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96-well PCR Free Library Construction on NIMBUS for Illumina Sequencing

I. Purpose

To provide specific guidelines for Plate based TruSeq Illumina PCR free genomic library construction (300-600bp) on Hamilton NIMBUS.

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 the Covaris LE220	LIBPR.0097
Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples	LIBPR.0017

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Document Title	Document Number
Operation and Maintenance of the Caliper Labchip GX for DNA samples using the High Sensitivity Assay	LIBPR.0051
Quantifying DNA samples using the Qubit Fluorometer	LIBPR.0030
Span-8 Pooling of DNA Samples	LIBPR.0093
Normalization of Nucleic Acid Concentration using the JANUS Automated Workstation	LIBPR.0113
Automated qPCR reaction setup	LIBPR.0125

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	F540L		✓
Fisherbrand Textured Nitrile gloves – various sizes	Fisher	270-058-53		✓
dNTPs, 10 mM each	Invitrogen	46-0519		✓
Ice bucket	Fisher	11-676-36		✓
Covaris LE220 with WCS and Chiller	Covaris	LE220	✓	
DNA *AWAY*	Molecular BioProducts	21-236-28		✓
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-F-180-LRS		✓
P50 (Universal) Tips, Presterile with Barrier, 50 µL, 96/rack, 10racks/case, CS960	Beckman	CABKA21586		✓
Plate, 96-Well reservoirs, diamond-bottom, Low-Profile	Seahorse	EK2036		✓
Plate, 96-Well reservoirs, diamond-bottom, Deep-Profile	Seahorse	S30014		✓
Plate, 96-Well reservoirs, 450uL EtOH & Waste	Axygen	P-96-450V-C		✓
AB1000 96-well 200µl PCR plate	Fisher	AB1000		✓
MagMax express 96 Deep Well plates	Applied Biosystems	4388476		✓
Storage Plate, 96-well, 1.2 mL, square well, U-bottomed	ABgene	AB1127		✓
Microlab NIMBUS	NIMBUS	Hamilton	✓	
Eppendorf Benchtop Centrifuge	Eppendorf	5810 R	✓	
70% Ethanol	In house	N/A	N/A	N/A
Qiagen Buffer EB – 250 mL	Qiagen	19086		✓
10 X TBE - 10 L	Invitrogen	15581-028		✓
1 X TBE	In House	N/A	N/A	N/A
UltraPure Distilled Water	Invitrogen	10977-023		✓
Nuclease Free 2.0 mL eppendorf tube	Ambion	12400		✓
5 mL Screw Cap tubes	Ultident	SCT-5ML-S		✓
TruSeq Indexed Adapters	IDT	NA		
TruSeq Universal Primers	IDT	NA		
Alpaqua Magnum FLX	Alpaqua Engineering	A000400	✓	
Ampure XP Beads, 450mL	Agencourt	A63882		✓
PCR Clean DX (ALINE beads)	ALINE Biosciences	C-1003-450		✓
NIMBUS P50 tips 50uL Clear Sterile Tips, 5760 tips/case	Hamilton Co.	235831		✓
NIMBUS P300 tips 300uL Clear Sterile Tips, 5760 tips/case	Hamilton Co.	235832		✓
Tween 20, 10%, for easy pipetting, 1L	Bio-Rad	161-0781		✓

These sequences are for internal use only:

TruSeq Indexed Adapters

GATCGGAAGAGCACACGTCTGAACTCCAGTCACNNNNNNATCACGATCTCGTATGCCGTCTTCTGCTT
G

TruSeq Universal Adapter

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

TruSeq Universal Primer (For Rescue)
AATGATACGGCGACCACCGACACTC
CAAGCAGAAGACGGCATACGAGAT

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. 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 2000 g 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. Positive and Negative Controls

3.1. The positive control template to be used for this protocol is HL60 genomic DNA. 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 measure 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.

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. The default dead volume required by the NIMBUS for each well of a Brew Source Plate is 5µL/well regardless of the number of plates being processed. 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.

6. General notes on NIMBUS programs

The following steps are generally followed:

A. Start Hamilton Run control
B. Open File/Production/LibraryConstructionv/LibraryConstruction-Scheduler .wfl
    a. Note that file type must have the.wfl extension.
C. Select PCRFree

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

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

Note: Bead to reaction ratios are defined at each step

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

2.1. Samples must be diluted in 62.5µL of Qiagen elution buffer prior to shearing. The minimum requirement is 250ng or 4.17ng/µL in 62.5µL. The maximum input is 1000ng or 16ng/µL in 62.5µL.

2.2. Normalize input as directed by your supervisor AND according to the following SOP:

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

3. Shearing

3.1. The NIMBUS has off-set pipetting capability; hence we can run any number of samples on a plate.

3.2. Samples need to be arrayed in the same order as the TruSeq adapter plate layout. Ensure that the source and destination plate layouts match for all plates, including the source DNA, Covaris shearing, brew and adapter plates.

3.3. To transfer normalized DNA into the Covaris plate or Covaris strip tubes, log into the NIMBUS computer with your phage login and password and follow Step 6 of Introduction and Guideline.

3.4. Log into the NIMBUS and select Shearing Setup:

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

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

3.6. After piercing, DNA is transferred into the Covaris plate/Covaris strip tubes using p50 tips.

3.7. Cover the Covaris plate with Covaris/VWR foil. Covaris strip tubes can be covered with Covaris foil strips or Covaris/VWR foil.

3.8. 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.

4. Agilent HS DNA QC after shearing – Spot Check

4.1. 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

4.2. The profile for sonicated DNA should have the peak close to 425bp. 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.

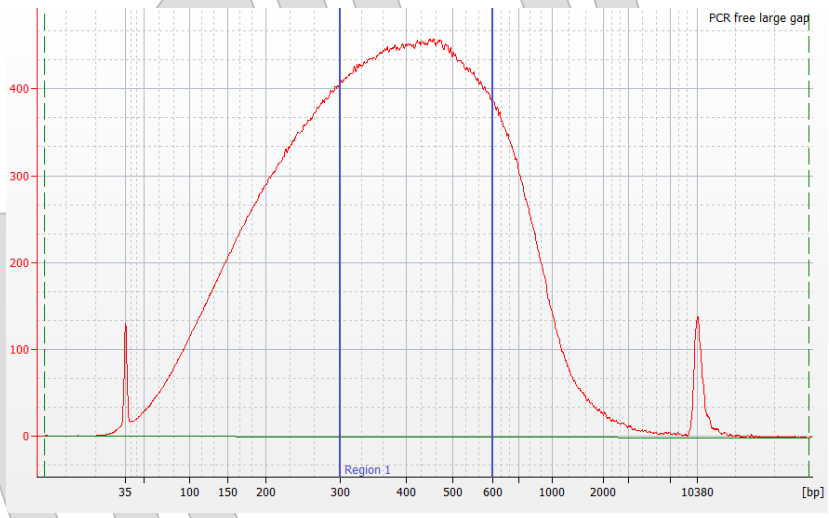


Figure 1: High Sensitivity DNA chip showing ideal shearing in the 300:600 bp range

5. Transferring DNA out of Covaris Plate or Covaris strip tubes

5.1. Log into the following NIMBUS program to transfer sheared gDNA to an AB1000 plate. Follow the deck layout and prompts. All sample volume is removed from the Covaris plate. Do note that NIMBUS does multiple transfers to ensure that maximum volume is transferred.

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

5.2. **Please note: you MUST remove the TOP foil cover prior to starting the transfer step.** The NIMBUS is not programmed to pierce the foil after shearing.

5.3. Visually inspect the source and destination wells to ensure that all of the sheared material has been transferred. Repeat the transfer out of Covaris procedure if template is remaining in the covaris tubes.

6. End-Repair and Phosphorylation Reaction

6.1. 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.

6.2. Match brew plate layout with the DNA plate. Use a Distriman repeater pipettor to dispense brew in as many wells as needed.

Solution	Volume (µL/well)
End Repair Premix (NEB)	40
DNA from Covaris plate	60
Total Reaction Volume	**100**

6.3. Log into NIMBUS Program as follows:

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

6.4. 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 4ºC for 1 minute. Inspect the reaction plate for any variations in volume.

6.5. Incubate End-Repair reaction plate at 20ºC for 30 minutes. Total reaction volume is 100µL.

Tetrad Program: Run > LIBCOR > ER

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

7. Size Selected End Repaired DNA

7.1. Log into the NIMBUS and select the pipeline-specific Size Selection method:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> PCRFree > **Size Select End Repaired DNA**

This step does upper and lower cut.

Upper Cut

Repaired DNA (µL)	Beads (µL)	Mix Volume (µL)	Supernatant (µL)
100	50	120	150

Lower Cut

Supernatant (µL)	Beads (µL)	Mix Volume (µL)	Supernatant Volume (µL)	EB Elution Volume (µL)	Transfer Volume (µL)
150	25	140	175	30	30

7.2. Note that end-repaired product can be stored at -20 °C after size selection

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

8.1. A-Tailing Premix must be thawed on ice, and then gently mixed prior to dispensing 20µL brew per well into the brew plate as described below. Note that aliquot volumes have to be exact. There is no dead volume.

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

8.2. Log into NIMBUS Program as follows:

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

8.3. 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 4ºC for 1 minute. Inspect the reaction plates for any variations in volume.

8.4. Incubate A-Tailing reaction plate at 37ºC for 30 minutes; 70ºC for 5 minutes; 4ºC for 5 minutes; 4ºC hold, using the following Tetrad program. Total reaction volume is 50ul.

Tetrad Program: Run > LIBCOR > ATAIL

8.5. During the incubation incubate the ligation brew on ice.

8.6. After the incubation, temporarily store the template on ice in preparation for adapter ligation. This is not a safe stop, ligation must occur on the same day as adenylation.

Note: DO NOT bead clean adenylated library.

9. Adapter Ligation

9.1. Indexed TruSeq adapters are pre-aliquoted into single use AB1000 plates. For partial plates, prepare an adapter plate by transferring 4µL of TruSeq adapters to the appropriate columns of an AB1000 plate.

9.2. It is crucial that you know indexes being used for the set of libraries you are making and that the TruSeq adapter plate layout matches your sample plate.

9.3. Confirm with your supervisor if you have any questions on how to proceed.

9.4. Thawed Ligation Master Mix premix must be mixed gently prior to dispensing into the brew plate using volumes described below. Note that aliquot volumes have to be exact. There is no dead volume.

9.5. The Ligation brew becomes the reaction plate. Template is first combined with the adapter plate and then the template/adapter mix is subsequently added to the Ligation brew plate. See the table below for reaction set-up.

Ligation Reaction Solution	Volume (µL/well)
NEB Ligation Master Mix	21
Adenylated template	50
TruSeq Indexed Adapters, 6.25 uM	4
Total Reaction Volume	**75**

9.6. Log into NIMBUS Program as follows:

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

9.7. Seal the plates and quick spin at 4ºC for 1 minute after the NIMBUS process has completed. Inspect the reaction plates for any variations in volume.

9.8. Incubate Ligation reaction plate(s) at 20ºC for 15 minutes, using the following Tetrad program:

Tetrad Program: Run > LIBCOR > LIGATION

9.9. While the ligation incubation is running, set up the NIMBUS for bead clean up. Bead clean up must occur immediately after the ligation reaction is completed.

9.10. **After the 15 minute incubation, quick spin the plate and then proceed immediately to Bead Clean Up after ligation**. Store plate on ice during set up.

10. Bead Clean Up after Ligation

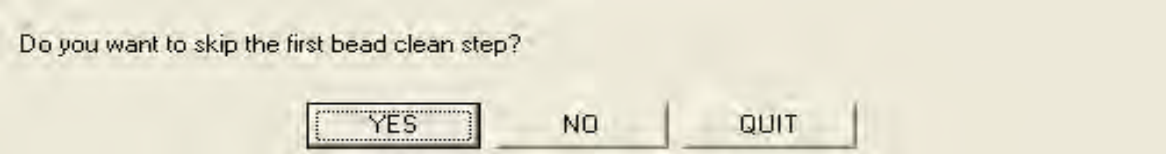
Note: Prior to the second bead clean elution, DO NOT remove the tip corresponding to the PCR brew control well from the tip box. EB-Tween will be used for PCR brew control.

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

10.2. Log into the following NIMBUS program:

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

10.3. Post-ligation bead cleanup is performed twice 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).



10.4. Samples can be stored at -20°C or you may proceed immediately to prepare samples for qPCR.

11. Dilute for qPCR

11.1. This step is designed to make serial dilutions for automated qPCR setup.

11.2. Prepare a solution of 0.05% Tween 20 in EB buffer by adding 75µL of Tween 20 (10%) solution to 15mL of Qiagen EB buffer. Mix well. For larger volumes, use 250µL of Tween 20 (10%) to 49.75mL of Qiagen EB Buffer.

11.3. Log into the NIMBUS as follows:

Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> PCRFree > **Dilute for QC and/or qPCR> *Select appropriate protocol**

11.4. Follow the deck layout. The NIMBUS will prepare the following dilution series: 2 in 38 (1/20 dilution), 2 in 48 (1/500 dilution) and 8 in 32 (1/2500 dilution). The

1/2500 dilution plate is used for qPCR. Refer to LIBPR.0125 - Automated qPCR reaction setup.

11.5. If the PCR free genome samples are to be pooled, use the average bp, 560 bp, to calculate the nM for sub-libraries based on qPCR results. Refer to your APC's instructions for normalization and pooling info. If normalization and pooling are done using Span-8, refer to the following SOP:

LIBPR.0093 Span-8 Pooling of DNA Samples

11.6. If the PCR-free genomes are not pooled, proceed to step 12.

12. Re-array into 1.5mL Tubes for Submission

12.1. Discuss with supervisor to see if libraries pass the acceptance criteria. If libraries pass the acceptance criteria, libraries can be re-arrayed into 1.5mL tubes for submission.

12.2. If rescue PCR is needed, please see Appendix B.

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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-PCR Free Library Construction - IDX pipeline
    - **Be sure to scan the NIMBUS equ ID**
    - **Avg_DNA_bp_size: 560**
4. Bioanalyzer Run or Caliper Run- QC Category: Post library construction size selection: **Only needed if doing Rescue PCR.**

    Enter the following attributes:
    1) Library_size_distribution_bp
    2) Avg_DNA_bp_size
5. PCR_Rescue – only run this protocol when PCR rescue is required.
6. qPCR Run generated in LIMS
7. qPCR quant
8. Final_Submission – TPE (no pooling); ITP (pooled) or DITP (Dual-indices pooling)

Appendix B: Rescue PCR

1. Rescue PCR for failed PCR-Free library

1.1. Libraries that do not meet the acceptance criteria may be rescued by doing 4 cycles PCR or more as specified by your supervisor.

1.2. The volume requirement is as follows:

Solution	1 rxn (µL)
Undiluted ligated template	8
5X Phusion HF Buffer	5
TruSeq Primer 1 & 2 Cocktail, 10 uM	2
10mM dNTP	0.5
100% DMSO	0.75
Hot Start Phusion Enzyme, 2 U/µL	0.25
Nuclease free water	8.5
Reaction total	**25**

PCR Brew (17µL)

1.3. Generate a brew calculator in LIMS as follows:

LIMS: Mix Standard Solution > **PCRFree-Rescue-PCR** > *follow the prompts* > Save Standard Solution

1.4. Use a single pipette or Gilson Distriman to dispense 17µL of PCR rescue brew into an AB1000 plate labeled "PCR rescue". Transfer 8 µL of Adapter-ligated template DNA into the "PCR rescue" plate. Cover the PCR plate with VWR foil seal and quick spin for 1 min.

1.5. Perform 4 cycle PCR using the following tetrad program:

Tetrad program: LIBCOR>PF'PCR4.

1.6. Rescue PCR is followed by 1:1 bead clean according as described below.

2. ALINE PCR Clean DX Bead Clean Up after PCR rescue

2.1. Clean up PCR rescue using ALINE beads as described in the following SOP:

LIBPR.0073- Manual Bead Clean using Ampure XP Beads

2.2. Specific volumes are highlighted below.

DNA volume (µL)	Bead Volume (µL)	Mixing Volume (µL)	Bead Binding Time (mins)	Magnet Clearing Time (mins)	Supernatant Volume (µL)	2x 70% EtOH Wash Vol (µL)	Ethanol Air Dry Time (mins)	EB Elution Volume (µL)	Elution Time (mins)	Magnet Elution Time (mins)	Transfer Volume (µL)
25	25	40	15	7	50	150	5	22	3	2	20

2.3. Quantify the rescued PCR products by Qubit quant according to the following protocol:

LIBPR.0030 Quantifying DNA Samples using the Qubit Fluorometer

2.4. Run 1µL aliquot of each PCR Rescued sample on a DNA1000 Agilent chip according to the following protocol:

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

2.5. Use the determined bp from the Agilent profile and the Qubit concentration to calculate the molarity of the rescued DNA.

2.6. Follow LIMS SOP as per Appendix A, step 10: PCR Rescue

Confirm the concentration with your APC and submit the sample for sequencing if the submission criteria for concentration are met.

Appendix C: Manual PCR free library construction

1. Shearing & QC

1.1. Transfer gDNA, to Covaris LE220 vessels
1.2. Covaris LE220, LIBPR.0097
1.3. QC: Agilent HS DNA Assay

2. End Repair

Solution	1 rxn (µL)
End-Repaired gDNA	60
NEB Adenylation Premix	40
Reaction volume	**100**

2.1. Transfer 40 µL of NEB End Repair Premix into wells of a destination plate.
2.2. Transfer 60 µL of sheared & repaired DNA to the brew, mix using 80% volume, 10X.
2.3. Tetrad Program **LIBCOR > PRECR/ER-** 20^{0}C for 45 minutes; hold 4C
2.4. Safe stopping point if stored at -20^{o}C.

3. Upper/Lower Size Selection

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

Upper Cut

DNA volume (µL)	Bead Volume (µL)	Mixing Volume (µL)	Bead Binding Time (mins)	Magnet Clearing Time (mins)	Supernatant Volume
100	50	120	15	7	150

Lower Cut

Supernatant Volume (µL)	Bead Volume (µL)	Mixing Volume (µL)	Bead Binding Time (mins)	Magnet Clearing Time (mins)	Ethanol Air Dry Time (mins)	EB Elution Volume (µL)	Elution Time (mins)	Magnet Elution Time (mins)	Transfer Volume (µL)
150	25	140	15	7	5	32	3	2	30

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

4. A-Tailing

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

4.1. Transfer 20 µL of NEB Adenylation Premix to 30 µL of size selected and repaired/phosphorylated DNA.

4.2. Tetrad Program: LIBCOR>ATAIL

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

5. Adapter Ligation

Solution	1 rxn (µL)
Adenylated template	**50**
NEB Ultra Premix 2X	21
TruSeq Adapter, 6.25uM	4
Reaction volume	**75**

5.1. Transfer Template to single use adapter plate containing 4uL of TruSeq adapter per well.
5.2. Transfer 21µL of ligation brew to 54 µL of template plus index adapter
5.3. Reset pipette to 80% total volume, mix 10X.
5.4. Select tetrad program: LIBCOR>LIGATION
5.5. Set a timer for 15 minutes. Quick spin plate and store on ice immediately after the 15 minute ligation.

6. Double Bead Clean post Ligation (1:1)

Bead clean #1

DNA volume (µL)	Bead Volume (µL)	Mixing Volume (µL)	Bead Binding Time (mins)	Magnet Clearing Time (mins)	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	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)	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	150	5	20	3	2	19

6.1. The ligated template can be stored at -20°C after the first or second bead clean up step.

7. Dilute for qPCR.

7.1. Samples should be diluted using the automated NIMBUS protocol.

> Hamilton Run Control: File> Open> Production> LibraryConstruction > Library Construction-Scheduler.wfl> PCRFree > **Dilute for QC and/or qPCR> *Select appropriate protocol**

7.2. Refer to LIBPR.0125 for qPCR set up.

7.3. If the PCR-free genome samples are to be pooled, use the average bp from either the Caliper or Agilent and concentration from Qubit or Quant-iT to calculate the nM for the sub-libraries.

7.4. For POG and specified HiSeqX genomes, pool tumour and normal in a 2:1 nM ratio and send the data to the APC for the pooled IX# generation and the instructions for qPCR setup on the pooled libraries. Add the "Avg_DNA_bp_size" attribute to the pooled library

7.5. For non-tumour and normal paired pooling, follow the APC's instructions regarding pooling and subsequent qPCR.

7.6. If the PCR-free genomes are not pooled, proceed to step 9 following the qPCR setup using LIBPR.0125

> LIBPR.0125 Automated PCR and qPCR reactions setup

8. Re-array into 1.5 mL Tubes for Submission

8.1. Discuss with supervisor to see if libraries pass the acceptance criteria. If libraries pass the acceptance criteria, libraries can be re-arrayed into 1.5mL tubes for submission.

8.2. Follow LIMS SOP, Appendix A.

8.3. Print all the barcodes, label tubes and affix barcodes before DNA transfer.

8.4. Submit the entire amount, there should be no need to dilute or hold any back.