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SLX-PET Protocol for Illumina Sample Prep

I. Purpose

To provide specific guidelines for preparing template from gDNA, cDNA or ChIP DNA for Illumina Paired-End Sequencing (PET=Paired-Ended Sequencing).

II. Scope

All procedures are applicable to the BCGSC Functional Genomics Library Construction Core.

III. Policy

All production procedures shall be documented and controlled by approved systems.

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Production Coordinator 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

SOP/Reference Title	SOP/Reference Number
Sample Preparation for Paired-End Sample Prep Kit from Illumina	Version 1.1
Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA Samples	LIBPR.0017
Scanning pre-pcr gel images with the GelG9Imager system	LIBPR_WorkInst.0004
Illumina Concentration Checked Protocol	LIBPR.0030

VI. 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.

VII. Materials and Equipment

Name	Supplier	Number: #	Model or Catalogue #	
Bulk Nucleic Acid Sample Prep Reagents for Illumina GA II	NEB	E6000YJ		✓

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Fisherbrand Textured Nitrile gloves - large	Fisher	270-058-53		✓
Ice bucket – Green	Fisher	11-676-36		✓
Wet ice	In house	N/A		N/A
Nuclease Free 2.0 ml eppendorf tube	Ambion	12400		✓
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		✓
Neptune barrier tips 10 ul	CLP	Bt10XL		✓
Neptune barrier tips 20 ul	CLP	Bt20		✓
Neptune barrier tips 1000 ul	CLP	Bt1000		✓
Galaxy mini-centrifuge	VWR	37000-700	✓	
VX-100 Vortex Mixer	Rose Scientific	S-0100	✓	
Large Kimwipes	Fisher	06-666-117		✓
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		✓
Qiaquick PCR Purification Kit (50)	Qiagen	28104		✓
MinElute PCR Purification Kit (50)	Qiagent	28004		✓
DNA*AWAY*	MBS	7010		✓
Mussel Glycogen (20mg)	Roche	10 901 393 001		✓
3 M Sodium Acetate	Sigma	EC 211-162-9		✓
Anhydrous Ethyl Alcohol (100% Ethanol)	Commercial	People Soft ID:		✓
Phenol/Chloroform/Isoamyl Alcohol	Fisher	BP1752-100		✓
2.0 mL Phase lock tubes	Brinkmann	955154011		✓
Mylar PET film, clear (40"x10'x 0.003")	McMaster-	8567K32		✓
Plastic wrap	In house			✓
NanoDrop ND-1000 Spectrophotometer	NanoDrop	ND-1000	✓	
Agilent DNA 1000 Series II Kit	Agilent	5067-1504		✓
DNA 1000 Gel Matrix	Agilent	5067-1504		✓
DNA 1000 Dye Concentrate	Agilent	5067-1504		✓
DNA 1000 Marker	Agilent	5067-1504		✓
DNA 1000 Ladder	Agilent	5067-1504		✓
Agilent DNA 1000 Chips	Agilent	6064-8230		✓
Agilent Chip Priming Station	Agilent	Chip Priming	✓	✓
IKA Works Vortexer	Agilent	MS2S9-Agilent-	✓	
22R Microfuge Centrifuge	Beckman	22R Centrifuge	✓	
GeneAmp PCR System 9700	ABI	PCR System 9700	✓	
Agilent Electrode Cleaner	Agilent	6064-8230		✓
Peltier Thermal Cycler	MJ Research	PTC-225	✓	
Power Supply, LVC2kW, 48VDCV	Tyco	RM200HA100	✓	
Spin-X Filter Tube	Fisher	CS008160		✓

Qubit Fluorometer	Invitrogen	Q32857	✓	
40% Polyacrylamide (37.5:1 acrylamide:bis)	BioRad	161-0148		✓
10% Ammonium Persulfate (APS)	BioRad	161-0800		✓
TEMED	BioRad	161-0700		✓
Penguin Owl Electrophorosis System	Owl Scientific	P9 DS3-CE	✓	✓
Gel casting	Owl Scientific	JGC-2	✓	
Power PAC	BioRad	Power PAC 200	✓	
50 X TAE	In House	N/A		
1 X TAE	In House	N/A		
100 bp Ladder	Invitrogen	15628-019		✓
Xcell SureLock Mini-Cell	Invitrogen	EI0001		✓
1 X TBE	In House	N/A		
Novex 8% TBE Gel, 1.0mm, 10 Well	Invitrogen	EC6215BOX		✓

These sequences are for internal use only:

PE adapters:
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT
3' GAGCCGTAAGGACGACTTGGCGAGAAGGCTAG

PE PCR Primers
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
5' CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

PE Sequencing Primer
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT
5' CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

VIII. Procedure

1. Retrieval of reagents and equipment preparation

1.1. Put on a clean pair of gloves and disposable lab coat (re-use till end of day).

1.2. Wipe down the assigned specific workstation, pipetors, and small equipment.

1.3. Lay down new benchcoat.

1.4. Change gloves.

1.5. Retrieve ice and all required reagents.

1.6. Thaw all reagents; vortex and pulse spin.

1.7. The samples to be used for this reaction could come post sonication, gel purification (for example ChIP) or cDNA synthesis.

1.8. If running a PAGE gel, go to step 2.1. If not running a gel (for example, purified 200bp DNA fraction), proceed to End-Repair, step 5.1 (discuss with your supervisor if not sure.)

Note: During library construction, attach sample labels / LIMS labels to the PET worksheets (Appendix B and Appendix C).

2. PAGE gel Electrophoresis

2.1. Use one pre-cast gel for one sample only. Cut open the Novex gel cassette pouch to remove the gel cassette, and drain away the gel packaging buffer. Handle the gel cassette by the edges only. Rinse the gel cassette with deionized water.

2.2. Peel off the tape covering the slot on the back of the gel cassette.

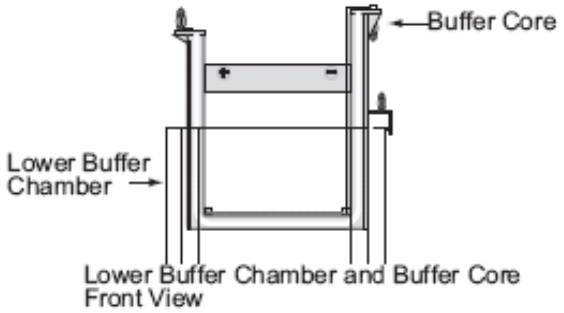
2.3. In one fluid motion, pull the comb out of the cassette.

2.4. Use a 1mL pipette to gently wash the cassette wells with 1X TBE gel running buffer. Repeat twice, and then fill the sample wells with running buffer.

It is important to wash wells thoroughly to remove preservative residue from wells that may impede sample running efficiently through gel.

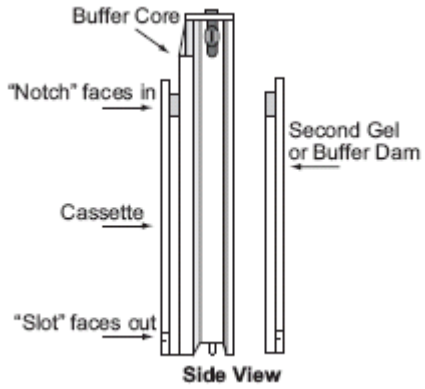
Assemble the gel apparatus as follows:

2.4.1. Lower the Buffer Core into the Lower Buffer Chamber so that the negative electrode fits into the opening in the gold plate on the Lower Buffer Chamber as shown in the figure.

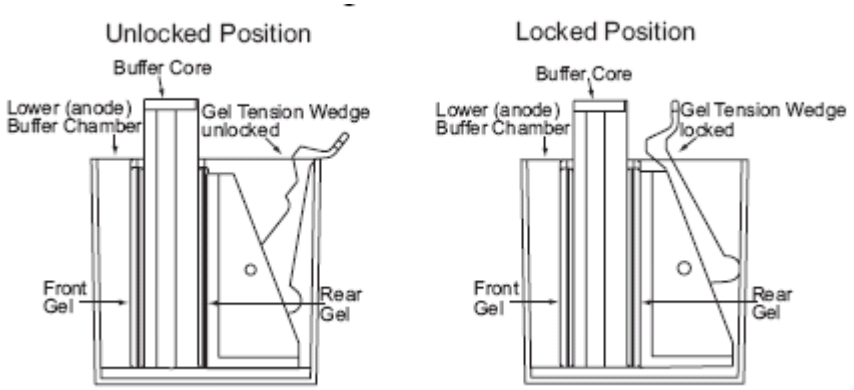


2.4.2. Insert the Gel Tension Wedge into the XCell *SureLock* cell behind the buffer core. Make sure the Gel Tension Wedge is in its unlocked position, allowing the wedge to slip easily into the XCell *SureLock* unit.

2.4.3. Insert the gel cassette into the lower buffer chamber in front of the core, with the well side of the cassette facing in towards the buffer core. The slot on the back of the cassette must face out towards the lower buffer chamber. Place the Buffer Dam behind the core.

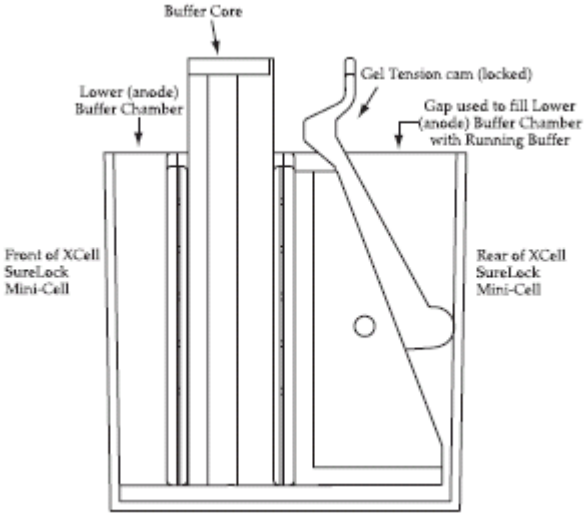


2.4.4. Pull forward on the Gel Tension Lever in a direction towards the front of the XCell *SureLock* unit until lever comes to a firm stop and the gel/buffer dam appear snug against the buffer core.



2.4.5. Fill the Upper Buffer Chamber with 200mL of the 1X TBE running buffer. Ensure that the Upper Buffer Chamber is not leaking. If the level of the running buffer drops, the apparatus will need to be reassembled.

2.4.6. Fill the Lower Buffer Chamber with approximately 200mL of running buffer through the gap between the Gel Tension Wedge and the back of the Lower Buffer Chamber as shown below:



2.4.7. Align the lid on the Buffer Core. The lid can only be firmly seated if the (-) electrode is aligned over the banana plug on the right.

Caution: Power must be off before connecting the XCell *SureLock* Mini Cell to the power supply.

2.5. Prepare your samples by adding appropriate amount of 10X loading dye.

2.6. Before loading the sample, carefully rinse out the wells with 1X TBE running buffer using a 1mL pipette tip.

2.7. Retrieve the sample as scheduled by a supervisor. Load the specified amount of sample in a well towards the centre of the gel. Be aware that some samples have a tendency to float out of the well, so load a very small volume to ensure that the sample doesn't float out. If floating does occur, add 1µl of glycerol to the rest of the sample and retest. The loading dye is made of bromophenol blue dye only. If desired, load some loading dye consisting of both bromophenol blue and xylene cyanol beside the sample lane so that when cutting out the gel fraction later, the xylene cyanol dye (runs at ~220bp) can be used as a reference.

2.8. For PET samples, load 5µl of the 100bp DNA Ladder (20ng/µl) at least 6 wells apart from the sample.

For ChIP-TS samples, do not load any ladder.

2.9. Replace the lid of the apparatus, and start the gel run.

2.10. Run the 8% TBE gel at 200V for 35 minutes. The bromophenol blue dye corresponds to the 25 nucleotide DNA marker, and it will have migrated approximately ¾ of the length of the gel. The xylene cyanol dye corresponds to the ~220 nucleotide DNA marker. If a larger size fraction (eg. 600bp) was used for library construction, discuss with supervisor to see how long to run the gel for.

2.11. Using colored tape, attach a label to the gel apparatus which states the library name, start time, finish time, date, and your initials.

2.12. Dispose of all waste.

3. Scanning gel with GelG9Imager and cutting DNA fraction from PAGE

3.1. Put on a clean pair of gloves and disposable lab coat.

3.2. Pre-chill a micro-centrifuge to 4°C.

3.3. Retrieve fresh ice and all reagents.

3.4. For shearing the gel slices, make a hole through the bottom of 0.5ml tubes with 18 gauge needle. Place each 0.5mL tube into a 2mL tube. You will need one of these shearing devices per fraction that you will be cutting out. If sample is loaded on multiple lanes, more shearing devices might be needed per fraction. Consult with supervisor to determine how many fractions to cut out.

3.5. Label each 2mL tube on the side with the library name, size fraction, date, and initials.

3.6. Cover the Dark Reader screen with a fresh sheet of plastic wrap. Wrap the right-angle ruler with plastic wrap.

3.7. Log onto GelG9Imager and wipe down imager bed by following LIBPR_WorkInst.0004: Scanning pre-pcr gel images with the GelG9Imager system.

3.8. Prepare fresh 1X TBE / SybrGreenI stain; 6µL stock in 60mL 1x TBE in a clean tray designated for staining pre PCR gels. Minimize exposure to light.

3.9. At the end of the gel run, turn off the power and disconnect the cables from the power supply. Remove the lid and unlock the Gel Tension Lever. The Gel Tension Wedge can be left in place.

3.10. Remove the gel cassette from the mini-cell. Handle gel cassettes by their edges only.

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3.11. Lay the gel cassette well side up on the benchtop. **Carefully** insert the Gel Knife's beveled edge into the narrow gap between the two plates of the cassette.

3.12. Push up and down gently on the knife's handle to separate the plates. You will hear a cracking sound which means you have broken the bonds which hold the plates together. Repeat until you have broken the bonds on one side. Rotate the cassette and repeat until the two plates are completely separated.

3.13. Upon opening the cassette, the gel may adhere to either side. Remove and discard the plate without the gel, allowing the gel to remain on the other plate. Place over the gel staining container with the gel facing downward. Remove the gel from the cassette plate by loosening one upper corner carefully by pulling away the gel and allow the gel to peel away from the plate.

3.14. Stain the gel for 1 minute with gentle agitation.

3.15. Wipe down a piece of mylar with water.

3.16. Place the stained gel onto the mylar sheet.

3.17. Scan gel according to LIBPR_WorkInst.0004: Scanning pre-pcr gel images with the GelG9Imager system.

3.18. Move the gel on the mylar sheet over to the dark reader.

3.19. Cut out different size fractions according to the following guideline:

3.19.1. **For Genome-PET libraries** cut out 400bp, 400+ and 400- fractions. Cut each fraction out with razor blade and use the 400bp fraction for library construction. The other fractions are for backup.

3.19.2. **For WTSS-PET libraries** cut out 200-300bp, 300-400bp, 400-500bp. Cut each fraction out with razor blade and use the 200-300bp fraction for library construction. The other fractions are for backup.

3.19.3. **For ChIP-PET libraries** cut out 130-180bp, 180-280bp, 280-330bp fractions. Cut each fraction out with razor blade and use the 130-180bp fraction for library construction. The other fractions are for backup.

3.19.4. **For ChIP-TS libraries** cut out 100-300bp, 300-600bp using the marked ruler. Cut each fraction out with razor blade and use the 100-300bp fraction for library construction. The 300-600bp fraction is for backup.

3.19.5. **For other library types**, consult supervisor on what fractions to cut out.

3.19.6. Label each size fractions correspondingly.

3.19.7. The above is only a general guideline for gel excision. Different fractions might have to be cut out depending on the sample (eg. 600bp library). Supervisors will provide the information regarding which fractions to cut out.

3.20. Transfer each fraction into a 0.5mL shearing device prepared earlier.

3.21. Discard stain into the liquid SYBR Green waste bucket and wipe down tray.

3.22. Discard mylar sheet with the leftover gel pieces into the SYBR Green waste bucket. Discard used blades in sharps container.

3.23. Change gloves. Repeat steps 3.4 to 3.22 for remaining PAGE gels (other samples).

3.24. With the lids tailing (left of tube position), spin all samples, including backup fractions if they were cut out, at 12,000 rpm @ RT for 3 minutes. The gel slices should shear through the holes and collect into the bottom of the 2mL tubes. Pull out unsheared material with a pipette tip and save it in the tubes of fractions.

3.25. For each gel slice that was sheared into the 2mL tubes, including backup fractions, add 200µL of elution buffer (5:1, LoTE:7.5M Ammonium Acetate). Ensure that there is sufficient elution buffer to cover the slurry, add more if necessary. Do not save leftover EB.

3.26. Mix well by vortexing. Pulse–spin.

3.27. Store all fractions in the corresponding "Gel Slurries and Starting Material" box in -20°C. If continuing with library construction next day, store the fraction that goes into library construction at 4°C instead of at -20°C.

3.28. If time permits, for the fraction that goes into library construction, incubate for 1 hour at 65°C and go directly to step 4.4.

3.29. Dispose all waste (including pipette tips waste bag), benchcoat, and partially used reagents aliquots.

3.30. Place used racks in **fresh** decontamination soak.

3.31. Wipe down the workstation, pipettes, and small equipment.

3.32. 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. Invert to air-dry.

3.33. Wipe down the PAGE workstation.

4. Precipitating the size fraction.

4.1. To help calculation, use the SLX-PET Day 1 worksheet (Appendix B).

4.2. Retrieve the gel slurries from -20°C or from 4°C. Vortex and pulse spin.

4.3. If gel slurries are retrieved from -20°C, incubate at 65°C for 1hr. If gel slurries are retrieved from 4°C, incubate at 65°C for 15 minutes. Pulse spin.

4.4. Transfer the contents of each tube into one Spin-X Filter Tube. Tap the slurry into the Spin Filter or use a new disposable spatula to aid transfer.

4.5. Change gloves.

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

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

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

4.9. Transfer the eluate to a single sterile 1.5mL tube and add the following. Adjust the reagent volumes proportionally but keeping mussel glycogen volume at 3µl, if the eluate is more than 200µL.

Reagent	***Volume***
Eluate	200 µL
3M Sodium Acetate	20 µL
Mussel Glycogen (20 mg/mL)	3 µL
100 % Ethanol	500 µL
TOTAL VOLUME	**723 µL**

4.10. Vortex and pulse spin.

4.11. Chill the tube at -20°C for 20 minutes.

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

4.13. Dispose all waste and partially used reagents aliquots.

4.14. Put on a clean pair of gloves.

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

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

4.17. After removing the final wash, dab the tube rims on a KimWipe to remove ethanol. Pulse-spin the tubes and carefully remove any residual ethanol by using a 10µL pipette.

4.18. Mark the outside bottom of the tube to better locate the pellets when resuspension later in Qiagen EB.

4.19. Air-dry the pellet for 5–10 minutes, or until the pellet is translucent. Do not over-dry the pellet.

4.20. Resuspend the pellet in a total volume of 40µL of Qiagen's elution buffer, EB. The resuspension of the pellet may be aided by repetitive pipetting using a 10µL pipette.

5. End-Repair and Phosphorylation

5.1. Before setting up the reaction dilute Klenow DNA Polymerase 1/5 in Nuclease free water on ice.

5.2. Set-up the following 50µL reaction:

Reagent	**Volume**
DNA	40 µL
10X Phosphorylation Buffer	5 µL
dNTP mix (10mM)	2 µL
T4 DNA polymerase (3U/µL)	1 µL
Klenow DNA Polymerase -1/5 diluted (5U/µL)	1 µL
T4 PNK (10U/µL)	1 µL
Total Volume	**50 µL**

5.3. Incubate for 30 minutes at room temperature.

5.4. While the incubation is proceeding, prepare one 2.0ml PLG tube for each library by spinning at 14000rpm for 1 minute at room temperature.

5.5. When the 30 minute incubation is complete, add 50µL of water to bring the volume up to 100µl.

5.6. Add sample to the pre-spun 2.0ml PLG tube.

5.7. Add 100µl of Phenol/Chloroform/Isoamyalcohol and shake well to mix until the mixture appears milky.

5.8. Spin for 5 minutes at 14000rpm at room temperature.

5.9. Transfer the aqueous phase to a clean 1.5mL microcentrifuge tube. Ethanol precipitate using 250µl (2.5X) cold 100% ethanol, 1µl of mussel glycogen and 10µl of sodium acetate (1/10).

5.10. Mix by inverting a few times and incubate for 20min at -20°C. Chill the centrifuge to 4ºC.

5.11. Centrifuge the sample for 30 minutes at 14000rpm at 4ºC.

5.12. Wash the pellet twice with 1ml of cold 70% ethanol and spin for 3 minutes at 14000rpm at 4°C.

5.13. Air-dry the pellet. Resuspend the pellet in 34µl of Qiagen EB.

If Section 6 will not be performed on the same day, store samples at -20°C, wipe down small equipment with DNAway.

6. Addition of an 'A' Base to the 3' End of the DNA Fragments

6.1. Prepare the following reaction mix:

Reagent	**Volume**
DNA from section 5	34 µl
Klenow buffer (10X)	5 µl
dATP (1mM)	10 µl
Klenow fragment (3' to 5' exo minus)	1 µl
Total Volume	**50 µl**

6.2. Incubate for 30 min at 37°C. Meanwhile, prepare a 2.0ml PLG tube for each library by spinning @ 14,000 rpm, 1 minute, RT.

6.3. Bring the volume up to 100µl with 50µl of water.

6.4. Add sample to a pre-spun 2.0ml PLG tube.

6.5. Add 100µl of Phenol/Chloroform/Isoamyalcohol and shake well to mix until the mixture appears cloudy.

6.6. Spin for 5 minutes at 14000rpm at room temperature.

6.7. Transfer the aqueous phase to a new 1.5mL microcentrifuge tube. Ethanol precipitate using 250µl (2.5X) cold 100% ethanol, 1µl of mussel glycogen and 10µl of sodium acetate (1/10).

6.8. Mix by inverting a few times and incubate for 20 minutes at -20^{o}C.

If Section 7 will not be done on the same day, leave samples overnight precipitating in ethanol at -20^{o}C. If Section 7 will be done on the same day, proceed with Step 6.9.

6.9. Chill the centrifuge to 4^{o}C.

6.10. Centrifuge the sample for a minimum of 20 minutes at 14000 rpm at 4^{o}C.

6.11. Wash the pellet twice with 1ml of cold 70% ethanol and spin for 3 minutes at 14000 rpm at 4^{o}C.

6.12. Air dry the pellet. Do not overdry the pellet. Resuspend the pellet in 10µl of Qiagen EB.

7. Ligation of Adapters to the Ends of the DNA Fragments

7.1. Prepare the following mix:

Reagent	**Volume**
DNA from section 6	10 µl
DNA ligase buffer (2X)	15 µl
PE adapters	1.0 µl
DNA Quick ligase (1U/µL)	4.0 µl
Total Volume	**30 µl**

7.2. Incubate for 15 min at room temperature.

7.3. Purify each reaction on a QIAquick column using the components of the QIAquick spin kit following these instructions, not the kit handbook.

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7.4. Pre-heat Qiagen elution buffer to 65°C by placing the Qiagen elution buffer aliquot in the 65°C heat block. For each ligation, label a fresh 1.5ml tube with library name, date and your initials. Set tubes aside until later.

7.5. Add 150µl of PBI to each ligation reaction and mix thoroughly.

7.6. Apply the sample to the QIAquick column and centrifuge at room temperature for 1 min at 13000rpm. Discard flow-through. Place the QIAquick column back into the same tube.

7.7. Add 0.75ml of Buffer PE to the column and centrifuge at room temperature for 1 min at 13000rpm. Discard flow-through and place the column back into the same tube.

7.8. Perform a second spin at room temperature for 2mins at 13000rpm.

7.9. Use a p200 pipette to aspirate any residual ethanol trapped on the inner rim of the Qiagen column. Transfer column to the pre-labeled tubes from step 7.4.

7.10. Let the column air dry for 1 minute before adding buffer EB (Qiagen).

7.11. Add 30µl of the pre-warmed EB (Qiagen) to the centre of the column and let sit for 1 minute prior to centrifugation. Spin at room temperature for 1 min at 13000rpm. Check volume of flow-through before discarding column. Be sure the microcentrifuge tube is accurately labeled before discarding the column.

8. Enrichment of adapter-modified DNA fragments by PCR

Note: Wipe down all surfaces and equipment with DNAway before and after PCR.

8.1. For each library, check with the supervisor to determine the appropriate PCR program to use (10 cycles or 15 cycles). Make enough brew mix for all libraries plus the "no template" control, using the SLX-PET Day 2 worksheet (Appendix C) to calculate the total volume needed for each reagent.

8.2. Prepare the following PCR reaction mix in the 5th Floor BSC:

Reagent	**Volume/Reaction**
Phusion High Fidelity Master Mix (2X Master Mix)	12.5 µl
PE PCR primer 1.0	1.0 µl
PE PCR primer 2.0	1.0 µl
Ultra pure dH_2O	0.5 µl
Total Volume	**15.00 µl**

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8.3. In the 5th floor BSC aliquot the brew mix into the appropriate number of 0.5mL PCR tubes. For the "No Template" control sample, add 10µL of water to the tube containing brew mix.

8.4. Transport the PCR tubes containing brew mix on ice to the 6th floor BSC.

8.5. In the 6th floor BSC, for each library, add 10µL of the modified template to the reaction tubes, including the "fraction" from the blank gel if scheduled.

8.6. Quick spin tubes containing fractions and brew mix for PCR.

8.7. Save the remaining 20µL of template in the appropriate "PCR Template" box.

8.8. Run Program TSPET10 and/or TSPET15 on Tetrad. Consult supervisor to see which PCR programs to run.

TSPET PCR Parameters

- 98°C 30 sec
- 98°C 10 sec
- 65°C 30 sec } 10/15 Cycles of PCR
- 72°C 30 sec
- 72°C 5 min
- 4°C ∞

8.9. When the PCR protocol is completed, purify all PCR reactions using the Qiagen MinElute Kit components but following the instructions below.

8.10. Pre-heat elution buffer to 65°C by placing the Qiagen elution buffer aliquot in the 65°C heat block. For each PCR product, label a fresh 1.5ml tube with library name, date and your initials. Set tubes aside until later.

8.11. Add 125µl of PBI to each PCR reaction and mix thoroughly.

8.12. Apply the sample to the Qiagen MinElute spin column and centrifuge at room temperature for 1 minute at 13000rpm. Discard flow-through. Place the Qiagen MinElute spin column back into the same tube.

8.13. Add 0.75ml of Buffer PE to the column and centrifuge at room temperature for 1 minute at 13000rpm. Discard flow-through and place the column back into the same tube.

8.14. Perform a second spin at room temperature for 2 minutes at 13000rpm. Use a p200 pipette to aspirate any additional ethanol trapped on the inner rim in the MinElute column. Transfer column to a clean tube from step 8.10.

8.15. Allow the column air dry for 1 minute before adding Elution Buffer.

8.16. Add 13µl of pre-warmed EB (Qiagen) to the centre of the column and let sit for 1 minute prior to centrifugation. Centrifuge at room temperature for 1 minute at 13000rpm to elute DNA. Check volume of flow-through before discarding column. The yield should be approximately 12µl. Be sure the microfuge tube is accurately labeled before discarding the column.

8.17. Run Agilent DNA 1000 Series II assay for quality assurance by following LIBPR.0017 – Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples; be sure to run the negative control as well.

8.18. Discuss the Agilent results with Supervisors to see if Post-PCR purification is required. If post PCR purification is not required, proceed to Step 12.2.

9. Post PCR Purification – Preparing and Running a Precast 8% TBE Gel

9.1. Refer to Step 2.1 to Step 2.7 for the set up of Precast 8% TBE Gel.

9.2. Prepare your samples by adding appropriate amount of 10X loading dye.

9.3. Before loading the ladder and sample, carefully rinse out the wells with 1X TBE running buffer using a 1mL pipette tip.

9.4. Aliquot 1mL 1X TBE to 2mL tube and set aside. This tube is for pipette tip washing when loading ladder.

9.5. Using a P10 tip, aspirate 10µl 100bp ladder. Wash pipette tip in the 1X TBE before loading the 10µl into a well on the left side of the gel. Leave a gap of 4-5 wells, and carefully load the sample into as many wells as required, keeping in mind that one well holds a maximum volume of 22µL.

9.6. Replace the lid of the apparatus, and start the gel run.

9.7. Run the 8% TBE gel at 200V for 35 minutes. The bromophenol blue dye corresponds to the 25 nucleotide DNA marker, and it will have migrated approximately ¾ of the length of the gel. If a larger size fraction other than 200bp was used for library construction, discuss with supervisor to see how long to run the gel for.

10. Post PCR Purification – Staining the TBE Gel and Excision of Fraction

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10.1. Prepare sets of tubes for shearing the gel slices. One tube is needed per lane. Make a hole through the bottom of a 0.5mL RNase-free non-stick tube with an 18 gauge needle and place on top of a 2mL RNase-free tube. Label the 2mL tubes on the side with the library/sample name, date, and initials.

10.2. Prepare fresh 1X TBE / SybrGreenI stain; 6µL stock in 60mL 1x TBE in a clean tray designated for staining post PCR purified gels. Minimize exposure to light

10.3. At the end of the gel run, turn off the power and disconnect the cables from the power supply.

10.4. Remove the lid and unlock the Gel Tension Lever. The Gel Tension Wedge can be left in place.

10.5. Remove the gel cassette from the mini-cell. Handle gel cassettes by their edges only.

10.6. Lay the gel cassette well side up on the benchtop. **Carefully** insert the Gel Knife's beveled edge into the narrow gap between the two plates of the cassette.

10.7. Push up and down gently on the knife's handle to separate the plates. You will hear a cracking sound which means you have broken the bonds which hold the plates together. Repeat until you have broken the bonds on one side. Rotate the cassette and repeat until the two plates are completely separated.

10.8. Upon opening the cassette, the gel may adhere to either side. Remove and discard the plate without the gel, allowing the gel to remain on the other plate. Place over the gel staining container with the gel facing downward. Remove the gel from the cassette plate by loosening one upper corner carefully by pulling away the gel and allow the gel to peel away from the plate.

10.9. Stain the gel for 1 minute with gentle agitation. **DO NOT SCAN GELS.**

10.10. Carefully remove the gel and lay onto a clean piece of mylar over a new sran wrap on the Dark Reader. Using a brand new razor blade cut out the appropriate fraction. Refer to the Agilent profile and cut a tight fraction at the darkest region of the smear. The darkest region should be ~120bp above the insert size.

For example: If 200bp insert was used for library construction, cut a tight band around 320bp. If 400bp insert was used for library construction, cut a tight band around 520bp. For ChIP-PET, if 130-180bp fraction was used, cut from 250-300bp. Confirm with supervisor if you are unsure of what range should be excised.

10.11. Transfer gel slices into the prepared 0.5mL tubes for gel shearing, using approximately one tube per lane loaded.

10.12. Close the 0.5mL tube lids to contain the gel slices, but leave the 2mL tube lids open to centrifuge the sample. Spin at 12,000 rpm at room temperature for 3 minutes. The gel slice should shear through the hole and collect into the bottom of the 2mL tubes.

10.13. After shearing the gel fractions, check that all of the gel has cleared the 0.5ml tubes. Discard the 0.5mL tubes and add 200µL of elution buffer (5:1, LoTE:7.5M Ammonium Acetate) to each gel slurry. Ensure that there is sufficient elution buffer to cover the slurry, otherwise add more if necessary.

10.14. Store the tubes in the gel slurry box in the 4°C fridge for overnight incubation or if time permits, incubate for 1 hour at 65°C and go directly to step 11.3.

10.15. Clean the XCell *SureLock* apparatus and the gel knife with tap water and 2% Micro90, and rinse thoroughly with tap water. Then rinse thoroughly with 18µM water.

11. Post PCR Purification – Precipitating the size fraction

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

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

11.3. Transfer the contents of each tube into one Spin-X Filter Tube. Tap the slurry into the Spin Filter or use a new disposable spatula to aid transfer.

11.4. Change gloves.

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

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

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

11.8. Transfer the eluate to a single sterile 1.5mL tube and add the following. Add 0.1X volume of 3M Sodium Acetate, 2.5X volume cold 100% Ethanol and 2µl Mussel Glycogen. For 200µl eluate volume, add the following:

Reagent	Volume
Eluate	200 µL
3M Sodium Acetate	20 µL
Mussel Glycogen (20mg/mL)	2 µL
Cold 100 % Ethanol	500 µL
Total Volume	**722 µL**

11.9. Vortex and pulse spin

11.10. Chill the tube at -20°C for 10 minutes.

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

11.12. Dispose all waste and partially used reagents aliquots.

11.13. Put on a clean pair of gloves and disposable lab coat.

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

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

11.16. After removing the final wash, dab the tube rims on a KimWipe to remove ethanol. Pulse-spin the tubes and carefully remove any residual ethanol by using a 10µL pipette.

11.17. Mark the outside bottom of the tube to better locate the pellets when resuspending later in Qiagen EB.

11.18. Air-dry the pellet for 5–10 minutes, or until the pellet is translucent. Do not over-dry the pellet.

11.19. Resuspend the pellet in a total volume of 9 - 13 µL* of Qiagen's elution buffer, EB. The resuspension of the pellet may be aided by repetitive pipetting using a 10µL pipette.
*Generally there is sufficient product to resuspend the pellet in 13µL volume. However occasionally, sample maybe in substantially low yield. In such a situation, a lower volume of Qiagen elution buffer should be used. If unsure of what volume to resuspend the sample in, consult with Supervisor.

12. Agilent and Qubit for QC

12.1. Run Agilent DNA 1000 Series II assay for quality assurance of post PCR purified product by following LIBPR.0017 – Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples. Be sure to run the negative control as well if one is available from additional PCR reactions performed.

12.2. Discuss with supervisor to see if sample is ready for submission. If sample is good for submission, quantify 1µl of each sample with Qubit as per LIBPR.0030 – Illumina Concentration Checked Protocol.

12.3. Determine the average size from the Agilent reading and calculate the nM based on the Qubit value (ng/µL) (remember to include the x 200 dilution factor). Use the calculator on Appendix C: SLX-PET Day 2 worksheet to calculate nM and the dilution factor necessary to reach ~8nM.

12.4. Dilute the DNA sample to a concentration of ~ 8nM in Buffer EB supplemented with 0.1% Tween-20.

Note: If possible, we should submit at least 8µl of the diluted sample. Based on the dilution factor and the library "**goal**", dilute accordingly. If unsure of what final volume is needed, discuss with Supervisor.

12.5. Do not label the tube with the concentration, that will be recorded by the person doing the "Illumina concentration checked" SOP.

12.6. Place it in the 6th floor, Qubit section of the "Samples to double check" box if not proceeding to quant diluted samples on Qubit. If proceeding with quanting of diluted samples on Qubit, refer to LIBPR.0030 – Illumina Concentration Checked Protocol.

12.7. Proceed to LIMS protocol in Appendix A.

CHANGE HISTORY

Description of Change	Rev. Number	Submitted by	Effective Date
1) Protocol drafted	1	Angela Tam	September 21, 2009

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Appendix A

LIMS SLX-gDNA size selection

1. Scanner Protocol

The following scanner protocol is for PET samples. If you are running TS samples through the protocol, select SLX-TS as the pipeline instead of SLX-PET, but still scanning in PE adapters.

1.1. Make sure you are under the Gene Expression tab, **scan the DNA sample**. **Save the tube set**.

1.2. Select "SLX gDNA size selection" from the dropdown menu and select "Continue Prep" button. Change pipeline to PET: SLX-PET. Select "Completed Aliquot to 1.5 ml Tube". A new barcode will be produced. **This barcode represents the aliquot that was loaded onto the gel. Paste it into your lab notes with the image of the gel**.

1.3. If the amount of starting material is known, enter the value in the appropriate field. If a sonication step has been performed, enter the Bioruptor or Sonicator equipment ID depending on which one was used and the length of time sonicated in the appropriate field. Select the "Completed Sonication equipment and time" button.

1.4. Make sure that the pipeline is "PET: SLX-PET". Select "Completed Transfer DNA to a 2 ml Tube". This represents the gel slice cut from the PAGE gel. Collect the new barcode from the printer. **This barcode will become the permanent barcode for the DNA template. It should be placed on the template tube**.

1.5. Enter the rack number of the Gel Slurries box.

1.6. Select "Completed store size fraction after purification".

1.7. If continuing directly with SLX-PET protocol, select the SLX-PET protocol from the dropdown menu and proceed to Step 2.3 below.

LIMS SLX-PET SOP

2. Scanner Protocol

Note: In any aliquoting or resuspension steps, if different volumes were used for different libraries, separate them by commas.
For any attribute, eg. Size fraction used, PCR cycles, Avg_DNA_bp_used, enter the value only. Do not enter any unit.

2.1. Scan the tube(s) that will be used for this protocol and save it as a new tube set.

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2.2. Now select the SLX-PET protocol, continue.

2.3. Click on "gDNA size selection protocol is completed". This is a reminder that the size selection protocol needs to be completed before running the SLX PET protocol. If size selection protocol is not done, click "skip gDNA size selection protocol is completed".

2.4. Enter the fraction size that was cut from the PAGE gel (eg. 200-300bp). Select the "Completed enter fraction size being used".

2.5. Then scan the following reagents as they come up.

- T4 DNA polymerase
- Klenow DNA polymerase
- T4 polynucleotide Kinase (T4 PNK)
- Klenow exo-
- PE adapter oligo mix
- DNA Quick ligase

2.6. Click on "Completed Decant" to offset any pre-existing volume.

2.7. Resuspend and store PCR template: Scan in Buffer EB. Enter 30µl (which is the PCR template volume). Enter the rack location for PCR template.

2.8. Aliquot to 1.5ml tube: Enter the volume of the **total** PCR template that was used for PCR. Eg. If two rounds of PCR were done, with 10µl and 5µl in the first and second rounds, enter 15µl as the volume to be aliquoted. **This will be the PPGP PCR product barcode.**

2.9. Scan in the following reagents as they come up:

- Phusion Master Mix
- PCR Primer PE 1.0
- PCR Primer PE 2.0

2.10. Click on "Completed Decant". The Decant step will set the PPGP PCR product volume to 0µl.

2.11. Resuspend and Store PPGP PCR Product: Scan in Buffer EB. Enter the volume of the PPGP PCR Product minus Agilent and Qubit QC volume. Eg. If PPGP PCR product was

resuspended in 13µl, subtract 2µl for Agilent and Qubit, then enter 11µl as the volume. Enter the rack location for the stock PPGP PCR product.

2.12. Enter the Agilent Run ID for the **pre–PPGP** PCR product.

2.13. Enter the Agilent Run ID for the **post-PPGP** PCR product.

2.14. Final DNA Quant (Agilent / Qubit)

- Enter the stock PPGP PCR product concentration according to Qubit (ng/µl)
- Enter the PCR cycle
- Enter the Library size distribution
- Enter the Average DNA bp size of the final PPGP PCR product.

2.15. Aliquot DNA to 1.5ml tube: Enter the volume of the stock PPGP PCR product that was aliquoted out to make the diluted sample for submission. **This will be the barcode for the sample for submission.**

2.16. Click on "Completed Decant". The Decant step will set the sample for submission volume to 0µl.

2.17. Resuspend Sample for Double Checking: Scan in Buffer EB. Enter in the final volume of the sample for submission.

2.18. Put the final barcode on the diluted sample. Store the diluted sample in the "Samples to Double Check" box on the 6th floor. Do not label the tube with a concentration; that will be done after the double check is completed.

Appendix B

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SLX-PET Day 1

Library Name:	**Date**	**Prepared by**	**Size Fraction**
1			
2			
3			
4			

Gel Purification	**Sol #**	**Volume**	**Added**
number of columns used: _____			
100% Ethanol		2.5X volume	
3M NaOAc		1/10 volume	
Mussel Glycogen		3 µL	
Qiagen Elution Buffer		40 µL	

End-Repair and Phosphroylation	**Sol #**	**Volume**	**Added**
DNA		40 µL	
10X Phosphorylation Buffer		5 µL	
dNTP mix		2 µL	
T4 DNA Polymerase		1µL	
Klenow DNA Polymerase (1/5 diluted)		1 µL	
T4 PNK		1 µL	
30 min incubation at room temp			
PCI/EtOH Precipitate/resuspend in EB		34µL	

Addition of an 'A' Base to the 3' End	**Sol #**		
DNA from section 1		34 µL	
10X Klenow Buffer		5 µL	
dATP (1mM)		10µL	
Klenow Fragment (3' to 4'' exo minus)		1µL	
30 min incubation at 37^0C			
PCI/EtOH Precipitate/resuspend in EB		10µL	

Ligation of Adapters	**Sol #**		
DNA from section 6		10 µL	
DNA ligase buffer (2X)		15 µL	
PE Adapters (1x concentration)		1 µL	
DNA Quick Ligase		4 µL	
15 min incubation at room temp			
Qiagen Column Clean-up Elute in EB		30 µL	

Appendix C

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SLX-PET Day 2

Library Name:	**Date**	**Prepared by**	**Size Fraction**
1			
2			
3			
4			

Enrichment PCR (solexa Regents)	**Sol #**	**per rxn (uL)**	**# of rxns**	**Vol. (uL)**	**Added**
DNA Template		10			
Phusion DNA Polymerase (Premix)		12.5	5	62.5	
PCR Primer PE 1.0		1.0	5	5	
PCR Primer PE 2.0		1.0	5	5	
Nuclease-free water		0.5	5	2.5	
	Total	25			

Determine Molarity		**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Library Name					
PCR Cycle yielding the best result (10 or 15)					
Qubit concentration	(ng/uL)				
determine bp from agilent	(bp)				
calculate molarity	(nM)	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
dilute sample to 8nM (dilution factor)		#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!