µl |  |

1. On ice, prepare the Adapter Ligation Master Mix (ALMM) in a microcentrifuge tube (1.5mL) according to the table. Pipette gently 5 times to mix. Then, transfer 40µl Low Input or 20µl High Input Adapter Ligation Master Mix (RDMM) into each well of an 8-well PCR strip tube. Centrifuge briefly and place on ice.
2. Using an 8-channel P10 pipette, add Adapter Ligation Master Mix (ALMM) to each DNA sample. Pipette gently 3 times to mix.
   1. 2.9 µl for Low Input
   2. 1.4 µl for High Input
3. Add barcoded EcoRI Adapter plate to each well of the restriction digested DNA plate. Pipette gently 3 times to mix.
   1. 2.0 µl for Low Input
   2. 1.0 µl for High Input
4. Seal the DNA plate with PCR adhesive film and centrifuge briefly.
5. In a thermal cycler, incubate the DNA plate at 16°C for 2 hours with heated lid at 20°C, followed by 4°C hold.
6. Remove the DNA plate from the thermal cycler. Briefly centrifuge the plate to bring its contents to the bottom of the wells. Remove the adhesive seal. Place on ice.
7. Dilute each reaction by adding 0.1X TE, pH7.5-8.0).
   1. 45.0 µl for Low Input
   2. 90.0 µl for High Input
8. Seal the DNA plate with PCR adhesive film and centrifuge briefly. Place on ice and proceed to the next step, or store at -20°C to -80°C for up to 3 months until ready to use. This is now your diluted restriction digested, adapter ligated DNA plate.

# PCR AMPLIFICATION OF ADAPTER-LIGATED LIBRARY

Two separate 20µl PCR amplification reactions are setup for each adapter ligated DNA product and pooled after the amplification is complete.

Thaw the PCR amplification reagents on ice. Be sure to minimize warming of the iProof High-Fidelity DNA Polymerase. Centrifuge briefly all components and return to ice until ready to use. If applicable, remove the restriction digested, adapter ligated DNA plate from the freezer. Briefly centrifuge the plate to thaw, and then place on ice.

|  |  |  |
| --- | --- | --- |
| PCR Amplification Master Mix (PCMM) | | |
| Component | **Amount for 2 x 20 µl reactions (µl)** | Amount for 2 x 96 reactions  plus 10% extra (µl) | |
| Water | 18.80 | 1985.3 | |
| 5X iProof HF Buffer | 8.00 | 844.8 | |
| dNTP mix, 10mM each | 0.80 | 84.5 | |
| MgCl2, 50mM | 0.80 | 84.5 | |
| Illpcr Primer Mix, 5µM each | 2.60 | 274.6 | |
| iProof Polymerase, 2U/µl | 0.40 | 42.2 | |
| DMSO, 100% | 0.60 | 63.4 | |
| Final Volume | 32.00 | 3379.2 | |
| \* Transfer 100 µl to each well of four (4) 8-well PCR strip tube | | | |

1. On ice, prepare the PCR Amplification Master Mix (PCMM) in a centrifuge tube (15mL) according to the table. Pipette gently with a P200 Pipette 5 times to mix. Avoid introducing bubbles. Then, transfer 100µl of the PCR Amplification Master Mix (PCMM) into each well of four 8-well PCR strip tubes. Centrifuge briefly and place on ice.
2. Using an 8-channel P100 Pipette, add 16µl of the PCR amplification Master Mix (PCMM) to each well of two new PCR plates.
3. Add 4µl of restriction digested, adapter ligated DNA to well containing PCR mix. Pipette gently 3 times to mix. Seal the PCR plates with PCR adhesive film and centrifuge briefly.
4. In a thermal cycler, incubate PCR plates using the following PCR profile: 98°C for 30 seconds; 30 cycles of 98°C for 20 seconds, 60°C for 30 seconds, and 72°C for 40 seconds; and a final extension at 72° C for 10 minutes with heated lid at 105°C; followed by a 4°C hold.
5. Remove the PCR plate from the thermal cycler. Briefly centrifuge the plate to bring its contents to the bottom of the wells.
6. Transfer the products of both PCR plates into a single centrifuge tube (15mL or 50mL). Place on ice, and proceed to the next step. This is your barcoded RAD Library ready for purification.

# PURIFICATION OF PCR AMPLIFIED LIBRARY

**The RAD library is purified with three successive rounds of AMpure XP Bead to improve DNA migration during Blue Pippin Size Selection. We first process multiple volumes in parallel and then collapse the recovered sample into a single volume for the remainder of the protocol.** Warm Agencourt AMpure XP to room temperature for 30 minutes. Gently vortex the bottle thoroughly to resuspend the magnetic beads.

1. Round 1: Prepare 2 volumes of 500 µl of RAD library in separate 2.0 mL microcentrifuge tubes.
2. Add 0.8X volume of Agencourt AMpure XP Reagent (400 µl) to each tube containing the RAD library.
3. Slowly pipette 10 times to mix the reagent and library. Incubate for 15 minutes at room temperature.
4. Place the tubes on the magnet for 5 minutes or longer until solution clears.
5. With the tube on the magnet,
   1. aspirate the cleared solution and discard;
   2. wash the beads with 1000 µl of 80% Ethanol. Incubate for 30 seconds at room temperature;
   3. aspirate the ethanol and discard. Repeat once more for a total of two washes;
   4. remove ALL ethanol. Wait 8 to 10 minutes for the beads to dry.
6. Remove the tube from the magnet. Add 127 µl of Nuclease-free water to each tube and pipette mix 20 times. Incubate at room temperature for 5 minutes.
7. Place the tube on the magnet for 5 minutes or longer until solution is clear.
8. Transfer 125 µl from the two (2) tubes into a single new 2.0 mL microcentrifuge tube.
   1. (Optional) Reserve 5µl for QC.
   2. The final volume is ~ 250µl of Round 1 purified RAD library.
9. Round 2: Add 0.8X volume of Agencourt AMpure XP Reagent (200 µl) to the 250µl of RAD library recovered during Round 1. Repeat Steps 3 through 8 except use only 500 µl 80% Ethanol, wait 2 to 5 minutes for beads to dry, and elute with 127 µl of Nuclease-free water and transfer 125 µl into a new single 2.0 mL microcentrifuge tube.
   1. (Optional) Reserve 5µl for QC.
   2. The final volume is ~ 125 µl of Round 2 purified RAD library.
10. Round 3: Add 0.8X volume of Agencourt AMpure XP reagent (100 µl) to 125 µl of RAD library recovered during Round 2. Repeat Steps 3 through 8 except use only 500 µl 80% Ethanol, wait 2 to 5 minutes for beads to dry, and elute with 37.5 µl of Nuclease free water. Transfer 35.0 µl into a 1.5 mL microcentrifuge tube.
    1. The final volume is 35.0 µl of Round 3 purified RAD library.
    2. This is your triple purified RAD library.
    3. Proceed to Size-Selection.

# AUTOMATED SIZE SELECTION

Quantify purified RAD Library Vortex briefly and centrifuge the triple purified library. Use 1.0 µl of library for the Qubit DNA High-Sensitivity Assay. Record the sample concentration. If concentration is too high, dilute 2.0 µl of library with 4.0 µl of nuclease-free water. Repeat the qubit assay with 1.0 µl of diluted library. Multiply the result by three (3) to determine concentration of the undiluted sample. Record library concentration as ng/µl. Based on the Qubit measurement, transfer 1.5 µg of triple purified RAD Library to a new 1.5mL microcentrifuge tube and bring to final volume of 30µl with nuclease-free water for automated size selection. (Optional: Record the concentration of library after 1 round and 2 rounds of purification.)

Automated size selection using Blue Pippin For complete instructions, refer the Blue Pippin User’s Guide and Manual.

Size selection protocol is briefly described here.

1. Combine 30µl RAD Library with 10µl R2 Marker. Vortex to mix and centrifuge briefly.
2. Program the instrument.
   1. Protocol Setup: First, select **1.5%DF Marker R2** for Cassette Definition. Set Reference markers as lane’s number. Set the size selection mode to **Range** and define the start and stop collection points as **700bp to 900bp**. The size selection program is optimized for recovery of 400bp-500bp fragments. Enter samples names. Save the protocol.
   2. Calibration: Calibrate the system to **0.60 mV** with the calibration tool.
   3. Cassette Inspection: Visually inspect the buffer levels in the reservoirs of the gel cassette and fill if not full. Confirm the gel columns are intact, and do not use if breaks are visually observed. Dislodge any bubbles by gently tapping the cartridge. Place the cartridge into the system. Carefully remove the adhesive seals.
   4. Replenish Elution Buffer: Remove buffer from elution modules and replace with 40µl of fresh buffer. Seal the elution modules with adhesive tape. Check that the sample well is completely filled with buffer.
   5. Perform Test: Confirm Separation and Elution currents are within continuity ranges.
   6. Load Sample: Remove 40µl buffer from sample well and load the prepared sample. Run the program and monitor the run. When run has completed, collect sample from the elution module by removing the adhesive seal and transferring the volume to a new 1.5 mL collection tube. The final volume is approximately 40 µl.
   7. Start Protocol. System will change to Idle when elution is complete.
   8. Recover Sample: Remove the adhesive seal from the elution module. Transfer eluted sample from the module to a new 1.5mL centrifuge tube.

Concentrate Size Selected RAD Library Use Agencourt AMpure XP Reagent to concentrate the Size Selected RAD library. Adjust eluted Size-Selected RAD library to 50ul with water. Add 0.8X AMpure XP Reagent (40 µl) to the size selected RAD Library. Mix by pipette 10 times and incubate for 10 minutes. Place on magnet for 2 minutes. Discard supernatant. Wash beads two times with 200 µl 70% Ethanol solution. Elute the RAD Library with 32.5 µl 1XTE. Incubate for 2 minutes and then place on the magnet. Transfer 30.0 µl to a new 1.5 mL tube.

# Determine Molar Concentration

Determine Average Fragment Length Use 2.0 – 3.0 µl of RAD library for the Bioanalyzer DNA 7500 DNA Assay to determine average fragment length (bp). (Reduce the DNA7500 Marker solution accordingly.) Expected average length is between 450 +/- 20bp.

Calculate Concentration by Qubit Measure 1.0 µl of the RAD library with the Qubit HS DNA Assay kit. Record concentration as ng/µl. The concentration should be at least 1.5 ng/µl. Calculate the molarity of the RAD library with this formula:

DNA molarity (nM ) =[(DNA conc.(ng/µl) \* 1000)/(649\*avg.size(bp)] \* 1000

Calculate Concentration by qPCR Dilute RAD library 15,000 times. Prepare 150X dilution with 2.0 µl of the RAD library and 298.0 µl of 0.1% Tween-20. Then, transfer 5.0 µl of 150X dilution to three (3) new 1.5mL microcentrifuge tubes containing 445.0 µl of 0.1% Tween-20 to make three replicates of 15,000X dilution.

Prepare three qPCR replicates for each sample and three (3) NTC replicates. Use the KAPA Quant Data Template to calculate sample concentration and molarity. Record ng/µl and nM. The molar concentration should be at least 2.0nM.

# APPENDIX

## ***Validation: Single & Double Restriction Digest***

Single and double restriction digest with EcoRI and MseI Digest genomic DNA with two enzymes: EcoRI and MseI. Perform Bioanalyer Smear Analysis to validate single and double restriction digest reactions.

*Required Amount of Genomic DNA*

* High Input: 0.6 – 1.8ug total
* Low Input: 40 – 70ng total

*Bioanalyzer Analysis of digested DNA*

* High Input: DNA7500 Chip
* Low Input: High-Sensitivity DNA Chip

### Restriction Digestion of DNA

Thaw the restriction digest reagents on ice. Keep enzymes in freezer until ready to use. Remove the prepared DNA strip tube from the freezer. Briefly centrifuge the strip tube. Place on ice.

1. Dilute genomic DNA to a final volume of 20ul with water.
   1. High Input: 30 – 90 ng/ul
   2. Low Input: 2 – 3.5 ng/ul
2. Transfer 6ul of diluted genomic DNA to three reaction tubes. Label as #1, #2, #3. (Use either PCR strip tubes or 96-well PCR plate). Briefly centrifuge the strip tube to bring its contents to the bottom of the wells. Store on ice or at -20°C to -80°C until ready to use.
   1. Note: Each well contains DNA from the same source, but a different restriction digest master mix is added to each.
3. According to the **Restriction Digest Table**, prepare three separate restriction digest master mixes. Gently pipette to mix.
   1. Only MseI
   2. Only EcoRI
   3. Both MseI and EcoRI
4. Add 2.6 µl of a different master mix to each DNA. Pipette 5 times to wash the pipette tip. Centrifuge briefly. Keep ice.
   1. #1 - Only MseI
   2. #2 – Only EcoRI
   3. #3 – both MseI and EcoRI
5. In a thermal cycler, incubate at 37°C for 2 hours, then at 65°C for 20 minutes with heated lid at 105°C, followed by a 4°C hold.
6. Remove the reactions from the thermal cycler. Briefly centrifuge to bring its contents to the bottom of the wells. Place on ice, and proceed to the next step. This is now the digested DNA.

|  |  |  |  |
| --- | --- | --- | --- |
| Restriction Digest Table | | | |
| **Component** | **Only MseI (µl)** | **Only EcoRI (µl)** | **Both MseI and EcoRI (µl)** |
| 10X T4 Buffer | 10.0 | 10.0 | 10.0 |
| NaCl, 1M | 5.2 | 5.2 | 5.2 |
| BSA, 1mg/mL | 5.2 | 5.2 | 5.2 |
| MseI enzyme, 10,000U/mL | 1.0 | --- | 1.0 |
| EcoRI enzyme, 20,000U/mL | --- | 2.5 | 2.5 |
| Water | 4.6 | 3.1 | 2.1 |
| Final Volume | 26.0 | 26.0 | 26.0 |
| NOTE: This is enough reagent for 8 reactions of each. | | | |

### Purification of Digested DNA

1. Add 21.5 µl of water to digested DNA from previous step. Final volume is now ~ 30 µl.
2. Add 1.65X volumes of Agencourt AMpure XP Reagent (50 µl) to each sample.
3. Slowly pipette 10 times to mix the reagent and sample. Incubate for 15 minutes at room temperature.
4. Place on the magnet for 5 minutes or longer until solution clears. Aspirate the cleared solution and discard.
5. Add 200 µl of 70% Ethanol. Incubate for 30 seconds at room temperature. Aspirate the ethanol and discard. Repeat steps 5b once more for a total of two washes. Remove ALL ethanol. Wait 2 to 5 minutes for the beads to dry.
6. Remove the tubes from the magnet. Add 13 µl of Nuclease-free water to each tube. Pipette mix 20 times. Incubate at room temperature for 5 minutes. Place the tube on the magnet for 5 minutes or longer until solution is clear.
7. Transfer 10 µl from the tubes into separate 1.5 mL microcentrifuge tube. Do not disturb the beads.
8. Proceed with next step.

*Determine Concentration by Qubit*

Low Input: Measure 3.0 µl of the RAD library with the Qubit HS DNA Assay kit. Record concentration as ng/µl.

High Input: Measure 1.0 µl of the RAD library with the Qubit HS DNA Assay kit. Record concentration as ng/µl.

*Evaluate Fragment Distribution*

Low Input: Load 1.0 µl of the RAD library into Bioanalyzer High-Sensitivity DNA Chip. Evaluate smear pattern.

High Input: Load 1.0 – 2.0 µl of the RAD library into Bioanalyzer DNA7500 Chip. (Reduce the DNA7500 Marker solution accordingly.) Evaluate smear pattern.

## Updates

* DNA sample volume increased to 12ul. Restriction digest and ligation master volumes adjusted to compensate.
* Three successive rounds of purification of amplified fragments (i.e. RAD Library) with AMpure XP Bead Reagent.
* Automated size selection of RAD Library includes purification of size-selected library with AMpure XP Bead Reagent.
* Quantitate final library with KAPA Library Quantification Kit – Illumina to estimate loading concentration.

## Reagents

* EcoRI (20,000 units/mL, P/N R0101L, New England BioLabs)
* MseI (10,000 units/mL, P/N R0525L, New England BioLabs)
* T4 DNA Ligase (400,000 units/mL, P/N M0202L, New England Biolabs)
* iProof High-Fidelity Polymerase (500 units, P/N 172-5302, Bio-Rad)
* dNTP Set [100mM] (4x0.25mL, P/N 786-460, G-Bioscience)
* Bovine Serum Albumin, BSA [10mg/mL] (provided with EcoRI and MseI)
* Sodium Chloride Solution, NaCl [5M] (multiple vendors)
* Dimethyl Sulfide, DMSO [100%] (5x10mL, P/N N182-5X10ML, Amresco)
* Tris-HCl, 1M, pH 9.0 (multiple vendors)
* EDTA Solution, 0.5M, pH 8.0 (multiple vendors)
* Nuclease-free water (multiple vendors)
* Ethanol, 200 Proof (multiple vendors)
* Tween-20
* Agencourt AMPure XP (5mL, P/N A63880, Beckmann Coulter)
* Qubit® dsDNA HS Assay Kit (100 rxn, P/N Q32851, Invitrogen)
* BluePippin Gel Cassette, 1.5%, and Marker R2 (Sage Science)
* Bioanalyer DNA 7500 DNA Kit (Agilent)
* Library Quantification Kit – Universal (500 x 20µl, P/N KK4824, KAPA Biosystems)

## Equipment

* Thermal Cycler, 96-well (multiple vendors)
* Benchtop Plate Centrifuge (multiple vendors)
* Microcentrifuge (multiple vendors)
* Benchtop Vortex (multiple vendors)
* Nandrop Spectrophotometer
* Qubit Fluorometer (Invitrogen Life Technologies)
* DynaMag-2 Magnet (P/N 12321D, Invitrogen Life Technologies)
* BluePippin, Automated DNA size selection system (Sage Science)
* Agilent Bioanalyzer 2100 Instrument
* CFX Real-Time PCR Detection System (BioRAD) or equivalent
* Pipette Set [P10, P20, P200, P1000] (multiple vendors)
* Pipette, 8-channel [P10 and P100] (multiple vendors)
* Pipette tips [P10, P20, P200, P1000] (multiple vendors)
* PCR Plates, 96-well, no-skirt, sterile (multiple vendors)
* Hard-Shell 96-Well PCR Plates (P/N HSP9601, BioRAD)
* Microfilm B (P/N MSB1001, BioRAD)
* PCR Adhesive Seals (100/PK, P/N AB0558, Fisher Scientific)
* PCR-strip tubes, 8-wells, sterile (multiple vendors)
* PCR tube, 0.2mL, sterile (multiple vendors)
* Microcentrifuge tubes, 1.5mL (multiple vendors)
* Centrifuge tubes, 15mL (multiple vendors)
* Centrifuge tubes, 50mL (multiple vendors

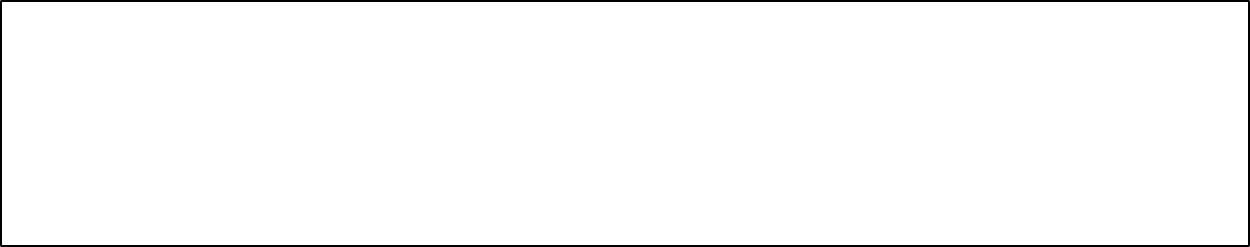
## Oligonucleotide Sequences

**Barcoded EcoRI Adapter** The structure of the barcoded EcoRI Adapter is unchanged between the original method and the Paired-end (PE) method. For more detail, refer to “Amplified restriction fragments for genomic enrichment by Parchman, Gompert, and Buerkle (version 2.3 August 2011).

Paired-end MseI Adapter The MseI Adapter is modified from the original sequence to facilitate Paired-end (PE) Sequencing.

*MseI Oligonucleotide-1* 5’-GCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3’

*MseI Oligonucleotide-2*  5’-TAGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATCTCGTATGCCGTCTTCTGCTTG-3’



**3’- GTCAGTGTAGTGCTAGAGCATACGGCAGAAGACG-5’ [Illumina PCR Primer 2]**

**3’- CTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTGTAGTGCTAGAGCATACGGCAGAAGACG-5’ [MseI Adapter 1]**

**5’-TAGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATCTCGTATGCCGTCTTCTGCTTG-3’[MseI Adapter 2]**

Figure 2.1: Oligonucleotide sequences modified to accommodate Paired-end (PE) sequencing strategy. The sequence of Paired-end TruSeq Sequencing Primer from the Illumina TruSeq Kit was added to the 3’-end of the MseI Adapter 1. Adjustments to the Illumnia Selective PCR Primer 2 were made to accommodate the addition of the sequencing primer site. MseI Adapter 2 is the reverse compliment of MseI Adapter 1 and the strand read during Paired-end (PE) sequencing. John Tan, University of Notre Dame, prepared modified MseI Adapter sequences.

Illumina PCR PrimersThe Illumina PCR Primers are designed to amplify only DNA fragments with the EcoRI and MseI Adaptor sequences. In addition, phosphothiolate bonds are added to the first two bases of the 5’-end of both the forward and reverse primer to inhibit endo- nuclease and exo-nuclease from acting on the adapter ligated DNA fragments during the PCR amplification reaction. These phosphothiolate bases are a variant of normal DNA; a sulfur molecule replaces one of the non-bridging oxygen molecules. The asterisks represent phosphothiolate-modified bases within the oligonucleotide sequence.

*Illpcr1* 5’- A\*A\*TGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3’

*Illpcr2* 5’- C\*A\*AGCAGAAGACGGCATACGAGATCGTGATGTGACTG-3’

## Working Solutions

**Restriction digest and ligation using 6ul DNA sample**

* **Bovine Serum Albumin, BSA [1mg/mL]** Combine 100 µl of BSA [10mg/mL] with 900 µl of water to a final volume of 1000 µl. Aliquot 100 µl volumes and store at -20̊C.
* **Sodium Chloride Solution, NaCl [1M]** Combine 100 µl of NaCl [5M] with 400 µl of water to a final volume of 500 µl. Aliquot 100 µl volumes and store at -20̊C.

**Restriction digest and ligation using 12ul DNA sample**

* **Bovine Serum Albumin, BSA [2mg/mL]** Combine 200 µl of BSA [10mg/mL] with 800 µl of water to a final volume of 1000 µl. Aliquot 100 µl volumes and store at -20̊C.
* **Sodium Chloride Solution, NaCl [2M]** Combine 200 µl of NaCl [5M] with 300 µl of water to a final volume of 500 µl. Aliquot 100 µl volumes and store at -20̊C.

Anneal EcoRI Adapter In a 96-well PCR Plate, mix 1µl of EcoRI barcoded oligonucleotide (100µM) from both Plate A and Plate B with 98µl of water. Heat to 95°C for 5 minutes with heated lid at 105°C and then cool to room temperature. This annealing step only needs to be performed once. The prepared EcoRI Adapter 96-well plate is enough annealed, double- stranded EcoRI adapter stock for up to 90 x 96-well plates.

Anneal MseI Adapter In a 1.5 mL tube, combine 13µl of MseI Oligonucleotide-1 (100µM) and 13µl of MseI Oligonucleotide-2 (100µM) with 104µl of water. Heat to 95°C for 5 minutes with heated lid at 105°C and then cool to room temperature. This annealing step only needs to be performed once. Note: The prepared 130µl stock is enough annealed, double-stranded MseI adapter stock for 96 DNA samples or a single 96-well plate.

Illumina PCR Primer Mix Combine 50µl of Illpcr1 (100µM) and 50µl of Illpcr2 (100µM) with 900µl of water to make 1000µl of Illumina PCR Primer Mix with 5 pmol/µl (5µM) of each PCR primer. Note: The prepared 1000µl Illpcr primer mix is enough working stock for six 96-well PCR plates.

**dNTP Mix, 10mM** Combine 100 µl of each dNTP (100 µM) and dilute with 600 µl Water to a final volume of 1000 µl. Aliquot 100 µl volumes and store at -20̊C.

**0.1X TE (Tris-HCl, EDTA) Solution, pH7.5-8.0** To prepare the primary stock (10X TE), combine 5 mL 1M Tris-HCl, pH 9.0 with 1 mL 0.5M ETDA, pH 8.0 and dilute with 44 mL of water to final volume of 50mL. Next, prepare the second stock (1X TE) by diluting 5mL of 10X TE with 45 mL of water to final volume of 50 ml. Finally, prepare the working stock (0.1X TE) by diluting 5 mL 1X TE with 45 mL of water to final volume of 50mL.

**Ethanol Solution, 70% v/v** Combine 35 mL Ethanol, 200 Proof with 15 mL of water to final volume of 50mL