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96-well PCR-enriched Library Construction for Illumina Sequencing

I. Purpose

To provide specific guidelines for 96-well PCR-enriched ss-cDNA/DNA library construction for Illumina Paired-End Sequencing

II. Scope

All procedures are applicable to the BCGSC Library Core and Library TechD 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 Quality Assurance Management to audit this procedure for compliance and maintain control of this procedure.

V. References

Reference Title	Reference Number
Sample Preparation for Paired-End Sample Prep Kit from Illumina	Version 1.1 (from Prep Kit)

VI. Related Documents

Document Title	Document Number
96-well DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR3V	LIBPR.0108
Operation of Covaris E-Series	LIBPR.0041
Operation of the Covaris LE220	LIBPR.0097
Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples	LIBPR.0017
Operation and Maintenance of the Caliper Labchip GX for DNA samples using the High Sensitivity Assay	LIBPR.0051

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Document Title	Document Number
Operation of the Invitrogen Egel iBase Power System Quantifying DNA samples using the Qubit Fluorometer	LIBPR_WORKINST.0012 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 #	
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		✓
Phusion Hotstart	Fisher	F540-L		✓
Fisherbrand Textured Nitrile gloves – various sizes	Fisher	270-058-53		✓
Ice bucket	Fisher	11-676-36		✓
Wet ice	In house	N/A	N/A	N/A
Covaris E210	Covaris	E210	✓	
Covaris LE220 with WCS and Chiller	Covaris	LE220	✓	
DNA *AWAY*	Molecular BioProducts	21-236-28		✓
AB1000 Plates	Thermo Scientific	SP-5201/150		✓
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	✓	
Diamond Filter tips DFL10	Mandel Scientific	GF -F171203		✓
Diamond Filter tips DF30	Mandel Scientific	GF-F171303		✓
Diamond Filter tips DF200	Mandel Scientific	GF-F171503		✓
Diamond Filter tips DF1000	Mandel Scientific	GF-F171703		✓
Galaxy mini-centrifuge	VWR	37000-700	✓	
VX-100 Vortex Mixer	Rose Scientific	S-0100	✓	
Black ink permanent marker pen	VWR	52877-310		✓
Clear Tape Sealer	Qiagen	19570		✓
Aluminum Foils seals	VWR	60941-126		✓
Aluminum foil tape, 3"x 60 yds	Scotch/3M	34000740		✓
Eppendorf BenchTop Refrigerated Centrifuge 5810R	Eppendorf	5810 R	✓	
Bench Coat (Bench Protection Paper)	Fisher	12-007-186		✓
Small Autoclave waste bags 10"X15"	Fisher	01-826-4		✓
Anhydrous Ethyl Alcohol (100% Ethanol)	CommercialAlcohols	00023878		✓
IKA Works Vortexer	Agilent	MS2S9-5065-4428	✓	
22R Microfuge Centrifuge	Beckman	22R Centrifuge	✓	
Peltier Thermal Cycler	MJ Research	PTC-225	✓	
Power Supply, LVC2kW, 48VDCV	Tyco Electronics	RM200HA100	✓	
P165B Tips, sterile, 10 racks of 96/box	Ultident	24-FXF-180-LRS		✓

P50 (Universal) Tips, Presterile with Barrier, 50 µL, 96/rack, 10racks/case, CS960	Beckman	CABKA21586		✓
P20B Tips, sterile, 10 racks of 96/box	Ultident	24-FXF-20-LRS		✓
Plate, 96-well v bottom (Ethanol/waste plates)	Utilident	P-96-450V-C		✓
Plate, 96-Well reservoirs, diamond-bottom, High-profile	Seahorse	S30014		✓
Biomek FX Liquid Handling System	Beckman	Biomek FX	✓	
Eppendorf Benchtop Centrifuge	Eppendorf	5810 R	✓	
70% Ethanol	In house	N/A	N/A	N/A
Qiagen Buffer EB – 250 mL	Qiagen	19086		✓
UltraPure Distilled Water	Invitrogen	10977-023		✓
Agilent 2100 Bioanalyzer	Agilent	G2943CA		✓
Caliper LabGX	Perkin Elmer	GX1016N0210		✓
Qubit Fluorometer	Invitrogen	Q32857		✓
Span-8	Beckman Coulter	717007	✓	✓
PE PCR Primer 1.0-	IDT	N/A		✓
AmpErase Uracil N-Glycosylase	Applied Biosystems	N8080096		✓
96 Deep Well Reservoir, Pyramid bottom	E & K Scientific	EK-2035		✓
96 Low Profile Reservoir, Pyramid bottom	E & K Scientific	EK-2036		✓
Indexed PCR Primer plate	IDT	N/A	N/A	N/A
Ampure XP Beads, 450mL	Agencourt	A63882		✓
PCR Clean DX (ALINE beads)	ALINE Biosciences	C-1003-450		✓
USER Enzyme	NEB	M5505L		✓

These sequences are for internal use only:

PE adapters:
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT
3' GAGCCGTAAGGACGACTTGGCGAGAAGGCTAG

PE PCR Primers
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
5'CAAGCAGAAGACGGCATACGAGATNNNNNNCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

IX. Introduction and Guidelines

1. General Guidelines

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 on the 5th Floor and 6th floor respectively.
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. Colour code: red fonts designate exceptions or protocol-specific steps.
1.6. Discuss with the APC/PC/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 Up to 4 plates can be processed at a time by one technologist using Biomek FX.
2.2 To avoid cross-well contamination, reaction plates should never be vortexed and plate seals should never be re-used. Use Biomek FX for mixing.
2.3 Use only VWR foil seals for both short term storage and tetrad incubations/USER digestion/PCR, and 3M aluminum foil seal for long term storage.
2.4 After completion of every incubation step, quick spin the plate(s) at 4ºC for 1 minute at 2000 g.
2.5 Sample plates can be stored at -20ºC overnight after every step except post "A" addition. **"A" addition and adapter ligation reactions must be performed on the same day.**
2.6 The reaction plates should be placed on ice throughout the day when not being worked on.

3. Positive and Negative Controls

3.1. The positive control template to be used for this protocol is HL60 genomic DNA or UHR cDNA. The yield of library products constructed from positive controls is expected to differ from those of collaborators' samples. However, the yield should not differ significantly from that of previously constructed positive controls.
3.2. The negative control template to be used for this protocol is Qiagen Elution Buffer. This control will ensure the absence of background products that result from the library construction process.

4. General Brew Preparation Guidelines

4.1. Double check the QA release and expiry date of each reagent and enzyme.
4.2. Thaw required reagents and place them on ice. Enzymes should be left in the freezer until ready to use. Each premix tube can be freeze thawed three times.
4.3. Reagents and enzymes should be well mixed, the former by pulse-vortexing and the latter by gentle flicking. After mixing, quick spin down in a mini-centrifuge.
4.4. All premixed and prepared 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 End-Repair and Ligation brews are particularly viscous.
4.5. All reactions require the preparation of a Brew Source Plate. The Biomek FX will be used to aliquot the brew from the Brew Source Plate into the reaction plates.

4.6. All brew calculators include excess volume to account for dead volume required by the Biomek FX in the Brew Source Plate and to account for pipetting loss.

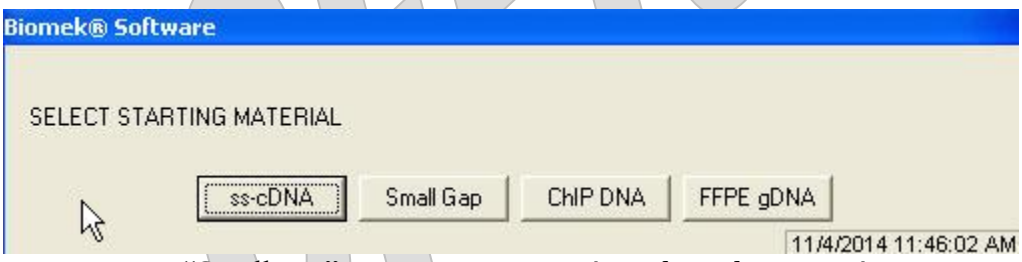
5. Biomek FX Handling Guidelines

5.1. Home all axes before starting each Biomek run
5.2. Reaction brews vary in viscosity, selecting the correct Biomek technique is therefore essential to ensure accurate volume transfer.
5.3. The default dead volume required by the Biomek FX in each well of a Brew Source Plate/Indexing Primer plate is 5µL/well regardless of the number of plates being processed.
5.4. The dead volume required by the Biomek FX in the 96-well reservoir is 25mL.
5.5. Confirm the plate and tip box locations on the Biomek deck matches the software deck layout on the computer screen.
5.6. Ensure plate seals and tip box covers are removed before starting the Biomek program.

6. General note on Biomek programs

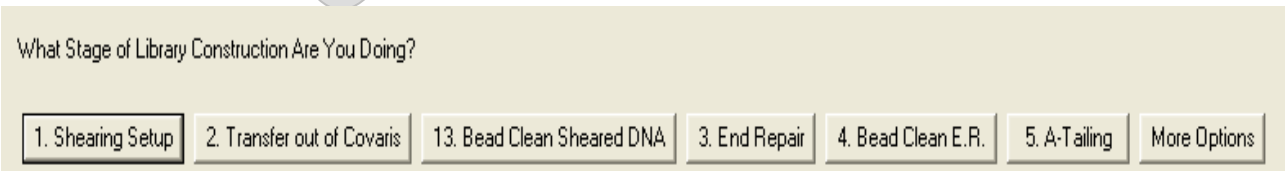
The following steps are generally followed:

A. Open project: LIBPR
B. Select and Run: LibraryConstruction
C. Select starting material type:



Note: "Small gap" encompasses gDNA, and amplicon DNA

D. Select Library Construction step. An example is shown below:



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

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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: Bead to reaction ratio are 1.8:1 for post-shearing, 2:1 for other pre-ligation clean ups and 1:1 for post-ligation reactions.

X. Procedure

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 96-well DNA Quantification using Quant-iT and VICTOR3

Note: this does not apply to ChIP DNA, ss-cDNA, ribopletion or if the libraries have previously been quantified.

1.2 This recommended range of input amounts is specified below:

Starting Material		Amount (µg)
ss-cDNA		NA
Small gap	gDNA	0.5-1.0
	Amplicon DNA	0.130-1.0
ChIP DNA		NA

2. Shearing

Note: this does not apply to ChIP DNA

A. To transfer DNA/cDNA into the Covaris plate, log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction> RUN> *Protocol X >**Shearing Setup**

*Select protocol type: ss-cDNA, Small Gap or ChIP DNA

B. Refer to the following SOP for shearing of all except amplicons:

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.

Refer to the following SOP for shearing of amplicons:

LIBPR.0041 Operation of the Covaris E-Series

3. Agilent HS DNA QC after shearing – Spot Check

Note: For clinical amplicon samples, refer to step 3.3.

3.1 For each 96 well plate of sheared samples, use 1 µL 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. Refer to the following SOP:

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

3.2 The following table shows the expected average size from the sheared material. Send the results to the APC for approval.

Starting Material	Average size
ss-cDNA	200-250bp
Small gap	250bp

Note: For ribo-depleted ss-cDNA and lower input poly(A)-based protocol, the products will not be visible on the HSDNA chip. Use the non-depleted UHR control and the 500ng UHR control, respectively, to QC accurate shearing.

3.3. For clinical amplicon samples, all samples and controls need to be QCed. QC after shearing can be done on either the Agilent or the Caliper depending on the number of samples that need to be QCed. Use the the Biomek program to transfer 2µl of DNA to 28µl of EB if using the Caliper for sonication QC. Refer to the following SOP:

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

Or

LIBPR.051 Operation and maintenance of the Caliper LabChip GX for DNA Samples using the High Sensitivity Assay

Send the results to APC for approval.

4. Transferring DNA/cDNA out of Covaris Plate

To transfer DNA/cDNA from Covaris plate to reaction plate (ie. 96-well AB1000 plate), log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction > RUN> Protocol X > **Transfer out of Covaris**

A. For ss-cDNA or ChIP DNA, there is no cleanup therefore proceed to End Repair.

B. For small gap/amplicons, log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction > RUN> Small Gap >**Bead clean sheared DNA**

5. End-Repair and Phosphorylation Reaction

Reminder: If an Amplicon positive control needs to be added to the plate, add it at this point before proceeding to End-Repair.

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

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

5.2. Dispense the appropriate amount of brew into an AB1000 plate. Brew volumes include 5µL DV required for Biomek processing.

	End Repair Premix (uL) per well

	End Repair Premix (uL) per well
Aliquot: 1 plate	28.5
Aliquot: 2 plates	52
Aliquot: 3 plates	75.5
Aliquot: 4 plates	99

5.3. Log into Biomek FX Program as follows:

Biomek: Project > LIBPR> LibraryConstruction > RUN> Protocol X >**End Repair**

5.4. The brew plate is the "Source" and the DNA plate(s) is the "Dest." After Biomek program completion, seal the plates and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

5.5. Incubate End-Repair reaction plate(s) at 20ºC for 30 minutes. The total reaction volume is 58.5 µL.

Tetrad Program: Run > LIBCOR > ER

Enter **'58'** for reaction volume and select **'Y'** for heated lid

6. Magnetic Bead Clean Up after End-Repair

6.1. Log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction > RUN> Protocol X > **Bead Clean E.R.**

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

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

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

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

7.2. Dispense the appropriate amount of brew into an AB1000 plate. Brew volumes include 5µL DV required for Biomek processing.

	A-Tailing Premix (uL) per well
Aliquot: 1 plate	25
Aliquot: 2 plates	45
Aliquot : 3 plates	65
Aliquot : 4 plates	85

7.3. Log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction > RUN> Protocol X > **A-tailing**

7.4. The brew plate is the "Source" and the DNA plate(s) is the "Dest." After Biomek program completion, seal the plates and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

7.5. Incubate A-tailed reaction plate(s) at 37ºC for 30 minutes; 70ºC for 5 minutes; 4ºC for 5 minutes, hold at 4ºC. Enter '50' for reaction volume and select 'Y' for heated lid.

Tetrad Program: Run > LIBCOR > ATAIL

7.6. After the incubation, proceed immediately to the next step. This is NOT a safe stopping point. Quick spin plate and store on ice while setting up the ligation reaction. **Adenylated products are not bead cleaned prior to ligation.**

8. Illumina PE Adapter Ligation Reaction

8.1. Thaw the PE Adapter stock aliquot in the Tissue Culture Room laminar flow hood on the 5th floor, room 511 and immediately place on ice.

8.2. Adapter Ligation brew (minus the PE adapter) must be made in the PCR Clean Room laminar flow hood on the 5th floor, room 510. Addition of PE adapter to the brew must be made in the Tissue Culture Room laminar flow hood on the 5th floor, room 511

8.3. The volume requirement for 1 reaction set up for SSTRA_3.0 and ChIP is as follows:

Solution	1 rxn (µL)
Adenylated template	50
2X Ligation Premix	21
dH_2O	3.6
PE Adapter (10 µM)	0.4
Reaction volume	**75**

Ligation_Brew_4pmol (25µL)

8.4. The volume requirement for Ribodepletion is as follows:

Solution	1 rxn (µL)
Adenylated template	50
2X Ligation Premix	21
dH_2O	3.0
PE Adapter (10 µM)	1.0
Reaction volume	**75**

Ligation_Brew_10pmol (25µL)

8.5. The volume requirement for 1 reaction set up for Small Gap is as follows:

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

Ligation_Brew_40pmol (25µL)

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

LIMS: Mix Standard Solutions > *X > *follow the prompts*> Save Standard Solution

*__X__=Ligation_Brew_4pmol **or** Ligation_Brew_10pmol or Ligation_Brew_40pmol

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

	Ligation_Brew (ul) per well
Aliquot: 1 plate	30
Aliquot: 2 plates	55
Aliquot : 3 plates	80
Aliquot : 4 plates	105

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

8.8.1. Add the PE adapter to the brew last, not more than 10 min before brew addition on Biomek. Make sure the brew is on ice at all times.

8.8.2. Dispense the appropriate amount of brew into an AB1000 plate. Brew volumes include 5µL DV required for Biomek processing

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

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

Log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction >RUN> Protocol X > **Adapter_Ligation**

8.9. The brew plate is the "Source" and the DNA plate(s) is the "Dest." After Biomek program completion, seal the plates and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

8.10. Incubate Adapter Ligation reaction plate(s) at 20ºC for 15 minutes. Enter '75' for reaction volume and select 'Y' for heated lid. Set a timer for 15 minutes.

Tetrad Program: LIBCOR> LIGATION

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

9. Magnetic Bead Clean Up after Adapter Ligation

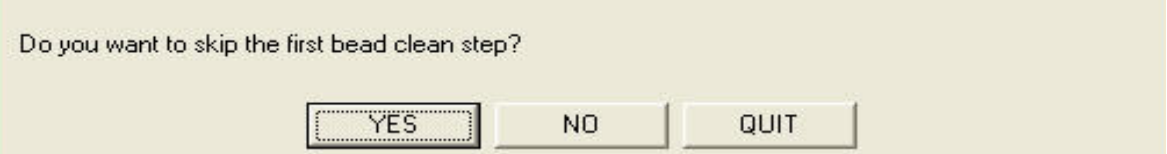
Note: Prior to the second bead clean elution, remove the tip corresponding to the PCR brew control well from the tip box to avoid carrying over elution buffer to the PCR brew control well.

9.1. The input volume for this step is 75 µL per well.

9.2. Log into the following Biomek FX program:

Biomek: Project > LIBPR> LibraryConstruction >RUN> Protocol X > **Bead clean Ligation (2x)**

9.3. Post-ligation bead cleanup is performed twice for all protocols and a safe stopping point 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 and pause, select "No". If you have already finished one round of bead clean and are continuing, select "Yes" (see Figure below). Pour beads back to falcon tube and shake before the 2nd bead cleanup



9.4. Note that template will be eluted in 20µL of EB for subsequent full template iPCR.

9.5. Remove EB contents of designated PCR Brew control well.

10. Indexed PCR Amplification Reaction Or "USER Digestion with PCR" for SSTRA_3.0 and "UNG Digestion with PCR" for Ribodepletion

10.1. Thaw the PE PCR primer 1.0 in the Tissue Culture Room laminar flow hood on the 5th floor, room 511 and immediately place on ice.

10.2. Thaw the Indexing Primer Plate in a working bench across from Biomek FX on the 5th floor, quick spin at 4ºC for 1 minute and immediately place on ice. **The small gap and clinical samples will use 1 time use index primer aliquots of 4µl.**

10.3. 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.

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

10.5. Ensure there is enough volume for the number of plates to be processed including the Biomek dead volume. Inspect the thawed index primer plate after spin down to ensure there are no cracked wells.

10.6. iPCR brew (minus the primers) must be made 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. Addition of the Indexing Primer Plate to the reaction plate is made by the Biomek FX.

10.7. The volume requirement for 1 reaction set up for ChIP and Small Gap is as follows:

Solution	1 rxn (µL)	
Adapter Ligated + BC DNA (Full Template)	19	
5X Phusion HF Buffer	10	PCR Brew (27µL)
10mM dNTP	1	
DMSO	1.5	
Hot Start Phusion (2 U/µL)	0.5	
PE PCR primer 1.0 (25 µM)	2	
dH_2O	12	
Indexed PCR primer plate (12.5µM)	4	
Reaction volume	**50**	

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

Solution	1 rxn (µL)	
Adapter Ligated + BC DNA (Full Template)	19	
5X Phusion HF Buffer	10	PCR Brew + UNG (27µL)
10mM dNTP	1	
DMSO	1.5	
Hot Start Phusion (2 U/µL)	0.5	
PE PCR primer 1.0 (25 µM)	2	
UNG	5	
dH_2O	7	
Indexed PCR primer plate (12.5µM)	4	
Reaction volume	**50**	

10.9. The volume requirement for 1 reaction set up for SSTRA_3.0 is as follows:

Solution	1 rxn (µL)
Adapter Ligated + BC DNA (Full Template)	19
5X Phusion HF Buffer	10
10mM dNTP	1
DMSO	1.5
Hot Start Phusion (2 U/µL)	0.5
PE PCR primer 1.0 (25 µM)	2
USER	3
dH_2O	9
Indexed PCR primer plate (12.5µM)	4
Reaction volume	**50**

PCR Brew + USER (27µL)

10.10. Generate the PCR Brew Mix calculator using LIMS:

LIMS: Mix Standard Solutions > **LibConst_IndexingPCR_Brew OR LibConst_IndexingPCR plus UNG OR LibConst_IndexingPCR plus USER** *follow the prompts* > Save Standard Solution

10.11. 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 Biomek FX.

10.12. Dispense the appropriate amount of brew into an AB1000 plate. Brew volumes include 5µL DV required for Biomek processing.

	Indexing PCR brew (ul) per well
Aliquot: 1 plate	32
Aliquot: 2 plates	59
Aliquot : 3 plates	86
Aliquot : 4 plates	113

Cover with plate seal and quick spin at 4ºC for 1 minute.

10.13. Log into the following Biomek FX program:

Biomek: Project > LibraryConstruction >RUN> Protocol X >**Index PCR**

10.14. The Biomek program for iPCR setup is as follows:

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

10.14.2. Transfer of PCR brew + index primer directly to ligated template (full template PCR).

10.14.3. For small gap and clinical samples only-The Biomek programs will add brew from the Brew Source plate to DNA and then add the DNA/Brew mix to the one time use index primer plate.

10.15. Add 19 µL of water to the PCR brew control well.

10.16. After Biomek program completion, seal the plate(s) and quick spin at 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

10.17. Run PCR program specified in the table below. Use a rubber pad on top of the reaction plate. For clinical samples, only use the clinically designated tetrad.

PCR parameters for SSTRA 3.0 or Ribodepletion:

- 37˚C 15 min
- 98˚C 1 min
- 98˚C 15 sec
- 65˚C 30 sec
- 72˚C 30 sec

} Total of 13 Cycles

- 72˚C 5min
- 4˚C ∞

PCR parameters for others (see table below):

- 98˚C 1 min
- 98˚C 15 sec
- 65˚C 30 sec
- 72˚C 30 sec

} *Total of 6 or 8 Cycles

- 72˚C 5min
- 4˚C ∞

*The number of PCR cycles is dependent on each of the protocol:

Starting Material	PCR cycles	Tetrad Program
ss-cDNA	13	SSCDNA13
Small gap	6	LCPCR-6
ChIP DNA	8	LCPCR-8

11. Post-LC Size Selection (2x 1:1)

11.1. For size selection, log into the following Biomek FX program

Biomek: Project > LibraryConstruction >RUN> Protocol X > **Bead Clean iPCR (2X)**

11.2. 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.

11.3. The final elution volume is 25µL.

12. Preparation of Diluted Library QC Plate

12.1. Prepare a 10x dilution QC plate using the following Biomek FX program:

Biomek: Project > LibraryConstruction >RUN> Protocol X > **Dilute for QC**

The Biomek 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.

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

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

LIBP.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

13.2. For Quant-iT, use the 10x dilution plate or undiluted library as source plates for the QC. Log into the following Biomek program:

Biomek: Project > LibraryConstruction >RUN> Protocol X > **Quant-iT**

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

14. Final HS Caliper QC

14.1. For all protocols, run the 10x dilution QC plate on the Caliper GX according to the following SOP and then calculate the nM quants using the average bp size from Caliper and the Qubit or Quant-iT: Send the results to the APC for approval:

LIBPR.0051 Operation and maintenance of the Caliper LabChip GX for DNA Samples using the High Sensitivity Assay

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

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

LIBPR.0093 Span-8 Pooling of DNA Samples

16. Qubit QC on Pooled Samples and Unpooled Samples for submission

16.1. Refer to the following SOPs:

LIBPR.0030 Quantifying DNA Samples using the Qubit Fluorometer

17. Sequencing Submission:

17.1. 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 and the result from the Quant-iT or Qubit to obtain the final size-corrected nM quant. The remaining final volume (after QCs) is ~20µl. The maximum submission concentration is 60nM. Minimum concentrations and volumes will vary by library type. The APC will confirm whether acceptable range for submission.

Appendix A: LIMS Protocol

1. Start of Plate Library Construction (Skip if doing ss-cDNA)
2. Bioanalyzer Run or Caliper Run – QC Category: Sonication QC (Skip if doing ChIP)
3. A-Library Construction – IDX pipeline
4. Plate_Indexed_PCR- IDX pipeline
5. Plate_PPBC_SizeSelection – IDX pipeline
6. Bioanalyzer Run or Caliper Run – QC Category: Post library construction size selection

   Note: For libraries going into multiplex capture, please select "Post-PCR QC" as the QC Category instead. No need to enter attributes and please skip remaining steps.

   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 or normalizing: Action: 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

Appendix C: Expert SOP: 96-well ss-cDNA library construction (v3.0)

Step	SOP; program name	Biomek protocol: LibraryConstruction	LIMS protocols
1) Transfer DNA to Covaris plate		ss-cDNA> **Shearing Setup**	Aliquot to new TRA to set to IDX pipeline
2) Shear DNA to 200-250 bp ss-cDNA (LE220)	**Plate_130sec_cDNA.e1proc** (in 40uL vol) LIBPR.0097		
3) QC sheared DNA: Agilent HS DNA assay	LIBPR.0017		Bioanalyser Run- Sonication QC
4) Transfer out of covaris plate		ss-cDNA> **Transfer out of Covaris**	
5) End Repair	ER (tetrad)	ss-cDNA> **End Repair**	A-Library Construction – IDX pipeline
6) Clean up End Repair		ss-cDNA> **Bead Clean E. R.**	
7) Adenylation	ATAIL (tetrad)	ss-cDNA> **A-Tailing**	
8) Ligation	LIGATION (tetrad)	ss-cDNA> **Adapter Ligation**	*Ligation_Brew_4pmol*
9) Adapter Clean up		ss-cDNA> **Bead Clean Ligation(2X)**	
10) Indexing PCR	SSCDNA13 (poly-A selected)	**ss-cDNA> Index PCR**	Plate_Indexed_PCR – IDX pipeline *Libconst_IndexingPCR plus USER_brew*
11) Post PCR size selection 2X, 1:1 bead:sample clean up		ss-cDNA> **Bead Clean iPCR(2X)**	Plate_PPBC_SizeSelection – IDX pipeline
12) Quantify final libraries Quant-iT HSDNA Assay	LIBPR.0108	ss-cDNA> **Quant-iT** (Undiluted and 10X dil.)	
13) QC Average size Caliper HSDNA assay	LIBPR.0051	ss-cDNA> **Library QC** (10X dil.)	Bioanalyser Run / Caliper Run- Post library construction size selection QC
14) Option: Span-8 equal molar pooling	LIBPR.0093	Biomek Span-8: Project > LibCore> **Pool_Samples_from_AB1000_or_Axgen PCR 96FS_to_microfugeTubes17mm**	Action: -Aliquot to create pooling TRA – rearray function to track IX pool
15) Option: Quantify pooled libraries Qubit HS DNA assay	LIBPR.0030		

Non Controlled Version

**Note: Controlled Versions of this document are subjected to change without notice*

16) Submit libraries			Final_Submission: IPE or PET pipeline

Solutions: *Ligation_Brew_4pmol*, *Libconst_IndexingPCR plus USER_brew*

Appendix D: Expert SOP: 96-well Small Gap library construction

Step	SOP; program name	Biomek protocol: LibraryConstruction	LIMS protocols
1) Transfer DNA to Covaris plate		Small Gap> **Shearing Setup**	Start of plate library construction
2) Shear DNA to 250 bp Amplicon DNA (E210) gDNA (LE220)	 LIBPR.0041 LIBPR.0097		
3) QC sheared DNA: QC 11/plate: Agilent HS DNA assay	LIBPR.0017		Bioanalyser Run- Sonication QC
4) Transfer out of covaris plate		Small Gap> **Transfer out of Covaris**	
5) Bead clean sheared DNA		Small Gap> **Bead clean sheared DNA**	
6) End Repair	ER (tetrad)	Small Gap> **End Repair**	A-Library Construction – IDX pipeline
7) Clean up End Repair		Small Gap> **Bead Clean E. R.**	
8) Adenylation	ATAIL (tetrad)	Small Gap> **A-Tailing**	
9) Ligation	LIGATION (tetrad)	Small Gap> **Adapter Ligation**	
10) Adapter Clean up 2X, 1:1 Ligation clean up		Small Gap> **Bead Clean Ligation**	
11) Indexing PCR	LCPCR-6 (tetrad)	Small Gap> **Index PCR**	Plate_Indexed_PCR – IDX pipeline
12) Post PCR size selection 2X, 1:1 bead:sample clean up		Small Gap> **Bead Clean iPCR (2X)**	Plate_PPBC_SizeSelection – IDX pipeline
13) Quantify final libraries Quant-iT HSDNA Assay	LIBPR.0108	Small Gap> **Quant-iT** (Undiluted and 10X dil.)	
14) QC Average size Caliper HSDNA assay	 LIBPR.0051	Small Gap> **Library QC** (10X dil.)	Bioanalyser Run / Caliper Run - Post library construction size selection QC
15) Option: Span-8 equal molar pooling	LIBPR.0093	Biomek Span-8: Project > LibCore > **Pool_Samples_from_AB1000_or_Axygen PCR 96FS_to_microfugeTubes17mm**	Action: Aliquot to create pooling TRA Rearray function to track IX pool

16) Option: Quantify pool Qubit HS DNA assay QC	LIBPR.0030		
17) Submit libraries			Final_Submission: IPE or PET pipeline

Solutions: *Ligation_Brew_40pmol; Libconst_IndexingPCR_brew*

Appendix E: Expert SOP: 96-well ChIP DNA library construction

Step	SOP; program name	Biomek protocol: LibraryConstruction	LIMS protocols
1) End Repair	ER (tetrad)	ChIP DNA> **End Repair**	Start of Plate Library Construction
2) Clean up End Repair		ChIP DNA> **Bead Clean E. R.**	A-Library Construction - IDX pipeline
3) Adenylation	ATAIL (tetrad)	ChIP DNA> **A-Tailing**	
4) Ligation	LIGATION (tetrad)	ChIP DNA> **Adapter Ligation**	
5) Adapter Clean up 2X, 1:1 Ligation clean up		ChIP DNA> **Bead Clean Ligation(2X)**	
6) Indexing PCR	LCPCR-8 (tetrad)	ChIP DNA> **Index PCR**	Plate_Indexed_PCR - IDX pipeline
7) Post PCR size selection 2X, 1:1 bead:sample clean up		ChIP DNA> **Bead Clean iPCR(2X)**	Plate_PPBC_SizeSelection – IDX pipeline
8) Quantify final libraries Quant-iT HSDNA Assay	LIBPR.0108	ChIP DNA> **Quant-iT** (Undiluted and 10X dil.)	
9) QC Average size Caliper HSDNA assay	LIBPR.0051	ChIP DNA> **Library QC** (10X dil.)	Bioanalyser Run / Caliper Run - Post library construction size selection QC
10) Option: Span-8 equal molar pooling	LIBPR.0093	Biomek Span-8: Project > LibCore > **Pool_Samples_from_AB1000_or_Axygen PCR 96FS_to_microfugeTubes17mm**	Action: Aliquot to create pooling TRA -Rearray function to track IX pool
11) Option: Quantify pooled libraries Qubit HS DNA assay	LIBPR.0030		
12) Submit libraries			Final_Submission – IPE or PET pipeline

Solutions: *Ligation_Brew_4pmol; Libconst_IndexingPCR_brew*