Procedure & Checklist - 10 kb to 20 kb Template Preparation and Sequencing with Low (100 ng) Input DNA

Before You Begin

To perform this procedure, you must have the PacBio®:

- Template Prep Kit
- AMPure® PB Beads
- DNA/Polymerase Binding Kit (P6 v2 or later)
- MagBead Kit
- DNA Sequencing Kit
- SMRT® Cells

This procedure can be used to prepare 10-20 kb libraries from 100 ng to 200 ng of sheared and concentrated DNA, minimally 100 ng into shearing. Note: for input amounts between 200 ng and 1 µg, the standard 10 kb library prep protocol may be used.

Insert Size Target	Insert Size Range	Input DNA Amount	Ligation	DNA Damage Repair
10 kb to 20 kb	8 kb to 22 kb	100 to 200 ng	Blunt	Required

Fragment and Concentrate DNA

Use a Covaris g-TUBE device to shear your DNA sample, following the g-TUBE user manual Covaris website. However, reduce the sample volume from 150 µL to 50 µL. After the first spin, open the tube to vent, and re-spin. If there is any remaining sample, add 10 µL Elution Buffer (EB) to the upper chamber, flick the g-TUBE or pipette up and down several times, and spin again. Depending upon the quality of your sample, approximately 20% to 50% sample loss is to be expected as a result of the shearing and concentration process.

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STEP	✓	Concentrate DNA	Notes
1		Add **0.5X** volume of AMPure PB magnetic beads. _____ µL of sample X **0.5X** = _____ 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. Consistent and efficient recovery of your sample is critical to successful SMRTbell™ template preparation. If using this protocol for the first time, we strongly recommend that you process a control sample first. Using the DNA shearing methods and subsequent AMPure PB bead purification steps described below, you should recover approximately 50%-80% of your input DNA (by mass). Typical yields, from pre-purified DNA (where smaller fragments are already eliminated as a result of the shearing process) are between 80-100%.	
2		Mix the bead/DNA solution thoroughly.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by mixing in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After mixing, the bead/DNA mixture should appear homogenous. We recommend using a VWR vortex mixer with a foam microtube attachment (see the relevant PacBio Guide for part numbers). If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.	
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. 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.	

STEP	✓	Concentrate DNA	Notes
10		Remove residual 70% ethanol and dry the bead pellet. – 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 to 60 seconds.	
13		Add **37 µL** of PacBio Elution Buffer to elute the beads. – Mix until homogeneous – Vortex for 1-2 minutes at 2000 rpm – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack – Carefully collect the eluted sample. – Discard the beads.	
14		Proceed to the next step. If necessary, store at -20ºC for longer duration.	

Repair DNA Damage

Use the following table to repair any DNA damage. If preparing more than one sample, pre-mix the Damage Repair Buffer, NAD+, ATP high, and dNTP.

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

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Sheared DNA	-		37µL	-		
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	-		

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 37ºC for 60 minutes or longer, 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 pipetting or flicking the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25ºC for 5 minutes (no longer), return the reaction to 4ºC.

STEP	✓	Purify DNA	Notes
1		Add **0.5X** volume (or **26.0 µL**) 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 thoroughly.	
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.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol and dry the bead pellet. – 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 tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **32-33 µL** Elution Buffer. – Mix until homogeneous – Vortex for 1-2 minutes at 2000 rpm – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack – Carefully collect the eluted sample. – Discard the beads.	
14		Proceed to the next step. If necessary, store at -20ºC for longer duration.	

Prepare Blunt-Ligation Reaction

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

1. In a LoBind microcentrifuge tube (on ice), add the following reagents in the order shown. 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. For multiple samples, pre-mix the Template Prep Buffer and ATP Low.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (End Repaired)	-		32.0 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 pipetting or flicking the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 25ºC for 60 minutes.
5. Incubate at 65ºC for 10 minutes to inactivate the ligase, then return the reaction to 4ºC. You must proceed with adding exonucleases after this step.

Add exonucleases 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		0.5 µL
ExoVII		10.0 U/µL		0.5 µL
Total Volume				41 µL

6. Mix the reaction well by pipetting or flicking the tube.
7. Spin down contents of tube with a quick spin in a microfuge.
8. Incubate at 37ºC for 1 hour, then return the reaction to 4ºC. Do not exceed 1 hour incubation time. You must proceed with purification after this step.

Purify SMRTbell™ Templates

There are 2 final purification steps using **0.45X** volume of AMPure PB beads.

STEP	✓	Purify SMRTbell™ Templates - First Purification	Notes
1		Add **0.45X** volume (**18.5 µL**) 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 thoroughly.	
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.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol and dry the bead pellet. – 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 tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **50 µL** of Elution Buffer. Mix for 10 minutes at 2000 rpm. – Mix until homogeneous – Vortex for 1-2 minutes at 2000 rpm – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack – Carefully collect the eluted sample. – Discard the beads.	

STEP	✓	Purify SMRTbell™ Templates - Second Purification	Notes
1		Add **0.45X** volume (**22.5 µL**) of AMPure PB beads to the **50 µL** of eluted DNA.	
2		Mix the bead/DNA solution thoroughly.	
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.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol and dry the bead pellet. – 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 tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **8-10 µL** of Elution Buffer. – Mix until homogeneous – Vortex for 1-2 minutes at 2000 rpm – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack – Carefully collect the eluted sample. – Discard the beads.	
14		Verify the library size and concentration with a Bioanalyzer instrument using a Hight Sensitivity DNA Analysis kit or check quantitation with the Qubit® dsDNA HS Assay kit. Alternatively, estimate concentration based on 10-20% yield of input amount into damage repair.	

Anneal and Bind SMRTbell™ Templates

To anneal sequencing primer and bind polymerase to SMRTbell templates, use of the **Binding Calculator**.

1. Enter the volume and estimated library concentration, assuming 10% library yield, and select the following:
   – Size selection: No
   – Protocol: **Magnetic bead OCPW**
   – Binding Kit: P6v2
   – Preparation protocol: Small scale
   – DNA control complex: No
   – Complex Reuse: No
   – Standard Concentration: **No**

2. In the **Annealing Primer** step, it is likely the volume of diluted sequencing primer will be too low to pipette. Prepare a pre-mix of the 10X primer buffer and diluted sequencing primer, or increase the volume of diluted primer, if the difference is small, by increasing the **Primer:Template Ratio** in **Custom Parameters** as necessary.

3. In the **Binding Polymerase to Templates** step:
   – Check that the final concentration of diluted polymerase is at least 8.0 nM. If necessary, increase the **Polymerase:Template Ratio** in **Custom Parameters** as to bring the diluted polymerase concentration up to 8.0 nM.
   – Increase binding at 30°C to **60** minutes.
4. Increase the **Concentration on Plate** in **Custom Parameters** to 0.012 – 0.024 nM. Some samples may need higher loading. To maximize the number of reads/sample, fine-tune the concentration on plate to use the entire bound complex. For example, if there is 19 µL of complex, and the desired loading concentration results in a maximum of 4 cells using 16 µL of complex, increase the on-plate concentration until nearly all 19 µL are used for 4 cells.

5. During **MagBead** binding and washing steps, process 1 to 2 samples at a time and keep samples on ice or at 4°C to minimize losses.