Procedure & Checklist - Very Low (10 ng) Input 2 kb Template Preparation and Sequencing with Carrier 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

Additional required materials:

- Carrier plasmid DNA, 10 µg (pBR322 or pUC, NEB or Thermo Scientific)
- Exo III, Exo VII, and Template prep buffer from the DNA Template Prep Kit may be used to prepare carrier DNA (below).

This procedure can be used to prepare 1 kb to 3 kb libraries from 5 ng to 50 ng of sheared and concentrated DNA, or from 1kb to 3kb amplicons. Note that when preparing libraries with such a low DNA, amount you must use MagBead loading for sequencing.

To obtain the highest yield and avoid sample loss on the tube walls, carry out all steps from shearing through polymerase binding in one day. Bound samples may be stored at 4°C for up to 48 hours; yields may be reduced with longer storage. Carrier DNA may be prepared beforehand and stored at -20°C. To maintain intact carrier DNA, avoid freeze-thaw cycles and aliquot into small volumes for a few samples.

Insert Size Target	Insert Size Range	Sheared and Concentrated DNA Amount	Ligation	DNA Damage Repair
2 kb	1 kb to 3 kb	5 to 50 ng	Blunt	Recommended

Prepare Carrier DNA

1. Quantify carrier DNA with a Nanodrop® quantitation platform
2. Prepare stock solution of carrier, 10 µg in 100 µL in Elution Buffer (EB)
3. Exo treatment – add reagents listed in the order below:

Reagent	Stock	Volume
Carrier	100 ng/µL	100 µL
Template Prep or ExoIII Buffer	10X	20 µL
ExoIII	100 - 200 U/µL	2 µL
Exo VII	10 U/µL	2 µL
Distilled H_2O		76 µL or to 200 µL total
Total		**200 µL**

4. Incubate 37°C for 1 hour, then return to 4°C
5. Purify and concentrate using 0.6X AMPure PB beads (see the Concentrate DNA section of this procedure); elute in 50 µL EB
6. Quantify the Exo-treated carrier with a Nanodrop spectrophotometer.

Fragment and Concentrate DNA

If preparing 1 kb to 3 kb amplicon libraries, proceed to Concentrate DNA, page 3.

The following conditions were used for shearing on the Covaris® S2 Focused-ultrasonicator. If other shearing devices are used, PacBio recommends testing the manufacturer's conditions for both 2 kb and 3 kb libraries. There may be significant sample loss (up to 50%) during the shearing and concentration process.

Target Shear Size	Tube	Sample Volume	Temp of Chiller	Temp of Bath	Duty Factor	Peak Incident Power (W)	Cycles per Burst	Time (s)	Amount of DNA in Tube
2 kb - 3 kb	Blue MiniTUBE	200 µL	20ºC	20ºC	20%	0.1	1000	10 cycles X 60 sec. (10 min.)	5 ng - 50 ng

STEP	✓	Concentrate DNA	Notes
1		Add **0.6X** volume of AMPure® PB magnetic beads. _____ µL of sample X **0.6X** = _____ 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 **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 **38 µL** of PacBio Elution Buffer volume to your beads. – Thoroughly resuspend beads by vortexing for 1 minute at 2000 rpm. If the beads appear over-dried or cracked, let the Elution Buffer sit on the beads for 2 to 3 minutes then vortex again. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Discard the beads.	
14		Perform qualitative and quantitative analysis using the Bioanalyzer high sensitivity kit, if input is sufficient. Note actual recovery per µL and total available sample material: ____________	
15		For best results, proceed directly to DNA damage repair.	

Repair DNA Damage

In order to obtain the highest possible yield, DNA Damage Repair is suggested for libraries >1 kb.

1. In a LoBind microcentrifuge tube, add the following reagents. Consider preparing a pre-mix of the DNA Damage Repair Buffer, NAD+, ATP high, and dNTP and add 11 µL of pre-mix per sample.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Sheared DNA	-		37 µL	-		
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 20 minutes or longer, then return the reaction to 4ºC for 1 to 5 minutes. Proceed to Repair Ends.

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, return the reaction to 4ºC. Do not incubate the End Repair reaction longer than 5 minutes. Proceed directly to the next step.

STEP	✓	Purify DNA	Notes
1		Add **0.6X** volume (or **31.5 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 µL** Elution Buffer. Mix until homogenous, then mix for 1 minute at 2000 rpm.	
14		For best results, proceed directly to ligation.	

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. A Master Mix may be prepared for the Template Prep Buffer and ATP Low (pre-mix these 2 reagents when preparing multiple samples). Other reagents (Blunt Adapter and Ligase) should be added separately in the order listed below and mixed well prior to the next step.

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
Carrier DNA, pre-treated with ExoIII and ExoVII		100 ng/µL		5.0 µL
Mix reaction well by pipetting				
ExoIII		100.0 U/µL		0.5 µL
ExoVII		10.0 U/µL		0.5 µL
Total Volume				46 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 37ºC for 1 hour, then return the reaction to 4ºC. You must proceed with purification after this step.

Purify SMRTbell™ Templates

There are 2 final purification steps using **0.6X** volume of AMPure PB beads.**For libraries ≥1.5 kb**, the second AMPure PB bead clean-up may be done with **0.5X** AMPure beads to reduce shorter fragments, if desired. Expect a somewhat lower yield.

STEP	✓	Purify SMRTbell™ Templates - First Purification	Notes
1		Add **0.6X** volume (or **27.6 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 1 minute at 2000 rpm.	
14		The eluted DNA in **50 µL** Elution Buffer should be taken into the second **0.6X** AMPure PB bead purification step.	

STEP	✓	Purify SMRTbell™ Templates - Second Purification	Notes
1		Add **30 µL** (**0.6X** volume) of AMPure PB beads to the **50 µL** of eluted DNA from the first AMPure PB bead purification step above. For better removal of shorter fragments from the ≥ 1.5 kb libraries, add **25 µL (0.5X**) volume of AMPure PB beads. Accurate pipetting is critical.	
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. Note: It is especially important to save the supernatant for **0.40X** volumes of AMPure PB purification steps. In case of low recovery, perform a **1X** AMPure PB purification step to recover the DNA.	
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 **10 µL** of Elution Buffer. Mix for 1 minute at 2000 rpm.	
14		Estimate the final library amount and concentration by assuming 20% overall yield. For example, if 5 ng was the starting amount (into DNA damage repair), 20% yield would be 1 ng/10 µL, or 0.1 ng/µL.	

Anneal and Bind SMRTbell™ Templates

Anneal sequencing primer and bind polymerase to SMRTbell templates, using the **Binding Calculator** with the following settings:

1. Enter the volume and estimated library concentration, assuming 20% 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 – 0.045 nM.** 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.

Sequence

To prepare for sequencing on the instrument, see the *Online Help* system or the relevant Pacific Biosciences Guides for more information. Note that you must have a DNA Sequencing Kit and SMRT Cells for standard sequencing.

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