

Bisulfite Library Construction on Hamilton NIMBUS for Illumina Sequencing	
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Bisulfite Library Construction on Hamilton NIMBUS for Illumina Sequencing

I. Purpose

To provide specific guidelines for 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.

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 (*enter group name here*) Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Systems Team to audit this procedure for compliance and maintain control of this procedure.

V. References

Document Title	Document Number
Sample Preparation for Paired-End Sample Prep Kit from Illumina EZ DNA Methylation Gold Kit Kapa HiFi Hot Start Uracil ⁺ Ready Mix	Version 1.1 D5005 KM2801

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VI. Related Documents

Document Title	Document Number
Manual Bisulfite Library Construction for Illumina Sequencing	LIBPR.0091
Operation of Covaris LE220	LIBPR.0097
Manual Bead Clean using Ampure XP Beads	LIBPR.0073
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		✓
DNAAWAY	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 Kit	Cedarlane	D5005		✓
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	✓	

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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		✓
		MS2S9-		
IKA Works Vortexer	Agilent	Agilent-5065-4428	✓	
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, 450µL	PerkinElmer	20815114		✓

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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/iMe-dC/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

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' CAAGCAGAAGACGGCATAACGAGATNNNNNNCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

Bisulfite Conversion Library Construction Work Flow

Quant-iT/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)

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“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)
Caliper/Agilent and Quant-iT/Qubit QC
Library Dilution
Pooling/Qubit QC if required

IX. Introduction and Guidelines

1. Introduction and Upstream set up

- 1.1. Ensure proper personal protective equipment is used when handling sample plates, reagents and equipment. Treat everything with clean PCR techniques.
- 1.2. Wipe down the assigned workstation, pipetman, tip boxes and small equipment with DNA AWAY. Ensure you have a clean working surface before you start.
- 1.3. Pre-PCR and Post-PCR work should be performed at the designated workstations.
- 1.4. Acronyms: NA stands for Not Applicable. Pre-LC refers to Pre-Library Construction. Post-LC refers to Post-Library Construction. BC refers to Bead Clean.
- 1.5. Discuss with the supervisor/designated trainer the results of every QC step. Report and record equipment failures and/or malfunctions and variations in reaction well volumes.

2. General Plate Guidelines

- 2.1. Only 1 plate can be processed at a time by one technologist using the Hamilton NIMBUS.

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- 2.2. To avoid cross-well contamination, reaction plates should never be vortexed and plate seals should never be re-used. Use NIMBUS for mixing and protocol "Resuspend Samples.med" to resuspend samples.
- 2.3. Use only VWR foil seals for both short term storage and tetrad incubations and 3M aluminum foil seal for long term storage.
- 2.4. Quick spin the plate(s) at 4°C for 1 minute at 2000 g before being placed on the NIMBUS and after incubation.
- 2.5. Sample plates can be stored at -20°C overnight after every step except post Adenylation and post Ligation. Adenylation and Ligation must be performed on the same day. At least one bead clean must be performed post Ligation.

3. Starting Material

- 3.1. A minimum of 1.0 ug of intact gDNA as measured by Qubit or Quant-iT is required. Your supervisor will specify if less than 1.0 ug can be used. HL60 genomic DNA (1 ug) will be used as a positive control and EB will be used as a negative control.

4. General Brew Preparation Guidelines

- 4.1. Double check the QA release and expiry date of each reagent.
- 4.2. Thaw required reagents and premixed brews and place them on ice. Enzymes should be left in the freezer until ready to use. Each premix is limited to freeze-thaw 3 times for library construction use.
- 4.3. Reagents and enzymes should be well mixed, the former by pulse-vortexing and the latter by gentle tapping of the tube or gentle repeat inversions. Treat premixed brews as enzymes. After mixing, quick spin down in a mini-centrifuge.
- 4.4. Once prepared, all brews should be well mixed by gentle, repeated pulse-vortexing to ensure equal distribution of all components and thus uniformity of enzymatic reactions across a plate. The Ligation brew is particularly viscous.
- 4.5. All reactions require the preparation of a Brew Source Plate.
- 4.6. All brews are aliquoted by the technician. No dead volume is required as DNA is added to the brew. It is imperative that utmost care is taken to aliquot exact volume.
- 4.7. Follow instructions in this SOP to determine the volume of premixed brew per well.

5. Hamilton NIMBUS Handling Guidelines

- 5.1. Reaction brews vary in viscosity, selecting the correct pipetting technique is therefore essential to ensure accurate volume transfer.
- 5.2. This SOP doesn't require any dead volume for brews. The dead volume required by the NIMBUS in the 96-well reservoir is 25mL.

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- 5.3. For each reaction setup, confirm the plate and tip box locations on the NIMBUS deck match the software deck layout on the computer screen.
- 5.4. Ensure plate seals are removed before starting the NIMBUS program.
- 5.5. The following steps are generally followed:
 - 5.5.1. Start Hamilton Run Control
 - 5.5.2. Open: File/Production/LibraryConstruction/LibraryConstruction-Scheduler.wfl
 - 5.5.3. Select Bisulfite
- 5.6. The NIMBUS bead cleanup modules in this SOP are based on the following conditions:

Bead Binding Time (mins)	1 st Magnet Clearing Time (mins)	2 X 70% EtOH Wash Vol (µL)	Ethanol Air-dry Time (mins)	Elution Volume (µL)	Elution time (mins)	2 nd 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 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.**

X. Procedure

Note: All version numbers for Nimbus protocols have been removed on this document. They are present when running the protocol. If you are unsure which version to use, consult your supervisor.

Note: ALINE beads (PCR Clean DX) and Ampure XP beads can be used interchangeably in the magnetic bead clean up steps

1. Initial QC

- 1.1. For each gDNA 96 well stock plate, quantify according to the following SOP:

LIBPR_WORKINST.0108 96-well DNA Quantification using Quant-iT and VICTOR3

2. Sample Normalization on the JANUS Automated Workstation

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- 2.1. Normalize input to 1ug as directed by your supervisor AND according to the following SOP:

LIBPR.0113 Normalization of Nucleic Acid Concentration using the JANUS automated workstation

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. The total volume of the sample with Lambda DNA spike in should equal 62.5µL. The HL60 gDNA control sample must also be spiked in with 10 ng of Lambda DNA per 1ug of gDNA material.
- 3.2. If processing several samples log into the following NIMBUS program for automated Lambda DNA spike in.
- 3.3. The robot will transfer 2µL of 5 ng/µL Lambda DNA from to the destination wells. The Lambda DNA must be arrayed in the same number of columns as the destination plate.

<u>Hamilton Run Control</u> : File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite > Spike-in Lambda

4. Covaris LE220 Series Shearing

- 4.1. The NIMBUS has off-set pipetting capability therefore 1 to 92 samples can be processed.
- 4.2. Log into the NIMBUS and select Shearing Setup to transfer gDNA + Lambda spike in to a Covaris plate or Covaris 8-strip plate.

<u>Hamilton Run Control</u> : File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite> Shearing Setup

- 4.3. NIMBUS pierces the Covaris foil one column at a time using p300 tips.

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- 4.4. After piercing, DNA is transferred into the Covaris plate/Covaris strip tubes using p50 tips.
- 4.5. Cover the Covaris plate with VWR foil. Covaris strip tubes can be covered with Covaris foil strips.
- 4.6. Refer to the following SOP for shearing conditions:

LIBPR.0097 Operation of the Covaris LE220

Make sure that you have performed the shearing twice with a spin in between according to the SOP above.

5. Agilent HS DNA QC after shearing – Spot Check

- 5.1. For each 96 well plate of sheared samples, use 1µL from 11 random samples (ensure that at least one of these samples is a positive control) to spot check on a High Sensitivity DNA Agilent Assay.

LIBPR.0017 Operation and Maintenance of the Agilent 2100
Bioanalyzer for DNA samples

- 5.2. The profile for sonicated DNA should have the peak close to 300bp. Consult with your supervisor to confirm the sonicated DNA profiles. Repeat shearing may be necessary in some cases, and if so ask your supervisor for the additional required sonication time.

6. Bead Clean Up after Shearing

- 6.1. Log into the following program to transfer sheared product to an AB1000 plate:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite> **Transfer out of Covaris**

- 6.2. Log into the following program to clean up sheared gDNA:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library

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Construction-Scheduler.wfl> Bisulfite> **Bead Clean Sheared DNA**

6.3. Dispense reagents according to the plate layout as described below. Dispense ethanol into a clean deep well bock immediately prior to each bead clean.

Reagent	Labware	Volume/well (µL)
70% Ethanol (2 nd BC)	2 mL MagMAX deep well plate	350
ALINE PCRDX Beads	AB1127 plate	112.5*
Qiagen Elution buffer	EK 2036 Low Profile Reservoir	Fill to line

*Includes 50 µL dead volume

7. End-Repair and Phosphorylation Reaction

7.1. End Repair Premix must be thawed on ice, and then gently mixed prior to dispensing brew into the brew plate using volumes described below. Note that aliquot volumes have to be exact, as there is no dead volume.

7.2. Use a Distriman repeater pipettor to dispense brew in as many wells as needed.

Solution	Volume (µL/well)
End Repair Premix (NEB)	23.5
DNA from Covaris plate	35
Total Reaction Volume	58.5

7.3. Log into NIMBUS Program as follows:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite> **End Repair**

7.4. The brew plate is the “REACTION” and the DNA plate is the “DNA Sample.” After NIMBUS program completion, seal the plate and quick spin at 4°C for 1 minute. Inspect the reaction plate for any variations in volume.

7.5. Incubate End-Repair reaction plate at 20°C for 30 minutes. The total reaction volume is 58.5 µL.

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Tetrad Program: Run > LIBCOR > ER

7.6. After the 30 minute incubation, store plate at -20°C or proceed to Size Selected End Repaired DNA

8. Bead Clean End-Repaired DNA

8.1. Log into the NIMBUS program as follows:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite> **Bead Clean ER**

8.2. Dispense reagents according to the plate layout as described below. Dispense ethanol into a clean deep well block immediately prior to each bead clean.

Reagent	Labware	Volume/well (µL)
70% Ethanol	2 mL MagMAX deep well plate	350
ALINE PCRDX Beads	AB1127 plate	108.5*
Qiagen Elution buffer	EK 2036 Low Profile Reservoir	Fill to line

*Includes 50µL dead volume

8.3. Note that end repaired product can be stored at -20C after the bead clean up step.

9. Addition of the an 'A' Base (A-Tailing) Reaction

9.1. A-Tailing Premix must be thawed on ice, and then gently mixed prior to dispensing 20 µL brew per well into the brew plate as described below.

Solution	Volume (µL/well)
dA Tailing Reaction Mix (NEB)	20
End-Repair + BC DNA	30
Total Reaction Volume	50

9.2. Log into NIMBUS Program as follows:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite> **A-Tailing**

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9.3. The brew plate is the “REACTION” and the DNA plate is the “DNA Sample.” After NIMBUS program completion, seal the plate and quick spin at 4°C for 1 minute. Inspect the reaction plates for any variations in volume.

9.4. Incubate A-Tailing reaction plate at 37°C for 30 minutes; 70°C for 5 minutes; 4°C for 5 minutes; 4°C hold, using the following Tetrad program. The total reaction volume is 50 µL.

Tetrad Program: Run > LIBCOR > ATAIL

9.5. Thaw the ligation brew and methylated adapters during the adenylation reaction.

9.6. After the incubation, immediately proceed to Adapter Ligation. **Note: do not bead clean adenylated library.**

10. Methylated PE Adapter Ligation Reaction

10.1. Prepare Methylated PE adapter ligation brew as described below:

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)

10.2. Generate the Ligation brew Mix calculator using LIMS:

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

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

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

10.3.2. Add the Methylated PE adapter to the brew last and not more than 10 minutes before brew addition to DNA. Store prepared brew on ice.

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10.4. Immediately after the brew is prepared, dispense 25 µL of brew into each well of a destination plate and store on ice.

10.5. Log into the following NIMBUS program.

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite> **Adapter Ligation**

10.6. The brew plate is the “REACTION” and the DNA plate is the “DNA Sample.” After Nimbus program completion, seal the plates and quick spin at 4°C for 1 minute. Inspect the reaction plates for any variations in volume.

10.7. Incubate Adapter Ligation reaction plate(s) at 20°C for 15 minutes. The total reaction volume is 75µL per well. 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: LIBCOR> LIGATION

10.8. As soon as the ligation reaction has completed, quick spin the plate and store on ice while preparing the Nimbus for post ligation bead clean up

11. Magnetic Bead Clean Up after Adapter Ligation

11.1. Log into the following NIMBUS program:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite> **Bead Clean Ligation (2x)**

11.2. Post Ligation bead clean up is performed twice and a safe stopping pint is after the first bead clean. A prompt will appear asking “Do you want to skip the first bead clean? Yes, No or Quit”. If you want to proceed to the first bead clean select “No”. If you have already finished the first bead clean post ligation you should select “Yes”.

11.3. Dispense reagents according to the plate layout as described below. Dispense ethanol into a clean deep well block immediately prior to each bead clean.

Reagent	Labware	Volume/well (µL)
70% Ethanol (1 st BC)	2 mL MagMAX deep well plate	350

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70% Ethanol (2 nd BC)	2 mL MagMAX deep well plate	350
ALINE PCRDX Beads	AB1127 plate	*175
Qiagen Elution buffer	EK 2036 Low Profile Reservoir	Fill to line

* Includes required volume both rounds of bead clean ups (including 50µL dead volume). For 1st bead clean only, dispense 125µL of beads (including 50µL dead volume). For 2nd bead clean only, dispense 100µL of beads (including 50µL dead volume).

11.4. Note that the template will be eluted in 25µL of EB for subsequent Bisulfite Conversion

12. Bisulfite Conversion

12.1. 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.

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

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

12.3. 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.

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

12.5. Pre-warm conversion reagent to 37°C 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).

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12.6. Dispense 130 μ L of prepared Bisulfite Conversion reagent into the appropriate wells of an empty AB1000 plate. Brew source wells must match the sample destination wells.

12.7. Log into the following NIMBUS program:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite> Bisulfite Conversion
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12.8. The robot will transfer all of the template to the destination wells containing 130 μ L of prepared Bisulfite Conversion reagent.

12.9. After the process is complete, cover the plate with a tetrad incubation pad and incubate at 98°C for 10 minutes, 64°C for 2.5 hours and 4°C for up to 20 hours. Enter '100' for reaction volume (max allowable) and select 'Y' for heated lid.

Tetrad Program: Run > LIBCORE > BSCONVER

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

13.1. 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.

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

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

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

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

13.6. Add 200 μ L 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

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incubation. After the incubation, centrifuge at full speed for 1 minute. Discard the flow-through.

- 13.7. 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.
- 13.8. 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.
- 13.9. Proceed to PCR enrichment or store samples at -20°C for later use. For long-term storage, store at -80°C .

14. Indexed PCR Amplification

Note: Prior to transferring template to the brew plate, remove the tip corresponding to the PCR brew control well from the tip box to avoid carrying over elution buffer to the PCR brew control well.

- 14.1. Thaw the PE PCR primer 1.0 in the Tissue Culture Room laminar flow hood on the 5th floor, room 511 and then store on ice.
- 14.2. Thaw the Indexing Primer Plate in a working bench, quick spin at 4°C 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. The maximum freeze-thaw cycles for the indexing primer plate are 5 times.
- 14.4. Ensure there is enough volume for the number of plates to be processed including the NIMBUS dead volume.
- 14.5. Methylated iPCR brew (minus the primers) must be aliquoted in the PCR Clean Room laminar flow hood on the 5th floor, room 510. Addition of PE PCR primer 1.0 to the brew must be made in the Tissue Culture Room laminar flow hood on the 5th floor, room 511. The Indexing Primer is added to the reaction plate using the NIMBUS.

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14.6. Prepare the Bisulfite iPCR brew as described below:

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 Ligation 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. The indexing primers will be added to the Brew Source Plate using the NIMBUS.

14.9. Carefully dispense 26μL of brew per well of an AB1000 plate using a DISTRIMANN pipette. The NIMBUS robot will transfer template directly to the brew therefore no dead volume is required in the brew plate

14.10. Log into the following NIMBUS program:

14.11. The NIMBUS program for iPCR setup is as follows:

14.11.1. Addition of index primers to the Brew Source Plate.

14.11.2. Addition of all of the template into the Brew/Index Plate

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

14.13. Seal the plate(s) with VWR foil seal and quick spin at 4°C for 1 minute. Inspect the reaction plates for any variations in volume.

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

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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

14.15. Proceed immediately to post PCR bead clean up or temporarily store the iPCR product at -20C.

15. Post PCR bead clean (2X)

15.1. To purify the iPCR product, log into the following NIMBUS program:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite> **Bead Clean iPCR(2x)**

15.2. Post PCR cleanup is performed twice for all protocols and a safe stopping point is after the first bead clean if desired. A prompt will appear asking “Do you want to skip the first bead clean? Yes, No or Quit”. If you want to proceed to the first bead clean and pause, select “No”. If you have already finished one round of bead clean and are continuing, select “Yes.” This is important as the volumes going into the first and second bead cleans are different.

15.3. Dispense reagents according to the plate layout as described below. Dispense ethanol into a clean deep well block immediately prior to each bead clean.

Reagent	Labware	Volume/well (µL)
70% Ethanol (1 st BC)	2 mL MagMAX deep well plate	350
70% Ethanol (2 nd BC)	2 mL MagMAX deep well plate	350
ALINE PCRDX Beads	AB1127 plate	*150
Qiagen Elution buffer	EK 2036 Low Profile Reservoir	Fill to line

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* Includes required volume both rounds of bead clean ups (including 50µL dead volume). Dispense 100µL if only processing one bead clean.

- 15.4. Proceed to preparing dilutions for QC or store final library product at -20°C in the designated post-PCR freezer.
- 15.5. The final elution volume is 25µL.

16. Preparation of Diluted Library QC Plate

- 16.1. Prepare a 10x dilution QC plate using the following Nimbus program. This step is only required for Quant-iT/Caliper GX QCs.

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite> **Dilute for QC**

- 16.2. The Nimbus will transfer 18 µL of Qiagen EB to a new plate and then transfer 2µL of final library product to the EB plate. This 10x dilution will be used first for Quant-iT (2µL) and the remaining 18µL will subsequently be used for Caliper.

17. Quant-iT/Qubit QC on Samples for pooling

- 17.1. Refer to the following SOPs for setting up the QC plate prior to pooling:

LIBPR.0108 96-well DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR3V
or
LIBPR.0030 Quantifying DNA Samples using the Qubit Fluorometer

- 17.2. For Quant-iT, use the 10x dilution plate as the source plate for the QC. Log into the following Nimbus program:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite> **Quant-iT**

- 17.3. For Qubit, use the undiluted DNA from post-lc size selection.

18. Final HS Caliper QC or Agilent QC

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18.1. Refer to the following SOPs for evaluating final library profiles by Agilent DNA 1000 or Caliper HSDNA assay.

LIBPR.0017 Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples LIBPR.0051 Operation and maintenance of the Caliper LabChip GX for DNA Samples using the High Sensitivity Assay

18.1.1. For Caliper, run the 10x dilution QC plate on the Caliper GX.

18.1.2. For Qubit, run the undiluted samples on the Agilent DNA 1000 assay.

18.2. Use a smear analysis using 200:1000 bp to calculate the average base pair size of the final library.

18.3. Calculate the nM quant using the average bp size from Caliper and the Qubit or Quant-iT: Send the results to the APC for approval:

19. Pooling Samples into 1.5ml Tubes on Span-8 (if needed) or Rearray Unpooled Samples into 1.5ml Tubes

19.1. Refer to the following SOP for pooling on Span-8:

LIBPR.0093 Span-8 Pooling of DNA Samples
--

20. Qubit QC of Pooled Samples for submission

20.1. Refer to the following SOP:

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

21. Sequencing Submission:

21.1. For each library, determine the corrected final molar concentration for submission to sequencing. Use the average base pair size previously obtained from the Caliper HS DNA profile or Agilent DNA 1000 profile and the result from the Quant-iT or Qubit to obtain the final size-corrected nM quant. The remaining final volume (after QCs) is



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~20 μ L. The maximum submission concentration is 100nM. Minimum concentrations and volumes will vary by library type. The APC will confirm whether acceptable range for submission.

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Appendix A: LIMS Protocol

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

Enter the following attributes:

- Library_size_distribution_bp (From Agilent or Caliper)
 - Avg_DNA_bp_size (From Agilent or Caliper)
 - DNA_concentration_ng_uL (From Quant-iT or Qubit)
7. If Pooling: Action: Aliquot pooling volume into a new TRA
 8. If Pooling: Pooling and/or Manual Rearray into tubes – IPE pipeline
 9. Final_Submission – PET (no pooling); IPE (pooled)

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Appendix B: Bisulfite Sequencing QCs

Post Shearing Profiles on Agilent HS DNA chip

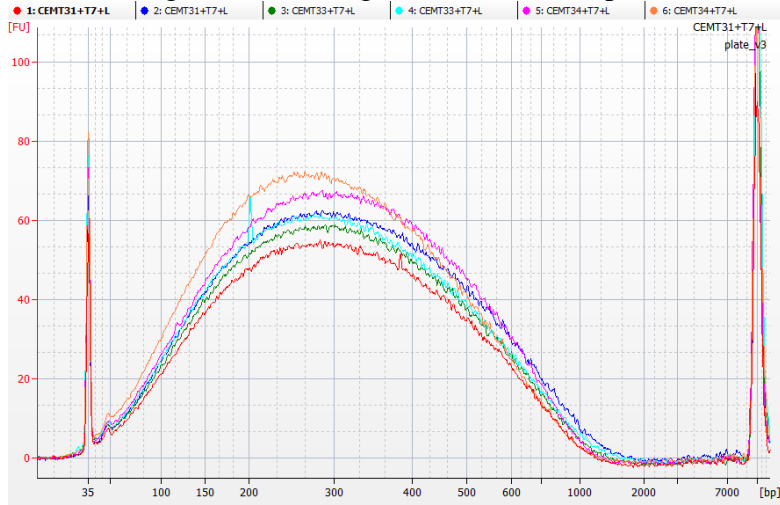
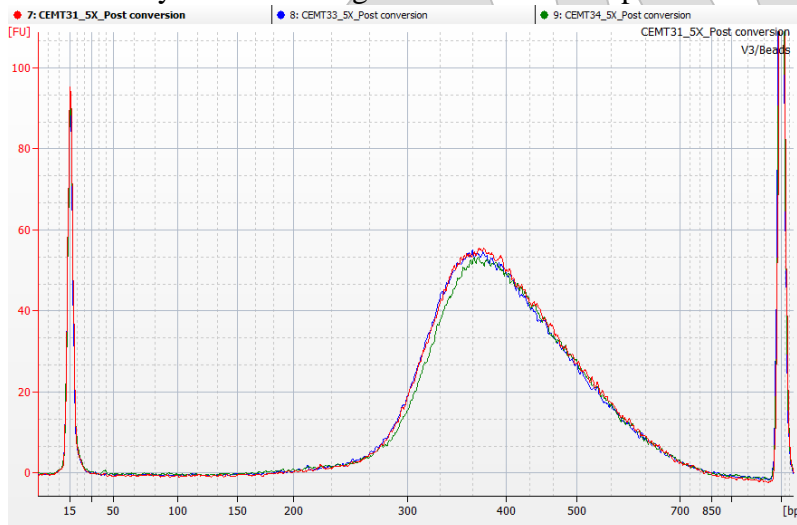


Fig 1. CEMT gDNA samples with spike in DNA post LE220 plate tube shearing (2*50s). Library profiles centre around 300bp on HSDNA chip. Libraries were diluted 6X prior to QC.

Final Library Profiles on Agilent DNA 1000 chip



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Fig 2. Final CEMT library profiles after bisulfite conversion and 5X cycles of enrichment using KAPA ura+ PCR enrichment kit. Library profiles peak ~375bp on DNA 1000 chip due to fragmentation during bisulfite conversion.

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