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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 Quality Systems associate.

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Library Core Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Systems 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	Version 1.1
EZ DNA Methylation Gold Kit	D5005
Kapa HiFi Hot Start Uracil^{+} Ready Mix	KM2801

VI. Related Documents

Document Title	Document Number
Operation of Covaris LE220	LIBPR.0097
Manual Bead Clean Up using Ampure XP or ALINE Beads	LIBPR.0073
Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA	LIBPR.0017

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Document Title	Document Number
samples	
Operation and Maintenance of the Caliper LabChip GX for DNA Samples using the High Sensitivity Assay	LIBPR.0051
Quantifying DNA Samples using the Qubit 4 Fluorometer	LIBPR.0153
Automated DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR^{3}V	LIBPR.0108
JANUS G3 Normalization and Pooling of DNA Samples	LIBPR.0146
Normalization of Nucleic Acid Concentration using the JANUS Automated Workstation	LIBPR.0113

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 safety data sheet (SDS) 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 (10mM)	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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Name	Supplier	Number	Model or Catalogue #	
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	Thermo scientific	4641010		✓
Finnpipette F1 2-20µL	Thermo scientific	4641060		✓
Finnpipette F1 20-200µL	Thermo scientific	4641080		✓
Finnpipette F1 100-1000µL	Thermo scientific	4641100		✓
Distriman repeater pipette	Gilson	GF-F164140		✓
Distriman tips 1250µL	Gilson	F164140		
Ice bucket	Fisher	11-676-36		✓
IKA Works Vortexer	Agilent	MS2S9-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	Thermo scientific	21377354		✓
Finntip Filter tips 20µL	Thermo scientific	21377353		✓
Finntip Filter tips 200µL	Thermo scientific	14386374		✓
Finntip Filter tips 1000µL	Thermo scientific	21377604		✓
Nuclease Free 1.5mL tube	Ambion	12400		✓
PCRmax Alpha Cycler 4	Froggabio/ Cole Parmer	AC496	✓	
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 10x15"	Fisher	01-826-4		✓

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Name	Supplier	Number	Model or Catalogue #	
Unmethylated Lambda DNA	Promega	D1521	✓	
Wet ice	In-house	N/A	N/A	N/A
VWR foil seals	VWR	60941-126		✓
Aluminum foil tape, 3"x 60 yds	Scotch/3M	34000740		✓
PCR Clean DX (ALINE beads)	ALINE Biosciences	C-1003-450		✓
AB1000 96-well plate	Thermo Scientific	FSSP9743245		✓
ABgene 1.2mL plate	Abgene	AB1127		✓
MagMax Microtiter 96 Deep well plate	Applied Biosystems	4388476		✓
Peltier adapter	In-house	N/A	N/A	N/A
96-well reservoir plate 450µL	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

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

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Bisulfite Conversion Library Construction Work Flow

Quant-iT/Qubit Quant the Starting Material (Biospecimen Core)

Normalize input to 1µg/well

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

Shear DNA on the Covaris (100 or 110 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)

Caliper/Agilent and Quant-iT/Qubit QC (dilution if required)

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, pipettes, tip boxes and small equipment with DNA *AWAY*. Ensure you have a clean working surface before you start.

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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.

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 4ºC for 1 minute at 2000g before being placed on the NIMBUS and after incubation.

2.5. Sample plates can be stored at -20ºC 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 1µg of intact gDNA (as measured by Qubit or Quant-iT) is required. Your supervisor will specify if less than 1µg can be used. HL60 genomic DNA (1µg) 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 3 times freeze-thaws for library construction use.

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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.

4.8. For preparation of PCR brew, the KAPA 2x HiFi Hot Start Uracil+ Ready Mix must be aliquoted in the PCR Clean Room laminar flow hood (5th floor) or in the Library Construction BSC (6th floor). PE PCR primer 1.0 must be thawed and added in the Blood Room laminar flow hood (5th floor) or on the bench in the Library Construction Room (6th floor).

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.

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

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5.5.3. Select Bisulfite v1

5.6. The NIMBUS bead cleanup modules 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 and 70% ethanol to equilibrate to room temperature by storing at room temperature for at least 30 minutes prior to use. Failure to do so may result in a reduction in yield.

X. Procedure

Note: If you are unsure of which Nimbus protocol version to use, please 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.0108 - Automated DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR^{3}V

2. Sample Normalization on the JANUS Automated Workstation

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

LIBPR.0113 - Normalization of Nucleic Acid Concentration using the JANUS Automated Workstation

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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 10ng of Lambda DNA per µg to 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 10ng of Lambda DNA per 1µg of gDNA material.

3.2. If processing more than 16 samples, log into the following NIMBUS program for automated Lambda DNA spike in. If processing 16 samples or less, add the spike in manually (refer to Appendix C).

3.3. The robot will transfer 2µL of 5ng/µL Lambda DNA from to the destination wells. The Lambda DNA must be arrayed in the same number of columns as the destination plate.

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl > Bisulfite v1 > **Spike-in Lambda**

4. Covaris LE220 Series Shearing

4.1. To transfer gDNA + Lambda spike in to a Covaris plate or a Covaris 8-strip tubes, log into the following NIMBUS program:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl > Bisulfite v1 > **Shearing Setup**

4.2. The NIMBUS pierces the Covaris foil one column at a time using P300 tips.

4.3. After piercing is complete, DNA is transferred into the Covaris plate/Covaris strip tubes using P50 tips.

4.4. Cover the Covaris plate with VWR foil seals. Covaris strip tubes can be covered with Covaris foil strips.

4.5. Refer to the following SOP for shearing instructions:

LIBPR.0097 - Operation of the Covaris LE220

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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 the positive and negative controls are selected) 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 300 bp (see Appedix B-Figure 1). Consult with your supervisor to confirm the sonicated DNA profiles. Repeat shearing may be necessary in some cases. If needed, 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 v1> **Transfer out of Covaris**

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

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite v1> **Bead Clean Sheared DNA**

6.3. 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 (2nd BC)	2mL MagMAX deep well plate	350
ALINE PCRDX Beads	AB1127 plate	112.5*
Qiagen Elution buffer	EK 2036 Low Profile Reservoir	Fill to line

*Includes 50µL dead volume

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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 pipette to dispense brew in as many wells as needed.

Solution	Volume (µL/well)
End-Repair Premix (NEB)	23.5
DNA	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 v1> **End Repair**

7.4. The brew plate is the "REACTION" and the DNA plate is the "DNA Sample." After completion of the Nimbus program, seal the plate and quick spin at 4ºC for 1 minute. Inspect the reaction plate for any variations in volume.

7.5. Incubate End-Repair reaction plate at 20ºC for 30 minutes. The total reaction volume is 58.5µL. Enter '58' for reaction volume.

Tetrad Program: Run > LIBCOR > ER

7.6. After the 30 minute incubation, store plate at -20ºC or proceed to bead clean.

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 v1> **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.

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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 bead clean End-Repaired product can be stored at -20ºC.

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

9.1. A-Tailing 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.

9.2. Use a Distriman repeater pipette to dispense brew in as many wells as needed.

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

9.3. Log into NIMBUS Program as follows:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite v1> **A-Tailing**

9.4. The brew plate is the "REACTION" and the DNA plate is the "DNA Sample." After completion of the Nimbus program, seal the plate and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

9.5. Incubate A-Tailing reaction plate at 37ºC for 30 minutes; 70ºC for 5 minutes; 4ºC for 5 minutes; 4ºC hold. Enter '50' for reaction volume.

Tetrad Program: Run > LIBCOR > ATAIL

9.6. Thaw the ligation brew and **Methylated PE adapter** during the adenylation reaction.

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9.7. After the incubation, immediately proceed to methylated adapter ligation. Quick spin plate and store on ice while setting up the ligation reaction.

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_Ligation_Brew_40pmol (25µL)
Methylated PE Adapter (10µM)	4	
Reaction volume	**75**	

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.

10.4. Immediately after the brew is prepared, use the Distriman repeater pipette to dispense 25µL 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 v1> **Adapter Ligation**

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

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10.7. Incubate Adapter Ligation reaction plate at 20ºC for 15 minutes. Enter '75' for reaction volume.

Tetrad Program: LIBCOR> LIGATION

After the ligation reaction has completed, quick spin plate and then proceed immediately to bead clean. This is NOT a safe stopping point.

11. 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 v1> **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 (1st BC)	2mL MagMAX deep well plate	350
70% Ethanol (2nd BC)	2mL 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.

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12. Bisulfite Conversion

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

12.2. Prepare the 10X CT Conversion reagent as described below:

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 -20ºC for up to one month.

12.5. 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).

12.6. Dispense 130µL 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 v1> **Bisulfite Conversion**

12.8. The robot will transfer the entire template to the destination wells containing 130µL of prepared Bisulfite Conversion reagent.

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12.9. After the process is complete, 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 (maximum allowable)..

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 24mL of 100% ethanol to the 6mL **M-Wash Buffer concentrate**. Indicate on the bottle that ethanol has been added.

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

13.3. Pipette each bisulfite converted library sample into its respective Zymo-SpinTM 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 100µL of **M-Wash Buffer** to the column. Centrifuge at full speed for 1 minute. Discard the flow-through.

13.6. Add 200µL 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.

13.7. Add 200µL 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 1.5mL micro-centrifuge tube. Add **22µL** 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.

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14. Indexed PCR Amplification

14.1. Thaw the PE PCR primer 1.0 and then store on ice.

14.2. Thaw the indexing primer plate in a working bench, 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. 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. Generate the appropriate PCR Brew LIMS calculator:

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

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

14.7. Prepare the Bisulfite iPCR brew as described below:

Solution	Volume (µL) per well	
KAPA 2X HiFi Ura+ Ready mix	25	Bisulfite_LibConst iPCR_Brew (26µL)
PE PCR primer 1.0 (25µM)	1	
Indexed PCR primer plate (12.5µM)	2	

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

14.9. The indexing primers will be added to the DNA source plate (from bisulfite conversion clean up) using the NIMBUS.

14.10. Before starting the program, remove EB contents from the designated PCR brew control well (from bisulfite conversion clean up) and replace it with 22µL of water.

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14.11. Log into the following NIMBUS program:

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

14.12.1. Addition of index primers to the DNA source plate.

14.12.2. Transfer of DNA + primers to the brew plate.

14.13. After completion of the Nimbus program, seal the plate and quick spin at 4ºC 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.

Tetrad Program: Run > LIBCOR > BS-5X

PCR Parameters

- 98˚C 1 min
- 98˚C 15 sec (Total of 5 cycles)
- 65˚C 30 sec (Total of 5 cycles)
- 72˚C 30 sec (Total of 5 cycles)
- 72˚C 5 mins
- 4˚C ∞

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

15. Post PCR bead clean (2x)

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

<u>Hamilton Run Control</u>: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite v1> **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

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

* 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 -20ºC 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 and Caliper QCs.

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> Bisulfite v1> **Dilute for QC**

16.2. The Nimbus will transfer 18µL 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 (2 µL) and the remaining 18µL will be subsequently used for Caliper.

17. Quant-iT or Qubit QC

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

LIBPR.0108 - Automated DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR^{3}V or LIBPR.0153 - Quantifying DNA Samples using the Qubit 4 Fluorometer

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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 v1> **Quant-iT**

17.3. For Qubit, use the undiluted samples from post-PCR BC (2x).

18. Final HS Caliper QC or Agilent QC

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

LIBPR.0017 - Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples or 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 Agilent, run the undiluted samples from post-PCR BC (2x) on the Agilent HS DNA assay.

18.2. Use a smear analysis using 100:1000 bp to calculate the average base pair size of the final library.

18.3. Calculate the nM quants using the average bp size from Caliper profile/Agilent HS DNA profile and the concentration from the Quant-iT/Qubit. Send the results to the APC for approval.

19. Pooling Samples into 1.5mL Tubes (if needed) or Rearray Unpooled Samples into 1.5mL Tubes on Janus G3

19.1. Refer to the following SOP for pooling on Janus G3:

LIBPR.0146 - JANUS G3 Normalization and Pooling of DNA Samples

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20. Qubit QC of Pooled Samples for Submission

20.1. Refer to the following SOP:

LIBPR.0153 - Quantifying DNA Samples using the Qubit 4 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/Agilent and the resultsfrom the Quant-iT/Qubit to obtain the final size-corrected nM quant. The remaining final volume (after QCs) is ~20µL. 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

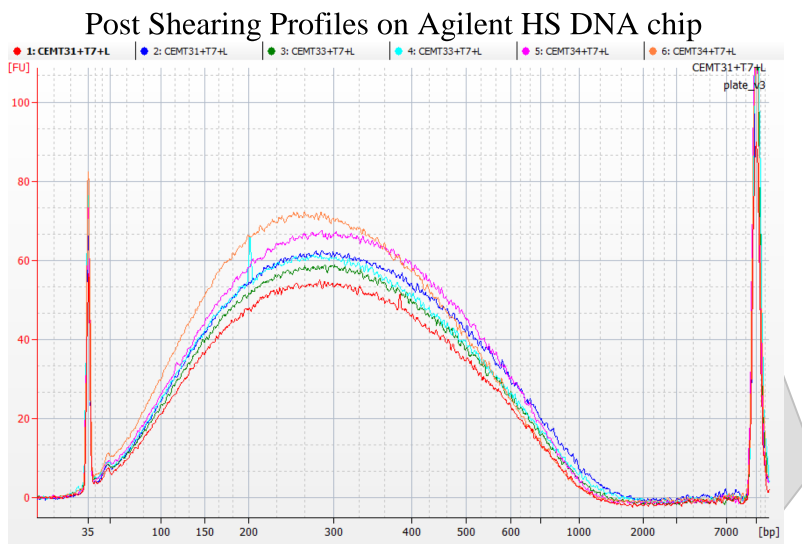

Figure 1: CEMT gDNA samples with spike in DNA post LE220 plate tube shearing (2*50s). Library profiles centre around 300bp on HSDNA chip. Libraries were diluted 6x prior to QC.

Final Library Profiles on Agilent DNA 1000 chip

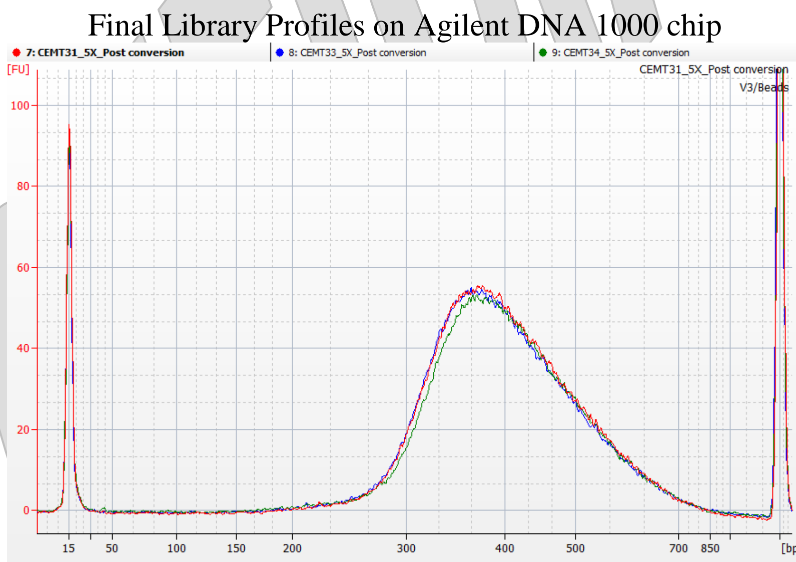

Figure 2: Final CEMT library profiles after bisulfite conversion and 5x cycles of enrichment using KAPA ura+ PCR enrichment kit. Library profiles peak ~375bp on DNA 1000 chip due to fragmentation during bisulfite conversion.

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Appendix C: Manual Bisulfite Library Construction

1. Add Unmethylated Lambda DNA to all samples

1.1. Add 10ng of unmethylated Lambda DNA per µg 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 10ng of Lambda DNA per 1µg of gDNA material.

2. Shearing

2.1. Transfer all gDNA to Covaris LE220 vessels

2.2. Covaris LE220 (LIBPR.0097)

2.3. QC: Agilent HS DNA Assay (LIBPR.0017)

3. Bead Clean Sheared gDNA

3.1. Ethanol and Magnetic beads must be incubated at room temperature for at least 30 minutes before use.

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

4. End Repair & Phosphorylation

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

4.1. Transfer 23.5µL of NEB End Repair Premix into wells of a destination plate.

4.2. Transfer 35µL of gDNA to End Repair Premix, mix using 80% volume, 10x.

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4.3. Tetrad Program: LIBCOR>ER

4.4. Safe stopping point if stored at -20ºC.

5. Bead Clean End Repaired & Phosphorylated Template

5.1. Ethanol and Magnetic beads must be incubated at room temperature for at least 30 minutes before use.

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

5.2. Note: This is a safe stopping point. Do not proceed to adenylation unless you have adequate time to perform ligation reaction as well.

6. A-Tailing

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

6.1. Transfer 20µL of NEB Adenylation Premix to 30µL of repaired/phosphorylated and bead cleaned DNA.

6.2. Tetrad Program: LIBCOR>ATAIL

6.3. Proceed directly to in-tandem ligation (**do not bead clean after Adenylation**). Store on ice while preparing Ligation premix and adapters.

7. Adapter Ligation

Methylated_Ligation_Brew_40pmol

Solution	1 rxn (µL)

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Solution	1 rxn (µL)
Adenylated template	50
NEB Ultra Premix 2X	21
Methylated PE Adapter (10 µM)	4
Reaction volume	**75**

Methylated_Ligation_Brew_40 pmol (25µL)

7.1. Transfer 25µL of Ligation brew to 50µL of adenylated template.
7.2. Reset pipette to 80% total volume, mix 10x.

7.3. Select tetrad program: LIBCOR>LIGATION

7.4. Set a timer for 15 minutes. After the ligation reaction has completed, quick spin plate and then proceed immediately to bead clean. This is NOT a safe stopping point.

8. Double Bead Clean post Ligation

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

8.1. The ligated template can be used for Bisulfite Conversion.

9. Bisulfite Conversion

9.1. 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 1µg of high quality gDNA and stringent removal of small inserts.

9.2. Prepare the 10x CT conversion reagent as described below:

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

9.3. 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.

9.4. 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.

9.5. 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).

9.6. Transfer 130µL 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 (maximum allowable) and select 'Y' for heated lid.

Tetrad Program: Run > LIBCORE > BSCONVER

10. Bisulfite Conversion Clean Up using Columns (EZ Methylation Gold Kit)

10.1. Prepare M-Wash buffer as described on the bottle. Add 24mL of 100% ethanol to the 6mL **M-Wash Buffer** concentrate. Note that ethanol has been added to the bottle.

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

10.3. 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.

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

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

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10.6. Add 200µL 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.

10.7. Add 200µL 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.

10.8. Place the column into a fresh 1.5mL micro-centrifuge tube. Add **22µL** of **M-Elution Buffer** directly to the column matrix. Centrifuge for 1 minute at full speed to elute the DNA.

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

11. Indexed PCR Amplification

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

Solution	1 rxn (µL)
Bisulfite converted DNA	22
KAPA 2X HiFi Ura+ Ready mix	25
PE PCR primer 1.0 (25µM)	1
Indexed PCR primer plate (12.5µM)	2

KAPA 2X HiFi Ura+ Ready mix + PE PCR primer 1.0 (25µM) → Bisulfite_Libconst_iPCR_Brew (26µL)

11.2. Remove EB contents from the designated PCR control well (from bisulfite conversion clean up plate) and replace it with 22µL of water.

11.3. Select 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 5 mins
- 4˚C ∞

(98˚C 15 sec, 65˚C 30 sec, 72˚C 30 sec: Total of 5 cycles)

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11.4. PCR-enriched template can be stored at -20°C or proceed immediately to bead clean PCR enriched template.

12. Double Bead Clean post iPCR

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

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

12.1. Template can be stored at -20ºC after the first or second bead clean up post PCR.

13. QC Final Library Products

13.1. Run 1µL of each final library product on Agilent HS DNA assay or perform a 1/10 dilution and run on HS Caliper GX.

13.2. Quantify each final library product by Qubit HS DNA assay or Quant-iT.

13.3. If required, pool samples manually or using Janus G3. Quantify the pool by Qubit HS DNA assay.

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