

Procedure and Checklist - 20 kb Template Preparation Using BluePippin™ Size-Selection System

Before You Begin

To perform this procedure, you must have the PacBio® Template Prep Kit and have reviewed the *User Bulletin - Guidelines for Preparing 20 kb SMRTbell™ Templates*.

This procedure can be used to prepare size-selected libraries from 5 µg of sheared and concentrated DNA using the BluePippin Size-Selection system. 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). If a BluePippin Size-Selection system is not available, see the *Procedure & Checklist - 20 kb Template Preparation Using AMPure® PB Beads*. Note that size selection using the AMPure PB beads does not completely remove all short SMRTbell templates. As a result, the average subread lengths may be shorter.

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%.

Insert Size Target	Insert Size Range	Sheared and Concentrated DNA Amount	Ligation	DNA Damage Repair
10 kb to 20 kb	15 kb to 20 kb (size-selected using BluePippin system)	5 µg	Blunt	Required

Fragment and Concentrate DNA

Before shearing your DNA sample, be sure to have assessed the quality and purified the sample according to the *User Bulletin - Guidelines for Preparing 20 kb SMRTbell™ Templates*. Note that prior to shearing, gentle mixing is recommended. This recommendation will hold for larger insert sizes, however, for 20 kb libraries no damage has been observed as a result of vortexing.

Use a Covaris® g-TUBE® device to shear your DNA sample. Review the recommendations listed in the *User Bulletin - Guidelines for Preparing 20 kb SMRTbell™ Templates*. Also, the most up-to-date guidance on how to use the g-TUBE device, along with recommended centrifuges and centrifugation speeds, can be found in the g-TUBE device 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 5 µg of sheared and concentrated DNA (140 ng/µL) for the subsequent repair steps.

STEP	✓	Concentrate DNA	Notes
1		<p>Add 0.45X volume of AMPure[®] PB magnetic beads.</p> <p>_____ μL of sample X 0.45X = _____ μL of beads</p> <p>Note that the beads must be brought to room temperature and all AMPure PB bead purification steps should be performed at room temperature.</p> <p>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.</p>	
2		Mix the bead/DNA solution thoroughly.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		<p>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.</p> <p>We recommend using a VWR vortex mixer with a foam microtube attachment (see the <i>Guide's</i> 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.</p>	
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		<p>With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.</p> <p>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.</p>	
8		<p>Wash beads with freshly prepared 70% ethanol.</p> <p>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.</p> <ul style="list-style-type: none"> – 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.	
10		<p>Remove residual 70% ethanol.</p> <ul style="list-style-type: none"> – 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 .	

STEP	✓	Concentrate DNA	Notes
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. $\text{_____ ng} \times 0.5 / (\text{_____ ng}/\mu\text{L}) = \text{_____ } \mu\text{L of Elution Buffer needed}$ The minimum DNA concentration required to proceed to the next step (End-Repair) is 140 ng/μL with preferred mass of at least 5 μg .	
14		Add the Pacific Biosciences® Elution Buffer volume (calculated in step 13 above) to your beads. <ul style="list-style-type: none"> – 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 0.45X AMPure PB bead purification), is approximately 50%-80% .	
16		The sheared DNA can be stored for up to 24 hours at 4°C or at -20°C for longer duration.	
17		Actual recovery per μL and total available sample material: _____	

ExoVII Treat DNA

Note that this is a new step to improve library quality. Use the following table to remove single-stranded ends from DNA fragments. 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 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		
ExoVII	●	10 U/µL	1.0 µL	0.2 U/µL		
H ₂ O	–		__ 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 37°C for 15 minutes, then return the reaction to 4°C.

Repair DNA Damage

Use the following table to prepare your reaction.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (ExoVII treated)	–		50 µL	–		
DNA Damage Repair Mix		25 X	2.0 µL	1X		
Total Volume			52.0 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 37°C for 20 minutes, return the reaction to 4°C for 1 to 5 minutes.

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)	–		52.0 µL	–		
End Repair Mix		20 X	2.5 µL	1X		
Total Volume			54.5 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 25°C for 5 minutes, return the reaction to 4°C.

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. <ul style="list-style-type: none"> – 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 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 4°C or at -20°C 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 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.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (End Repaired)	–		19.0 µL to 20.0 µL			
Annealed Blunt Adapter (20 µM)		20 µM	10* µL	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 ₂ O	–	–	___ µL to adjust to 40.0 µL	–		
Total Volume	–	–	40.0 µL	–		

*Note that this increase in adapter during ligation minimizes the incidence of chimeras. Adapter dimers are then efficiently removed during size selection in the BluePippin System. This is not recommended for libraries which are not being size selected using the BluePippin System.

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 25°C overnight.
5. Incubate at 65°C for 10 minutes to inactivate the ligase, then return the reaction to 4°C. 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 tube with a quick spin in a microfuge.
2. Incubate at 37°C for 1 hour, then return the reaction to 4°C. You must proceed with purification after this step.

Purify SMRTbell™ Templates

STEP	✓	Purify SMRTbell™ Templates	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. <ul style="list-style-type: none"> – 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 31 µL of Elution Buffer. Vortex for 1 minute at 2000 rpm.	

BluePippin™ Size Selection

For size selection cutoff (BP start) recommendations, see the Pacific Biosciences *User Bulletin - Guidelines for Preparing 20 kb SMRTbell™ Templates*. The size selection strategy depends on the mass and fragment distribution.

If the library contains < 0.5 µg, or if there is a broad size distribution to <4 kb, then a 0.40X AMPure purification step is recommended instead of BluePippin to avoid losing the library.

STEP	✓	Prepare DNA Samples for Each Lane	Notes
1		If necessary, dilute up to 5 µg SMRTbell template into a final volume of 30 µL Elution Buffer. Run 500 ng to 5 µg SMRTbell templates per lane. It's not recommended to start with less than 500 ng per lane.	
2		Bring the Loading Solution to room temperature, then add 10 µL of the Loading Solution to the 30 µL DNA sample. The Loading Solution is viscous so pipet slowly to ensure it is completely transferred into the DNA sample. Mix thoroughly by gently pipetting; do not vortex. Spin briefly to collect the contents at the bottom.	
3		Follow the manufacturer's recommendations for run set up and calibration. Select the appropriate protocol in the "Table of Recommendations" and start the run. Note that the protocol selection depends on the mass and fragment distribution. Pacific Biosciences <i>User Bulletin - Guidelines for Preparing 20 kb SMRTbell™ Templates</i>	
4		Enter BP end = 50000 bp for all samples.	
5		Collect eluate into a 1.5 or 2.0 mL LoBind microcentrifuge tube. Note that volumes may vary from approximately 40 to 60 µL. Proceed directly to AMPure purification at this point. Note: It is highly recommended to wait at least 45 minutes after the run terminates before removing the eluted DNA. This has shown an increase in recovery of SMRTbell libraries.	

Concentrate size-selected SMRTbell templates in a 1.5 or 2.0 mL LoBind microcentrifuge tube using 1X AMPure PB beads.

STEP	✓	Concentrate and Quantify Size-Selected Templates	Notes
1		Measure volume of eluate and overlay sample with an equal volume of AMPure PB beads.	
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. <ul style="list-style-type: none"> – 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 DNA from beads by pipetting 10 μ L EB onto beads. Close the tube and tap with finger to mix. Do not pipet to mix or spin down and place in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature. NOTE: For up to 5 μ g input DNA, elute in 10 μ L. For > 5 μ g input DNA (if multiple lanes were pooled for this step), scale the elution volume proportionately.	
14		Spin briefly to collect the contents at the bottom of the tube. Let stand at room temperature for 1-2 minutes, or vortex for 1 minute at 2000 rpm and re-spin to collect contents.	
15		Return sample to magnetic rack and let stand until beads are well-separated.	
16		Transfer supernatant containing size-selected SMRTbell template to a new LoBind microcentrifuge tube.	
17		Use 1 μ L of purified SMRTbell template to make a 1:5 dilution in EB, and measure the DNA concentration using a Qubit fluorometer. Retain the remaining 4 μ L of diluted sample for QC by FIGE. NOTE: Typical yields of size-selected, ~20 kb SMRTbell library from 500 ng - 5 μ g input material are 20-40%.	

Anneal and Bind BluePippin™ Size-Selected SMRTbell™ Templates

Before adding the primer to the SMRTbell template, the primer must go through a melting step at 80°C. This avoids exposing the sample to heat. The template and primer mix can then be incubated at 20°C 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 30°C 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*.

Prepare for MagBead Loading

Optimal loading of size-selected ~20 kb templates can be achieved with ~0.125 nM on-plate concentration. An initial loading test of 0.125 nM on-plate concentrations is highly recommended.

For efficient binding to Magnetic Beads, bound complexes (at 0.500 nM concentration) must be diluted in the appropriate ratio of MagBead Binding Buffer and MagBead Wash Buffer. Follow the Binding Calculator instructions to dilute your sample for MagBead binding.

Control Complex Dilution

If you will be using the PacBio Control Complex, dilute the DNA Control Complex according to the volumes and instructions specified in the Calculator.

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.

When sequencing size-selected ~20 kb SMRTbell templates prepared by this method, be sure to indicate the magnetic bead collection protocol, a 20,000 bp insert size, stage start, and 240 minute movies when setting up your run protocol in RS Remote.

