GBS Library Construction Using MagNA Bead Clean Up

## Work Flow (per 2x96 well plates)

1. Normalize DNA and aliquot 200ng – 1.5 hours
2. Digest DNA – 3 hours
3. Adapter Ligation – 1.5 hours
4. Quantify and Pool DNA – 1 hour
5. MagNA Clean up # 1 – 1 hour
6. Adapter Fill in – 30 minutes
7. Size selection clean up – 1 hour
8. Indexing PCR (add Illumina primers) – 40 minutes
9. MagNA clean up # 3 – 1 hour
10. BioAnalyzer QC check – 1 hour

# Pre-work

### MagNA recipe (in-house replacement of SPRI beads)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Reagent** | **50mL end Volume** | **200mL end volume** | **750mL end volume** | **1L end volume** |
| carboxyl-coated Ser-Mag SpeedBeads | 1 mL | 4 mL | (makes 15 falcon tubes) | (makes 20 falcon tubes) |
| PEG 8000 | 9g | 36g | 135g | 180g |
| 5M NaCl | 10mL | 40mL | 150mL | 200mL |
| 1M Tris-HCL pH 8.0 | 500uL | 2mL | 7.5mL | 10mL |
| 0.5M EDTA | 100uL | 400uL | 1.5mL | 2mL |
| 10% Tween | 250uL | 1mL | 3.75mL | 5mL |

\* Bring up to volume with H2O

*Note: Recipe makes varied number of 50mL falcon tubes. Store MagNA kit in the refrigerator in the dark and vigorously mix before use. Kit is stable for at least 3 months.*

1. Make buffer in 50mL falcon tubes
2. Re-suspend the SpeedBeads by vortexing, allow the solution to reach room temperature.
3. Transfer 1mL of SpeedBeads into a 2mL tube
4. Because the beads contain sodium azide, they are washed 3 x with 1xTE in the magnetic rack
   1. Add 1mL of beads into tube
   2. Remove supernatant by pipetting out liquid on magnetic holder
   3. Remove from magnet and add 1mL of 1xTE and mix well (pipetting up and down)
   4. Repeat steps b + c 2 more times
5. Be sure to remove the TE buffer completely from beads (pipette out)
6. beads are gradually re-suspended in the buffer and placed in the 50mL falcon (Submit 40ml buffer in 50ml falcon, then wash out the tube of cleaned beads with the remaining 10mL of buffer – beads tend to stick to tube, so a few washes of 1mL buffer at a time to get all the beads)
7. Keep refrigerated (4C). Prepared beads are good for at least 3 months (stick with expiry date from beads manufacturer)

## Normalize sample PCR plates to 20ng/uL in TE buffer.

1. Follow the [PicoGreen](onenote://C:/Users/JamesBC/Documents/OneNote%20Notebooks/Protocols/Protocols%20-%20Validated/PicoGreen.one#section-id={7E95D579-C709-4AA6-B9CD-EAB75B8140D5}&end) protocol to quantify DNA plates
2. Transfer 10uL of normalized DNA (200ng total DNA) into a new 96 well PCR plate
3. Foil seal the original plate, temp seal the 200ng DNA plate

*\* Plates can be frozen (-20) at this point for later*

# End Pre-work

## DNA digestion using PstI and MspI restriction enzymes

**Required supplies:**

DNA PCR plate (20ng/uL = 200ng total)

10X CutSmart

PstI-HF (20,000 units/ml)

MspI (20,000 units/ml)

### Digestion Master Mix Recipe

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Reagent** | **Individual sample (uL)** | **Half plate (uL)** | **Plate of 96 (uL)** | **2 PCR plates (uL)** |
| 10X CutSmart | 4.0 | 208 | 416 | 832 |
| Pstl-HF (8 units) | 0.7 | 36.4 | 72.8 | 145.6 |
| Mspl (8 units) | 0.7 | 36.4 | 72.8 | 145.6 |
| H2O | 4.6 | 239.2 | 478.4 | 956.8 |
| Total | 10 | 520 | 1040 | 2080 |

*\* Avoid vortexing after the addition of enzymes*

1. Add the 10uL of master mix to 10uL of normalized DNA sample
2. Digest at 37C for 2 hours; 65C for 20 minutes; hold at 8C **(program RES-LIG)**

## Adapter Ligation

**Required supplies:**

18 uL Restriction enzyme + DNA (PCR plate)

Adapter Mix working stock - (0.02uM Adapter 1 = 1 0.1pmol, 3uM Adapter 2 = 15pmol)

T4 DNA ligase buffer 10X (found in freezer)

T4 DNA ligase (400,000 U/mL -- freezer)

50% PEG 4000 (found in fridge)

### Ligation Mastermix Recipe

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Reagent** | **Per sample (uL)** | **½ plate (uL)** | **1 Plate (uL)** | **2 Plates (uL)** |
| Water | 10 | 550 | 1100 | 2200 |
| T4 DNA Ligase Buffer (10X) | 4 | 220 | 440 | 880 |
| PEG 4000 (50%) | 4 | 220 | 440 | 880 |
| T4 ligase (400,000 U/mL) | 0.0125 | 0.7 | 1.4 | 2.8 |
| Total Volume Mastermix | 18 | 990.7 | 1981.4 | 3962.8 |

*\*\* some added buffer to account for dead volume for plate volumes\*\**

1. Be sure to mix the ligation mastermix well, and check the bottle for any precipitates (If the ligase buffer has a white precipitate, heat at 37 ° C, vortex until clear again). PEG is also very viscous, so mix well.
2. Combine 20μl DNA/enzyme mastermix (PCR Plate) with 18uL of ligation mastermix and mix well.
3. Add 2uL of adapter working stock to each well (each well is unique, if pooling 2 plates, be sure to add a different adapter set to each plate) -- pulse on centrifuge to mix
4. Thermocycler - 1 h at 22C; 8C hold **(program LIGATE - HOLD)**

## Measure the concentrations and Pool DNA

1. Quantify the DNA using the [PicoGreen](onenote://C:/Users/JamesBC/Documents/OneNote%20Notebooks/Protocols/Protocols%20-%20Validated/PicoGreen.one#section-id={7E95D579-C709-4AA6-B9CD-EAB75B8140D5}&end) protocol
2. Transfer 10ng from each well into a 2mL tube **(10(ng) / Concentration (ng/uL) = uL to transfer)**
3. Write how much DNA is being transferred on side of tube, as that value is needed for next step

*Note: Storage at -20 if needed*

## MagNA Purification

**Required supplies:**

2mL tube of DNA (variable volume)

MagNA Bead solution (Falcon tubes from Pre-Work section)

70% ethanol

### MagNA Bead and EB Volumes for Clean-Up

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Clean Up #** | **Sample volume (uL)** | **Bead volume/tube (uL)** | **total volume/tube (uL)** | **Elution Volume (uL)** |
| 1 | 384 | 691 | 1075 | 150 |
| 2 | 400 | 720 | 1120 | 60 |

1. Re-suspend the stock of MagNA beads by briefly vortexing the tube.
2. Add MagNA bead suspension to the pooled DNA as follows (1.8x volume of sample).
3. Mix the tube by pipetting up and down until the beads have dissolved well.
4. Let the tubes stand at room temperature for 5 minutes
5. Place the tubes on a magnetic rack, and let it stand for 5 minutes to separate the beads from the solution. Pipette off and discard the supernatant without removing the beads (beads will be pulled to sides of tube, so pipette from bottom-center)
6. **Leave the tubes on the rack**, add 1mL of 70% ethanol to the tube and mix by pipetting up and down 10 times (be sure all the beads are washed off side of tube). Let stand for 30 seconds. Place back on rack and allow beads to separate from ethanol for 1 minute, then remove the supernatant by pipetting it off. Repeat 2 more times (3 total washes)
7. Leave the tubes on the rack, and allow to air dry for at least 1 hour to completely remove the ethanol from the beads (if the beads are starting to crack or changed to a light brown color, they are ready for elution)
8. Take the plate off the magnetic rack and add elution buffer, mixing well by pipetting up and down 10 times (make sure all the beads have been washed into the EB from the side of the tube.
9. Incubate at room temperature for 1 minute
10. Place the tube back onto the magnetic rack and let sit for 5 minutes to separate beads and eluant. Transfer **just the eluted DNA supernatant** to a new 2mL tube (avoid the beads).

*Note: Storage at -20 if needed*

*Option - run 2uL of eluted DNA at this point on Qubit as a QC checkpoint*

## Adapter Fill in

**Required supplies:**

50uL adapter fill in + DNA (150uL in tube) = 200uL final volume

dNTPs - 100mM mixture (freezer)

Bst polymerase, large Fragment (8U/uL - in freezer)

### Adapter master mix recipe

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Volume (uL) per tube** | **Final concentration in 200uL reaction** |
| H2O | 17.5 |  |
| ThermoPol reaction buffer (10X) | 20 | 1X |
| dNTPs | 5 | 250uM each |
| Bst polymerase, large fragment (8 U/uL) | 7.5 | 0.3 U/uL |
| Total Volume | 50 |  |

1. Add 50uL of adapter mastermix to the sample DNA tube. Mix well by pipetting up and down.
2. Briefly spin down the sample to ensure everything is at bottom of tube
3. Incubate for 20 minutes at 37C on Eppendorf ThermoStat Plus

## Size Selection Using magnetic beads

**Required materials:**

Magnetic beads (MagNA or AMPure)

Ethanol (70%)

Elution Buffer (Tris-CL)

2mL tubes

#### Left side size selection

1. Add 180uL (0.9x the volume of sample) of beads to the tube containing the DNA and adapter master mix. Either pipette up and down a few times or briefly vortex/pulse the sample to homogenize the beads.
2. Allow the beads and sample to incubate for ~5 minutes at room temperature.
3. Place sample onto magnetic rack and allow beads to separate to side until clear. Remove the supernatant.
4. Add 400uL of ethanol to the sample (do not remove from rack) and allow to incubate at RT for 1 minute
5. Remove ethanol without disrupting the beads. Remove tube from rack once all ethanol removed.
6. Add 100uL of elution buffer to tube, mix well to elute all beads and allow to incubate at RT for 1 minute
7. Place sample back on rack to separate beads to one side, and allow the sample to clear up (about 1 minute)
8. Transfer the supernatant to a new tube (this is a left side size selected sample), throw away old tube and beads

#### Right side size selection

1. Add 56uL (0.56x the volume of sample) of beads to the tube containing the DNA and adapter master mix. Either pipette up and down a few times or briefly vortex/pulse the sample to homogenize the beads.
2. Allow the beads and sample to incubate for ~5 minutes at room temperature.
3. Place sample onto magnetic rack and allow beads to separate to side until clear.
4. Transfer the supernatant to a new tube (this is the right side size selected sample).

#### Clean up the now size-selected sample

1. Add 214uL (1.8x the volume of sample – original ratio for right side size selection) of beads to the tube containing the DNA and adapter master mix. Either pipette up and down a few times or briefly vortex/pulse the sample to homogenize the beads.
2. Allow the beads and sample to incubate for ~5 minutes at room temperature.
3. Place sample onto magnetic rack and allow beads to separate to side until clear. Remove the supernatant.
4. Add 400uL of ethanol to the sample (do not remove from rack) and allow to incubate at RT for 1 minute
5. Remove ethanol without disrupting the beads. Remove tube from rack once all ethanol removed.
6. Add 75uL of elution buffer to tube, mix well to elute all beads and allow to incubate at RT for 1 minute
7. Place sample back on rack to separate beads to one side, and allow the sample to clear up (about 1 minute)
8. Transfer the supernatant to a new 2mL tube (final size selected and cleaned sample)

*Note: Storage at -20 if needed*

*Option - run 2uL of eluted DNA at this point on Qubit as a QC checkpoint*

## Indexing PCR

**Required supplies:**

Size selected DNA (75uL total)

Phusion HF Buffer 5X - Freezer

dNTPs - 100mM mixture (freezer)

Illumina primers (10uM) - Freezer

Phusion Hot Start HF DNA polymerase (2U/uL - in freezer)

### Indexing Mastermix Recipe

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Volume/well (uL)** | **Total volume/tube (uL)** | **Final conc. in 50uL** |
| H2O | 29.1 | 261.9 |  |
| Phusion HF Buffer (5X) | 10 | 90 | 1X |
| dNTPs (25 mM each) | 0.4 | 3.6 | 200 uM each |
| 10uM Forward and Reverse Primers @ 10uM ("Illumina\_PE") | 1 | 9 | 200 nM |
| Phusion Hot Start High-Fidelity DNA Polymerase (2 U/uL) | 0.5 | 4.5 | 0.02 u/uL |
| total | 41 | 369 |  |

\*\* one well extra for total volume/tube to account for dead volume\*\*

1. Make 8 PCR reactions for each library by transferring 9uL of the eluted DNA from the tube to a well (from each tube, make a full column in a PCR plate)
2. Add 41uL of the indexing mastermix to the DNA sample in each well (41 x 8 = 328uL of mastermix per tube)
3. Run the new plate (50uL/well total now) on a PCR cycle shown below **(program WISSENSCHAFT)**:

|  |  |  |  |
| --- | --- | --- | --- |
| **Cycles** | **Step** | **Temp** | **Time** |
| 1 cycle | Initial Denaturation | 98C | 30 sec |
| 16 cycles | Denaturation | 98C | 10 sec |
| 16 cycles | Annealing | 60C | 20 sec |
| 16 cycles | Elongation | 72C | 20 sec |
| 1 cycle | Final Extension | 72C | 10 min |
| 1 cycle | Hold | 8C | indefinitely |

1. Pool the 8 reactions into a new 2mL tube after the cycle is complete move directly on to the final purification.
2. **Repeat MagNA purification (steps 11-20) following bead and elution volumes for clean up # 2.**

*Option - run 2uL of eluted DNA at this point on Qubit as a QC checkpoint*

*Note -- Final elution volume of 60uL (final library).*

## QC check library on Agilent BioAnalyzer

1. Load samples into the BioAnalyzer using the DNA 1000 chip.
2. Check the fragment size composition of the library by selecting the region of the peaks. Make note of the concentration and the fragment size of the library.

## Submit library for sequencing

1. Label a new tube for each library (1mL tube) and aliquot 20uL of size selected library into the labelled tubes for submitting for sequencing. Store in -20 freezer in “completed libraries” box.
2. Fill out the NRC “[Sequencing request Template](file:///L:\MolecularGroup\Molecular\Parkin%20Lab\Brian%20James\Genetic%20Diversity%20Program\6.%20%20NRC%20forms\library%20submissions)”