Target Sequence Capture Using IDT Library with PacBio® Barcoded Adapters

Before You Begin

This document describes the enrichment process for sample libraries using IDT Libraries which are then subsequently sequenced on a PacBio System.

Workflow

The workflow includes the following:

1. Preparing the library using linear barcoded adapter for singleplex or multiplex.
2. Capturing target regions by hybridizing the sample library with the IDT Library.
3. Constructing SMRTbell™ libraries and sequencing using a PacBio System.

Materials Needed

Item	Vendor	Part Number
KAPA Hyper Prep Kits for Illumina sequencing	KAPA Biosystems	KK8503
xGen Lockdown Reagents	IDT	
Invitrogen Human Cot-1 DNA	Life Technologies	15279-011
Takara LA Taq DNA Polymerase Hot-Start version	Clontech	RR042A
Dynabeads M-270 Streptavidin	Life Technologies	65305
AMPure PB Beads	PacBio	
Template Prep Kit	PacBio	
PacBio Universal Sequence /5Phos/gcagtcgaacatgtagctgactcaggtcac	IDT	N/A

Recommended PacBio Barcoded Adapters

	Barcoded Adapter Pairs	Sequence
1	Univ.V3_lbc0001_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacTCAGACGATGCGTCATggtagT
	Univ.V3_lbc0001_rev_comp	/5phos/ctaccATGACGCATCGTCTGAgtgacctgagtcagctacatgttcgactgc
2	Univ.V3_lbc0009_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCTGCGTGCTCTACGACggtagT
	Univ.V3_lbc0009_rev_comp	/5phos/ctaccGTCGTAGAGCACGCAGgtgacctgagtcagctacatgttcgactgc
3	Univ.V3_lbc0017_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCATAGCGACTATCGTGggtagT
	Univ.V3_lbc0017_rev_comp	/5phos/ctaccCACGATAGTCGCTATGgtgacctgagtcagctacatgttcgactgc
4	Univ.V3_lbc0026_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCGAGCACGCGCGTGTGggtagT
	Univ.V3_lbc0026_rev_comp	/5phos/ctaccCACACGCGCGTGCTCGgtgacctgagtcagctacatgttcgactgc
5	Univ.V3_lbc0038_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacTGCTCGCAGTATCACAggtagT
	Univ.V3_lbc0038_rev_comp	/5phos/ctaccTGTGATACTGCGAGCAgtgacctgagtcagctacatgttcgactgc
6	Univ.V3_lbc0040_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCAGTGAGAGCGCGATAggtagT
	Univ.V3_lbc0040_rev_comp	/5phos/ctaccTATCGCGCTCTCACTGgtgacctgagtcagctacatgttcgactgc
7	Univ.V3_lbc0048_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacTCACACTCTAGAGCGAggtagT
	Univ.V3_lbc0048_rev_comp	/5phos/ctaccTCGCTCTAGAGTGTGAgtgacctgagtcagctacatgttcgactgc
8	Univ.V3_lbc0052_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacGCAGACTCTCACACGCggtagT
	Univ.V3_lbc0052_rev_comp	/5phos/ctaccGCGTGTGAGAGTCTGCgtgacctgagtcagctacatgttcgactgc
9	Univ.V3_lbc0058_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacAGATATCATCAGCGAGggtagT
	Univ.V3_lbc0058_rev_comp	/5phos/ctaccCTCGCTGATGATATCTgtgacctgagtcagctacatgttcgactgc
10	Univ.V3_lbc0059_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacGTGCAGTGATCGATGAggtagT
	Univ.V3_lbc0059_rev_comp	/5phos/ctaccTCATCGATCACTGCACgtgacctgagtcagctacatgttcgactgc
11	Univ.V3_lbc0062_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacGACAGCATCTGCGCTCggtagT
	Univ.V3_lbc0062_rev_comp	/5phos/ctaccGAGCGCAGATGCTGTCgtgacctgagtcagctacatgttcgactgc
12	Univ.V3_lbc0070_T_overhang_for	gcagtcgaacatgtagctgactcaggtcacCTGCGCAGTACGTGCAggtagT
	Univ.V3_lbc0070_rev_comp	/5phos/ctaccTGCACGTACTGCGCAGgtgacctgagtcagctacatgttcgactgc

- The primers should be standard desalt purified. HPLC purification is not necessary.
- We recommend 100 µM scale synthesis.
- The "**lbc00XX**" in the name denotes PacBio Barcodes.

<table>
<tr><th>STEP</th><th>✓</th><th>Anneal Barcoded Adapters</th><th>Notes</th></tr>
<tr><td>1</td><td></td><td>The single-stranded barcoded adapters must 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>Primer Annealing (PacBio Template Prep Kit)</td><td>10X</td><td>2 µL</td></tr>
<tr><td>Oligo 1</td><td>100 µM</td><td>2 µL</td></tr>
<tr><td>Oligo 2</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></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°C</td><td>2 minutes</td></tr>
<tr><td>2</td><td>25°C
Ramp to 25°C</td><td>1 second
0.1 °C /sec</td></tr>
<tr><td>3</td><td>25°C</td><td>1 second</td></tr>
<tr><td>4</td><td>4°C</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 at -20°C 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>Dilute 2 µg of genomic DNA (gDNA) to 150 µL total volume in molecular biology grade water, TE or PacBio Elution Buffer (EB).</td><td></td></tr>
<tr><td>2</td><td></td><td>Load the 150 µL diluted sample 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 singleplex, we recommend shearing the gDNA to 10 kb.
For multiplex, we recommend shearing the gDNA to 6 kb.
<table><tr><th></th><th>Shear Target Size</th><th>RPM (5415D)</th></tr><tr><td>Singleplex</td><td>10 kb</td><td>6000</td></tr><tr><td>> 2-plex</td><td>6 kb</td><td>7000</td></tr></table>
Other centrifuges may be used but speed should be optimized to achieve proper gDNA shearing.
Check for any residual DNA in the upper chamber. If some sample still remains at the top, pulse the sample up to speed (7,200 rpm) for 5 seconds. Repeat pulses until all of the sample is at the bottom of the tube.</td><td></td></tr>
<tr><td>4</td><td></td><td>Invert the g-TUBE device and spin the sample at same speed and duration. If some sample still remains at the top, pulse the sample up to 7200 rpm for 5 seconds. Repeat pulses until all of the sample is at the bottom of tube.</td><td></td></tr>
<tr><td>5</td><td></td><td>Recover the sample into a new 1.5 mL LoBind tube.</td><td></td></tr>
</table>

STEP	✓	Clean and Concentrate Genomic DNA	Notes
1		Add 120 µL (0.8X) AMPure PB beads to each sheared gDNA sample. Mix thoroughly by tapping the LoBind tube until the sample is homogeneous.	
2		Incubate at room temperature for 15 minutes. Place on a magnetic bead rack until solution clears.	
3		Remove and discard supernatant.	
4		With LoBind tube still on the magnetic bead rack, add 200 µL freshly prepared 70% ethanol to the tube containing the beads and DNA.	
5		Remove and discard the 70% ethanol.	
6		Repeat steps 4 to 6 for a total of two washes with 70% ethanol.	
7		Let the beads air-dry for 1 minute. (Note - over drying the beads will result in reduced DNA yield.)	
8		Add 34 µL EB. Mix by tapping the tube gently until the sample is homogeneous and incubate at room temperature for 2 minutes.	
9		Place tube back on magnetic bead rack. When the solution clears, transfer 32 µL supernatant into a new 1.5 mL LoBind tube.	
10		Determine the DNA concentration using a Qubit quantitation platform or similar DNA quantification assay.	
11		Run 1 µL of sample on an Agilent DNA 12000 chip according to manufacturer's instructions.	

<table>
<tr><th>STEP</th><th>✓</th><th>End-Repair and A-tailing</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 previous page for annealing barcoded adapters.</td><td></td></tr>
<tr><td>1</td><td></td><td>Note: When “mixing or pipette mixing” is indicated at a step, hand mixing by gently tapping the tube should be performed in order to reduce damage to the DNA.
Use a minimum 200 ng of sheared gDNA diluted to 50 µL in EB as input into the End Repair and A-tailing reaction.
For single-plex, prepare two reactions.
For multiplex, prepare a single reaction for each sample.
<table>
<tr><th>Component</th><th>Volume</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.

4. Mix thoroughly and centrifuge briefly.
5. Incubate in a thermocycler with the following thermal profile:
<table>
<tr><th>Step</th><th>Temp</th><th>Time</th></tr>
<tr><td rowspan="2">End Repair & A-Tailing</td><td>20 °C</td><td>30 min</td></tr>
<tr><td>65 °C</td><td>30 min</td></tr>
<tr><td>HOLD</td><td>4 °C</td><td>∞</td></tr>
</table>
6. Proceed immediately to the next step.</td><td></td></tr>
</table>

<table>
<tr><th>STEP</th><th>✓</th><th>Adapter Ligation</th><th>Notes</th></tr>
<tr><td>1</td><td></td><td>Prepare the number of reactions:
For single-plex, two separate reactions are required to generate enough DNA for the subsequent reactions.
For multiplex, prepare a single reaction 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.
Proceed immediately to the next step.</td><td></td></tr>
<tr><td>2</td><td></td><td>Purify the sample with 0.50 X AMPure PB beads.

a. Mix thoroughly by tapping the tube until the sample is homogeneous.
b. Incubate at room temperature for 15 min to bind DNA to the beads.
c. Place the tube on a magnetic bead rack to capture the beads.
d. After solution clears, carefully remove and discard the supernatant.
e. Keeping the tube on the magnet, add 70% ethanol to the rim of the tube.
f. Remove the 70% ethanol.
g. Keeping the tube on the magnet, add 70% ethanol to the rim of the tube.
h. Remove the 70% ethanol. Try to remove all residual ethanol without disturbing the beads.
i. Let the beads dry for 60 seconds.
j. Air-dry the beads at room temperature for 1 minute. Caution: over-drying the beads may result in dramatic yield loss.
k. Remove the tube from the magnet.</td><td></td></tr>
<tr><td>3</td><td></td><td>Elute the DNA with 52 µL EB. Mix thoroughly by tapping the LoBind tube until the sample is homogenous (while avoiding splashes to the side of the tube). Incubate at room temperature for 2 minutes to elute DNA off the beads.</td><td></td></tr>
<tr><td>4</td><td></td><td>Place tube back on the magnet bead rack to capture the beads. After solution clears, transfer supernatant to a new 1.5 mL LoBind tube and proceed to the amplification step.</td><td></td></tr>
</table>

<table>
<tr><th>STEP</th><th>✓</th><th>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
100 µM PacBio Universal Primer</td><td></td></tr>
<tr><td>1</td><td></td><td>For each sample, prepare the following mix.

<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>
Split the above into two, 100 µL reactions.</td><td></td></tr>
<tr><td>2</td><td></td><td>Amplify using the following PCR conditions:

<table>
<tr><th>Step</th><th>Temp</th><th>Time</th></tr>
<tr><td>1</td><td>95°C</td><td>2 minutes</td></tr>
<tr><td>2</td><td>95°C</td><td>20 seconds</td></tr>
<tr><td>3</td><td>62°C</td><td>15 seconds</td></tr>
<tr><td>4</td><td>68°C</td><td>10 minutes</td></tr>
<tr><td>5</td><td colspan="2">Repeat Step 2, 6 times</td></tr>
<tr><td>6</td><td>68°C</td><td>5 minutes</td></tr>
<tr><td>7</td><td>4°C</td><td>Hold</td></tr>
</table>
The extension time can be modified. As a general rule of thumb, for every 1 kb add 1 minute to the extension time.</td><td></td></tr>
</table>

STEP	✓	Post Amplification Clean-Up	Notes
1		Pool the two 100 µL reactions for each sample and purify using 0.5X AMPure PB beads. Mix thoroughly by tapping the tube until the sample is homogenous.	
2		Incubate at room temperature for 15 minutes. Place on a magnetic bead rack until the solution clears.	
3		Remove and discard supernatant	
4		With the tube still on the magnet, add 70% ethanol to the rim of the tube.	
5		Remove and discard 70% ethanol.	
6		Repeat steps 4 to 6 for total of two washes with 70% ethanol.	
7		Let beads air-dry for 1 minute. (Note - over drying the beads will result in reduced DNA yield.)	
8		Add 32 µL water and remove LoBind tube from the magnet. Mix by tapping the tube gently until the sample is homogeneous and incubate at room temperature for 2 minutes.	
9		Place back on the magnet. When the solution clears, remove 32 µL supernatant into new 1.5 mL LoBind tube.	
10		Determine DNA concentration using Qubit quantitation platform or similar quantification assay.	
11		Run 1 µL of sample on an Agilent DNA 12000 chip according to manufacturer's instructions.	
12		Prepare the DNA samples for size selection.	

STEP	✓	Size-Select Amplified Samples	Notes
1		Prepare the 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 collection mode, "Range" with start size 4500 bp and end size 50000 bp for each lane. ● 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 sample.	
7		Concentrate the sample by performing a 1X AMPure bead purification step. The DNA should be eluted in 20 µL EB.	
8		Determine DNA concentration using a Qubit quantitation platform or similar quantification assay and run 1 µL on a Bioanalyzer system 12000 chip.	
9		The hybridization step requires a total of 1.5 µg – 2.0 µg for single-plex or multiplex. **For multiplex hybridization, pool samples using equal mass**.	

<table>
<tr><th>STEP</th><th>✓</th><th>Prepare the Hybridization Sample</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
xGen Hybridization Buffer Enhancer
IDT Library (target 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>Note that 1.5 µg to 2 µg of the size-selected library is required for hybridization. Add the size-selected DNA 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/Blocking Oligos in a DNA vacuum concentrator (SpeedVac).</td><td></td></tr>
<tr><td>6</td><td></td><td>To each dried-down DNA 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>Pipette up and down to mix, and transfer to a low-bind 0.2 ml PCR tube.</td><td></td></tr>
<tr><td>8</td><td></td><td>Place the tube in a +95°C thermocycler with a heated lid for 10 minutes to denature the DNA. This minimizes evaporation.</td><td></td></tr>
<tr><td>9</td><td></td><td>Quick spin the tube at maximum speed at room temperature. This allows the mix to cool at room temperature before the addition of the probes. It’s important that probes are never be added at 95°C.</td><td></td></tr>
<tr><td>10</td><td></td><td>Add 4 µL of the IDT probe (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 at maximum speed.</td><td></td></tr>
<tr><td>12</td><td></td><td>Incubate in a thermocycler with a heated lid at +65°C for 4 hours. The thermocycler’s heated lid should be turned on and set to maintain +75°C (10°C above the hybridization temperature).</td><td></td></tr>
</table>

<table>
<tr><th>STEP</th><th>✓</th><th>Wash and Recover Captured DNA Sample</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 fresh bead wash buffers (Volumes are for one capture):

<table>
<tr><th>Buffer Stock</th><th>vol</th><th>Water</th><th>Total Volume of 1X Buffer*</th></tr>
<tr><td>xGen 2X Bead Wash Buffer</td><td>250</td><td>250 µL</td><td>500 µL</td></tr>
<tr><td>xGen 10X Wash Buffer 1</td><td>30</td><td>270 µL</td><td>300 µL</td></tr>
<tr><td>xGen 10X Wash Buffer 2</td><td>20</td><td>180 µL</td><td>200 µL</td></tr>
<tr><td>xGen 10X Wash Buffer 3</td><td>20</td><td>180 µL</td><td>200 µL</td></tr>
<tr><td>xGen 10X Stringent Wash Buffer</td><td>40</td><td>360 µL</td><td>400 µL</td></tr>
</table>
a. Preheat the following wash buffers to +65°C in a heat block:
o 400 µL of 1X Stringent Wash Buffer
o 100 µL of 1X Wash Buffer I

b. Store remaining 1X Buffers at room temperature.</td><td></td></tr>
</table>

2		Prepare the capture beads: a. Allow the Dynabeads M-270 Streptavidin, to warm to room temperature for 30 minutes prior to use. b. Mix the beads thoroughly by vortexing for 15 seconds. c. Aliquot 100 µL beads for each capture into a single 1.5 mL LoBind tube. Enough beads for six captures can be prepared in a single tube. d. Place the LoBind tube in a magnetic rack. When the liquid becomes clear remove and discard the liquid being careful to leave all of the beads in the tube. e. While the LoBind tube is in the magnetic rack, add 200 µL of 1X Bead Wash Buffer. f. Remove the tube from the magnetic rack and vortex for 10 seconds. g. Place the LoBind tube back in the magnetic rack to bind the beads. Once clear, remove and discard the liquid. h. Repeat steps e - g for a total of two washes. i. After removing the buffer following the second wash, resuspend by vortexing the beads in 100 µL1X Bead Wash Buffer j. Aliquot 100 µL of resuspended beads into new 0.2 mL LoBind tubes. k. Place the tube in the magnetic rack to bind the beads. Once clear, remove and discard the liquid. l. The capture beads are now ready to bind the captured DNA. Proceed immediately to the next step as quickly as possible. Do not allow the capture beads to dry out. Small amounts of residual Bead Wash Buffer will not interfere with binding of DNA to the capture beads.	
3		Bind DNA to the capture beads: a. Transfer the hybridization samples to the prepared beads in the previous step. b. Mix thoroughly by tapping the tube until the sample is homogeneous. c. Incubate in a thermocycler set to +65°C for 45 minutes (heated lid set to +75°C). Hand mix by gently tapping the tube every 12 minutes during the 65°C incubation.	

4		Wash the capture beads and bound DNA (65°C Wash): a. After the 45-minute incubation, add 100 µL preheated 1X Wash Buffer I to capture beads. b. Mix thoroughly by tapping the tube until the sample is homogeneous. c. Transfer the entire contents of each 0.2 mL tube to a 1.5 mL LoBind tube. d. Vortex briefly. e. Place the tubes in the magnetic rack to bind the beads. Remove and discard the liquid once clear. f. Remove the tubes 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°C 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°C. Wash the capture beads and bound DNA (Room Temperature Wash): a. Add 200 µL of room temperature 1X Wash Buffer I. Hand mix by gently tapping the tube. If liquid has collected in the tube's cap, tap the tube gently to collect the liquid into the tube's bottom before continuing to the next step. b. Place the tubes in the magnetic rack to bind the beads. Remove and discard the liquid once clear. c. Add 200 µL of room temperature 1X Wash Buffer II and mix thoroughly by tapping the tube until sample is homogeneous. d. Place the tubes in the magnetic rack to bind the beads. Remove and discard the liquid once clear. e. Add 200 µL of room temperature1X Wash Buffer III and mix thoroughly by tapping the tube until sample is homogeneous. f. Place the tubes in the magnetic rack to bind the beads. Remove and discard the liquid once clear. g. Remove the tubes from the magnetic rack and add 50 µL EB to each tube of bead-bound captured sample.	

<table>
<tr><th>STEP</th><th>✓</th><th>Amplification of Captured DNA Sample</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.

<table><tr><th>Component</th><th>Volume</th></tr><tr><td>Captured Library</td><td>50 µL</td></tr><tr><td>Water</td><td>110.8 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>Total volume</td><td>200 µL</td></tr></table>Split the mix into two 100 µL reactions.</td><td></td></tr>
<tr><td>2</td><td></td><td>Amplify using the following PCR protocol:

<table><tr><th>Step</th><th>Temp</th><th>Time</th></tr><tr><td>1</td><td>95°C</td><td>2 minutes</td></tr><tr><td>2</td><td>95°C</td><td>20 seconds</td></tr><tr><td>3</td><td>62°C</td><td>15 seconds</td></tr><tr><td>4</td><td>68°C</td><td>10 minutes</td></tr><tr><td>5</td><td colspan="2">Repeat Step 2, 24 times</td></tr><tr><td>7</td><td>68°C</td><td>5 minutes</td></tr><tr><td>8</td><td>4°C</td><td>Hold</td></tr></table></td><td></td></tr>
</table>

STEP	✓	Post Amplification Clean UP	Notes
1		Add 0.5 X AMPure PB beads to each sample. Mix thoroughly by tapping the LoBind tube until the sample is homogeneous.	
2		Incubate at room temperature for 15 minutes. Place on a magnetic bead rack until solution clears.	
3		Remove and discard supernatant.	
4		With the tube still on the magnet rack, add 200 µL freshly prepared 70% ethanol to the rim of the tube.	
5		Remove and discard 70% ethanol.	
6		Repeat steps 4 to 6 for total of two washes with 70% ethanol.	
7		Let beads air dry for 1 minute. (Note - over drying the beads will result in reduced DNA yield.)	
8		Elute DNA in 32 µL EB. Mix thoroughly by tapping the tube until the sample is homogeneous. Then incubate at room temperature for 2 minutes.	
9		Place back on magnet. When the solution clears, transfer 32 µL supernatant into a new 1.5 mL LoBind tube.	
10		Determine DNA concentration using a Qubit quantitation platform.	
11		Run 1 µL of sample on an Agilent DNA 12000 chip according to manufacturer's instructions.	

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
Sheared 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 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 End-Repair 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 by letting it sit at room temperature 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 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 30 to 60 seconds.	
13		Elute the DNA off the beads in **30 µL** EB. Mix by gently tapping the LoBind tube until homogenous, then let stand at room temperature for 2 minutes.	
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 system instrument with the DNA 12000 Kit. Note that typical yield at this point of the process (following End-Repair and one **0.45X** AMPure PB bead purification) is approximately between **80-100%** of the total starting material.	
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			
Annealed 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	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 letting the sample sit at room temperature for 15 minutes.	
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		Elute the DNA off the beads in **32 µL** of EB. Mix thoroughly by gently tapping the LoBind tube and let sit at room temperature for 2 minutes.	
14		The eluted DNA in **32 µL** EB should be taken into the second **1X** AMPure PB bead purification step.	

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 collection mode, "Range" with start size 4500 bp and end size 50000 bp for each lane of sample. ● 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 AMPure PB beads to each amplified library sample. Mix thoroughly by tapping the LoBind tube until the sample is homogeneous.	
2		Incubate at room temperature for 15 minutes. Place on magnetic bead rack until solution clears.	
3		Remove and discard supernatant.	
4		With the tube still on magnet, add freshly prepared 70% ethanol to rim of the tube containing beads plus DNA.	
5		Remove and discard 70% ethanol.	
6		Repeat steps 4 to 6 for total of two washes with 70% ethanol.	
7		Let beads air dry for 1 minute. (Note - over drying the beads will result in reduced DNA yield.)	
8		Add 10 µL EB and remove the tube from the magnet. Mix thoroughly by tapping the tube until the sample is homogeneous. Then incubate at room temperature for 2 minutes.	
9		Place back on magnet. When the solution clears, transfer 10 µL supernatant into new 1.5 mL LoBind tube.	
10		Determine DNA concentration using a Qubit quantitation platform or similar quantification assay.	
11		Run 1 µL of sample on Agilent DNA 12000 chip according to manufacturer's instructions.	

Control Complex Dilution

You must have the PacBio Control Complex for this step. Dilute the Control Complex according to the volumes and instructions specified in the Calculator.

Anneal and Bind SMRTbell™ Templates

Before adding the primer to the SMRTbell template, the primer must go through a melting step at 80ºC. This avoids exposing the sample to heat. The template and primer mix can then be incubated at 20ºC for 30 minutes. Note that you must have the PacBio DNA/Polymerase Kit and use LoBind microcentrifuge tubes for this step.

For polymerase binding, incubation at 30ºC for 30 minutes is sufficient. Instructions for polymerase binding are provided by the calculator.

The recommended loading concentration is 7.5 pM on plate. For more information about using the Binding Calculator, see the Pacific Biosciences *Template Preparation and Sequencing Guide* and *QRC - Annealing and Binding Recommendations*.

Sequence

To prepare for sequencing on the instrument, refer to the *RS Remote Online Help* system or *Pacific Biosciences Software Getting Started Guide* for more information. Follow the touchscreen UI to start your run. Note that you must have a DNA Sequencing Kit and SMRT Cells for standard sequencing.