

Strand Specific 96-well Library Construction for Illumina Sequencing	
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Strand Specific 96-well Library Construction for Illumina Sequencing

I. Purpose

To provide specific guidelines for 96-well, strand specific (coding strand only) RNA-seq (WTSS) library construction from cDNA template for Illumina Paired-End Sequencing

II. Scope

All procedures are applicable to the BCGSC Library TechD and the Library Core groups.

III. Policy

This procedure will be controlled under the policies of the Genome Sciences Centre, as outlined in the Genome Sciences Centre High Throughput Production Quality Manual (QM.0001). Do not copy or alter this document. To obtain a copy see a QA associate.

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Group Leaders to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Assurance team 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
Strand Specific 96 Well cDNA Synthesis	LIBPR.0079
DNA Transfer to Covaris Tube Rack and Sheared DNA Transfer to 96-well Plate Using the Biomek FX	LIBPR.0045
96-well DNA Purification Using Ampure Magnetic Beads and Biomek FX	LIBPR.0047
Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples	LIBPR.0017
Quantifying DNA Samples using the Qubit Fluorometer	LIBPR.0030
Manually pour agarose gels for Barracuda DNA size selection	LIBPR.0055
DNA Size Selection on the Barracuda Robot II	LIBPR.0090

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Document Title	Document Number
Clean and Concentrate DNA using a QIAGEN MinElute 96 UF PCR Filter Plate	LIBPR.0080
96-well DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR ³ V	LIBPR.0108
Operation and Maintenance of the Caliper Labchip GX for DNA samples using the High Sensitivity Assay	LIBPR.0051
Span-8 Pooling of DNA Samples	LIBPR.0093
Operation of the Invitrogen Egel iBase Power System	LIBPR_WORKINST.0012
Operation of the Covaris LE220	LIBPR.0097

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 Kit	NEB	E6000B-25		✓
Fisherbrand Textured Nitrile gloves – various sizes	Fisher	270-058-53		✓
Ice bucket – Green	Fisher	11-676-36		✓
Wet ice	In house	N/A	N/A	N/A
AB1000 96-well 200µl PCR plate	Fisher	AB1000		✓
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	GF-F171203		✓
Diamond Filter tips DF30	Mandel	GF-F171303		✓
Diamond Filter tips DF200	Mandel	GF-F171503		✓
Diamond Filter tips DF1000	Mandel	GF-F171703		✓
Galaxy mini-centrifuge	VWR	37000-700	✓	
VX-100 Vortex Mixer	Rose Scientific	S-0100	✓	
AMPure XP, 450mL Beads	Agencourt	000132		✓
Black ink permanent marker pen	VWR	52877-310		✓
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		✓
MinElute PCR Purification Kit (50)	Qiagent	28004		✓

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DNA Away	VWR	53509-506		✓
Mussel Glycogen (20mg)	Roche Scientific	10 901 393 001		✓
3 M Sodium Acetate	Sigma	EC 211-162-9		✓
(EB Buffer) 10mM Tris-HCl pH 8.0	Qiagen	19570		✓
Covaris E210 Sample Preparation System	Covaris	E210	✓	
Anhydrous Ethyl Alcohol (100% Ethanol)	Commercial Alcohol	People Soft ID:		✓
Mylar PET film, clear (40"x10"x 0.003")	McMaster-Carr	8567K32		✓
Plastic wrap	In house	N/A		✓
22R Microfuge Centrifuge	Beckman	22R Centrifuge	✓	
Peltier Thermal Cycler	MJ Research	PTC-225	✓	
Power Supply, LVC2kW, 48VDCV	Tyco Electronics	RM200HA100	✓	
Spin-X Filter Tube	Fisher	CS008160		✓
VICTOR ³ V	Perkin Elmer	1420-040	✓	
Uracil N-Glycosylase	Applied Biosystems	N808-0096		✓
P2-20 Rainin Lite Manual 12-channel	Rainin	L12-20	✓	
P20-200 Rainin Lite Manual 12-channel	Rainin	L12-200	✓	
P200 Barrier Rainin tips	Rainin	RT-L200F	✓	
P20 Barrier Rainin tips	Rainin	RT-L10F	✓	
Power PAC	BioRad	Power PAC 200	✓	
Biomek FX Liquid Handling System	Ultident	24-FXF-180-	✓	
10 X TBE	Invitrogen	15581-028	N/A	N/A
1 X TBE	In House	N/A	N/A	N/A
100 bp Ladder	Invitrogen	15628-019	N/A	N/A
MicroAmp clear adhesive film	ABI	4306311		✓
Foil Tape, 3" x 60yds, 12rolls/caseVWR	General FastenersVWR	3400074060941-		✓
Clear Tape Sealer	Qiagen	19570		✓
Aluminum Foils	VWR	60941-126		✓

IX. Procedure

1. Introduction and Upstream Set Up

- 1.1. Put on a clean pair of gloves and a lab coat. Wipe down the assigned specific workstation, pipetman, and small equipment. Lay down new bench coat and retrieve ice and all required reagents.
- 1.2. It is important to wear gloves when handling sample plates, reagents and equipment, and to treat everything with clean PCR techniques.
- 1.3. General guidelines:

A. *Never re-use plate sealing tape.*

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- B. To avoid cross-well contamination, reaction plates should never be vortexed.*
- C. Plate mixing steps are only to be done using Biomek FX or manual 12-channel pipette.*
- D. All buffers should be well mixed before addition to the brew. All brews should be well mixed in tubes before dispensing into reaction plates to assure equal distribution of all components and thus uniformity of enzymatic reactions across a plate.*

- 1.4. Up to 3 plates can be processed at a time by one technician using the Biomek FX.
- 1.5. Plates can be stored at -20°C overnight after every step except after “A” addition and after dUTP strand digestion.

Note: “A” addition and adapter ligation reactions should be performed on the same day. Similarly, dUTP digestion and PCR should be performed on the same day as well.

- 1.6. Store “A” tailed reactions and dUTP digested product at 4°C (or on ice) until ready to proceed with same day XP bead clean-up.
- 1.7. All pre-PCR work should be done on the 5th Floor. Post PCR work is to be performed on the 6th floor.
- 1.8. Single use aliquots of PE Illumina adapter once taken out of the freezer should be kept on ice and never refrozen.
- 1.9. Indexing PCR primers are allowed to be thawed only up to 5 times. Each time the plate is taken out of the freezer and thawed it should be marked even if it was not used (including when plates are taken out during freezer maintenance).
- 1.10. The input material for this procedure is double stranded, strand specific cDNA generated using SOP: “LIBPR.0079 - Strand Specific 96well cDNA Synthesis”.
 - 1.10.1. Prior to starting this procedure the quality and quantity of cDNA template should be assessed using HS Agilent Assay *at least* for positive controls and random samples from each plate. For cDNA quality control acceptance criteria refer to “LIBPR.0079 - Strand Specific_ 96well cDNA Synthesis” document.
- 1.11. Use Qiagen tape seals for short term storage, MicroAmp plate seals for tetrad incubations (except for UNG digestion and PCR), VWR aluminum foil for PCR and dUTP digestion with UNG, and foil tape for long term storage.

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2. Covaris LE-series shearing

2.1. Adjust the volume of cDNA to 60uL/well using Qiagen EB buffer. Log onto the 5th floor Biomek FX and follow the prompts.

Biomek: LIBPR > P50B_LIBPR-Transfer to AB1000

2.2. When the program is complete, seal the plates with clear tape sealer and quick spin in an Eppendorf centrifuge using Program 2 (2000g for 1 minute at 4°C). Keep the plates on ice.

2.3. For Covaris LE-series shearing set-up refer to protocols:

LIBPR.0045 DNA Transfer to Covaris Tube Rack and Sheared DNA Transfer to 96-well Plate Using the Biomek FX

LIBPR.0097 Operation of the Covaris LE220, program Plate_130sec_cDNA.e1proc

2.4. Shearing conditions for strand specific PET library construction using Covaris LE-series are:

Duty cycle: 30

PIP: 450

Treatment: 2 x 65 seconds

Cycles/burst: 200

2.5. To assess the sonication efficiency and desired size range, run a High Sensitivity DNA agilent chip on specified or random samples and include the positive control. For this QC, take a 1.5ul aliquot from each of the wells. Refer to

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

2.5.1. Confirm with your Supervisor whether sufficient sonication has been done to achieve the desired size range. The sonicated cDNA should have a peak at 250bp.

2.6. Transfer samples back into a new AB1000 plate.

LIBPR.0045 DNA Transfer to Covaris Tube Rack and Sheared DNA Transfer to 96-well Plate Using the Biomek FX

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2.7. Clean up DNA after sonication. The DNA volume going into this step is 60µL/well.

LIBPR.0047 – 96-well DNA Purification Using Ampure Magnetic Beads and Biomek FX

2.8. **Please complete LIMS for this section, Plate_Shear_DNA and create Bioanalyzer runs for the Agilent QC (Appendix A steps 1-2)**

3. End-Repair and Phosphorylation

Note: Reaction brew must be made in the PCR Clean Room laminar flowhood on the 5th floor, room 510.

3.1. Retrieve and thaw 5x Ligase Buffer and 10mM dNTPs at room temperature then place them on ice. Leave enzymes in the freezer until you are ready to add them to the brew.

3.2. Generate the End Repair brew mix using LIMS:

LIMS: Mix Standard Solutions > **LibConst_End_Repair_Brew** > enter the number of plates and solution numbers > Save Standard Solution

3.3. Prepare brew in an appropriate sized tube according to the printed calculator label, checking off reagents as they are added. Store the brew on ice.

3.4. Mix each reagent (buffers by pulse-vortexing and enzymes by gentle flicking of the tube) and spin down in a minifuge before addition to the brew.

3.5. Mix the brew very well by gentle, repeated pulse-spin vortexing. Store the brew on ice.

3.6. Using a Gilson Pipetman, on ice, dispense the following volume/well into one 96-well AB-1000 Brew Source Plate:

For one plate: 19µL/well (15µL+4µL dead volume)

For two reaction plates: 34µL/well (15µL*2 +4µL dead volume)

For 3 reaction plates: 49µL/well (15µL*3 +4µL dead volume)...etc

3.7. Cover brew source plate with plate seal and quick spin at 4°C, 2000g for 1 minute. Keep it on ice.

3.8. Wipe the Biomek and bench with ddH₂O followed by 70% EtOH.

3.9. Log onto 5th floor Biomek FX and follow the prompts.

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Biomek: FG Indexing > P50B_Lib_Construction_Reaction_Setup_AB1000 > EndRepair

Note: **Reaction brews vary in viscosity. In order to assure the accuracy of transferred volume it is very important to select the correct Biomek technique, in this case End Repair.**

- 3.10. After the program is completed, seal the plates and quick spin in an Eppendorf centrifuge on Program 2 (2000g at 4°C for 1 minute). Discard tips and recycle the boxes.
- 3.11. Check the brew source plate for the remaining dead volume. There should be about 4uL or less of the brew left in every well. Record in your lab notebook if there are any variations in the left over volume and notify your supervisor. Discard the source brew plate.
- 3.12. Incubate End Repair reaction plates at room temperature for 30 minutes.

4. Ampure Magnetic Bead Clean Up after End Repair

Note: Use Biomek FX-5 on the 5th floor for Pre-PCR work. Do not use the 6th floor Biomek.

- 4.1. To clean up DNA after end repair reaction, follow protocol. The DNA volume going into this step is 50µL/well.

LIBPR.0047 – 96-well DNA Purification Using Ampure Magnetic Beads and Biomek FX

- 4.2. **Please complete the LIMS portion of the protocol (Plate_End_Repair). See Appendix A, step 3.**

5. Addition of an 'A' Base to the 3' End of the cDNA Fragments

Note: Ensure that the "A" Addition reaction is done early enough to accommodate the Ligation reaction at the end of the day, as "A" Addition to Ligation steps MUST be performed on the same day. "A" addition after bead clean is not stable.

Note: Brew must be made in the PCR Clean Room laminar flowhood on the 5th floor, room 510.

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5.1. Retrieve and thaw 10x Klenow Buffer and 10mM dATP at room temperature then place them on ice. Leave enzymes in the freezer until you are ready to add them to the brew.

5.2. Generate the A-Addition brew mix using LIMS:

LIMS: Mix Standard Solutions > **LibConst_A_addition_Brew** > enter the number of plates and solution numbers > Save Standard Solution

5.3. Using a Gilson Pipetman, on ice, dispense the following volume/well into one 96-well AB-1000 Brew Source Plate:

For one plate: 19uL/well (15uL+4uL dead volume)

For two reaction plates: 34uL/well (15uL*2 +4uL dead volume)

For 3 reaction plates: 49uL/well (15uL*3 +4uL dead volume)...etc

5.4. Cover brew source plate with plate seal and quick spin at 4°C, 2000g for 1 minute. Keep it on ice.

5.5. Log onto 5th floor Biomek FX and follow the prompts

Biomek: FG Indexing > P50B_Lib_Construction_Reaction_Setup_AB1000 > A_Addition

Note: Reaction brews vary in viscosity and in order to assure an accurate volume transfer it is very important to select here the correct Biomek technique, in this case **dA_Addition.**

5.6. After the program is completed, cover reactions with PCR cover and quick spin in an Eppendorf centrifuge on Program 2 (2000g at 4°C for 1 minute). Discard tips and recycle the boxes.

5.7. Check the source brew plate for the uniformity of the remaining dead volume. There should be about 4uL or less left in every well. Record in your lab notebook if there are any variations in the left over volume and notify your supervisor. Discard the source brew plate.

5.8. Turn on tetrad machine on 5th floor to incubate reaction plates at 37°C for 30 minutes. The final reaction volume is 50uL.

Tetrad: LIBCORE > PCR program "37"

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6. Ampure Magnetic Bead Clean Up after A-Tailing

6.1. To clean up DNA after A-tailing follow protocol. The DNA volume going into this step is 50µL/well.

LIBPR.0047 – 96-well DNA Purification Using Ampure Magnetic Beads and Biomek FX

6.2. Proceed to the next step. This is NOT a safe stopping point.

7. Ligate Illumina PE Adapter to DNA Fragments

Note: Brew must be made in the PCR Clean Room laminar flowhood on the 5th floor, room 510.

7.1. Retrieve and thaw 5X Ligase Buffer and PE Adapters at room temperature then place them on ice. Leave enzymes in the freezer until you are ready to add them to the brew.

Note: Thaw PE Adapter in the Tissue Culture Room laminar flowhood on the 5th floor, room 511 and add it last to the reaction mix.

7.2. Generate the Adapter Ligation brew mix using LIMS. **You will be using 1uL of the 1uM adapter for this reaction**

LIMS: Mix Standard Solutions > **LibConst_1uM_Adapter_Ligation_Brew** > enter the number of plates and solution numbers > Save Standard Solution

7.3. Prepare brew in an appropriate sized tube according to printed calculator label, checking off reagents as they are added. Store the brew on ice.

7.4. Using a Gilson Pipetman, on ice, dispense the following volume/well into one 96-well AB-1000 Brew Source Plate:

For one plate: 29uL/well (25uL+4uL dead volume)

For two reaction plates: 54uL/well (25uL*2 +4uL dead volume)

For 3 reaction plates: 79uL/well (25uL*3 +4uL dead volume)...etc

7.5. Cover brew source plate with tape seal and quick spin 1min. Keep plate on ice until placed on Biomek FX-5 to transfer 25µL brew mix into each reaction plate.

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NOTE: In order to minimize adapter-adapter product it is very important to add DNA to the ligation brew as soon as possible.

7.6. Use the 5th floor Biomek FX and P50 barrier tips to add DNA to the reaction plates.

Biomek: FG Indexing > P50B_Lib_Construction_Reaction_Setup_AB1000 > Adapter Ligation

7.7. Wipe the Biomek and bench with ddH₂O followed by 70% EtOH.

7.8. Confirm the deck layout and click OK to start the program.

7.9. After the program is completed, cover all plates with clear tape sealer. Quick spin plates in an Eppendorf centrifuge on Program 2 (2000g at 4°C for 1 minute).

7.10. Incubate reaction plates at room temperature **OVERNIGHT**.

7.11. Discard source plates, the tips and recycle the tip boxes.

7.12. This is a safe stopping point. **Please complete the LIMS portion of the protocol. See Appendix A, number 4 (Plate_A_Addition).**

8. Ampure Magnetic Bead Clean Up after Adapter Ligation

8.1. To clean up DNA after Adapter Ligation reaction follow protocol. The DNA volume going into this step is 60µL/well.

LIBPR.0047 – 96-well DNA Purification Using Ampure Magnetic Beads and Biomek FX

8.2. **Please complete the LIMS portion of the protocol. See Appendix A, number 5 (Plate_Adapter_Ligation).**

9. dUTP Strand Digestion Reaction with UNG enzyme

Note: dUTP Strand Digestion and PCR reaction MUST be performed on the same day.

9.1. Retrieve UNG (Uracil-N-Glycosylase) enzyme from -20°C freezer. Thaw it on ice.

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9.2. When processing a full plate, on ice, using Gilson pipetman, aliquot 48µL of the UNG enzyme into **one row** (12 wells) of AB-1000 plate. Cover the plate with plastic seal and spin it down at 4°C, 2000g, for 1min. Keep the plate on ice.

9.3. If processing 3 rows of samples or less, aliquot the appropriate volume of UNG enzyme plus dead volume into **one row** of AB1000 plate, based on the number of rows:

For one row: 10µL per well

For two rows: (5µL*2 rows) + 5µL dead volume per well = 15µL/well.

Seal the plate with plastic seal and spin it down at 4°C, 2000g, for 1min. Keep it on ice.

9.4. Using Manual 12-channel Rainin P2-20 pipette, add 5uL of the UNG enzyme to each row of previously adapted and Ampure XP bead cleaned DNA. Mix 5 times after each addition. Change tips between rows and place a check mark on the side of the plate after each row addition to make sure no rows are skipped or repeated. **Seal the plate with VWR aluminum foil** and spin down at 4°C, 2000g, for 1min.

9.5. Use a rubber pad on top of the reaction plate. Run PCR program on the 5th floor tetrad:

Tetrad: LIBCORE > PCR program "UNG"

37°C for 30 minutes

95°C for 15min

4°C forever

9.6. After the incubation is completed, spin the plate at 4°C, 2000g for 1min then proceed to the Bead Clean.

10. Ampure Magnetic Bead Clean Up after dUTP strand digestion.

Note: Use Biomek FX-5 on the 5th floor for Pre-PCR work. Do not use the 6th floor Biomek.

10.1. To clean up DNA after dUTP strand digestion, follow protocol. The DNA volume going into this step is 45µL/well.

LIBPR.0047 – 96-well DNA Purification Using Ampure Magnetic Beads and Biomek FX

10.2. Proceed to the next step. This is not a safe stopping point. PCR amplification should be performed on the same day.

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11. PCR amplification

Note: PCR Brew must be made in the PCR Clean Room laminar flowhood on the 5th floor, room 510.

Note: Thaw PE PCR primer 1.0 in the Tissue Culture Room laminar flowhood on the 5th floor, room 511.

Note: After dUTP digestion, the DNA is single stranded and not stable; therefore to use up all the PCR template, **two PCR reactions are done for each source plate.**

11.1. Retrieve and thaw at room temperature: 5X Phusion HF Buffer, 10mM dNTPs, DMSO, PE PCR primer 1.0 (two aliquots of 125uL per plate), and Indexing primer plate. Once thawed, immediately place all reagents on ice. Leave enzymes in the freezer until you are ready to add them to the brew.

11.2. Make sure that the Indexing primer plate contains enough volume for the amount of plates you are processing:

For one plate the minimum Index primer plate volume is 10µL/well.

For 2 plates the minimum Index primer volume is 15µL/well

For 3 plates the minimum Index primer plate volume is 20µL/well.

11.3. Once thawed, mark off the Indexing primer plate to keep track of freeze-thaw cycles. Spin the plate at 4°C, 2000g, for 1min and keep it on ice.

11.4. Generate the Indexing PCR brew mix using LIMS:

LIMS: Mix Standard Solutions > **LibConst_IndexingPCR_Brew** > enter the number of plates, solution numbers and DNA volume (default = 19uL) > Save Standard Solution

11.5. Prepare PCR brew in an appropriate sized tube according to printed calculator label, checking off reagents as they are added. Mix each reagent before addition to the brew. Mix the brew very well by gentle repeated pulse-vortexing. Store the brew on ice. Keep the Ultrapure water used in making brew to later add as the negative control.

11.6. Move to the laminar flowhood in room 511 to add PE primer 1.0 and for dispensing brew.

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11.7. When processing a full plate or multiple plates, on ice, use a Gilson Distriman to dispense:

1 reaction plate: $27\mu\text{L} * 1 \text{ plates} + 5\mu\text{L} \text{ dead volume} = 32\mu\text{L/well}$ of brew source plate.

2 reaction plates: $27\mu\text{L} * 2 \text{ plates} + 5\mu\text{L} \text{ dead volume} = 59\mu\text{L/well}$ of brew source plate.

3 reaction plates: $27\mu\text{L} * 3 \text{ plates} + 5\mu\text{L} \text{ dead volume} = 86\mu\text{L/well}$ of brew source plate.

11.8. Cover brew source plate with plastic seal and quick spin 1min (4°C , 2000g). Keep the plate on ice.

11.9. Spin down both the Indexing primer plate and PCR source brew plate at 4°C for 1min, 2000g.

11.10. Log into Biomek FX-5:

Biomek: FG Indexing > P50B_Lib_Construction_iPCR_Setup

11.11. Enter the number of DNA plates being processed. **Make sure to enter the correct number of plates** as the volume of Index primers added to the PCR brew plate depends on the number of plates.

11.12. Confirm that “Y” is selected to add 96 Indexed Primers to PCR brew source plate.

11.13. After the program pauses, cover the PCR Brew plate with plastic seal and spin it down at 4°C , 2000g, for 1min.

11.14. After the program is completed cover all Dest plates with **VWR aluminum foil** seal. Discard tips and recycle the boxes.

11.15. Discard empty Source DNA Plates.

11.16. Quick spin PCR reaction plates using Program 2 (2000g at 4°C for 1 minute).

Note: At this point, PCR reactions plates are brought to the 6th floor and PCR amplified using tetrads in the post-PCR area.

11.17. Run PCR program TSPET13 on the thermocycler for DNA amplification. Use a rubber pad on top of the reaction plate

Tetrad: LIBCORE > TSPET13

1. 98°C 1 min
2. 98°C 15 sec

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3. 65°C 30 sec
4. 72°C 30 sec
Go to step 2, 12 more times.
5. 72°C 5min
6. 4°C ∞

11.18. When the PCR program is completed, remove reaction plates from the tetrad and quick spin in an Eppendorf centrifuge on Program 2 (2000g at 4°C for 1 minute).

12. Caliper QC of Indexed Libraries

12.1. The QC of PCR products will be done using the Caliper unless otherwise indicated by your Supervisor. QC is done on only one of the two PCR reactions done for each source plate.

LIBPR.0051 Operation and Maintenance of the Caliper Labchip Gx for DNA samples using the High Sensitivity Assay.

12.2. Set up Caliper QC plates using the 6th floor Biomek-FX.

Biomek: LIBPR > P50B_LIBPR-Transfer to AB1000

12.3. Use this program to transfer 28uL of EB into each caliper QC plate.

12.4. Restart the program to transfer 2uL of the PCR-amplified product to each QC plate. Use fresh tips for each transfer.

12.5. Mix caliper QC plates and spin them down in an Eppendorf centrifuge on Program 2 (2000g at 4°C for 1 minute) before loading onto the Caliper.

12.6. If the Caliper is not used, 12 samples from each plate including the negative and positive controls are run using an Agilent DNA1000 assay. Discuss with your supervisor if additional samples need to be run. Follow protocol

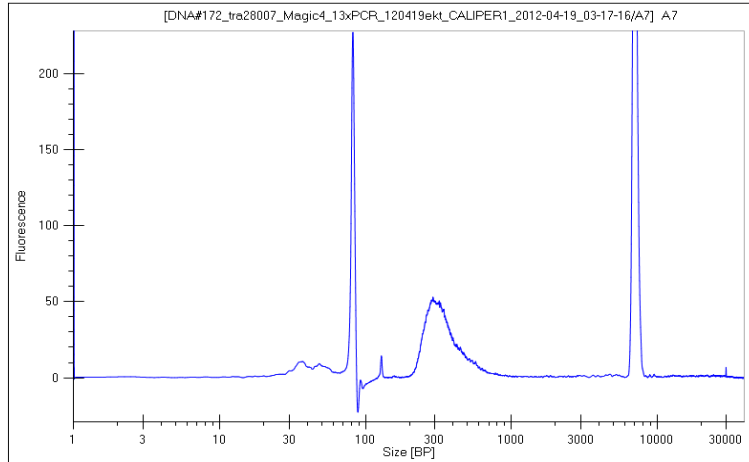
LIBPR.0017 – Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples.

12.7. Show the QC profiles to your supervisor to determine how to proceed with pooling and post-PCR gel purification. An example of a good PCR caliper profile is shown below:

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12.8. In cases of very limited PCR material, the additional PCR reaction may also be used for gel purification. Please confirm if this is the case with your Supervisor. Store the second PCR reaction plate at -20C if it is not required. Scan rac location in LIMS.

12.9. **Please complete the LIMS portion of the protocol. See Appendix A, step 6 (Plate_Uracil_N_Glycosylase_Digestion) and step 7 (Plate_Indexed_PCR).**

Note: If your samples are to be pooled for size selection first, then proceed to section 15, Manually Pool Indexed DNA. If your samples will be size selected before pooling then proceed to the next section (Section 13). In some cases, manual size selection may be needed (see Appendix B). Your supervisor will let you know if this is the case.

13. Unpooled DNA Samples

13.1. The samples that do not need to be pooled before size selection will be size selected by the Barracuda size selection Robot.

13.2. Proceed to pouring gels for Barracuda Size selection.

LIBPR.0055 Manually pour agarose gels for Barracuda DNA size selection

13.3. For preparing and running your PCR plate for Barracuda size selection, refer to

LIBPR.0090 DNA Size Selection on the Barracuda Robot II

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- 13.4. After processing your plate, store it at -20°C in the appropriate rack until you are ready to MinElute filter. *Please complete LIMS, "Plate_Barracuda_Size_Selection" (Appendix A, Section 2) of LIBPR.0090 or Appendix A step 8 (Plate_Barracuda_Size_Selection) of this SOP.*
- 13.5. For concentration of the Barracuda size selected product, please refer to below SOP. *Please complete LIMS, "Plate_MinElute_PPGP" (Appendix A) of LIBPR.0080 or Appendix A step 9 (Plate_MinElute_PPGP) of this SOP.*

LIBPR.0080 Clean and Concentrate DNA using a QIAGEN MinElute 96 UF PCR Filter Plate

- 13.6. Proceed to Section 14 "Check DNA Quant and Quality of Gel Purified Indexed DNA".

14. Check DNA Quant and Quality of Gel Purified Indexed DNA

- 14.1 For plate samples that have gone through barracuda size selection and MinElute filtration, QC samples using the Caliper.

LIBPR.0051 -Operation and Maintenance of the Caliper Labchip Gx for DNA samples using the High Sensitivity Assay

- 14.1.1 Transfer $1\mu\text{L}$ of each sample into a new AB1000 plate containing $29\mu\text{L}$ of EB using a multichannel pipette. (The Biomek FX may be used to add the $29\mu\text{L}$ of EB to the AB1000 plate).

Biomek > FG Indexing > P50B_LIBPR-Transfer to AB1000

- 14.2 For already pooled PPGP samples or if specified by your Supervisor, use $1\mu\text{L}$ of each sample and QC via Agilent DNA 1000 Series II assay. Create Bioanalyzer runs.

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

- 14.2.1 Fill in the following attributes when you are "analyzing" your Agilent runs:

DNA_concentration_ng_uL = the concentration of your sample from the - Qubit quant

Avg DNA bp size = the average size of the sample from the Agilent profiles

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Library_size_distribution_bp = the size of your sample

- 14.3 Use 1 μ L of each sample for Qubit quant.
- 14.4 Please make sure the volumes for the stock PPGP samples are entered so LIMS can calculate the molarity for you. This molarity is the stock nM concentration.
- 14.5 Discuss the results with your supervisor before the libraries are submitted. If the libraries are good, determine the bp size range and average size from the Agilent/Caliper profile. The Bioanalyzer run should calculate the nM based on the Qubit results (ng/ μ L) for you.
- 14.6 Dilute the DNA sample to a concentration of 2.1(in 15uL) - 60nM in buffer EB supplemented with 0.1% Tween-20.
- 14.7 If pooling of your samples is required and has not already been done, normalize the sample based on the molarity.

LIBPR.0031 SLX-PET Protocol for Illumina Sample Prep, Appendix D

- 14.8 Also you will need to manually pool the samples in LIMS after normalization (see section 15). If your samples do not need pooling proceed to the section 16.
- 14.9 **Please complete the LIMS portion for this section. See Appendix C, step 11-12 (Bioanalyzer run + Plate_PPGP_QC).**

15 Pooling Indexed DNA

- 15.1 For automated pooling of samples refer to

LIBPR.0093 Span-8 Pooling of DNA samples

- 15.2 For manual pooling of samples arrayed on a plate, refer to your Supervisor's instructions on which wells and what volume to pool for each sample. Use a Gilson single-channel pipette and mark off each well once it has been pooled into a clean eppendorf tube.
- 15.3 If pooled samples did not go through Barracuda size selection and need to be run on a Precast 8% TBE gel for post-PCR gel purification, samples may need to be Ethanol precipitated before they are loaded onto the gel. The resuspension volume for the pooled sample in EB will depend on how many lanes on the Precast TBE gel you choose to load.

ie. For EtOH precipitation of 200uL pooled sample:

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Reagent	Volume
Eluate	200 μ L
3M Sodium Acetate (1/10 volume)	20 μ L
Mussel Glycogen (20 mg/mL)	2 μ L
100 % Ethanol (2.5x volume)	500 μ L
TOTAL VOLUME	773μL

- 15.4 Generate pools in LIMS with IX name provided by your Supervisor.

LIMS: Pool/Rearray > tube > Manually Set up ReArray> enter plate barcode > ReArray/Pool Wells > choose desired wells and click in Target Plate(s)/Tubes(s) field > Click Rearray/Pool Selected Samples if multiple pools > Submit Rearray/Pool Request

16 QC Quant Final Product

- 16.1 DNA Qubit quant 1 μ L of each sample to confirm that you have sufficient product for submission for sequencing.

LIBPR.0030 – Quantifying DNA Samples using the Qubit Fluorometer

- 16.2 Record the plate set number and retrieve the 2D barcodes from the barcode printer. Record the concentration (nM) on the barcodes. Scan and place the tubes in the appropriate Next PET Run boxes.
- 16.3 **Please complete LIMS portion of this section. See Appendix C, step 13 (Plate_8nM_Final_Product).**

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Appendix A: LIMS Protocols

1. Plate_Shear_DNA LIMS: pipeline IDX
2. Sonication QC Bioanalyzer Run: There are no attributes to enter.
3. Plate_End_Repair LIMS: pipeline IDX
4. Plate_A_Addition LIMS: you can skip the steps that require QC data. pipeline IDX
5. Plate_Adapter_Ligation LIMS: pipeline IDX
6. Plate_Uracil_N_Glycosylase_Digestion LIMS: pipeline IDX
7. Plate_Indexed_PCR LIMS: pipeline IDX
8. Plate_Barracuda_Size_Selection LIMS for **unpooled** samples: pipeline IDX.
9. Plate_MinElute_PPGP LIMS for **unpooled** samples: pipeline IDX
10. Plate_Manual_PPGP LIMS for **pooled** samples: pipeline IDX
11. Create Bioanalyser run if samples QC'd by Agilent
12. Plate_PPGP_QC LIMS: If you have generated Bioanalyzer runs in LIMS for your samples, you should not fill in the values for the **Post PCR Agilent Run ID, DNA_concentration_ng_uL, Avg DNA bp size and Library_size_distribution attributes** in this protocol.
13. Plate_8nM_Final_Product LIMS : For pooled samples the pipeline is IPE: SLX-IPET. If samples are not pooled then the pipeline is PET: SLX-PET.

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Appendix B:

1. Size Selection by Precast 8% TBE gels

- 1.1 Put on a clean pair of gloves. Pre-chill a centrifuge to 4°C. Retrieve fresh ice and all reagents.
- 1.2 Set up sets of 0.5mL and 2mL tubes for shearing the gel slices: Make a hole through the bottom of 0.5mL tubes with an 18 gauge needle. Place each 0.5mL tube into a 2mL tube.
- 1.3 Label each 2mL tube on the side with the tra#, well position, date, and initials.
- 1.4 Cover the Dark Reader screen with a fresh sheet of plastic wrap. Wrap the right-angle ruler with plastic wrap.
- 1.5 Prepare fresh stain: 6µL SYBR Green in 60mL 1X TBE. Minimize exposure to light.
- 1.6 Stop the gel run after about 45-50 minutes. The first dye marker should be out of the gel and the second dye marker should still be visible toward the bottom of the gel. Dismantle the PAGE apparatus.
- 1.7 For completed runs, remove the gel cassette and transfer the corresponding label (with well notation) from the gel apparatus.
- 1.8 Using a post-PCR dedicated tray, processing 4 gels at a time, stain the gel (each in a separate tray) for 3 minutes. Set the timer.
- 1.9 After 3min, retrieve the gel from the staining solution and place onto the Mylar sheet. Also transfer the corresponding tape label.
- 1.10 Log onto the computer and scan image on high sensitivity setting. Save the image in R:\Library Core\QC\Gel. Name the file with tra#, plate well locations of the two samples_pipeline_PCR_DateInitials: Tra26984_A11_A12_Strand Specific_PCR_070315mm.gel
- 1.11 Lay the Mylar sheet with the gel on top onto the Dark Reader. For every sample cut out 280-400bp size fraction (see image below). The upper cut is slightly above 400bp ladder

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mark; the bottom cut is about $\frac{1}{4}$ down from the 300bp (see where the midpoint is between 200bp and 300bp ladder and go half that distance up for the lower cut). Do not cut below 280bp as we want to avoid the possibility of the concatenated 260bp adapter product.

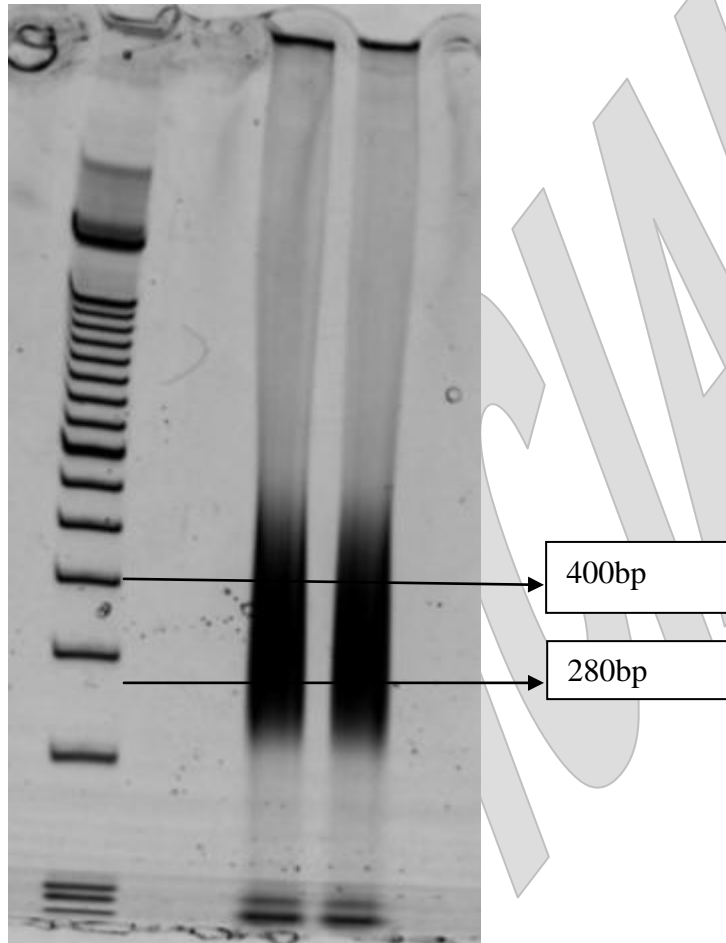


Figure 1. 280-400bp size fraction

- 1.12 Transfer gel fractions *for the same* sample into one of the 0.5mL tubes prepared and labeled earlier for shearing. Make sure that the labeling on the tube matches the labeling of the size selected sample.
- 1.13 Discard stain, spray with water, and wipe down tray. Discard Mylar sheet and remainder of the gel. Discard used blade in sharps container. Tidy up area. Change gloves.
- 1.14 Spin tubes at 12,000rpm at room temperature for 3 minutes. The gel slices should shear through the holes and collect into the bottom of the 2mL tubes.

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1.15 After shearing the gel fractions, check that all of the gel has cleared the 0.5mL tubes. If no gel remains, discard the 0.5mL tubes and add 200µL of Elution buffer (5:1, LoTE:7.5M Ammonium Acetate) to each gel slurry. Mix well by vortexing. Pulse spin.

1.15.1 If 200µL of Elution buffer is not sufficient enough to cover the gel fraction, add more Elution buffer until the gel slurry has a more liquid consistency. Note the total volume of EB that has been added to the gel slurry.

1.16 If time permits, incubate for 1 hour at 65°C to elute DNA and proceed to step 17 for Gel extraction.

1.17 If there is insufficient time to continue, incubate overnight at 4°C.

1.18 Clean PAGE apparatus: Run tap water over PAGE apparatus for 2 minutes; wipe down with 2% micro90; run water over PAGE apparatus for another 2 minutes. Wipe down the PAGE workstation.

2. Gel Extraction and Precipitating Pooled Indexed DNA.

2.1 Retrieve the gel slurries from 4°C. Vortex and pulse spin.

2.2 Incubate at 65°C for 15 minutes. Pulse spin.

2.3 Transfer the contents of each tube into one Spin-X Filter Tube. Tap the slurry into the Spin Filter or use a clean pipette tip to aid transfer. Change gloves.

2.3.1 Multiple Spin-X Filter Tubes may be used if the volume of gel slurry is too large to spin in one tube. Combine the eluates at the precipitation step.

2.4 Spin at 12,000rpm at 4°C for 3 minutes.

2.5 Check each Spin-X Filter Tube and ensure that the entire volume of buffer has spun through the filter. Re-spin the tubes if there is still liquid trapped in the gel material.

2.6 Remove and discard the filter column containing the gel material.

2.7 Transfer the eluate to a single sterile tube and add the following. Adjust the reagent volumes appropriately if the eluate is less/greater than 200 µL.

Reagent	Volume
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Eluate	200 µL
3M Sodium Acetate (1/10 volume)	20 µL
Mussel Glycogen (20 mg/mL)	2 µL
100 % Ethanol (2.5x volume)	500 µL
TOTAL VOLUME	773µL

2.8 Vortex and pulse spin.

2.9 Chill the tube at -20°C for 30 minutes.

2.10 Spin at 14,000rpm at 4°C for 30 minutes.

2.11 Dispose all waste and partially used reagents aliquots.

2.12 Carefully decant the supernatant into clean microcentrifuge tube. Keep an eye on the pellet so that it doesn't slide out.

2.13 Wash the pellet **two times** with 1 mL cold 70% ethanol. Spin at 14,000 rpm at 4°C for 3 minutes between each wash. Carefully decant the supernatant into a new microcentrifuge tube as a backup.

2.14 After removing the final wash, dab the tube rims on a Kimwipe to remove ethanol. Pulsespin the tubes and carefully remove any residual ethanol.

2.15 Mark the outside bottom of the tube to better locate the pellets.

2.16 Air-dry the pellet until translucent. Do not over-dry the pellet. Resuspend the pellet in a total volume of 12µL of Qiagen's elution buffer, EB.

2.17 **Please complete the LIMS portion for this section. See Appendix A, number 10 (Plate_Manual_PPGP).**