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# Manual Bisulfite Library Construction for Illumina Sequencing

## I. Purpose

To provide specific guidelines for manual plate-based Library Construction and Bisulfite Conversion for Illumina Paired-End Sequencing using NEB premix library construction reagents, Zymo EZ DNA Methylation Gold kits and KAPA Biosystems HiFi Hot Start Uracil Ready PCR enrichment kits. This SOP is intended for use in the case of small numbers of samples (12 or fewer).

## II. Scope

All procedures are applicable to the BCGSC Library Technology Development and Library Core Groups.

## III. Policy

This procedure will be controlled under the policies of the Genome Sciences Centre, as outlined in the Genome Sciences Centre High Throughput Production Quality Manual (QM.0001). Do not copy or alter this document. To obtain a copy see a QA associate.

## IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of the Quality Assurance team to audit this procedure for compliance and maintain control of this procedure.

## V. References

| SOP/Reference Title | SOP/Reference Number |
|---|---|
| Sample Preparation for Paired-End Sample Prep Kit from Illumina | Version 1.1 |
| EZ DNA Methylation Gold MagPrep Kit | D5042 |
| Kapa HiFi Hot Start Uracil$^+$ Ready Mix | KM2801 |

## VI. Related Documents

| Document Title | Document Number |
|---|---|
| Operation of Covaris LE220 | LIBPR.0097 |
| Manual Bead Clean using Ampure XP Beads | LIBPR.0073 |

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| Document Title | Document Number |
|---|---|
| Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples | LIBPR.0017 |
| Quantifying DNA Samples using the Qubit Fluorometer | LIBPR.0030 |

## VII. Safety

All Laboratory Safety procedures will be complied with during this procedure. The required personal protective equipment includes a laboratory coat and gloves. See the Material Safety Data Sheet (MSDS) for additional information.

## VIII. Materials and Equipment

| Name | Supplier | Number: # | Model or Catalogue # | |
|---|---|---|---|---|
| Sorvall Legend RT Centrifuge | Beckman Coulter | 717004 | ✓ | |
| Bench Coat (Bench Protection Paper) | Fisher | 12-007-186 | | ✓ |
| Black ink permanent marker pen | VWR | 52877-310 | | ✓ |
| NEB Paired-End Sample Prep Premix Kit – End Repair | NEB | E6875B-GSC | | ✓ |
| NEB Paired-End Sample Prep Premix Kit – A Tail | NEB | E6876B-GSC | | ✓ |
| NEB Paired-End Sample Prep Premix Kit – Ligation | NEB | E6877B-GSC | | ✓ |
| DNA*AWAY* | MBS | 7010 | | ✓ |
| dNTP Mix (10 mM) | Invitrogen | 18427-088 | | ✓ |
| GSC Methylated PE Adapters | IDT | In-house annealed | | ✓ |
| Eppendorf Benchtop Centrifuge 5424 | Eppendorf | 5424 | ✓ | |
| EZ DNA Methylation Gold MagPrep Kit | Cedarlane | D5042 | | ✓ |
| EZ DNA Methylation Gold Kit | Cedarlane | D5005 | | ✓ |
| CT conversion reagent (10 reactions) for added flexibility | Cedarlane | D5001-1 | | ✓ |
| Fisherbrand Textured Nitrile gloves (various sizes) | Fisher | 270-058-53 | | ✓ |
| Soft touch gloves (various sizes) | Ultident | 296359683 | | ✓ |
| Galaxy mini-centrifuge | VWR | 37000-700 | ✓ | |
| 20µL Pipet-Lite | Rainin | L12-20 | ✓ | |
| 200µL Pipet-Lite | Rainin | L12-200 | ✓ | |
| Barrier Rainin tips 10µL | Rainin | RT-L10F | | ✓ |
| Barrier Rainin tips 200µL | Rainin | RT-L200F | | ✓ |
| Gilson P2 pipetman | Mandel | GF-44801 | | ✓ |
| Gilson P10 pipetman | Mandel | GF-44802 | | ✓ |
| Gilson P20 pipetman | Mandel | GF23600 | | ✓ |
| Gilson P200 pipetman | Mandel | GF-23601 | | ✓ |

| Gilson P1000 pipetman | Mandel | GF-23602 | | ✓ |
|---|---|---|---|---|
| Finnpipette F1 0.2-2µL, micro | Thermoscientific | 4641010 | | ✓ |
| Finnpipette F1 2-20µL | Thermoscientific | 4641060 | | ✓ |
| Finnpipette F1 20-200µL | Thermoscientific | 4641080 | | ✓ |
| Finnpipette F1 100-1000µL | Thermoscientific | 4641100 | | ✓ |
| Ice bucket | Fisher | 11-676-36 | | ✓ |
| IKA Works Vortexer | Agilent | MS2S9- | ✓ | |
| Large Kimwipes | Fisher | 06-666-117 | | ✓ |
| Diamond Filter Tips 10µL | Mandel | GF-F171203 | | ✓ |
| Diamond Filter Tips 30µL | Mandel | GF-F171303 | | ✓ |
| Diamond Filter Tips 200µL | Mandel | GF-F171503 | | ✓ |
| Diamond Filter Tips 1000µL | Mandel | GF-F171703 | | ✓ |
| Finntip Filter Tips 10µL, micro | Thermoscientific | 21377354 | | ✓ |
| Finntip Filter Tips 20µL | Thermoscientific | 21377353 | | ✓ |
| Finntip Filter Tips 200µL | Thermoscientific | 14386374 | | ✓ |
| Finntip Filter Tips 1000µL | Thermoscientific | 21377604 | | ✓ |
| Nuclease Free 1.5 mL tube | Ambion | 12400 | | ✓ |
| Peltier Thermal Cycler | MJ Research | PTC-225 | ✓ | |
| Kapa HiFi Hot Start Uracil+ Ready Mix | Kapa Biosystems | KM2801 | | ✓ |
| PE primer 1.0 | In house | N/A | N/A | N/A |
| PE indexed primers | IDT | N/A | | |
| Small Autoclave waste bags 10"X15" | Fisher | 01-826-4 | | ✓ |
| Unmethylated Lambda DNA | Promega | D1521 | ✓ | |
| Wet ice | In house | N/A | N/A | N/A |
| VWR foil seals | VWR | 60941-126 | | ✓ |
| PCR Clean DX (ALINE beads) | ALINE Biosciences | C-1003-450 | | ✓ |
| AB1000 96-well plate | Thermo | FSSP9743245 | | ✓ |
| Abgene 1.2mL plate (for conversion reactions) | Abgene | AB1127 | | ✓ |
| MagMax Microtiter 96 Deep well plate (waste) | Applied Biosystems | 4388476 | | ✓ |
| Peltier adapter/heater (for bead drying) | In-house build | N/A | N/A | N/A |
| Plate, 96-Well reservoirs, 450uL | PerkinElmer | 20815114 | | ✓ |

**These sequences are for internal use only:**

**Cytosine Methylated PE Adapters (for ordering)**

**A/iMe-dC/A/iMe-dC/T/iMe-dC/TTT/iMe-dC//iMe-dC//iMe-dC/TA/iMe-dC/A/iMe-dC/GA/iMe-dC/G/iMe-dC/T/iMedC/TT/iMe-dC//iMe-dC/GAT/iMe-dC/*T**

* Phosphorothioate Bond

**/5Phos/GAT/iMe-dC/GGAAGAG/iMe-dC/GGTT/iMe-dC/AG/iMe-dC/AGGAATG/iMe-dC//iMe-dC/GAG**

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**In plain sequence (for reading):**
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'
3' GAGCCGTAAGGACGACTTGGCGAGAAGGCTAG 5'

**PE PCR Primers**
PE PCR 1.0:
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
PE PCR 2.0 with index
5' CAAGCAGAAGACGGCATACGAGATNNNNNNCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

**Bisulfite Conversion Library Construction Work Flow**

Qubit Quant the Starting Material (Biospecimen Core)

Normalize input to 1000ng/well

Spike-in Lambda DNA at 10ng per 1ug library sample

Shear DNA on the Covaris for 110 (2x55) seconds

Agilent QC

Bead clean (1:1 Bead Ratio)

End Repair Reaction

Bead clean (1:1 Bead Ratio)

"A" Addition Reaction

Methylated PE Adapter Ligation

Bead Clean (1:1 Bead Ratio x 2)

Bisulfite Conversion using
EZ DNA Methylation Gold Kits

5 cycle PCR with Kapa 2x HiFi Hot Start
Uracil+ Ready Mix

Bead Clean (1:1 Bead Ratio x 2)

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| Agilent and Qubit QC<br><br>Library Dilution<br><br>Pooling/Qubit QC if required |
|---|

**Starting Material Requirement**

A minimum of 1.0 µg DNA as measured by Qubit or Quant-iT is required. Your supervisor will specify if less than 1.0µg of DNA is to be used.

**Note:**

- LIMS must be done at the end of each day. Refer to Appendix A.
- 1.0µg HL60 genomic DNA will be used as a positive control all the way up to post PCR size selection QC
- EB will be used as a negative control all the way up to post PCR size selection QC

## IX. Procedure

Note: ALINE beads can be used as a direct replacement of Ampure XP magnetic beads in steps that specify the use of Ampure XP magnetic beads.

### 1. Introduction and Upstream Set Up

1.1. Put on a clean pair of gloves and a lab coat. In the 6th floor library construction room, wipe down the assigned specific workstation, pipettors, and small equipment with DNA away. Lay down new bench coat and retrieve ice and all required reagents.

Note: Always wear gloves when handling sample plates, reagents and equipment, and treat everything with clean PCR techniques.

1.2. General guidelines for library construction:

A. *Never re-use plate sealing tape.*
B. *To avoid cross-well contamination, reaction plates should never be vortexed.*
C. *Plate mixing steps are only to be done using manual 12-channel pipette, or single channel pipette.*
D. *All premixed brews should be thawed on ice and then should be well mixed in tubes by inversion and flicking the tube before dispensing into reaction plates to assure equal distribution of all components and uniformity of enzymatic reactions across a plate.*

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E. *All pre-PCR work is done in the 6th floor library construction room. Post PCR work is done in the 6th floor PPGP area.*

1.3. Plates can be stored at -20°C overnight with foil seal after every step except after "A addition and ligation.

NOTE: "A" addition and adapter ligation reactions must be completed on the same day.

1.4. Store "A" addition reactions at 4°C (or on ice) until ready to proceed (within the same day) to adapter ligation. **Note that A-tailed reactions are not bead cleaned prior to ligation.**

1.5. Single-use aliquots of GSC Methylated PE Illumina adapters should be kept on ice and never refrozen.

1.6. PCR reaction brews may be made in the 5th floor laminar hoods or in the 6th floor Library Construction Room's biological safety cabinet. Template may be added at a pre-PCR workstation.

1.7. Indexing PCR primers can be thawed up to 4 times and then must be discarded.

## 2. General note on bead clean up module:

The bead cleanup modules employed in this SOP are based on the following conditions:

| Bead Binding Time (mins) | 1st Magnet Clearing Time (mins) | 2 X 70% EtOH Wash Vol (µL) | Ethanol Air-dry Time (mins) | Elution Volume (µL) | Elution time (mins) | 2nd Magnet Clearing time (mins) |
|---|---|---|---|---|---|---|
| **15** | **7** | **150** | **5** | **20-52** | **3** | **2** |

Note: The bead to reaction ratio is 1:1 for all steps to ensure that the final library size is maximized.

**Allow ALINE beads or Ampure XP beads and 70% ethanol to equilibrate to room temperature by storing at room temperature for at least 15 minutes prior to use. Failure to do so may result in a reduction in yield.**

The elution volumes are specified below for each of the steps:

| Step | Elution Volume (µL) |
|---|---|
| Post-Shearing | 37 |

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| Step | Elution Volume (µL) |
|---|---|
| Post-End repair | 32 |
| Post-A addition | N/A |
| Post-ligation 1st cleanup | 52 |
| Post-ligation 2nd cleanup | 25 |
| Post-iPCR 1st cleanup | 52 |
| Post-iPCR 2nd cleanup | 25 |

## 3. Unmethylated Lambda DNA Spike-In

3.1. In order to track the efficiency of bisulfite conversion, it is necessary to spike in unmethylated Lambda DNA to the starting material. Add 10 ng of Lambda DNA per microgram of starting material. This needs to be done before shearing.

3.2. Transfer samples to Covaris MicroTubes for shearing.

## 4. Covaris LE220 Series Shearing

Note: HL60 positive control and EB negative control will be treated as samples and therefore will be included at the sonication step.

4.1. For this protocol, samples (including the positive control) should contain 1µg of DNA per well (as quantified by Qubit Assay). The sample is topped up with Qiagen EB buffer for a total volume of 60 µL. Refer to the schedule given by the APCs to determine the amount required for sonication.

4.2. Refer to the following protocol for shearing.

| LIBPR.0097 Operation and Maintenance of the Covaris LE220 |
|---|

4.3. Run one (1) High Sensitivity Agilent chip for QC according to the following protocol. If more than 11 samples are processed, spot check 1 chip's worth of samples. Ensure to include the HL60 control. To prevent over-loading on the assay dilute all samples 5X prior to running the assay.

| LIBPR.0017 Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA Samples |
|---|

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4.4. Confirm with your supervisor whether sufficient sonication has been done to achieve the desired size range (300bp). If more sonication is required, repeat steps from LIBPR.0097 Operation and Maintenance of the Covaris LE220 and LIBPR.0017 Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA Samples and report a process deviation. The process deviation will initiate a change to the SOP if shearing conditions consistently result in under or over sheared gDNA.

## 5. Bead Clean up after shearing.

5.1. Clean up sheared gDNA with Lambda DNA spike in as described in the following SOP:

| LIBPR.0073- Manual Bead Clean using Ampure XP Beads |
|---|

5.2. Specific volumes are highlighted below. This pipeline uses 1:1 Bead:Sample ratio to increase the final library size without jeopardizing the final library yield.

| DNA volume (µL) | Bead Volume (µL) | Mixing Volume (µL) | Bead Binding Time (mins) | Magnet Clearing Time (mins) | Supernatant Volume (µL) | 2x 70% EtOH Wash Vol (µL) | Ethanol Air Dry Time (mins) | EB Elution Volume (µL) | Elution Time (mins) | Magnet Elution Time (mins) | Transfer Volume (µL) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 60 | 60 | 96 | 15 | 7 | 120 | 150 | 5 | 37 | 3 | 2 | 35 |

## 6. End Repair and Phosphorylation

6.1. The volume requirement for 1 reaction set up is as follows:

6.2. Dispense **23.5 µL of End Repair Premix** into each well of a destination plate.

| Solution | 1 rxn (µL) |
|---|---|
| DNA | 35 |
| NEB End Repair Premix | 23.5 |
| **Reaction volume** | **58.5** |

6.3. Transfer DNA to each well of the destination plate containing the reaction brew. Mix 10 times. Cover with plate seal and quick spin at 4ºC for 1 minute.

6.4. Incubate End-Repair reaction plate at 20ºC for 30 minutes.

**Tetrad Program: Run > LIBCOR > ER**

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## 7. Magnetic bead clean after End Repair

7.1. The input volume for this step is 58.5 µL per well.

7.2. Clean up End Repaired DNA using Magnetic beads as described in the following SOP:

LIBPR.0073- Manual Bead Clean using Ampure XP Beads

7.3. Specific volumes are highlighted below.

| DNA volume (µL) | Bead Volume (µL) | Mixing Volume (µL) | Bead Binding Time (mins) | Magnet Clearing Time (mins) | Supernatant Volume (µL) | 2x 70% EtOH Wash Vol (µL) | Ethanol Air Dry Time (mins) | EB Elution Volume (µL) | Elution Time (mins) | Magnet Elution Time (mins) | Transfer Volume (µL) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 58.5 | 58.5 | 93 | 15 | 7 | 117 | 150 | 5 | 32 | 3 | 2 | 30 |

**Note that end repaired product can be stored at -20°C after the bead cleanup**.

## 8. Addition of an 'A' Base (A-Tailing) Reaction

8.1. The volume requirement for 1 reaction set up is as follows:

| Solution | 1 rxn (µL) |
|---|---|
| End-Repair + BC DNA | 30 |
| NEB Adenylation Premix | 20 |
| **Reaction volume** | **50** |

8.2. Dispense **20 µL of Adenylation Premix** into each well of a destination plate. Cover with plate seal and quick spin at 4ºC for 1 minute.

8.3. Transfer DNA to each well of the destination plate containing the reaction brew. Mix 10 times. Cover with plate seal and quick spin at 4ºC for 1 minute.

8.4. Incubate A-tailed reaction plate(s) at 37ºC for 30 minutes, 70 ºC for 5 minutes, 4 ºC for 5 minutes; hold at 4 ºC using the following tetrad program:

**Tetrad Program: Run > LIBCOR > ATAIL**

8.5. Once the plate is held at 4C, proceed immediately to the next step. This is NOT a safe stopping point. **Do not bead clean Adenylated products. Proceed immediately to methylated adapter ligation.**

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## 9. Methylated PE Adapter Ligation Reaction

9.1. Thaw the Methylated PE Adapter stock aliquot at a designated Pre-PCR work bench then immediately place on ice.

9.2. Adapter Ligation brew (minus the methylated PE adapter) must be made in a designated laminar flowhood. Add the methylated PE adapter to the brew at a designated pre-PCR work bench.

9.3. The volume requirement for 1 reaction is as follows:

| Solution | 1 rxn (µL) |
|---|---|
| A-Tail reaction | 50 |
| 2X NEB Ligation Premix | 21 |
| Methylated PE Adapter (10 µM) | 4 |
| **Reaction volume** | **75** |

Methylated_ Ligation_Brew_40pmol (25µL)

9.4. Generate the Ligation-Brew Mix calculator using LIMS:

<u>LIMS</u>: Mix Standard Solution > **Methylated_Ligation_Brew_40pmol**> *follow the prompts*> Save Standard Solution

9.5. To minimize adapter-adapter ligation, work quickly on ice and proceed as follows:

9.5.1. Prepare the Ligation brew in an appropriate sized tube according to the chemistry calculator.

9.5.2. Add the Methylated PE adapter to the brew last, not more than 10min before brew addition to DNA. Make sure the brew is on ice all the time.

9.5.3. Immediately after the brew is prepared, dispense 25 µL of brew into each well of a destination plate.

9.5.4. Transfer Adenylated DNA to each well of the destination plate containing the reaction brew. Mix 10 times. Cover with plate seal and quick spin at 4ºC for 1 minute.

9.5.5. Cover the brew source plate with plate seal and quick spin at 4ºC for 1 minute.

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9.5.6. Keep plates on ice but *proceed quickly* to the next step.

9.6. Incubate Adapter Ligation reaction plate(s) at 20ºC for 15 minutes. Do not let the incubation go longer or hold at 4C. Quick spin plate and then proceed immediately to magnetic bead clean after adapter ligation.

**Tetrad Program: Run > LIBCOR > LIGATION**

## 10. Magnetic Bead Clean Up after Adapter Ligation

10.1. Clean up ligated DNA using Magnetic beads as described in the following SOP.

| LIBPR.0073- Manual Bead Clean using Ampure XP Beads |
|---|

10.2. Specific volumes are highlighted below. Note that two bead cleans are performed post ligation. Note that the EB elution volumes are different for the first and second bead clean.

**Bead clean #1**

| DNA volume (µL) | Bead Volume (µL) | Mixing Volume (µL) | Bead Binding Time (mins) | Magnet Clearing Time (mins) | Supernatant Volume (µL) | 2x 70% EtOH Wash Vol (µL) | Ethanol Air Dry Time (mins) | EB Elution Volume (µL) | Elution Time (mins) | Magnet Elution Time (mins) | Transfer Volume (µL) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 75 | 75 | 120 | 15 | 7 | 150 | 150 | 5 | 52 | 3 | 2 | 50 |

**Bead clean #2**

| DNA volume (µL) | Bead Volume (µL) | Mixing Volume (µL) | Bead Binding Time (mins) | Magnet Clearing Time (mins) | Supernatant Volume (µL) | 2x 70% EtOH Wash Vol (µL) | Ethanol Air Dry Time (mins) | EB Elution Volume (µL) | Elution Time (mins) | Magnet Elution Time (mins) | Transfer Volume (µL) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 | 50 | 80 | 15 | 7 | 100 | 150 | 5 | 25 | 3 | 2 | 23 |

## 11. Bisulfite Conversion

11.1. The Zymo Gold kits are optimized for 200-500 ng of template DNA for optimal conversion efficiency. The current library construction pipeline yields 400-500ng in 20µL post ligation given 1ug of high quality gDNA and stringent removal of small inserts.

11.2. Prepare the 10X CT Conversion reagent as described below (Catalog D5001-1).

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| Solution | Volume (uL) |
|---|---|
| Dried CT Conversion Reagent | NA |
| Ultrapure water | 900 |
| M-Dissolving Buffer | 50 |
| M-Dilution Buffer | 300 |

11.3. Mix prepared CT Conversion reagent with frequent vortexing or shaking for 15 minutes prior to taking an aliquot. Conversion reagent must be protected from light. Cover aliquots with foil or use light blocking tubes.

11.4. Prepared CT conversion reagent should be used immediately after preparation. If it is not being used immediately store the reagent at -20ºC for up to one month.

11.5. Pre-warm conversion reagent to 37ºC if using a previously frozen aliquot of the reagent. Double check the expiry date on the tube prior to use (one month expiry after preparation).

11.6. Transfer 130 µL of prepared Bisulfite Conversion Reagent to each well containing up to 500 ng of template. Mix 10X, cover with a foil seal and then quick spin plate. Cover the plate with a tetrad incubation pad and incubate at 98ºC for 10 minutes, 64ºC for 2.5 hours and 4ºC for up to 20 hours. Enter '100' for reaction volume (max allowable) and select 'Y' for heated lid.

**Tetrad Program: Run > LIBCORE > BSCONVER**

## 12. Bisulfite Conversion Clean Up using Columns (EZ Methylation Gold kit)

12.1. Column purification is intended for processing up to 12 samples. If requested by your APC, you may be asked to perform purification using the bead-based method. Please refer to step 13 for automated bisulfite conversion clean up.

12.2. Prepare M-Wash buffer as described on the bottle. Add 24 mL of 100% ethanol to the 6 mL M-Wash Buffer concentrate (D5005). Note that Ethanol has been added to the bottle.

12.3. Place a column into a provided collection tube and add 600 µL of M-Binding buffer to a Zymo-Spin TM IC column. Label columns with library name.

12.4. Pipette each bisulfite converted sample into a Zymo-SpinTM IC column containing the M-Binding Buffer. Close the cap and mix by inverting the column several times.

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12.5. Centrifuge at full speed (>10,000 x g) for 30 seconds. Discard the flow-through.

12.6. Add 100 µL of M-Wash Buffer to the column. Centrifuge at full speed for 1 minute. Discard the flow-through.

12.7. Add 200 µL of M-Desulphonation Buffer to the column and let stand at room temperature (20-30°C) for 15 minutes. Set a timer and do not go above the 15 minute incubation. After the incubation, centrifuge at full speed for 1 minute. Discard the flow-through.

12.8. Add 200 µL of M-Wash Buffer to the column. Centrifuge at full speed for 1 minute. Discard the flow-through. **Repeat the wash for a total of two washes.** Discard the flow-through.

12.9. Place the column into a fresh 1.5 mL micro-centrifuge tube. Add **21 µL** of M-Elution Buffer directly to the column matrix. Centrifuge for 1 minute at full speed to elute the DNA.

12.10. Proceed to PCR enrichment (Step 14) or store samples at -20°C for later use. For long-term storage, store at -80°C.

**13. Bisulfite Conversion Clean Up on Biomek FX (EZ Methylation Gold MagPrep Kit)**

13.1. The Biomek FX was programmed to allow for conversion clean up of 1 to 96 samples. Conversion clean up must be performed on Biomek FX located on the 5th floor.

13.2. Add 288 mL of anhydrous Ethanol to 72 mL wash buffer concentrate prior to starting Bisulfite Conversion Clean up. Indicate on the bottle that Ethano[l] has been added and record the date. The prepared solution has a six month expiry.

13.3. Prepare M-Elution buffer by aliquoting 50µL of M-Elution buffer per well of an empty AB1000 plate. Ensure that the wells match the active wells undergoing bisulfite conversion. Cover the plate will a foil seal, quick spin, and then load on a tetrad and incubate at 55ºC. The elution buffer will hold at 55C until samples are ready for their final elution.

**Tetrad Program: Run > LIBCOR > 55Hold**

13.4. Set a Peltier heat block with the 1.2mL plate adapter to 55ºC.

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13.5. Prepare Binding buffer by adding 10µL of MagBinding Beads to 600µL of M-Binding Buffer for each reaction as described below in an appropriate container for the number of reactions being processed. Mix well by inversion.

| | MagBinding Beads (µL) | M-Binding Buffer (mL) | Total volume (mL) |
|---|---|---|---|
| Per Reaction | 10 | 0.600 | 0.610 |
| 12 samples | 132 | 7.92 | 8.05 |
| 1 plate | 1056 | 63.4 | 64.4 |

13.6. Bisulfite conversion clean up is processed on Biomek FX. Log into the following Biomek FX Program:

| Biomek: Project > LIBPR> LibraryConstruction> RUN> Bisulfite >**Clean Up Conversion** |
|---|

13.7. The Biomek will ask if you would like the FX to pre-dispense wash buffer. Select yes if you are processing a full plate. If not, dispense M-Wash Buffer (with Ethanol added) into the appropriate wells of three 450µL plates using a Distrimann as described below:

| | M-Wash Buffer (µL/well) |
|---|---|
| Wash 1 | 480 |
| Wash 2 | 480 |
| Wash 3 | 250 |

13.8. Dispense 240µL of desulphonization buffer to the appropriate wells of a 450µL plate.

13.9. Follow the prompts to complete the deck layout. There are two layers. This process requires **Alpaqua MAGNUM FLX, 96M Ex magnet, alternate magnets are not compatible.**

13.10. Pulse-vortex Bead/M-Binding buffer and dispense 610µL per well of a 1.2mL Abgene plate and load plate onto the appropriate ALP on the Biomek. Remove plate covers and press play after double-checking the deck layout.

13.11. Note that desulphonization must not exceed 20-25 minutes as per the manufacturer. The Biomek process including mixing, binding and clearing totals <20minutes. If the Biomek fails during this step you must intervene and process the bead clean manually to prevent damage to your sample. Note a process deviation if this does occur.

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13.12. The total Biomek process will take about 1.5 hours. Samples will be transferred and bound to beads in buffer and then cleared and washed. Samples will then undergo desulphonization and two wash steps. Samples are dried on the custom Peltier plate heater with the 1.2mL adapter for 5 minutes set at 55ºC prior to hot elution.

13.13. At the end of the process, cover your bisulfite-converted DNA plate with an appropriate plate cover. Samples can be immediately enriched or the template can be stored at -20ºC until PCR enrichment.

## 14. Indexed PCR Amplification

14.1. Thaw the PE PCR primer 1.0 at a designated work bench and then store on ice.

14.2. Thaw the Indexing Primer Plate in a working bench then quick spin at 4ºC for 1 minute and immediately place on ice.

14.3. To keep track of freeze-thaw cycles, mark off the indexing primer plate each time the plate is thawed even if it is not used.

14.4. The maximum freeze-thaw cycles for the indexing primer plate are 5 times.

14.5. Methylated iPCR brew (minus the primers) must be aliquoted in a designated laminar flow hood. Add PE PCR primer 1.0 to the brew at a designated work bench.

14.6. Prepare the Bisulfite iPCR brew as described below:

**Bisulfite_Libconst_iPCR_Brew**

| Solution | Volume (µL) per well |
|---|---|
| KAPA 2X HiFi Ura+ Ready mix | 25 |
| PE PCR primer 1.0 (25uM) | 1 |
| Indexed PCR primer plate (12.5µM) | 2 |

Bisulfite_LibConst iPCR_Brew (26µL)

14.7. Generate the appropriate Bisulfite iPCR brew LIMS calculator:

> LIMS: Mix Standard Solution > **Bisulfite_LibConst_iPCR_Brew** > follow the prompts > Save Standard Solution

14.8. Obtain the 1D large Solution/Box/Kit Label and Chemistry Label. Prepare the brew in an appropriate sized tube according to the chemistry calculator.

14.9. Carefully dispense 26µL per well of an AB1000 plate using a Distrimann pipette.

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14.10. Add 22µL of template and 2µL of index primers directly to the Bisulfite iPCR brew.

14.11. Add 22 µL of water to the PCR brew control well.

14.12. Mix reaction 10X with a multichannel pipette, seal the plate(s) with VWR foil seal and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

14.13. Load the plate on a tetrad and run the following program. Enter '50' for reaction volume and select 'Y' for heated lid.

**Tetrad Program: Run > LIBCOR > BS-5X**

PCR Parameters

- 98˚C 1 min
- 98˚C 15 sec
- 65˚C 30 sec
- 72˚C 30 sec
- 72˚C 5min
- 4˚C ∞

Total of 5 cycles

14.14. Once the PCR program is complete, quick spin the plate and store the enriched template at -20ºC or proceed immediately to post PCR bead clean.

**15. Magnetic Bead Clean Up after indexed PCR enrichment**

**Note: Post PCR work must be performed in the PPGP/PPBP area in the 6th floor lab**

15.1. Clean up enriched template using Magnetic beads as described in the following SOP.

LIBPR.0073- Manual Bead Clean using Ampure XP Beads

15.2. Specific volumes Specific volumes are highlighted below. Note that two bead cleans are performed post PCR. Note that the EB elution volumes are different for the first and second bead clean.

**Bead clean #1**

| DNA volume (µL) | Bead Volume (µL) | Mixing Volume (µL) | Bead Binding Time (mins) | Magnet Clearing Time (mins) | Supernatant Volume (µL) | 2x 70% EtOH Wash Vol (µL) | Ethanol Air Dry Time (mins) | EB Elution Volume (µL) | Elution Time (mins) | Magnet Elution Time (mins) | Transfer Volume (µL) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 | 50 | 80 | 15 | 7 | 100 | 150 | 5 | 52 | 3 | 2 | 50 |

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**Bead clean #2**

| DNA volume (µL) | Bead Volume (µL) | Mixing Volume (µL) | Bead Binding Time (mins) | Magnet Clearing Time (mins) | Supernatant Volume (µL) | 2x 70% EtOH Wash Vol (µL) | Ethanol Air Dry Time (mins) | EB Elution Volume (µL) | Elution Time (mins) | Magnet Elution Time (mins) | Transfer Volume (µL) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 50 | 50 | 80 | 15 | 7 | 100 | 150 | 5 | 25 | 3 | 2 | 23 |

## 16. Agilent and Qubit QCs

16.1. Run 1 µl of sample PPBC product on an Agilent DNA 1000 assay according to the following protocol.

| LIBPR.0017 Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA Samples |
|---|

16.2. Discuss your results with your supervisor to determine whether the libraries can be sent for sequencing. The desired size range is from 300 to 600bp. See Appendix B for typical profiles expected from the protocol.

16.3. While the Agilent is running, quantify 1 µl of each sample with Qubit, according to the following protocol.

| LIBPR.0030 Quantifying DNA Samples using the Qubit Fluorometer |
|---|

16.4. Once the libraries have passed QC, determine the average size from the Agilent reading using 200:1000 smear analysis and calculate the nM based on the Qubit value (ng/µL).

16.5. Dilute the DNA sample if above 75nM in Buffer EB. Qubit the diluted DNA sample.

16.6. Label the tubes with the name of library, date and initial, and concentration.

16.7. For LIMS protocol, refer to Appendix A.

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## Appendix A

1. Start of Plate Library Construction– IDX pipeline
2. Bioanalyzer Run – QC Category: Sonication QC
3. M-Bisulfite Library Construction - IDX pipeline
4. Plate_Indexed_PCR – IDX pipeline
5. Plate_PPBC_SizeSelection – IDX pipeline
6. Bioanalyzer Run – QC Category: Post library construction size selection QC.

   Enter the following attributes:

   - Library_size_distribution_bp (From Agilent)
   - Avg_DNA_bp_size (From Agilent)
   - DNA_concentration_ng_uL (From Qubit)
7. If pooling: Aliquot pooling volume into a new TRA
8. If pooling: Pooling and/or Manual Rearray into tubes – IPE pipeline
9. Final_Submission – IPE or PET pipeline

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## Appendix B

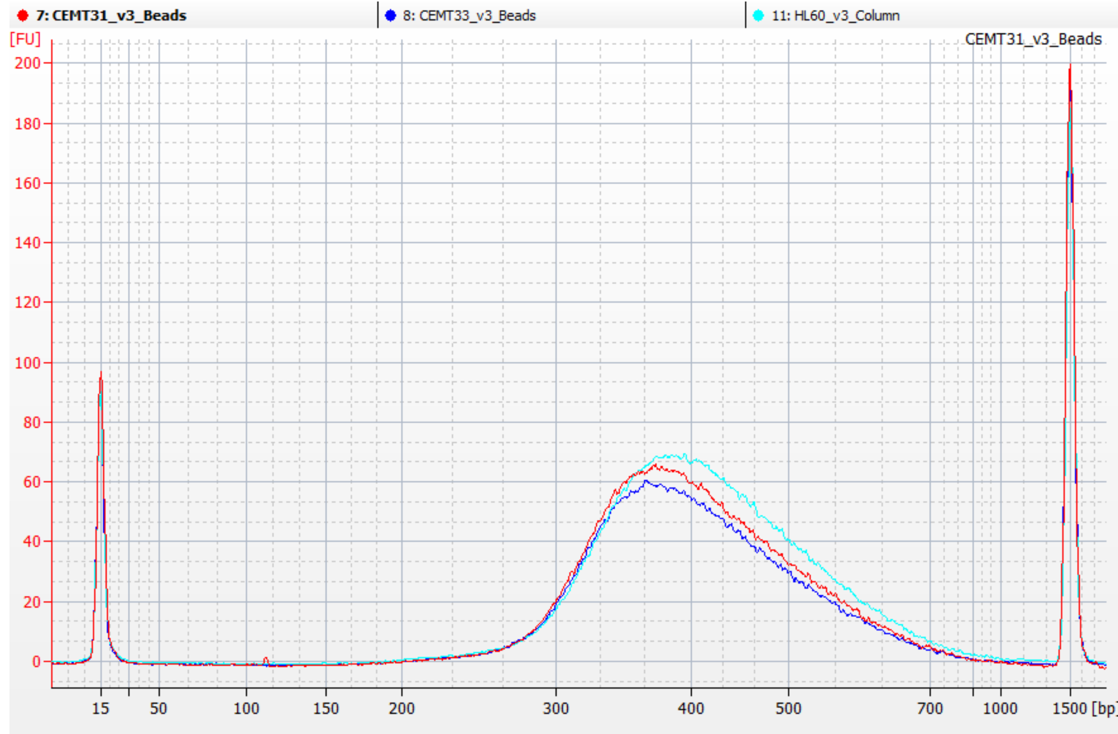


Figure 1. Typical profile on AB1000 assay of final library product after bisulfite conversion and 5X methylated iPCR enrichment. Note that these libraries were made using human gDNA. Plant samples may produce lower yields if shearing is inhibited but size distribution should be similar.

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# Appendix C
## Bisulfite Conversion Clean up

**Bead Binding**
Transfer 150µL (all) of the converted DNA to 610µL of Bead Binding solution. Set a multichannel pipette to 160µL and mix 10X.

| Conversion Mixture (µL) | Bead Binding Solution Volume (µL) | Mixing volume (µL) | # Mixes | Binding (min) | Magnet (min) | Supernatant Removal (uL) |
|---|---|---|---|---|---|---|
| 150 | 610 | 160 | 10 | 15 | 7 | 760 |

**Wash 1**
Remove the plate from the magnet and add 133µL of M-Wash buffer to each well, mix 10X using a multichannel pipette. Repeat two times to a total of 399µL.

| M-Wash Buffer (µL) | # Mixes | Binding (min) | Magnet (min) | Supernatant Removal (uL) | Dry time (min) |
|---|---|---|---|---|---|
| 3x133uL | 10 each | 1 | 1 | 400 | none |

**Desulphonization**
Remove the plate from the magnet and add 200µL of desuphonization buffer, mix 10X using a multichannel pipette.

| Desulphonization Buffer (µL) | # Mixes | Binding (min) | Magnet (min) | Supernatant Removal (uL) |
|---|---|---|---|---|
| 200 | 10 | 15 | 5 | 200 |

**Wash 2**
Remove the plate from the magnet and add 133µL of M-Wash buffer, mix 10X using a multichannel pipette. Repeat 3X to a total of 399µL.

| M-Wash Buffer (µL)* | # Mixes | Binding (min) | Magnet (min) | Supernatant Removal (uL) |
|---|---|---|---|---|
| 3x133uL | 10 each | 1 | 1 | 400 |

**Wash 3**
Remove the plate from the magnet and add 200µL of M-Wash buffer, mix 10X

| M-Wash Buffer (µL) | # Mixes | Binding (min) | Magnet (min) | Supernatant Removal (uL) | Dry time at 55C (min) |
|---|---|---|---|---|---|
| 200uL | 10 | 1 | 1 | 200 | 5 |

**Final Elution** Add pre-heated M-Elution buffer to dried beads, cover, quick spin and then incubate 4 minutes at 55ºC on the custom Peltier heater with 1.2mL adapter. Samples can be stored at -20ºC.

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| M-Elution buffer (uL) | Binding time at 55C (min) | Magnet time (min) | Transfer volume (µL) |
|---|---|---|---|
| 25 | 4 | 2 | 24 |

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