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96-well TruSeq 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 group.

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

Reference Title	Reference Number
TruSeq DNA PCR-Free Reference Guide	AM.0105
Illumina Adaptor Sequences	1000000002694 v16

VI. Related Documents

Document Title	Document Number
Automated DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR3V/VICTOR X3	LIBPR.0108
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
Quantifying DNA samples using the Qubit 4 Fluorometer	LIBPR.0153
JANUS G3 Normalization and Pooling of DNA Samples	LIBPR.0146
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 safety data sheet (SDS) for additional information.

VIII. Materials and Equipment

Name	Supplier	Number: #	Model or Catalogue #	
TruSeq DNA PCR-Free Library Prep (96 Samples)	Illumina	20015963		✓
IDT for Illumina TruSeq DNA UD Indexes (96 indexes, 96 samples)	Illumina	20023784		✓
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)	Commercial Alcohols	00023878		✓
IKA Works Vortexer	Agilent	MS2S9-5065-4428	✓	

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Name	Supplier	Number: #	Model or Catalogue #	
22R Microfuge Centrifuge	Beckman	22R Centrifuge	✓	
PCRmax Alpha Cycler 4	Froggabio/Cole Parmer	AC496	✓	
Plate, 96-Well reservoirs, diamond-bottom, Low-Profile	Thomas Scientific	1149J14		✓
AB1000 96-well 200µL PCR plate	Fisher	AB1000		✓
MagMax express 96 Deep Well plates (EtOH and waste)	Applied Biosystems	4388476		✓
Storage Plate, 96-well, 1.2mL, 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 – 250mL	Qiagen	19086		✓
UltraPure Distilled Water	Invitrogen	10977-023		✓
Nuclease Free 2.0mL eppendorf tube	Ambion	12400		✓
5 mL Screw Cap tubes	Ultident	SCT-5ML-S		✓
Alpaqua Magnum FLX	Alpaqua Engineering	A000400	✓	
NIMBUS P50 tips 50µL Clear Sterile Tips, 5760 tips/case	Hamilton Co.	235831		✓
NIMBUS P300 tips 300µL Clear Sterile Tips, 5760 tips/case	Hamilton Co.	235832		✓
Tween 20, 10%, for easy pipetting, 1L	Bio-Rad	161-0781		✓
ALPS 50V Microplate Heat Sealer	Thermo Scientific	AB-1443	✓	
EZPierce 20µM Thermal foil	ThermoFisher	AB1720		✓

These sequences are for internal use only:

Barcoded as IDT for Illumina TruSeq DNA UD indexes

The dual indexed adapter sequences are proprietary and their design is depicted below.

Index 1 (i7) Adapters
GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**[i7]**ATCTCGTATGCCGTCTTCTGCTTG
Index 2 (i5) Adapters
AATGATACGGCGACCACCGAGATCTACAC**[i5]**ACACTCTTTCCCTACACGACGCTCTTCCGATCT

Where the i7 and i5 are 10 bp unique dual indices.

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IDX Cap Universal Primer (For Rescue PCR and 10X PCR QC)
Note: 10 cycle PCR QC is for troubleshooting purpose only. Instruction is not listed on this SOP.
5' IDX CAP: AATGATACGGCGACCACCG
3' IDX CAP: CAAGCAGAAGACGGCATACGAG

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 in the 5th Floor and 6th floor laboratories, 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. Up to 2 plates 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 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.

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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.

4.8. Allow Resuspension Buffer (RSB) and Sample Purification Beads (SPB) 30 minutes to reach room temperature before use.

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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 5 µL/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 25 mL.

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/ /Illumina TruSeq LibraryConstruction- Scheduler.wfl
Note: file type must have the.wfl extension.
C. Select TruSeq PCR-Free

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)
5	**8**	**150**	**5**	**18**	**2**	**2-5**

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.

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1. Initial QC

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

LIBPR_WORKINST.0108 Automated DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR3V/VICTOR X3

2. Sample Normalization on the JANUS Automated Workstation

2.1. Samples must be diluted in 62.5 µL of Resuspension Buffer (RSB) prior to shearing. The minimum requirement is 250 ng or 4 ng/µL in 62.5 µL. The ideal input is 1000 ng or 16 ng/µL in 62.5 µL.

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 IDT for Illumina TruSeq DNA UD Index 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 > > Illumina TruSeq Library Construction.wfl > TruSeq PCR-Free > Shearing Setup

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

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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 1 µL from 11 random samples (ensure that at least one of these samples is a positive control and one sample is a negative 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 425 bp. 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.

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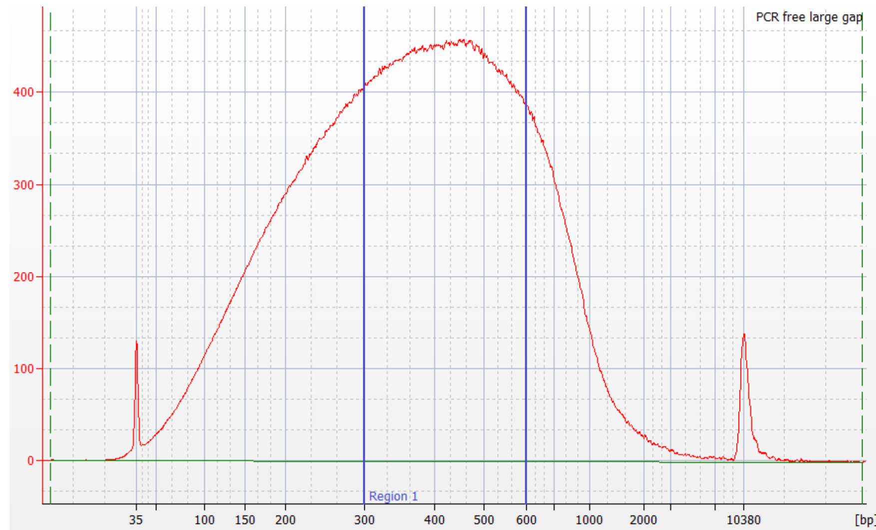

Figure 1: High Sensitivity DNA chip showing ideal shearing in the 300:600 bp range

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 > > Illumina TruSeq LibraryConstruction v1.0.wfl > TruSeq PCR-Free > **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 Mix 2 (ERP 2) 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.

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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 Mix (ERP 2)	40
DNA from Covaris plate	60
Total Reaction Volume	**100**

6.3. Log into NIMBUS Program as follows:

Hamilton Run Control: File > Open > Production > > Illumina TruSeq LibraryConstruction v1.0.wfl > TruSeq PCR-Free > **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 30ºC for 30 minutes using the following tetrad program.

Tetrad Program: Run > > ER30

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 > > Illumina TruSeq LibraryConstruction v1.0.wfl> TruSeq PCR-Free > **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

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Lower Cut

Supernatant (µL)	Beads (µL)	Mix Volume (µL)	Supernatant Volume (µL)	RSB Elution Volume (µL)	Transfer Volume (µL)
150	25	140	175	18	30

7.2. Note that end-repaired product can be stored at -20ºC 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 12.5 µL 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)
A-Tailing Mix 2 (ATL 2)	12.5
End-Repair + BC DNA	17.5
Total Reaction Volume	**30**

8.2. Log into NIMBUS Program as follows:

Hamilton Run Control: File > Open > Production > > Illumina TruSeq LibraryConstruction v1.0.wfl> TruSeq PCR-Free > **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.

Tetrad Program: Run > > ATAIL

8.5. During the incubation thaw the Ligation Mix 2 (LIG 2) 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.

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Note: DO NOT bead clean adenylated library.

9. Adapter Ligation

9.1. Diluted (1:1) IDT for Illumina TruSeq DNA UD Indexes adapters are pre-aliquoted into single use AB1000 plates. See Appendix E on how to make the diluted adapter plate and single use adapter plates (5 µL).

9.2. It is crucial that you know indexes being used for the set of libraries you are making and that the 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 Mix 2 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. Return Ligation Mix 2 immediately to the freezer after use.

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)
Ligation Mix 2 (LIG 2)	2.5
Adenylated template	30
Index adapter (1:1 diluted)	5
Total Reaction Volume	**37.5**

9.6. Log into NIMBUS Program as follows:

Hamilton Run Control: File> Open> Production> > Illumina TruSeq LibraryConstruction v1.0.wfl> TruSeq PCR-Free >**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 30ºC for 10 minutes, using the following Tetrad program:

Tetrad Program: Run > > LIG30

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.

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9.10. **After the 10 minute incubation, quick spin the plate and then proceed immediately to Bead Clean Up after ligation**. Store plate on ice during set up.

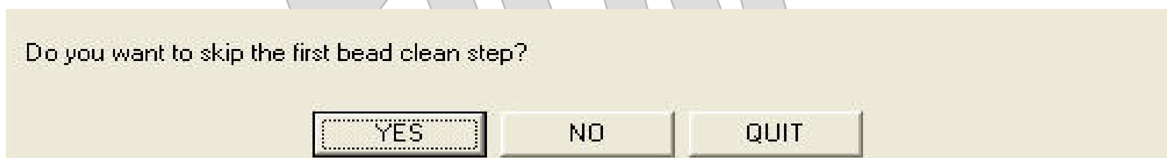
10. Bead Clean Up after Ligation

10.1. The input volume for this step is 37.5 µL per well.

10.2. Log into the following NIMBUS program:

Hamilton Run Control: File> Open> Production> > Illumina TruSeq LibraryConstruction v1.0.wfl> TruSeq PCR-Free > **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 75 µL of Tween 20 (10%) solution to 15 mL of Qiagen EB buffer. Mix well. For larger volumes, use 250 µL of Tween 20 (10%) to 49.75 mL of Qiagen EB Buffer.

11.3. Log into the NIMBUS as follows:

Hamilton Run Control: File> Open> Production> > Illumina TruSeq LibraryConstruction v1.0.wfl> TruSeq PCR-Free > **Dilute for QC and/or qPCR> *Select appropriate protocol**

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11.3.1. Select the appropriate protocol: 3-step dilute: Set up for qPCR only

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 JANUS G3, refer to the following SOP:

LIBPR.0146 JANUS G3 Normalization and Pooling of DNA Samples

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

12. Re-array into 1.5 mL 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 1.5 mL 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-TruSeq 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) or DITP (Dual-indices pooling)

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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
NEBNext Ultra II Q5 Master Mix	12.5
10µM 5' IDX_CAP	2
10µM 3' IDX_CAP	2
Nuclease free water	0.5
Reaction total	**25**

PCR Brew (17µL) (Master Mix, 5' IDX_CAP, 3' IDX_CAP, Nuclease free water)

1.3. Generate a brew calculator in LIMS as follows:

LIMS: Mix Standard Solution > **PCRfree-Rescue-Q5** > *follow the prompts* > Save Standard Solution

1.4. Use a single pipette or Gilson Distriman to dispense 17 µL of PCR rescue brew into an AB1000 plate labeled "PCR rescue". Transfer 8 µL of Adapter-ligated template DNA into the "PCR rescue" plate.

*Heat seal the plate using Adhesive foil EZPierce 20 µM Thermal foil (Cat. No. AB1720, Thermo Fisher). The equipment used for this is ALPS 50V Microplate Heat Sealer (Cat. No. AB-1443, Thermo Scientific). Please see Appendix D for instructions.

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

Tetrad program: >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:

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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.0153 Quantifying DNA Samples using the Qubit 4 Fluorometer

2.4. Run 1 µL 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

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

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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
End Repair Mix 2 (ERP 2)	40
Reaction volume	**100**

2.1. Transfer 40 µL of End Repair Mix 2 (ERP2) 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 > **ER30 for** 30°C at 30 minutes: hold 4°C

2.4. Safe stopping point if stored at -20°C.

3. Upper/Lower Size Selection

3.1. Ethanol and Sample Purification Beads (SPB) 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	5	7	150

Lower Cut

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Supernatant Volume (µL)	Bead Volume (µL)	Mixing Volume (µL)	Bead Binding Time (mins)	Magnet Clearing Time (mins)	Ethanol Air Dry Time (mins)	RSB Elution Volume (µL)	Elution Time (mins)	Magnet Elution Time (mins)	Transfer Volume (µL)
150	25	140	5	7	5	18	3	2	18

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	17.5
A-Tailing Mix 2 (ATL 2)	12.5
Reaction volume	**30**

4.1. Transfer 12.5 µL of A-Tailing Mix 2 (ATL 2) to 17.5 µL of size selected and repaired/phosphorylated DNA.

4.2. Tetrad Program: >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

5.1. **Dilute Adapters 1:1 unless using pre-aliquoted plate that is at working concentration**

Solution	1 rxn (µL)
Adenylated template	**30**
Ligation Mix 2 (LIG 2)	2.5
IDT for Illumina TruSeq DNA UD Index (1:1 Diluted)	5
Reaction volume	**37.5**

5.2. Transfer Template to single use adapter plate containing 5 µL of pre-diluted IDT for Illumina TruSeq DNA UD Index adapter per well.

5.3. Transfer 2.5 µL of ligation brew to 30 µL of template plus index adapter.

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5.4. Reset pipette to 80% total volume, mix 10X.

5.5. Select tetrad program: > LIG30

5.6. Set a timer for 10 minutes. Quick spin plate and store on ice immediately after the 10 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)	RSB Elution Volume (µL)	Elution Time (mins)	Magnet Elution Time (mins)	Transfer Volume (µL)
37.5	37.5	60	5	8	150	5	52	2	5	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)	RSB Elution Volume (µL)	Elution Time (mins)	Magnet Elution Time (mins)	Transfer Volume (µL)
50	50	80	5	8	150	5	22	3	2	20

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.

<u>Hamilton Run Control</u>: File> Open> Production> -> 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 560 bp to calculate the nM for the sub-libraries.

7.4. Follow the APC's instructions regarding pooling and subsequent qPCR.

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7.5. If the PCR-free genomes are not pooled, proceed to step 8 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.

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Appendix D: ALPS 50V Microplate Heat Sealer

NOTE: The seals should be stored in the foil seal packaging to maintain proper orientation. Failure to orient the foil seal with the adhesive side down in the plate sealer will result in the seal adhering to the instrument rather than the plate.

1. Turn on the ALPS 50V heat sealer and allow the instrument to warm up. The Heat on/off LED will flash during this time and stay on once the desired temperature is reached. The sealer should be pre-set for 165°C, 3 second seal time.

2. Place the foil seal on top of the input plate (shiny side up).

3. Place the plate on the plate carrier so that well A1 is in the back left corner. Avoid touching the heating surface while loading the sample plate to prevent injury.

4. Grasp the handle and lower to thermally compress the foil seal onto the input plate. Do NOT apply more pressure to the handle than necessary. When the correct pressure is achieved, an audible tone will sound and the timer will count down to zero.

5. Once the timer reaches zero, another audible tone will sound. Raise the handle to release the heater plate.

6. Rotate the plate so that well A1 is in the front right corner (H12 will be in the back left corner) and repeat the sealing steps 4 and 5.

7. Use a roller seal to ensure that all wells are properly sealed.

8. Put a thermal pad on top of the output plate, then close and tighten the lid.

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Appendix E - Host Seq Indexed Adapter 1:1 Dilution Plate

1. Thaw the IDT for Illumina TruSeq DNA UD index plate on ice. Centrifuge the plate at 4ºC to bring liquid down. Visually check the volumes across the whole plate. Store on ice until ready for dilution steps.
2. Using a multichannel pipette fitted with fresh tips, manually pierce one column at a time. Do not pierce next column until post transfer.
3. Using the 2-20 uL multichannel pipette fitted with fresh tips, pipette up and down to mix prior to transferring **10 uL** to empty AB1000 plate.
4. Once all 12 columns of UD indexed adapters have been transferred to the AB1000 plate, seal the plate and quick spin. Check the 10 uL volume transfers are consistent throughout the plate
5. Using the NIMBUS, select **Transfer or Distribute Samples or Reagent v1.3** program.
   a. Select **Transfer brew or samples**.
   b. Choose **Low Profile Reservoir** as source plate and **AB-1000** as destination plate.
   c. Select **Brew/Beads (Viscous)** as liquid type to be transferred
   d. Enter the number of columns (**12**)
   e. Select Destination plate first column (**1**)
   f. Select Source plate first column (**1**)
   g. Enter current volume in Destination plate (**10 uL**)
   h. Enter transfer volume (**10 uL**)
   i. Place labware according the deck layout. Fill the reservoir with EB to line.
   j. Run the program.
6. After running the program, quick spin the newly diluted 1:1 plated. Check the plate for 20uL volumes throughout the plate.

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7. Again, use the NIMBUS, select **Transfer or Distribute Samples or Reagent v1.3** program for 5uL one use aliquot plates.
    a. Select **Transfer brew or samples**.
    b. Choose **AB-1000** as source plate and **AB-1000** as destination plate.
    c. Select **Brew/Beads (Viscous)** as liquid type to be transferred
    d. Enter the number of columns (**12**)
    e. Select Destination plate first column (**1**)
    f. Select Source plate first column (**1**)
    g. Enter current volume in Destination plate (**0 uL**)
    h. Enter transfer volume (**5 uL**)
    i. Select (**NO**) to rescue samples from source plate.
    j. Place labware according the deck layout.
    k. Run the program.
    l. Foil seal; quick spin; **5 uL** volume check throughout the plate; barcode; and store at -20°C.
8. Repeat **step 7** for another 2 times to produce a total of 3 useable index plates.

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