250 bp Amplicon Library Preparation and Sequencing

Before You Begin

To perform this procedure, you must have the PacBio® Template Prep Kit.

This procedure is optimized for SMRTbell™ template preparation from PCR amplicons of less than 250 bp. Although sheared DNA can be used in this procedure, we find that yields are significantly lower than when starting with PCR products.

Insert Size Target	Insert Size Range	Sheared and Concentrated DNA Amount	Ligation	DNA Damage Repair
250 bp	< 250 bp	250 ng	A/T	Required

The minimum required DNA concentration for End-Repair is 15 ng/µL with preferred mass of at least 250 ng. If your sample is diluted, proceed with concentrating the DNA according to the table on page 2.

If preparing larger amounts of DNA, scale all the reaction volumes proportionally (e.g., if the input amount of DNA is double the amount set forth in this procedure, then double all the reaction volumes listed in the tables). Note that if you will be using Circular Consensus Sequencing with sheared libraries, we recommend you shear DNA to 1 kb to 2 kb fragments and use the 1 kb or 2 kb procedures (for more information, see *Procedure & Checklist, 1 kb Template Preparation and Sequencing* or *Procedure & Checklist, 2 kb Template Preparation and Sequencing*).

Required Materials

- Klenow exo- from Thermo Fisher Scientific (5 µL)
- Overhang Adapter (20 µM) (P/N 001-560-522) (Request from PacBio Field Application Scientist or representative. Overhang Adapter will arrive with the next paid order.)
- SMRTbell™ Template Prep Kit 1.0

STEP	✓	Concentrate DNA	Notes
1		Add **1.8X** volume AMPure® PB magnetic beads. _____ µL of sample X **1.8X** = _____ 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 shaking 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 vortexing, 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 bead 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 **0.8** / (____ng/µL) = _____ µL of Elution Buffer needed The minimum DNA concentration required to proceed to the next step (End-Repair) is **12 ng/µL** with preferred mass of at least **250 ng**.	
14		Add the Pacific Biosciences® Elution Buffer volume (calculated in step 13 above) 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. – 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		Perform qualitative and quantitative analysis using a Bioanalyzer® instrument. Note that the Bioanalyzer instrument has different kits in its offering and the appropriate kit, based on insert size, should be used. 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 specific kit you might be using. Note that typical yield, at this point of the process (i.e. post-shearing and after one **1.8X** AMPure PB bead purification), is approximately **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.

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

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Sheared DNA	–		__ µL for 250 ng DNA	–		
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 tube contents with a quick spin in a microfuge.
4. Incubate at 37ºC for 20 minutes, then return reaction to 4ºC for 1 minute.

Repair Ends

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

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

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA	-		__ µL for 250 ng	-		
Template Prep Buffer		10X	3.0 µL	1X		
ATP Hi		10 mM	3.0 µL	1 mM		
dNTP		10 mM	1.2 µL	0.4 mM		
End Repair Mix		20X	1.5 µL	1X		
H_2O	-		__ µL to adjust to 30.0* µL	-		
Total Volume			30.0 µL	-		

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

6. Mix the reaction well by pipetting or flicking the tube.
7. Spin down tube contents with a quick spin in a microfuge.
8. Incubate at 25ºC for 15 minutes, then return the reaction to 4ºC until ready for purification.

STEP	✓	Purify DNA	Notes
1		Add **1.8X** 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 **20 µL** Elution Buffer. Mix until homogenous, then vortex for 1 minute at 2000 rpm.	
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. Note that typical yield, at this point of the process (following End-Repair and one 0.6X AMPure PB bead purification), is approximately **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: _____	

A-Tailing Reaction

1. In a microcentrifuge tube (on ice), add the following reagents:.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (End Repaired)	-	-	19 µL to 20 µL	-		
Template Prep Buffer	◯	10X	3.0 µL	1X		
dATP	●	10 mM	1.2 µL	0.4 mM		
Klenow (exo -) (From Thermo Fisher)	●	5 U/µL	1.2 µL	0.2 U/µL		
H2O	-	-	__ µL to adjust to 30.0 µL	-		
Total Volume	-	-	30.0 µL	-		

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin down tube contents with a quick spin in a microfuge.
4. Incubate your sample at 37ºC for 60 minutes, then return the reaction to 4ºC until purification.

STEP	✓	Purify A-Tailed DNA	Notes
1		Add **1.8X** volume of AMPure PB beads to the A-Tailed 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 **20 µL** Elution Buffer. Mix until homogenous, then vortex for 1 minute at 2000 rpm.	
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. Note that typical DNA yield, at this point of the process (following A-Tailed reaction and one **1.8X** AMPure PB purification), is approximately between **80-100%** of the total starting material going into the A-Tailed reaction.	

Prepare A-Tailed Ligation Reaction

Use the following table to prepare your A-Tailed ligation reaction:

1. In a LoBind microcentrifuge 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 (A-Tailed)	-		19 µL to 20 µL			
Overhang Adapter (20 µM)	-	20 µM	2.0 µL	1.3 µM		
Mix before proceeding						
Template Prep Buffer	○	10X	3.0 µL	1X		
ATP Lo		1 mM	1.5 µL	0.05 mM		
Mix before proceeding						
Ligase		30 U/µL	1.0 µL	1.0 U/µL		
H_2O			__ µL to adjust to 30.0 µL			
Total Volume	-	-	30.0 µL	-		

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin down tube contents with a quick spin in a microfuge.
4. Incubate at 25ºC for 16 hours. Note that this time can be reduced, however, there will be some yield loss. 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 exonucleases to remove failed ligation products.

Reagent	Tube Cap Color	Stock Conc.	✓	Volume
Ligated DNA				30 µL
ExoIII		100.0 U/µL		0.5 µL
ExoVII		10.0 U/µL		0.5 µL
Total Volume	-	-		31 µL

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down tube contents 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

STEP	✓	Purify SMRTbell™ Templates	Notes
1		Add **1.8X** volume of AMPure PB beads to the exonuclease-treated ligation 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 **10 µL** of Elution Buffer. Vortex for 1 minute at 2000 rpm.	
14		Verify your DNA amount and concentration with either a Nanodrop or Qubit quantitation platform reading. For general library yield expect 20% total yield from End Repair input. If your yield concentration is below **12 ng/µL**, use the Qubit system for quantitation. To estimate your final concentration: (____ ng of DNA going into End Repair X 0.1) / ___ of Elution Buffer =____ng/µL	
15		Perform qualitative and quantitative analysis using a Bioanalyzer instrument. Note that typical DNA yield, at this point of the process (following overhang ligation, exonuclease treatment and one **1.8X** AMPure PB bead purification), is between approximately **10-15%** of the total starting material going into the ligation reaction.	

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.

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.