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

Before You Begin

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

- Template Prep Kit
- DNA/Polymerase Binding Kit
- MagBead Kit
- DNA Sequencing Kit
- Control Complex
- SMRT® Cells for standard sequencing

This procedure can be used to prepare 10 kb libraries from 1 µg up to 5 µg of sheared and concentrated DNA. If preparing libraries with DNA input amounts greater than 5 µg, scale all the reaction volumes proportionally (e.g., if the input amount of DNA is 10 µg, which is double the maximum input amount set forth in this procedure, then double all the reaction volumes listed in the tables). Note that when preparing 10 kb libraries using only 1 µg of sheared DNA input, you must use MagBead loading for sequencing.

Insert Size Target	Insert Size Range	Sheared and Concentrated DNA Amount	Ligation	DNA Damage Repair
10 kb	8 kb to 12 kb	1 to 5 µg	Blunt	Required

Fragment and Concentrate DNA

Use a Covaris® g-TUBE® device to shear your DNA sample. The most up-to-date guidance on how to use the g-TUBE, along with recommended centrifuges and centrifugation speeds, can be found in the g-TUBE user manual available for download from the Covaris website. 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. Therefore, be sure to have sufficient amounts of starting DNA in order to have at least 1 µg of sheared and concentrated DNA (30 ng/µL) for the Damage Repair reaction.

Note that a Hydroshear® Shearing Device can also be used to shear your DNA sample. However, because hydrodynamic shearing performance can vary with each shearing assembly, we recommend optimizing the shearing whenever a new shearing assembly is used.

STEP	✓	Concentrate DNA	Notes
1		Add **0.45X** volume of AMPure® PB magnetic beads. _____ µL of sample X **0.45X** = _____ 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 *Guide's* Overview section 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		Calculate appropriate volume of Elution Buffer. _____ ng X **0.5** / (____ng/µL) = _____ µL of Elution Buffer needed The maximum DNA volume required to proceed to the next section (DNA Damage Repair/End-Repair) is **37 µL**. For example, for an input amount of **1 µg**, the minimum concentration is (**1000 ng/37 µL**) = **27 ng/µL**.	
14		Add the Pacific Biosciences' Elution Buffer volume (calculated in step 13 above) to your beads. – Thoroughly resuspend beads by mixing 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 mix again. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Perform concentration measurements. Verify your DNA concentration using a Nanodrop® or Qubit® quantitation platform. If the DNA concentration is estimated to be equal to or below **12 ng/µL**, a Qubit system reading is required. When performing a Qubit system reading, ensure that your sample is within the range of the Qubit kit you are using. For proper concentration calculations, incorporate the dilution factor (used when diluting your sample) to be within range of the Qubit kit and the dilution factor when diluting your sample with the working solution. The latter part of this dilution factor can be calculated automatically by the Qubit system. – Discard the beads.	
15		Using a Bioanalyzer® platform, perform qualitative sizing analysis of the sheared and concentrated DNA prepared above. Note that the Bioanalyzer platform has different kits in it's offering and the appropriate kit, based on insert size, should be used. Dilute the samples appropriately before loading on the Bioanalyzer platform 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 specific kit you might be using. Note that typical yield, at this point of the process (i.e. post-shearing and after one **0.45X** AMPure PB bead purification), is approximately 50%-80%.	
16		The sheared DNA can be stored for up to 24 hours at 4ºC or at -20ºC for longer duration.	
17		Actual recovery per µL and total available sample material: _____	

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 microcentrifuge tube, add the following reagents:

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Sheared DNA	–		__ µL for 1.0 to 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 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 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, 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 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 **30 µL** Elution Buffer. Mix until homogenous, then mix for 1 minute at 2000 rpm.	
14		Optional: Verify your DNA amount and concentration using a Nanodrop or Qubit quantitation platform, as appropriate. 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.	
15		The End-Repaired DNA can be stored overnight at 4ºC or at -20ºC for longer duration.	
16		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 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. 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 pipetting or flicking the tube.
3. Spin down contents of 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 to maximize ligation efficiency 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 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		1.0 µL
ExoVII		10.0 U/µL		1.0 µL
Total Volume				42 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 **0.45X** volume of AMPure PB beads.

STEP	✓	Purify SMRTbell™ Templates - First Purification	Notes
1		Add **0.45X** 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 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 **100 µL** of Elution Buffer. Mix for 1 minute at 2000 rpm.	
14		Verify your DNA amount and concentration with either a Nanodrop or Qubit quantitation platform reading. If recovery is sufficient to allow for an additional 25% loss in the final AMPure PB bead purification step (more if the library contains a high number of small fragments), and it is desirable to increase the stringency of size selection, consider using **0.40X** volumes of AMPure PB beads. This will remove most fragments <1.5 kb which will dominate loading, if present. Otherwise, proceed to the third **0.45X** volumes of AMPure PB bead purification step. Note that yield from **0.40X** is typically ~ 10% lower than **0.45X** volumes of AMPure PB bead purification.	

STEP	✓	Purify SMRTbell™ Templates - Second Purification	Notes
1		Add **45 µL** (**0.45X** volume) or **40 µL** (**0.40X** volume) of AMPure PB beads to the **100 µL** of eluted DNA. Note that for **0.40X** volume, it is critical to accurately pipet the desired volume of AMPure PB bead solution; there is a steep drop-off in recovery for concentrations < **0.40X**.	
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		Verify your DNA amount and concentration with a Qubit quantitation platform reading. For general library yield, expect 15% total yield from the Damage Repair input. To estimate your final concentration: (____ ng of DNA going into Damage Repair X 0.15) / ___ of Elution Buffer =____ng/µL	
15		Perform qualitative sizing analysis using a Bioanalyzer platform.	

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.

For more information about using the Binding Calculator, see the Pacific Biosciences *Template Preparation and Sequencing Guide* and *QRC - Annealing and Binding Recommendations*.

Bind Complex to MagBeads

To maximize yield, use MagBead loading for libraries made using this Procedure. The Calculator provides recommended sample concentrations for large scale and custom (small) scale binding; however, it is recommended that users titrate samples to determine optimal loading concentrations for specific libraries. For information on how to prepare and sequence using MagBeads, see the *Pacific Biosciences Procedure & Checklist - Preparing MagBeads for Sequencing*.