Procedure & Checklist – Multiplex Genomic DNA Target Capture Using IDT xGen® Lockdown® Probes

Before You Begin

This procedure describes capture and enrichment of regions of interest by using IDT xGen® Lockdown® probes. The captured and enriched fragments are constructed into SMRTbell® libraries and subsequently sequenced on a PacBio® Sequel® System.

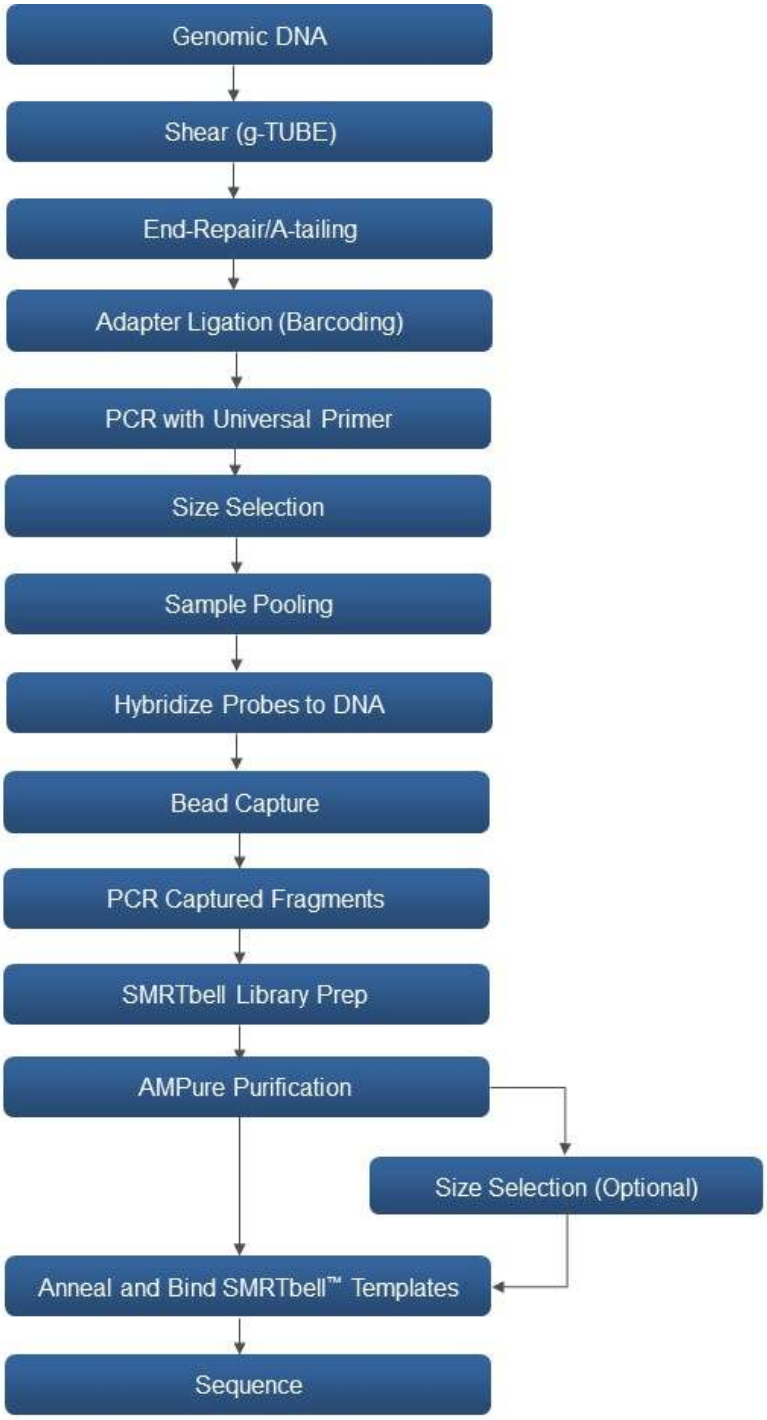
Materials Needed

Item	Usage	Vendor	Part Number/ Catalog Number
KAPA Hyper Prep Kits for Illumina sequencing	A-tailing and Ligation with linear barcoded adapters	KAPA Biosystems	KK8503
Forward and Reverse Barcoded Universal Adapter Oligos (Recommended sequences below)	Barcoded linear adapters	IDT	
xGen Lockdown Reagents	Hybridization	IDT	
PacBio Universal Primer /5Phos/gcagtcgaacatgtagctgactcaggtcac 100 µM, TE pH 8.0	Blocking and Amplification	IDT	
Human Cot-1 DNA	Blocking	Life Technologies	15279-011
Dynabeads M-270 Streptavidin	Bead capture	ThermoFisher Scientific	65305
Takara LA Taq DNA Polymerase Hot-Start version	Amplification	Clontech/Takara Bio	RR042A
AMPure PB Beads	Purification	PacBio	
Template Prep Kit	SMRTbell library Construction	PacBio	
Gel Cassettes and S1 Marker	BluePippin size selection	Sage Science	BLF7510
0.2 mL DNA LoBind PCR Tubes	PCR	Eppendorf	Multiple PN

Workflow

The workflow includes the following:

1. Ligating linear barcoded adapters to a single sheared gDNA sample or multiple samples.
2. Capturing target regions by hybridizing the barcoded samples with xGen Lockdown probes.
3. Constructing SMRTbell libraries with the captured samples and sequencing using a PacBio Sequel System.



Recommended PacBio Linear Barcoded Adapter Oligos with Universal Sequences

Please note that the linear barcoded adapter oligo pairs must be annealed first before use. See "Anneal Barcoded Adapters" section.

	Barcoded Adapter Oligo Pairs	Sequence
1	Univ.V3_bc1001_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCACATATCAGAGTGCGggtagT
	Univ.V3_bc1001_rev_comp	/5phos/ctaccCGCACTCTGATATGTGgtgacctgagtcagctacatgttcgactgc
2	Univ.V3_bc1002_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacACACACAGACTGTGAGggtagT
	Univ.V3_bc1002_rev_comp	/5phos/ctaccCTCACAGTCTGTGTGTgtgacctgagtcagctacatgttcgactgc
3	Univ.V3_bc1003_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacACACATCTCGTGAGAGggtagT
	Univ.V3_bc1003_rev_comp	/5phos/ctaccCTCTCACGAGATGTGTgtgacctgagtcagctacatgttcgactgc
4	Univ.V3_bc1004_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCACGCACACACGCGCGggtagT
	Univ.V3_bc1004_rev_comp	/5phos/ctaccCGCGCGTGTGTGCGTGgtgacctgagtcagctacatgttcgactgc
5	Univ.V3_bc1005_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCACTCGACTCTCGCGTggtagT
	Univ.V3_bc1005_rev_comp	/5phos/ctaccACGCGAGAGTCGAGTGgtgacctgagtcagctacatgttcgactgc
6	Univ.V3_bc1006_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCATATATATCAGCTGTggtagT
	Univ.V3_bc1006_rev_comp	/5phos/ctaccACAGCTGATATATATGgtgacctgagtcagctacatgttcgactgc
7	Univ.V3_bc1007_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacTCTGTATCTCTATGTGggtagT
	Univ.V3_bc1007_rev_comp	/5phos/ctaccCACATAGAGATACAGAgtgacctgagtcagctacatgttcgactgc
8	Univ.V3_bc1008_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacACAGTCGAGCGCTGCGggtagT
	Univ.V3_bc1008_rev_comp	/5phos/ctaccCGCAGCGCTCGACTGTgtgacctgagtcagctacatgttcgactgc
9	Univ.V3_bc1009_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacACACACGCGAGACAGAggtagT
	Univ.V3_bc1009_rev_comp	/5phos/ctaccTCTGTCTCGCGTGTGTgtgacctgagtcagctacatgttcgactgc
10	Univ.V3_bc1010_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacACGCGCTATCTCAGAGggtagT
	Univ.V3_bc1010_rev_comp	/5phos/ctaccCTCTGAGATAGCGCGTgtgacctgagtcagctacatgttcgactgc
11	Univ.V3_bc1011_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCTATACGTATATCTATggtagT
	Univ.V3_bc1011_rev_comp	/5phos/ctaccATAGATATACGTATAGgtgacctgagtcagctacatgttcgactgc
12	Univ.V3_bc1012_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacACACTAGATCGCGTGTggtagT
	Univ.V3_bc1012_rev_comp	/5phos/ctaccACACGCGATCTAGTGTgtgacctgagtcagctacatgttcgactgc
13	Univ.V3_bc1013_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCTCTCGCATACGCGAGggtagT
	Univ.V3_bc1013_rev_comp	/5phos/ctaccCTCGCGTATGCGAGAGgtgacctgagtcagctacatgttcgactgc
14	Univ.V3_bc1014_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCTCACTACGCGCGCGTggtagT
	Univ.V3_bc1014_rev_comp	/5phos/ctaccACGCGCGCGTAGTGAGgtgacctgagtcagctacatgttcgactgc
15	Univ.V3_bc1015_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCGCATGACACGTGTGTggtagT
	Univ.V3_bc1015_rev_comp	/5phos/ctaccACACACGTGTCATGCGgtgacctgagtcagctacatgttcgactgc

16	Univ.V3_bc1016_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCATAGAGAGATAGTATggtagT
	Univ.V3_bc1016_rev_comp	/5phos/ctaccATACTATCTCTCTATGgtgacctgagtcagctacatgttcgactgc
17	Univ.V3_bc1017_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCACACGCGCGCTATATggtagT
	Univ.V3_bc1017_rev_comp	/5phos/ctaccATATAGCGCGCGTGTGgtgacctgagtcagctacatgttcgactgc
18	Univ.V3_bc1018_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacTCACGTGCTCACTGTGggtagT
	Univ.V3_bc1018_rev_comp	/5phos/ctaccCACAGTGAGCACGTGAgtgacctgagtcagctacatgttcgactgc
19	Univ.V3_bc1019_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacACACACTCTATCAGATggtagT
	Univ.V3_bc1019_rev_comp	/5phos/ctaccATCTGATAGAGTGTGTgtgacctgagtcagctacatgttcgactgc
20	Univ.V3_bc1020_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCACGACACGACGATGTggtagT
	Univ.V3_bc1020_rev_comp	/5phos/ctaccACATCGTCGTGTCGTGgtgacctgagtcagctacatgttcgactgc
21	Univ.V3_bc1021_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCTATACATAGTGATGTggtagT
	Univ.V3_bc1021_rev_comp	/5phos/ctaccACATCACTATGTATAGgtgacctgagtcagctacatgttcgactgc
22	Univ.V3_bc1022_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCACTCACGTGTGATATggtagT
	Univ.V3_bc1022_rev_comp	/5phos/ctaccATATCACACGTGAGTGgtgacctgagtcagctacatgttcgactgc
23	Univ.V3_bc1023_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCAGAGAGATATCTCTGggtagT
	Univ.V3_bc1023_rev_comp	/5phos/ctaccCAGAGATATCTCTCTGgtgacctgagtcagctacatgttcgactgc
24	Univ.V3_bc1024_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCATGTAGAGCAGAGAGggtagT
	Univ.V3_bc1024_rev_comp	/5phos/ctaccCTCTCTGCTCTACATGgtgacctgagtcagctacatgttcgactgc

- The linear barcoded adapter oligos should be standard desalt-purified. HPLC purification is not required.
- PacBio recommends using an adapter oligo stock concentration of 100 µM in the manufacturer's recommended Buffer (TE, pH 8) and storing the oligo stock at -20ºC.
- The "bcXXXX" in the adapter oligo name denotes PacBio Barcodes.

<table>
<tr><th>STEP</th><th>✓</th><th>Anneal Linear Barcoded Adapters</th><th>Notes</th></tr>
<tr><td>1</td><td></td><td>The single-stranded linear barcoded adapters must first be annealed to a final concentration of 10 µM prior to ligation.</td><td></td></tr>
<tr><td>2</td><td></td><td>Dilute the barcoded adapters to 100 µM in water.</td><td></td></tr>
<tr><td>3</td><td></td><td>Prepare the following reactions:

<table><tr><th>Component</th><th>Stock Conc.</th><th>Volume</th></tr><tr><td>10X Primer Buffer v2*</td><td>10X</td><td>2 µL</td></tr><tr><td>Barcoded Adapter (forward)</td><td>100 µM</td><td>2 µL</td></tr><tr><td>Barcoded Adapter (reverse comp)</td><td>100 µM</td><td>2 µL</td></tr><tr><td>Water</td><td></td><td>14 µL</td></tr><tr><td>Total Volume</td><td></td><td>20 µL</td></tr></table>
*If a 10X Primer Buffer V2 is not available, please use a buffer with 1M NaCl, 0.1 M Tris pH 7.5.</td><td></td></tr>
<tr><td>4</td><td></td><td>Incubate in a thermocycler with the following thermal profile:

<table><tr><th>Step</th><th>Temp</th><th>Time</th></tr><tr><td>1</td><td>80℃</td><td>2 minutes</td></tr><tr><td>2</td><td>25℃</td><td>1 second Ramp to 25℃
0.1 ℃ /sec</td></tr><tr><td>3</td><td>25℃</td><td>1 second</td></tr><tr><td>4</td><td>4℃</td><td>Hold</td></tr></table></td><td></td></tr>
<tr><td>5</td><td></td><td>Place on ice until ready to use. Store annealed linear barcoded adapters at -20℃ for long term storage. To thaw, tap the tube gently. Do not vortex.</td><td></td></tr>
</table>

<table>
<tr><th>STEP</th><th>✓</th><th>Shear Genomic DNA</th><th>Notes</th></tr>
<tr><td>1</td><td></td><td>For each sample, dilute 2 µg of genomic DNA (gDNA) to 150 µL total volume in Elution Buffer (EB). Alternatively, you can use Qiagen Elution Buffer (EB).</td><td></td></tr>
<tr><td>2</td><td></td><td>Load the diluted sample (150 µL) to the top of the g-TUBE device and close the cap firmly.</td><td></td></tr>
<tr><td>3</td><td></td><td>Shearing recommendations:
For a single sample, PacBio recommends shearing the gDNA to 10 kb.
For a multiplexed sample, PacBio recommends shearing the gDNA to 6 kb. Using a shorter target shear size helps increase the yield of barcoded subreads during sequencing.
<table><tr><th>Sample Type</th><th>Target Shear Size</th><th>RPM (Eppendorf 5415D Centrifuge)</th><th>Spin Time (min)</th></tr><tr><td>Single sample</td><td>10 kb</td><td>6000</td><td>2</td></tr><tr><td>Each sample for multiplex (≥ 2-plex)</td><td>6 kb</td><td>7000</td><td>2</td></tr></table>
Other centrifuges may be used, however, the RPM speed should be optimized to achieve proper gDNA shearing.
Check for any residual DNA sample remaining in the upper chamber of the g-Tube. If some sample liquid still remains at the top, pulse the sample at higher speed (7,200 rpm) for 5 seconds. Repeat the high speed pulses until all of the sample is at the bottom chamber of the g-Tube.</td><td></td></tr>
<tr><td>4</td><td></td><td>Invert the g-TUBE device and spin the sample at the same RPM speed and duration. If some sample liquid remains at the top, pulse the sample up to 7200 rpm for 5 seconds. Repeat pulses until the sample is at the bottom of the g-Tube.</td><td></td></tr>
<tr><td>5</td><td></td><td>Place the sample into a new 1.5 mL Eppendorf LoBind tube.</td><td></td></tr>
</table>

STEP	✓	Concentrate Genomic DNA	Notes
1		Add **0.80X** volume of AMPure PB beads to the sheared gDNA. _____µL of sample X **0.80X** =_____µL of beads Note that the beads must be brought to room temperature and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.	
2		Mix bead/DNA solution thoroughly by tapping the tube gently. Do not pipet to mix.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.	
7		With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet. If the DNA is not recovered at the end of this Procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 - 60 seconds.	

13		Add **51 µL** Elution Buffer volume to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 mL Lo-Bind tube. – Discard the beads.	
14		Verify your DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit dsDNA BR Assay kit (or Qubit dsDNA HS Assay kit) according to the manufacturer's recommendations. Library recovery yield up to this step should be approximately 80% for a high-quality input gDNA sample.	
15		Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit to verify the fragment size distribution. Dilute the samples appropriately before loading on the Bioanalyzer chip so that the DNA concentration loaded falls well within the detectable minimum and maximum range of the assay. Refer to Agilent Technologies' guides for specific information on the range of the DNA 12000 kit.	
16		The sheared DNA can be stored for up to 24 hours at 4℃ or at -20℃ for longer duration.	
17		Actual recovered DNA concentration (ng/µl) and total available sample material (ng): _____	

<table>
<tr><th>STEP</th><th>✓</th><th>A-tailing and Ligation with Barcoded Adapters</th><th>Notes</th></tr>
<tr><td></td><td></td><td>In this section, you will need the following:
KAPA Hyper Prep Kit for Illumina sequencing.
Annealed Barcoded Adapter (10 µM); see Anneal Linear Barcoded Adapters section for annealing barcoded adapters.</td><td></td></tr>
<tr><td>1</td><td></td><td>Add the following reagents in the order shown below.
Use a minimum of 200 ng of purified, sheared gDNA diluted to 50 µL in EB as input into the End Repair and A-tailing reaction.
For a single sample, set up two replicates of the reaction (2 x 60 µL) shown in the table below. (A single reaction is not enough to proceed with the procedure.)
For multiplexed samples, prepare a single reaction for each sample (1 x 60 µL) as shown in the table below for each sample.
<table>
<tr><th>Component</th><th>Volume for 1 Reaction</th></tr>
<tr><td>Sheared DNA</td><td>50 µL</td></tr>
<tr><td>End Repair & A-Tailing Buffer†</td><td>7 µL</td></tr>
<tr><td>End Repair & A-Tailing Enzyme Mix†</td><td>3 µL</td></tr>
<tr><td>Total Volume</td><td>60 µL</td></tr>
</table>
† The buffer and enzyme mix may be pre-mixed and added in a single pipetting step. Premixes are stable for ≤24 hours at room temperature, for ≤1 week at 4°C, and for ≤3 months at -20°C.
Mix gently by tapping the tube, and then centrifuge briefly.
Incubate the reaction in a thermal cycler with the following temperature program:
<table>
<tr><th>Step</th><th>Temp</th><th>Time</th></tr>
<tr><td rowspan="2">End Repair & A-Tailing</td><td>20 ℃</td><td>30 min</td></tr>
<tr><td>65 ℃</td><td>30 min</td></tr>
<tr><td>HOLD</td><td>4 ℃</td><td>∞</td></tr>
</table>
Proceed immediately to the next step (Adapter Ligation).</td><td></td></tr>
</table>

<table>
<tr><th>STEP</th><th>✓</th><th>Barcoded Adapter Ligation</th><th>Notes</th></tr>
<tr><td>1</td><td></td><td>Prepare the reactions below:
For a single sample, two separate reactions (2 x 110 µL) are required to generate enough DNA for the subsequent reactions.
For multiplexed samples, prepare a single reaction (1 x 110 µL) for each sample:
<table>
<tr><th>Component</th><th>Stock Conc.</th><th>Volume</th></tr>
<tr><td>End Repair & A-Tailing reaction product</td><td></td><td>60 µL</td></tr>
<tr><td>PCR-grade water†</td><td></td><td>5 µL</td></tr>
<tr><td>Ligation Buffer†</td><td></td><td>30 µL</td></tr>
<tr><td>DNA Ligase†</td><td></td><td>10 µL</td></tr>
<tr><td>Annealed Barcoded Adapter</td><td>10 µM</td><td>5 µL</td></tr>
<tr><td>Total volume</td><td></td><td>110 µL</td></tr>
</table>
†The water, buffer and ligase enzyme may be pre-mixed and added in a single pipetting step. Premixes are stable for ≤24 hours at room temperature, for ≤1 week at 4°C, and for ≤3 months at -20°C.
Mix thoroughly and centrifuge briefly.
Incubate at 20°C for 15 min on the benchtop or in a thermal cycler.
Proceed immediately to the next step (AMPure PB Bead Purification) to purify each sample separately.</td><td></td></tr>
</table>

STEP	✓	AMPure® PB Bead Purification	Notes
1		Add **0.5X** volume of AMPure PB beads.	
2		Mix bead/DNA solution thoroughly by tapping the tube gently. Do not pipet to mix.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear.	
7		With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet. If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Let beads separate fully. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 - 60 seconds.	
13		Add **51 µL** of Elution Buffer volume to your beads. Tap the tube with finger gently to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 mL Lo-Bind tube. – Discard the beads.	
14		Verify your DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit dsDNA HS Assay kit according to the manufacturer's recommendations.	

<table>
<tr><th>STEP</th><th>✓</th><th>PCR Amplification Using Universal Primer</th><th>Notes</th></tr>
<tr><td></td><td></td><td>In this section, you will need the following:
Takara LA Taq DNA Polymerase Hot-Start Version from Clontech/Takara Bio
100 µM PacBio Universal Primer</td><td></td></tr>
<tr><td>1</td><td></td><td>For each sample, prepare the following mix for a total reaction volume of 200 µL.
<table>
<tr><th>Component</th><th>Stock Conc.</th><th>Volume</th></tr>
<tr><td>Eluted Sample</td><td></td><td>50 µL</td></tr>
<tr><td>Water</td><td></td><td>118.8 µL</td></tr>
<tr><td>LA PCR Buffer</td><td>10X</td><td>20 µL</td></tr>
<tr><td>dNTPs</td><td>2.5 mM each</td><td>8 µL</td></tr>
<tr><td>PacBio Universal Primer</td><td>100 µM</td><td>2 µL</td></tr>
<tr><td>Takara LA Taq DNA polymerase</td><td>5 U/µL</td><td>1.2 µL</td></tr>
<tr><td>Total</td><td></td><td>200 µL</td></tr>
</table>
It is highly recommended to perform the PCR amplification in 100 µL volumes. Transfer 100 µL aliquots into separate 0.2 ml PCR tubes so that there are two 100 µL PCR reactions per sample.</td><td></td></tr>
<tr><td>2</td><td></td><td>Amplify each sample using the following PCR program:
<table>
<tr><th>Step</th><th>Temp</th><th>Time</th></tr>
<tr><td>1</td><td>95℃</td><td>2 minutes</td></tr>
<tr><td>2</td><td>95℃</td><td>20 seconds</td></tr>
<tr><td>3</td><td>62℃</td><td>15 seconds</td></tr>
<tr><td>4</td><td>68℃</td><td>10 minutes</td></tr>
<tr><td>5</td><td colspan="2">Repeat steps 2 through 4, 6 times</td></tr>
<tr><td>6</td><td>68℃</td><td>5 minutes</td></tr>
<tr><td>7</td><td>4℃</td><td>Hold</td></tr>
</table>
The extension time can be modified depending on the DNA fragment size. As a general rule, for every 1 kb of DNA, add 1 additional minute to the extension time in Step 4 and Step 6 in the above table.</td><td></td></tr>
</table>

STEP	✓	AMPure® PB Bead Purification	Notes
1		Pool the two 100 µL PCR reactions for each sample and purify using **0.5X** AMPure PB beads.	
2		Mix bead/DNA solution thoroughly by tapping the tube gently. Do not pipet to mix.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear.	
7		With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet. If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Let beads separate fully. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 - 60 seconds.	
13		Add **30 µL** of Elution Buffer volume to your beads. Tap the tube with finger gently to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 mL Lo-Bind tube. – Discard the beads.	
14		Verify your DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit dsDNA HS Assay kit according to the manufacturer's recommendations.	
15		Proceed to size-selection. For a multiplexing workflow, it is highly recommended to perform size selection for each sample. The size-selected samples are then pooled prior to probe hybridization.	

STEP	✓	Size-Selection of PCR Amplified Samples	Notes
1		Prepare the PCR amplified DNA samples to run on a 0.75% BluePippin™ gel cassette (BLF7510) according to the manufacturer's instructions. Add 10 µL of loading buffer to the 30 µL of sample.	
2		Program the BluePippin system: • In the Protocol Editor Tab, choose cassette type: **0.75% DF Marker S1 High Pass 6-10 Kb Vs 3** • Choose BP start = 4500, BP end = 50000. • Determine which reference lane to add the S1 marker, enter it into "Reference Lane" field and select "Apply Reference to all Lanes" button.	
3		Calibrate the optics as outlined in the manufacturer's instructions.	
4		Prepare a 0.75% BluePippin cassette, load samples and run according to manufacturer's instructions.	
5		After the run, remove the **40 µL** of sample from each elution well.	
6		At this point, wells can be washed with an additional 40 µL electrophoresis buffer. Wash the well by pipetting up and down and recover the wash. Combine the 40 µL wash with the 40 µL of sample recovered in Step 5 above. Proceed directly with AMPure PB Bead Purification of the sample below.	

STEP	✓	AMPure® PB Bead Purification	Notes
1		Add **1X** volume of AMPure PB beads.	
2		Mix bead/DNA solution thoroughly by tapping the tube gently. Do not pipet to mix.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear.	
7		With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet. If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Let beads separate fully. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 - 60 seconds.	
13		Add **20 µL** of Elution Buffer volume to your beads. Tap the tube with finger gently to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 mL LoBind tube. – Discard the beads.	
14		Verify your DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit dsDNA HS Assay kit according to the manufacturer's recommendations.	
15		**For multiplex hybridization, pool the barcoded samples using equimolar** pooling so that the total mass is 1.5 - 2.0 µg. The hybridization step requires a total of 1.5 µg – 2.0 µg for a single sample or a multiplexed sample.	

<table>
<tr><th>STEP</th><th>✓</th><th>Hybridization of Probes</th><th>Notes</th></tr>
<tr><td></td><td></td><td>In this section, you will need the following:
COT Human DNA
100 µM PacBio Universal Primer
xGen 2X Hybridization Buffer (included in the xGen Lockdown reagent kit)
xGen Hybridization Buffer Enhancer (included in xGen Lockdown reagent kit)
xGen Lockdown probes</td><td></td></tr>
<tr><td>1</td><td></td><td>Add 5 µL COT Human DNA (1 mg/mL) to a new 1.5 mL LoBind tube.</td><td></td></tr>
<tr><td>2</td><td></td><td>Add 1.5-2.0 µg of single or multiplex size-selected DNA sample to the LoBind tube containing the 5 µL COT Human DNA.</td><td></td></tr>
<tr><td>3</td><td></td><td>Add 10 µL of 100 µM PacBio Universal Primer to the LoBind tube containing the DNA/COT Human DNA mixture.</td><td></td></tr>
<tr><td>4</td><td></td><td>Close the tube’s lid and make a hole in the top of the tube’s cap with an 18 – 20 gauge or smaller needle.</td><td></td></tr>
<tr><td>5</td><td></td><td>Dry the DNA Sample Library/COT Human DNA/PacBio Universal Primer in a DNA vacuum concentrator (Speed Vac). Do not apply heat.</td><td></td></tr>
<tr><td>6</td><td></td><td>To the dried-down sample, add the following:<table><tr><th>Component</th><th>Stock Conc.</th><th>Volume</th></tr><tr><td>xGen 2X Hybridization Buffer</td><td>2X</td><td>8.5 µL</td></tr><tr><td>xGen Hybridization Buffer Enhancer</td><td></td><td>2.7 µL</td></tr><tr><td>Nuclease-Free Water</td><td></td><td>1.8 µL</td></tr></table>Incubate at room temperature for 5-10 minutes.</td><td></td></tr>
<tr><td>7</td><td></td><td>Mix gently by tapping the tube, quick spin and transfer to a low-bind 0.2 mL PCR tube to be incubated in a thermal cycler.</td><td></td></tr>
<tr><td>8</td><td></td><td>Place the tube in a thermal cycler set at +95℃ for 10 minutes to denature the DNA. The thermal cycler’s heated lid should be turned on using default temperature so that evaporation is minimized during incubation.</td><td></td></tr>
<tr><td>9</td><td></td><td>Quick spin the tube in a centrifuge at maximum speed at room temperature for 5 seconds. This allows the mix to cool at room temperature before the addition of the hybridization probes. It’s important that probes are never added at 95℃.</td><td></td></tr>
<tr><td>10</td><td></td><td>Add 4 µL of the xGen Lockdown probes to the tube (Refer to IDT’s recommendations on diluting the probe set to a working solution.)</td><td></td></tr>
<tr><td>11</td><td></td><td>Spin the tube at maximum speed in a minicentrifuge.</td><td></td></tr>
<tr><td>12</td><td></td><td>Incubate the tube in a thermal cycler at +65℃ for 4 hours. The thermocycler’s heated lid should be turned on and set to maintain +75℃ (10℃ above the hybridization temperature).</td><td></td></tr>
</table>

<table>
<tr><th>STEP</th><th>✓</th><th>Prepare Capture Beads</th><th>Notes</th></tr>
<tr><td></td><td></td><td>In this section, you will need the following:
xGen 2X Bead Wash Buffer, xGen 10X Wash Buffer 1, xGen 10X Wash Buffer 2, xGen 10X Wash buffer 3 and xGen 10X Stringent Wash Buffer
M-270 Streptavidin Beads</td><td></td></tr>
<tr><td>1</td><td></td><td>Prepare 1x concentrations of the following buffers (Volumes shown below are for one capture reaction):
<table>
<tr><th>Buffer Stock</th><th>Volume (µL)</th><th>Water (µL)</th><th>Total Volume of 1X Buffer</th></tr>
<tr><td>2x Bead Wash Buffer</td><td>250</td><td>250</td><td>500</td></tr>
<tr><td>10x Wash Buffer I (1)</td><td>30</td><td>270</td><td>300</td></tr>
<tr><td>10x Wash Buffer II (2)</td><td>20</td><td>180</td><td>200</td></tr>
<tr><td>10x Wash Buffer III (3)</td><td>20</td><td>180</td><td>200</td></tr>
<tr><td>10x Stringent Wash Buffer</td><td>40</td><td>360</td><td>400</td></tr>
</table>
a. Preheat the following wash buffers to +65℃ in a heat block (the colors below can be used to track the various buffers):
o 400 µL of 1X Stringent Wash Buffer
o 100 µL of 1X Wash Buffer I
b. Store the remaining 1X Buffers at room temperature.</td><td></td></tr>
<tr><td>2</td><td></td><td>Prepare the capture beads:<ol type="a">
Allow the Dynabeads M-270 Streptavidin to warm to room temperature for 30 minutes prior to use.
Mix the beads thoroughly by vortexing at 2000 RPM for 15 seconds.
Aliquot 100 µL of beads for each capture reaction into a single 1.5 mL LoBind tube. (Enough beads for up to six captures can be prepared in a single tube.)
Place the LoBind tube in a magnetic rack. Once clear, remove and discard liquid (careful to leave all of the beads in the tube).
While the LoBind tube is in the magnetic rack, add 200 µL of 1X Bead Wash Buffer.
Remove the tube from the magnetic rack and vortex for 10 seconds.
Place the LoBind tube back in the magnetic rack to bind the beads. Once clear, remove and discard the liquid.
Repeat steps e - g for a total of two washes.
Resuspend beads by adding 100 µL1X Bead Wash Buffer and mix by vortexing for 10 seconds.
Aliquot 100 µL of resuspended beads into new 0.2 mL LoBind PCR tubes.
Place the tube in the magnetic rack to bind the beads. Once clear, remove and discard the liquid. Do not allow the beads to dry out. Residual wash buffer does not impact binding.
The washed beads are now ready to bind to the probes that are hybridized to the target DNA. Proceed immediately to the next step (Bead Capture and Wash).</td><td></td></tr>
</table>

STEP	✓	Bead Capture and Wash	Notes
1		Bind hybridized DNA samples to the capture beads: a. Add the hybridized sample/probe mix from the Hybridization of Probes section. b. Mix gently by tapping the tube until the sample is homogeneous. c. Incubate in a thermocycler set to +65℃ for 4 5 minutes (heated lid set to +75℃). Hand mix by gently tapping the tube **every 12 minutes** during the 65℃ incubation period.	
2		Wash the capture beads and bound DNA (**65℃ Wash**): a. After the 45-minute incubation, add 100 µL of preheated 1X Wash Buffer I to the bead/sample mix. b. Mix gently by tapping the tube until the sample is homogeneous. c. Transfer the entire contents of the 0.2 mL tube to a 1.5 mL LoBind Eppendorf tube. d. Quick spin. e. Place the tube in the magnetic rack. Once clear, remove and discard the liquid. f. Remove the tube from the magnetic rack and add 200 µL of preheated 1X Stringent Wash Buffer. Pipet up and down 10 times. Do not create any bubbles during pipetting. g. Incubate at +65℃ for 5 minutes. h. Place the tube in the magnetic rack. Allow the beads to separate. Discard the supernatant. i. **Repeat steps f-h for a total of two washes using** 1X Stringent Wash Buffer **heated to +65℃** .	
3		Wash the capture beads and bound DNA (**Room Temperature Wash**): a. Add 200 µL of room temperature 1X Wash Buffer I and mix gently by tapping the tube until the sample is homogeneous. If any liquid has collected in the tube cap, tap the tube gently to collect the liquid into the bottom of the tube before continuing to the next step. b. Place the tube in the magnetic rack. Once clear, remove and discard the liquid. c. Add 200 µL of room temperature 1X Wash Buffer II and mix gently by tapping the tube until sample is homogeneous. d. Place the tube in the magnetic rack. Once clear, remove and discard the liquid. e. Add 200 µL of room temperature1X Wash Buffer III and mix gently by tapping the tube until sample is homogeneous. f. Place the tube in the magnetic rack. Once clear, remove and discard the liquid. g. Remove the tube from the magnetic rack and add 50 µL of EB to the tube of bead-bound captured sample. This is enough for two PCR reactions required in the next section. h. Store the beads plus captured sample at -15 to -25℃ or proceed to the next step. It is not necessary to separate the beads from the eluted DNA. The bead/sample mix can be added to the PCR reaction directly.	

<table>
<tr><th>STEP</th><th>✓</th><th>Amplification of Captured DNA Fragments</th><th>Notes</th></tr>
<tr><td></td><td></td><td>In this section, you will need the following:
Takara LA Taq DNA Polymerase Hot-Start Version
100 µM PacBio Universal Primer</td><td></td></tr>
<tr><td>1</td><td></td><td>For each sample, prepare the following mix for a total of 200 µL.
<table>
<tr><th>Component</th><th>Volume</th></tr>
<tr><td>Captured Library</td><td>50 µL</td></tr>
<tr><td>10X LA PCR Buffer</td><td>20 µL</td></tr>
<tr><td>2.5 mM each dNTPs</td><td>16 µL</td></tr>
<tr><td>100 µM PacBio Universal Primer</td><td>2 µL</td></tr>
<tr><td>Takara LA Taq DNA polymerase</td><td>1.2 µL</td></tr>
<tr><td>Water</td><td>110.8 µL</td></tr>
<tr><td>Total volume</td><td>200 µL</td></tr>
</table>
It is highly recommended to perform the amplification in 100 µL volumes. Transfer 100 µL aliquots into two 0.2 ml low-bind PCR tubes.</td><td></td></tr>
<tr><td>2</td><td></td><td>Amplify using the following thermal profile:
<table>
<tr><th>Step</th><th>Temp</th><th>Time</th></tr>
<tr><td>1</td><td>95℃</td><td>2 minutes</td></tr>
<tr><td>2</td><td>95℃</td><td>20 seconds</td></tr>
<tr><td>3</td><td>62℃</td><td>15 seconds</td></tr>
<tr><td>4</td><td>68℃</td><td>10 minutes</td></tr>
<tr><td>5</td><td colspan="2">Repeat steps 2 through 4, 15 times</td></tr>
<tr><td>7</td><td>68℃</td><td>5 minutes</td></tr>
<tr><td>8</td><td>4℃</td><td>Hold</td></tr>
</table></td><td></td></tr>
</table>

STEP	✓	Post Amplification Clean UP	Notes
1		Pool the PCR reactions and add **0.45X** volume of AMPure PB beads.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 - 60 seconds.	
13		Add **37 µL** of Elution Buffer to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 mL Lo-Bind tube. – Discard the beads.	
14		Perform DNA quantitation using Qubit and assess the size of the eluted sample using a Bioanalyzer instrument with the DNA 12000 Kit.	
15		Proceed with SMRTbell library construction in the next section (Repair DNA Damage)	

Repair DNA Damage

Use the following table to repair any DNA damage. If preparing larger amounts of DNA, scale the reaction volumes accordingly (i.e., for 10 µg of DNA scale the total volume to 100 µL). Do not exceed 100 ng/µL of DNA in the final reaction.

1. In a LoBind microcentrifµge tube, add the following reagents:

Reagent	Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Amplified DNA			__ µL for 5.0 µg			
DNA Damage Repair Buffer		10 X	5.0 µL	1 X		
NAD+		100 X	0.5 µL	1 X		
ATP high		10 mM	5.0 µL	1 mM		
dNTP		10 mM	0.5 µL	0.1 mM		
DNA Damage Repair Mix			2.0 µL			
H_2O			__ µL to adjust to 50.0* µL			
Total Volume			50.0 µL			

*To determine the correct amount of H_2O to add, use your actual DNA amount noted in the Notes column.

2. Mix the reaction well by gentle mixing.
3. Spin down contents of the LoBind tube with a quick spin in a microfuge.
4. Incubate at 37ºC for 20 minutes, then return the reaction to 4ºC for 1 minute.

Repair Ends

Use the following table to prepare your reaction then purify the DNA.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (Damage Repaired)			50 µL			
End Repair Mix		20 X	2.5 µL	1X		
Total Volume			52.5 µL			

1. Mix the reaction well by gentle mixing.
2. Spin down contents of LoBind tube with a quick spin in a microfuge.
3. Incubate at 25ºC for 5 minutes, return the reaction to 4ºC.

STEP	✓	Purify DNA	Notes
1		Add **0.45X** volume of AMPure PB beads to the Damage Repair reaction.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 - 60 seconds.	
13		Add **30 µL** of Elution Buffer to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 mL Lo-Bind tube. – Discard the beads.	
14		Optional: Verify your DNA amount and concentration using a Nanodrop or Qubit quantitation platform, as appropriate.	
15		Optional: Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit.	
16		The End-Repaired DNA can be stored overnight at 4ºC or at -20ºC for longer duration.	
17		Actual recovery per µL and total available sample material: _____	

Prepare Blunt-Ligation Reaction

Use the following table to prepare your blunt-ligation reaction:

1. In a LoBind microcentrifuge LoBind tube (on ice), add the following reagents in the order shown. Note that you can add water to achieve the desired DNA volume. If preparing a Master Mix, ensure that the adapter is NOT mixed with the ligase prior to introduction of the inserts. Add the adapter to the well with the DNA. All other components, including the ligase, should be added to the Master Mix.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.		Notes
DNA (End Repaired)			29.0 µL to 30.0 µL			
Blunt Adapter (20 µM)		20 µM	1.0 µL	0.5 µM		
Mix before proceeding						
Template Prep Buffer		10 X	4.0 µL	1X		
ATP low		1 mM	2.0 µL	0.05 mM		
Mix before proceeding						
Ligase		30 U/µL	1.0 µL	0.75 U/µL		
H_2O			_____ µL to adjust to 40.0 µL			
Total Volume			40.0 µL			

2. Mix the reaction well by gentle mixing.
3. Spin down contents of LoBind tube with a quick spin in a microfuge.
4. Incubate at 25ºC for 15 minutes. At this point, the ligation can be extended up to 24 hours or cooled to 4ºC (for storage of up to 24 hours).
5. Incubate at 65ºC for 10 minutes to inactivate the ligase, then return the reaction to 4ºC. You must proceed with adding exonuclease after this step.

Add exonuclease to remove failed ligation products.

Reagent	Tube Cap Color	Stock Conc.	✓	Volume
Ligated DNA				40 µL
Mix reaction well by pipetting				
ExoIII		100.0 U/µL		1.0 µL
ExoVII		10.0 U/µL		1.0 µL
Total Volume				42 µL

1. Spin down contents of LoBind tube with a quick spin in a microfuge.
2. Incubate at 37ºC for 1 hour, then return the reaction to 4ºC. You must proceed with purification after this step.

Purify SMRTbell® Templates

STEP	✓	Purify SMRTbell Templates – First Purification	Notes
1		Add **0.45X** volume of AMPure PB beads to the exonuclease-treated reaction.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 - 60 seconds.	
13		Add **100 µL** of Elution Buffer to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 mL Lo-Bind tube. – Discard the beads.	

STEP	✓	Purify SMRTbell Templates – Second Purification	Notes
1		Add **0.45X** volume of AMPure PB beads.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 - 60 seconds.	
13		Add **10 µL** of Elution Buffer to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 mL Lo-Bind tube. – Discard the beads.	
14		Verify your DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit dsDNA BR Assay kit and the dsDNA HS Assay kit according to the manufacturer's recommendations.	
15		Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit. If your library is contaminated with short insert SMRTbell templates, an optional size selection step may be performed. Proceed to the **Size-Select SMRTbell Library** section below.	
16		If an optional size selection step is not performed, proceed to the "**Anneal and Bind SMRTbellTemplates**" section.	

Optional Size-Selection:

A size selection step may be necessary if your library is contaminated with short insert SMRTbell templates. Follow the procedure below to size select your SMRTbell library.

STEP	✓	Size-Select SMRTbell Library	Notes
1		Prepare the DNA samples to run on a 0.75% BluePippin gel cassette (BLF7510) according to the manufacturer's instructions. Proceed with the procedure if there is >500 ng DNA. Add 10 µL of Loading Solution to 30 µL of the eluted sample.	
2		Program the BluePippin system: • In the Protocol Editor Tab, choose cassette type **0.75% DF Marker S1 High Pass 6-10kb vs3**. • Choose BP start = 4500, BP end = 50000 • Determine which reference lane to add the S1 marker, enter into "Reference Lane" field and select "Apply Reference to all Lanes" button.	
3		Calibrate the optics as outlined in the manufacturer's instructions.	
4		Prepare a 0.75% BluePippin cassette, load samples and run according to manufacturer's instructions.	
5		After the run, remove the 40 µL of sample from each elution well.	
6		At this point wells can be washed with an additional 40 µL electrophoresis buffer. Combine the 40 µL wash with the 40 µL eluted sample.	

STEP	✓	Post Size-Selection Clean-Up	Notes
1		Add **1X** volume of AMPure PB beads to the exonuclease-treated reaction. (For detailed instructions on AMPure PB bead purification, see the Concentrate DNA section.)	
2		Mix the bead/DNA solution by tapping the tube.	
3		Quickly spin down the LoBind tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the LoBind tube (for 1 second) to collect beads.	
6		Place the LoBind tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol and dry the bead pellet. – Remove the LoBind tube from the magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the LoBind tube back on the magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the LoBind tube from the magnetic bead rack and allow beads to air-dry (with LoBind tube caps open) for 60 seconds.	
13		Add **10 µL** of Elution Buffer to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 mL Lo-Bind tube. – Discard the beads.	
14		Verify your DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit dsDNA BR Assay kit and the dsDNA HS Assay kit according to the manufacturer's recommendations.	
15		Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit.	
16		The library is ready for primer annealing and polymerase binding.	

Anneal and Bind SMRTbell Templates

For primer annealing, follow the instructions in SMRT Link Sample Setup.

For polymerase binding, follow the instructions in SMRT Link Sample Setup.

Sequencing

We recommend performing loading titrations to determine the appropriate loading concentration. For more information, refer to *Quick Reference Card – Diffusion Loading and Pre-extension Time Recommendations for the Sequel System*.

Revision History (Description)	Version	Date
Initial release.	01	October 2017
Fixed typo in title.	02	December 2017
Removed loading specifics and referenced “Quick Reference Card – Diffusion Loading and Movie Time Recommendations for the Sequel System” for more information.	03	February 2018
The procedure is updated to align with the “Procedure & Checklist – Multiplex Genomic DNA Target Capture Using SeqCap EZ Libraries”. The procedures are similar except for the required reagents for hybridization, hybridization volume, and incubation temperatures.	04	July 2018