Procedure & Checklist - Preparing HiFi SMRTbell® Libraries using SMRTbell Express Template Prep Kit 2.0

This document describes a method for constructing HiFi SMRTbell libraries for generating high-accuracy long reads on the Sequel II System using PacBio's SMRTbell Express Template Prep Kit 2.0.

High quality genomic DNA (gDNA) can be sheared using a Megaruptor instrument (Diagenode) or g-TUBEs (Covaris). Depending on your project requirements, SMRTbell libraries are size-selected using a SageELF system (Sage Science), BluePippin system (Sage Science) or AMPure PB Beads (PacBio). Table 1 is a summary of recommendations for constructing HiFi long reads for specific applications.

Application	Size-Selection Method	Number of Collected Fractions	Note
HiFi for Variant Detection	SageELF	5 (~11 kb, ~13 kb, ~15 kb, ~17 kb, >19 kb)	Reads may also be used for *de novo* assembly
	BluePippin	2 (11-13 kb, 13-20 kb)	Reads may also be used for *de novo* assembly
HiFi for *de novo* Assembly	AMPure PB Beads	1 (5-20 kb, depending on shear distribution)	Reads are not suitable for variant detection. Removes <5 kb and reduces <10 kb SMRTbells from final library.

Table 1: Library construction recommendations for applications requiring HiFi long reads

This procedure describes construction of HiFi libraries from sheared gDNA with a mode size of 15 kb or larger. Table 2 summarizes DNA input, quality and DNA shear mode requirements for specific size-selection options. The final SMRTbell library yield (%) of the collected and purified HiFi fractions depends on the quality of the starting genomic DNA and distribution of the DNA shear.

To increase the recovery yield of larger fraction sizes (>20 kb), the target shear size distribution must be adjusted so that the mode is 20 kb. Always perform test shears prior to starting SMRTbell library construction.

Size-Selection Method	Required Input gDNA Amount	Required Input gDNA Quality (Mode Size)	Target Sheared Fragment Size Distribution Mode	Shearing Method
SageELF	15 µg	>40 kb	>15-20 kb	g-TUBE or Megaruptor
BluePippin	15 µg	>40 kb	>15-20 kb	g-TUBE or Megaruptor
AMPure PB	15 µg	>40 kb	>20 kb	g-TUBE or Megaruptor

Table 2: DNA requirements and recommended shearing methods for constructing HiFi libraries.

Required Materials

DNA QC (one of the following)	
CHEF Mapper XA	Bio-Rad 170-3670
Pippin Pulse	Sage Science PP10200
Femto Pulse	Agilent Technologies, Inc. P-0003-0817
DNA Quantitation	
Qubit 3.0 Fluorometer	Life Technologies Q33216
dsDNA HS Assay Kit	Life Technologies Q32854
DNA Shearing (one of the following)	
Megaruptor	Diagenode B06010001
Long Hydropores	Diagenode E07010002
Hydrotubes	Diagenode C30010018
g-TUBE	Covaris 520079
Microcentrifuge	Any MLS
SMRTbell Library Preparation	
SMRTbell Express Template Prep Kit 2.0	Pacific Biosciences 100-938-900
AMPure® PB Beads	Pacific Biosciences 100-265-900
SMRTbell Enzyme Cleanup Kit	Pacific Biosciences 101-746-400
Sequencing Primer V2	Pacific Biosciences 101-847-900
Rotator	Any MLS
100% Ethanol, Molecular Biology Grade	Any MLS
Wide Orifice Tips (Tips LTS W-O 200UL Fltr RT-L200WFLR)	Rainin 17014294
2.0 mL DNA Lo-Bind Tubes	Eppendorf 022431048
Fractionation (one of the following if not using AMPure PB Beads for size-selection)	
SageELF System	Sage Science ELF0001
0.75% Agarose Cassettes	Sage Science ELD7510
BluePippin	Sage Science BLU0001
0.75% Agarose Cassettes, S1 Marker	Sage Sciences BLF7510

HiFi Express TPK 2.0 Workflow

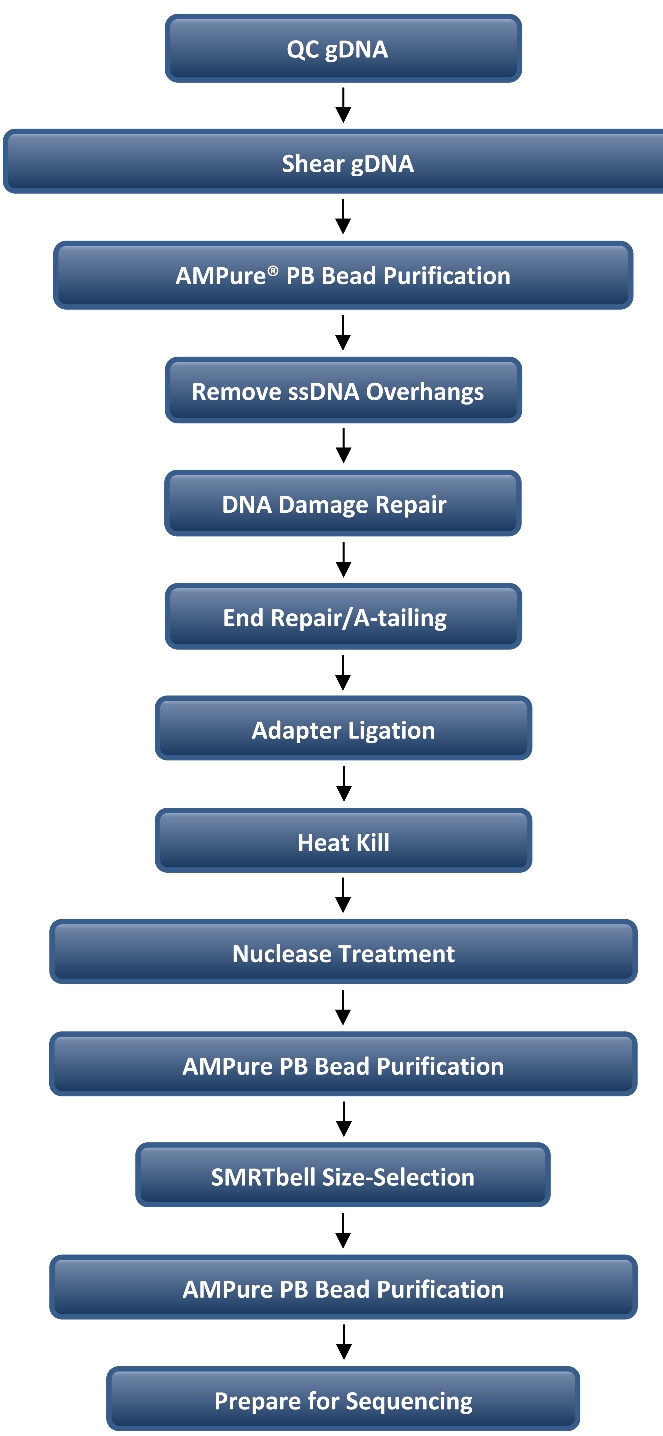

Figure 1: Workflow for preparing libraries for HiFi sequencing using SMRTbell Express Template Prep Kit 2.0.

Reagent Handling

Several tubes in the kits (shown in Table 3 below) are sensitive to temperature and vortexing. We highly recommend:

- Never leave tubes at room temperature.
- Always work on ice at all times when preparing master mixes.
- Finger-tap followed by a quick spin prior to use.

Reagent	Where Used
DNA Prep Additive	Remove single-strand overhangs
DNA Prep Enzyme	Remove single-strand overhangs
DNA Damage Repair Mix v2	DNA Damage Repair
End Prep Mix	End-Repair/A-tailing
Overhang Adapter v3	Ligation
Ligation Mix	Ligation
Ligation Additive	Ligation
Ligation Enhancer	Ligation
Enzyme A	Nuclease Treatment
Enzyme B	Nuclease Treatment
Enzyme C	Nuclease Treatment
Enzyme D	Nuclease Treatment

Table 3: Temperature sensitive reagents

Evaluate Genomic DNA (gDNA) Quality

This procedure requires high quality, high molecular weight gDNA with the majority of the DNA fragments >40 kb as determined by pulsed-field gel or capillary electrophoresis. Any of the three commercially available systems listed in Table 4 below may be used to evaluate gDNA quality. Links to recommended procedures for each are also provided. In Figure 2, there are examples of gDNA quality assessment using Bio-Rad's CHEF Mapper (2A) and Agilent Technologies' Femto Pulse (2B). Lanes A3 and B1 correspond to high quality gDNA samples that are suitable for use in this procedure. Lanes A4 and B2 show degraded gDNA samples that not suitable for use in this procedure.

Method	Procedure
Bio-Rad® CHEF Mapper® XA Pulsed Field Electrophoresis System	Procedure & Checklist - Using the BIO-RAD® CHEF Mapper® XA Pulsed Field Electrophoresis System
Agilent Technologies, Inc. Femto Pulse	Agilent Technologies, Inc.
Sage Science Pippin Pulse	Procedure & Checklist - Using the Sage Science Pippin Pulse Electrophoresis Power Supply System

Table 4. gDNA Quality Evaluation Methods and Procedures.

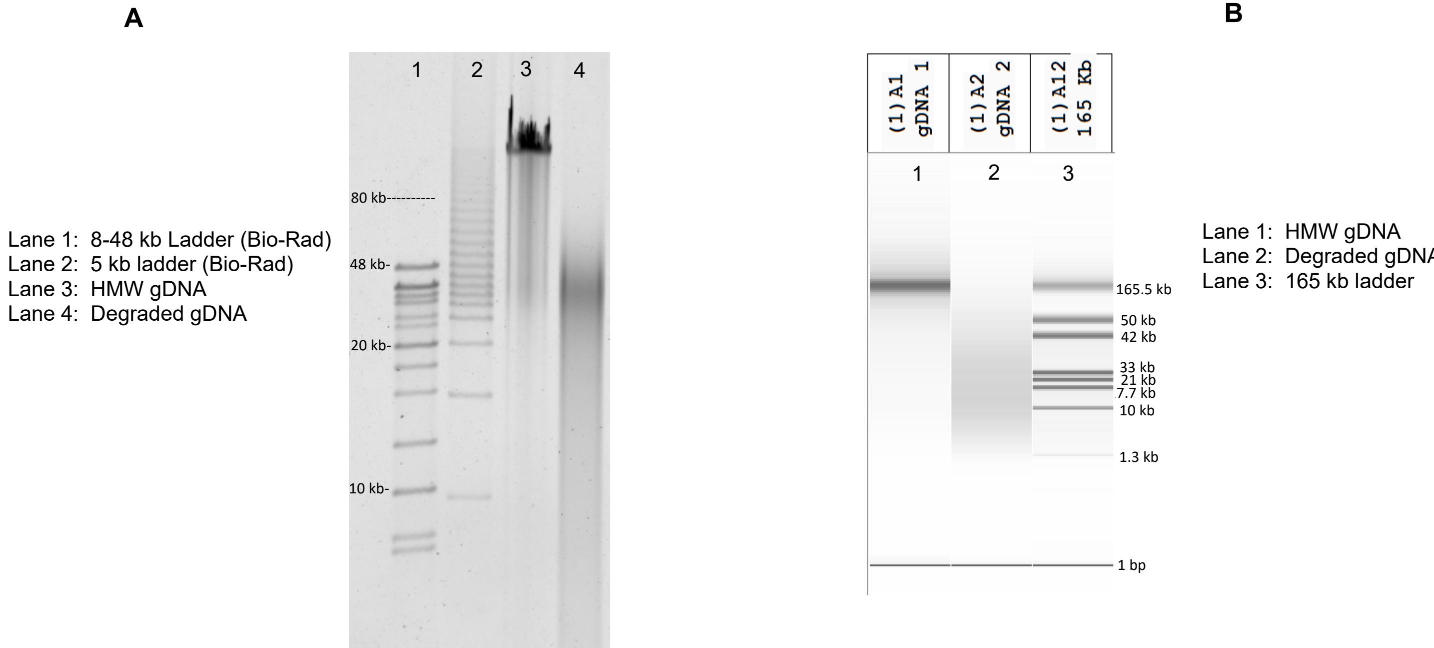

Figure 2: Evaluation of gDNA quality using two systems. A) Bio-Rad CHEF Mapper and B) Agilent Technologies' Femto Pulse.

Shear gDNA

This procedure describes the construction of HiFi libraries from DNA shears with a mode size of 15 kb or larger. Genomic DNA can be sheared using Diagenode's Megaruptor or Covaris' g-TUBEs. The response of individual gDNA samples to recommended shearing parameters may differ, so small scale test shears are highly recommended**.** Under- or over-shearing gDNA will result in low yields of the final, size-fractionated library. For high quality gDNA, typical yields of sheared and concentration DNA are approx. ≥70%. Because you will need 10 µg of sheared gDNA for the subsequent enzymatic steps, we recommend you start the shearing procedure with at least 15 µg of input gDNA.

Please note that if the goal of your project is to sequence larger HiFi fraction sizes (>20 kb), the shear distribution must be adjusted so that the mode is 20 kb.

Shearing Using Diagenode's Megaruptor

To shear gDNA using Diagenode's Megaruptor, generally follow the manufacturer's recommendations. We recommend initial small-scale test shears (for example, using a 50 µL or larger aliquot volume at a DNA concentration of 50 ng/µL) to evaluate the response of each gDNA sample to shearing parameters.

STEP	✓	Shear DNA	Notes
1		Dilute **15 µg** of high molecular weight gDNA in 1x Elution Buffer to a concentration of 50 ng/µL in a final volume of **300 µL**. It is important not to exceed this DNA concentration during shearing or you may clog the hydropore. Before shearing, remove a 1 µL aliquot (un-sheared sample) for sizing QC.	
2		Shear gDNA with long hydropores using the following recommended settings in the Megaruptor software. Mode — Megaruptor Software Setting 11-13 kb — 15 kb* 15-18 kb — 20 kb* *Perform small-scale test shears first.	
3		Proceed to the next step to concentrate the sheared gDNA using AMPure PB beads. Note that the Megaruptor 1 and 2 dilute DNA during shearing, so empirically measure the volume of the sheared gDNA sample before AMPure PB bead purification.	

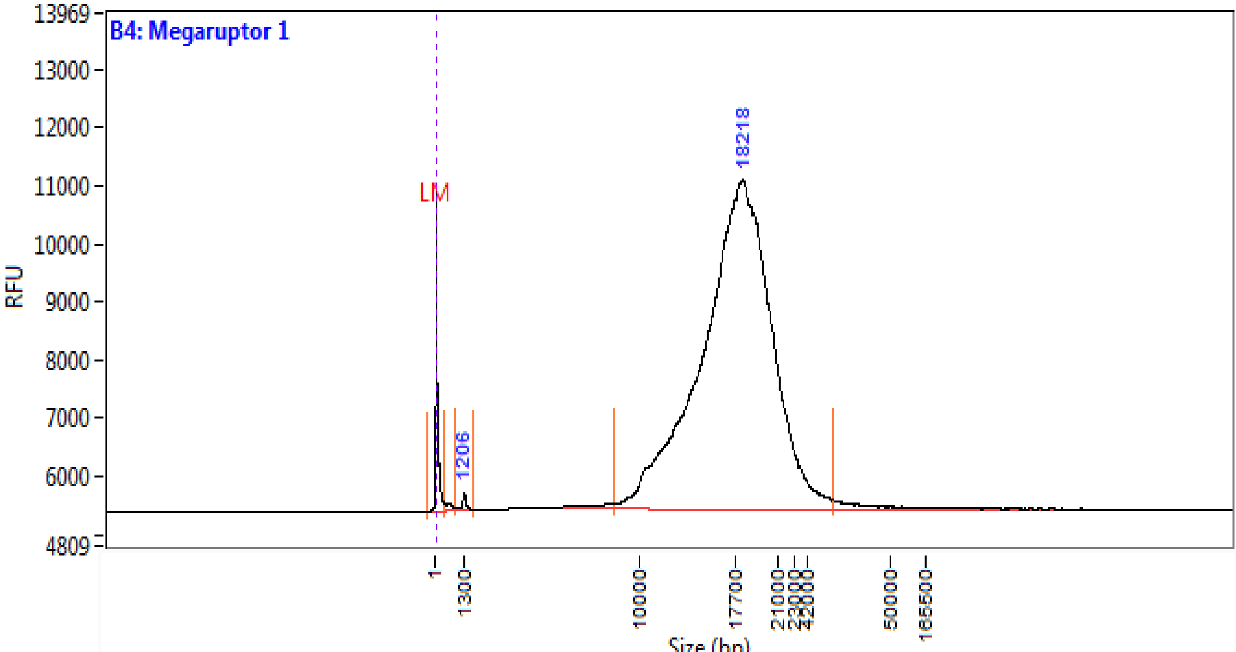

Figure 3: Example of a human genomic DNA sample sheared to 15-18 kb using the Megaruptor System.

Shearing Using Covaris g-TUBEs

Specific guidance for sheared gDNA using Covaris g-TUBEs (modified from the Covaris User Manual) is provided below and should be strictly followed to achieve the desired target fragment size. The response of individual gDNA samples to recommended shearing parameters may differ, so small scale test shears are highly recommended**.** If the DNA does not respond well to the procedure below, you may try concentrations between 100-200 ng/ul with spin speeds of 1073-2029 x g.

STEP	✓	Shear DNA	Notes
1		Dilute 15 µg of gDNA in 1x Elution Buffer to a concentration of **83.3 ng/µL** in a final volume of **180 µL.**	
2		Transfer gDNA to a g-TUBE and shear the gDNA at 2029 x *g* (5500 rpm in the Eppendorf MiniSpin Plus) for 2 minutes.	
3		Check for any residual sample remaining in the upper chamber. If present, re-spin for another 2 minutes. Repeat spin until entire gDNA sample has passed through the orifice.	
4		Invert and spin 2029 x *g* (5500 rpm in the Eppendorf MiniSpin Plus) until entire gDNA sample has passed through the orifice.	
5		Repeat step 4 FOUR times for a total of SIX passes through the orifice.	
6		Transfer the sheared gDNA to a fresh 2.0 mL DNA Lo-bind microfuge tube.	
7		Proceed to the "Concentrate DNA using AMPure PB Beads" section to concentrate the sample using AMPure PB beads.	

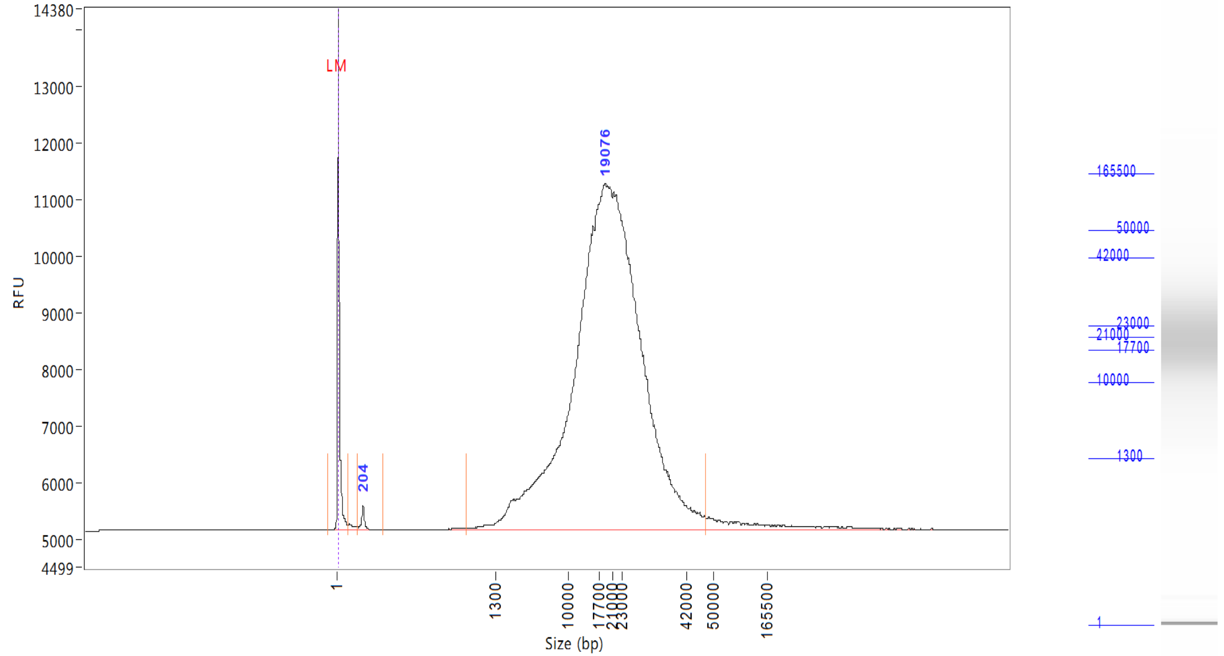

Figure 4: Example of a human genomic DNA sample sheared using a Covaris g-TUBE device.

Concentrate DNA Using AMPure® PB Beads

PacBio highly recommends using 2 mL DNA Lo-Bind tubes for this step.

STEP		Concentrate DNA	Notes
1		Add **0.45X** volume of AMPure PB magnetic beads to the sheared gDNA. 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.	
2		Mix bead/DNA solution thoroughly by tapping the tube gently.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by gentle rotation at room temperature for 30 minutes.	
5		Quickly 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, 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 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% 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 80% ethanol to fill the tube. Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat step 8.	
10		Remove residual 80% ethanol. – Remove tube from magnetic bead rack and quickly spin to pellet beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack.	
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 - 60 seconds.	

13		Elute in **100 µL** Elution Buffer. Finger-tap the tube to mix until beads are uniformly re-suspended. – Elute the DNA by gentle rotation at room temperature for 10-15 minutes. – Quickly spin the tube to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 2.0 mL Lo-Bind tube. Discard the beads.	
14		Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using the Qubit dsDNA HS Assay kit according to the manufacturer's recommendations.	
15		Use the remaining 9 µL of 1:10 diluted sample to determine the size distribution of the sheared gDNA by using any of the methods listed in Table 4. The mode size of sheared gDNA must be >15 kb - 20 kb to ensure sufficient yields of final size-fractionated SMRTbell library. Sheared DNA can be stored for up to 24 hours at 4°C or at -20°C for longer duration.	

Prepare SMRTbell Templates Using Express Template Prep Kit 2.0

Always work in ice. If starting with more than 5 µg of sheared gDNA, scale the reaction volumes proportionally (i.e., for a mass between 5-10 µg of DNA, scale the total reaction volume to 110 µL for the Remove Single-Strand Overhangs step).
Note that wide bore pipette tips are required when constructing large insert libraries (>15 kb).

Remove Single-Strand Overhangs

Use the following table to set up a reaction to remove single-strand overhangs using up to 5 µg of input sheared gDNA. Dilute the sheared gDNA sample to a DNA concentration of 111 ng/µL before beginning the procedure below.

For the Sequel System II, we recommend starting this step with at least 10 µg of input sheared gDNA and scaling all reagent volumes indicated two-fold (i.e., for a mass of 10 µg of DNA, scale the total reaction volume to 110 µL).

Follow the steps below for removal of single-strand overhangs:

1. Dilute the stock DNA Prep Additive.

Reagent	Tube Cap Color	Volume	✓	Notes
Enzyme Dilution Buffer		4.0 µL		
DNA Prep Additive		1.0 µL		
Total Volume		5.0 µL		

The diluted DNA Prep Additive should be used immediately and should not be stored.

2. Prepare the following reaction.

Reagent (Reaction Mix 1)	Tube Cap Color	Volume	✓	Notes
DNA Prep Buffer		7.0 µL		
Sheared DNA (111 ng/µL)		45.0 µL		
NAD		1.0 µL		
Diluted DNA Prep Additive (see step 1)		1.0 µL		
DNA Prep Enzyme		1.0 µL		
Total Volume		55.0 µL		

3. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips.
4. Spin down contents of tube with a quick spin in a microfuge.
5. Incubate at 37°C for 15 minutes, then return the reaction to 4°C.
6. Proceed to the next step.

Repair DNA Damage

Use the following table to set up a reaction to repair DNA damage using up to 5 µg of input sheared gDNA. If starting with more than 5 µg of sheared gDNA, scale the reaction volumes proportionally.

Reagent (Reaction Mix 2)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 1		55.0 µL		
DNA Damage Repair Mix v2		2.0 µL		
Total Volume		57.0 µL		

1. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37°C for 30 minutes, then return the reaction to 4°C.
4. Proceed to the next step.

End-Repair/A-tailing

Use the following table to set up a reaction to A-tail DNA ends using up to 5 µg of input sheared gDNA. If starting with more than 5 µg of sheared gDNA, scale the reaction volumes proportionally.

Reagent (Reaction Mix 3)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 2		57.0 µL		
End Prep Mix		3.0 µL		
Total Volume		60.0 µL		

1. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 20°C for 10 minutes.
4. Incubate at 65°C for 30 minutes, then return the reaction to 4°C.
5. Proceed to the next step.

Adapter Ligation

Use the following table to set up a ligation reaction using up to 5 µg of input sheared gDNA. If starting with more than 5 µg of sheared gDNA, scale the reaction volumes proportionally.

Reagent (Reaction Mix 4)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 3		60.0 µL		
Overhang Adapter v3		5.0 µL		
Ligation Mix		30.0 µL		
Ligation Additive		1.0 µL		
Ligation Enhancer		1.0 µL		
Total Volume		97.0µL		

1. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips. It is important to mix well.
2. Incubate at 20°C for 1 hour. [OPTIONAL: The Ligation reaction may also be left at 20°C overnight.]
3. Incubate at 65°C for 10 minutes to inactivate the ligase, then return the reaction to 4°C.
4. Proceed to the next step.

Nuclease Treatment of SMRTbell Library

Use the following table to set up a reaction to remove damaged or non-intact SMRTbell templates using up to 5 µg of input sheared gDNA. Scale the reaction volumes proportionally for input sheared gDNA amounts >5 µg.

To the ligated SMRTbell libraries, add the following components:

Reagent (Reaction Mix 5)	Tube Cap Color	Volume	✓	Notes
Ligated SMRTbell library		97.0 µL		
Enzyme A		4.0 µL		
Enzyme B		1.0 µL		
Enzyme C		1.0 µL		
Enzyme D		2.0 µL		
Total Volume		105.0 µL		

1. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips. It is important to mix well.
2. Incubate at 37°C for 1 hour.
3. Proceed immediately to the next step.

Purify SMRTbell Library Using 0.45X AMPure® PB Beads

PacBio highly recommends using 2 mL DNA Lo-Bind tubes for this step.

STEP	✓	AMPure PB Bead Purification	Notes
1		Add **0.45X** volume of AMPure PB beads to the nuclease-treated ligation reaction.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by gentle rotation at room temperature for 30 minutes.	
5		Quickly 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 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% 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 80% ethanol to fill the tube. Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat step 8.	
10		Remove residual 80% ethanol. – Remove tube from magnetic bead rack and quickly spin to pellet beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 80% 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 - 60 seconds.	
13		Elute in **31 µL** 1x Elution buffer. Add the Pacific Biosciences Elution Buffer volume to the beads. Finger-tap the tube to mix until beads are uniformly re-suspended. – Elute the DNA by gentle rotation at room temperature for 10-15 minutes. – Quickly spin the tube to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 2.0 mL DNA Lo-Bind tube. – Discard the beads.	
14		Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations. The remaining 9 µL of 1:10 diluted sample may be used for QC.	
15		Proceed with Size Fractionation or store the sample at -20C for future use.	

Size-fractionation of SMRTbell Library

For variant detection and *de novo* assembly requiring HiFi reads, PacBio highly recommends the SageELF or the BluePippin System. Typical recovery yields after fractionation are 15-30% and are highly dependent on the size distribution of the starting SMRTbell library. See Figures 6 and 7 for examples of SMRTbell libraries fractionated with SageELF and BluePippin, respectively.

While the SageELF and BluePippin systems are gold standard for generating HiFi fractions, AMPure PB Beads may be an option for size-selection. However, it is important to note that the success of this method highly depends on the size distribution of the sheared DNA. It is, therefore, recommended to optimize the shearing condition so that the final size distribution has a mode of 20 kb or larger, with little-to-no presence of <10 kb fragments. High presence of fragments <10 kb results in high abundance of short reads which impacts assembly (fragmented assembly). AMPure PB bead size-selected HiFi libraries is an option for *de novo* assembly only.

The AMPure PB bead size selection efficiently removes short SMRTbell templates <5 kb and effectively, reduces levels of SMRTbell templates ~5 kb – 10 kb in the final purified library. Please refer to the Procedure & Checklist - Using AMPure PB Beads for Size-Selection. The AMPure-based size selection procedure requires dilution of the AMPure PB Bead stock solution to 35% (v/v) with Elution Buffer prior to use.

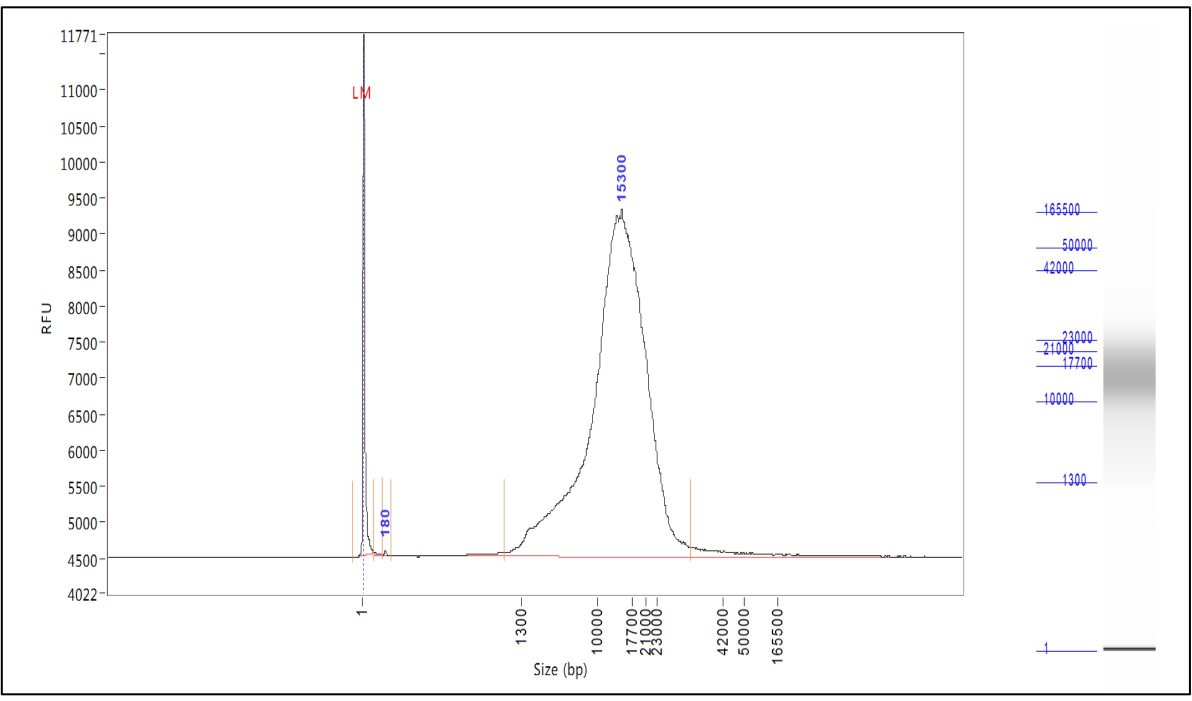

Figure 5: Example of a 15 kb SMRTbell library before size fractionation.

Size Selection Using the SageELF System

Sage Science's SageELF system may be used to fractionate SMRTbell libraries for HiFi long read sequencing. For the latest SageELF User Manual and guidance on size-selection protocols, please contact Sage Science (www.sagescience.com).

STEP	✓	Running the SageELF System	Notes
1		Follow the SageELF manufacturer's instructions to calibrate the instrument. A new calibration is recommended before each run.	
2		Inspect the gel cassette (using Sage Science's SageELF instructions). – Ensure that the buffer wells are full. – Ensure that there is no separation of the gel from the cassette.	
3		Prepare the gel cassette: – While the cassette is sealed, remove all bubbles from the elution buffer chamber by tilting the cassette and tapping it until all air bubbles move into the buffer chamber. – Hold the cassette firmly on the bench top and carefully remove the plastic seals on the cassette. – Remove the buffer from the elution well and fill with **30 µL** of fresh Electrophoresis Buffer. • Keep the pipette down the center of the well and avoid creating a vacuum in the well. • The bottom of the well is okay to touch. • If the well "bubbles" over when adding the buffer to the well, remove buffer and try again. – Cover the elution wells with a clear adhesive tape and verify that it is tightly sealed. – Remove the buffer from the sample well and fill with 70 µL of fresh Electrophoresis Buffer. Do not touch the sides and bottom of the sample well. – Carefully place the gel cassette in the SageELF System. – Verify that the buffer in the "moat" on both sides of the cassette that connects the electrode reservoirs is at the correct level: add additional electrophoresis buffer to fill the moat completely, then remove 1 mL from one side of the moat. – Close the lid and perform a Current Test.	
4		Prepare samples for loading. – Add **30 µL** of sample containing ~5 µg of size-fractionated HiFi library to a clean tube. – Add **10 µL** of Sage Science's Marker 75. Mix well and do a quick spin down.	
5		Load samples: – Remove **40 µL** of buffer from the sample well. – Load all **40 µL** of the sample prepared in step 4 into the sample well. – If necessary, top off well with additional Electrophoresis Buffer. Do not overfill the well.	

6		Set up the run Protocol: – In the “Protocol Editor” tab, click on the “New Protocol” button. – Select the “**0.75% 1-18kb v2**” in the cassette definition menu. – Select “size-based” for separation mode. – Enter **3450** in the “Target Value” field and move the bar slider to select well #12. – Save as new protocol. – On the Main screen, clear previous run data, select cassette description, cassette definition and protocol, enter sample ID(s). – Select in the Nest Selector the cartridge that will be run.	
7		Start the run.	
8		Once the run is complete, (approximately 4.5 hours), collect **30 µL** of the respective fractions from the elution wells. Fractions of interest are typically **~11 kb, ~13 kb, ~15 kb, ~17 kb, and >19 kb**. – Rinse each well by adding **30 µL** of fresh Elution Buffer into the empty elution well. Rinse by pipetting up and down 10 times with wide-orifice pipette tips and then collecting the rinse into the same tube.	
9		Repeat the rinse step for a total of 2 rinses.	
10		Quantify the DNA in each fraction using the Qubit dsDNA HS assay kit. Measure 1 µL of each fraction plus washes directly without further dilution.	
11		Check the sizes of all 12 fractions by loading on a Femto Pulse. To determine the **average library size**, perform a smear analysis by selecting the region of interest by defining the start and end points of the fractions.	
12		Proceed to the 0.5X AMPure PB Bead purification step with fractions of the desired size or store fractions at -20°C for future use.	

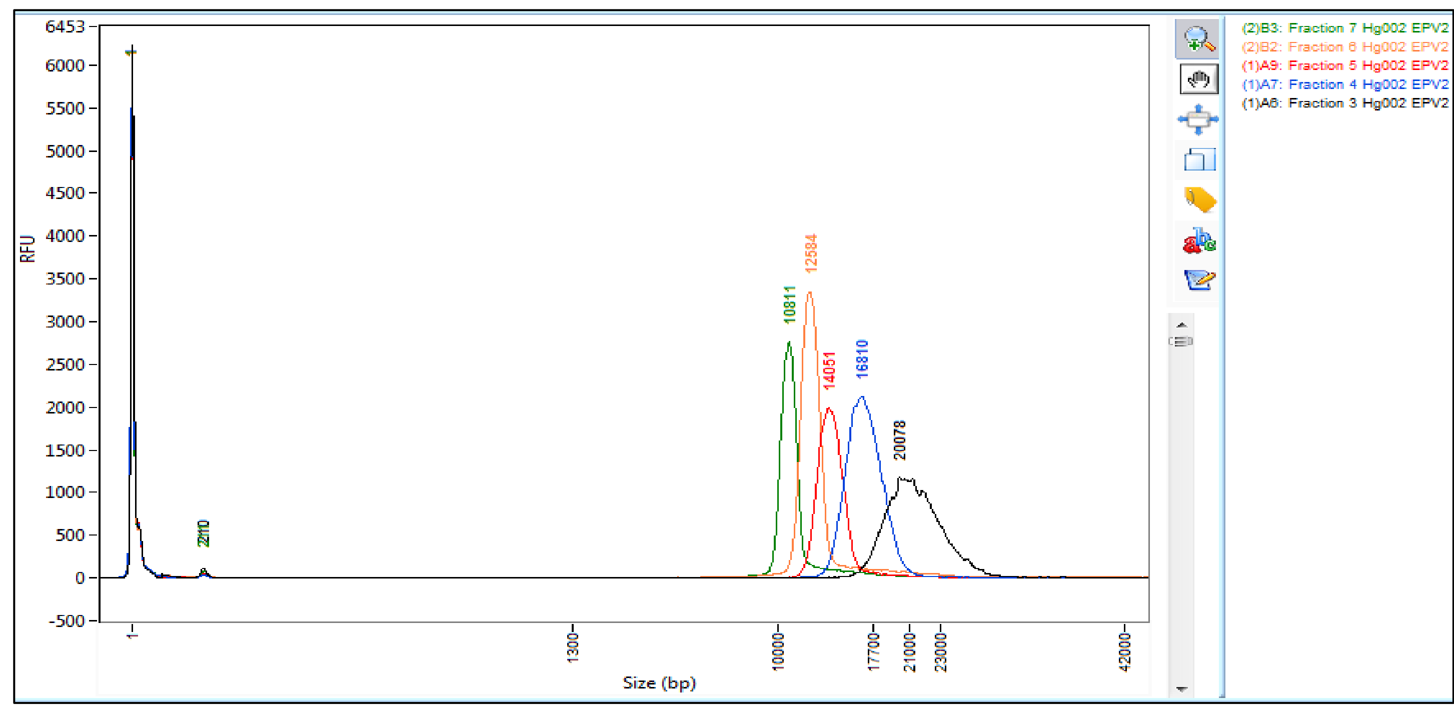

Figure 6: Example of a 15 kb SMRTbell library fractionated using Sage Science’s SageELF System. Each collected fraction is suitable for high accuracy long read sequencing.

Size Selection Using the BluePippin System

Sage Science's BluePippin may also be used to size-select two fractions**, 9-13 kb and >15 kb** for HiFi long read sequencing. For the latest BluePippin User Manual and guidance on size-selection protocols, please contact Sage Science (www.sagescience.com). **Note**: Visit Sage's website (http://www.sagescience.com) to verify that your BluePippin software is up to date. The current version is v6.31.

STEP	✓	BluePippin Size Selection	Notes
1		Prepare up to **5 µg** of SMRTbell templates in a final volume of **30 µL** of Elution Buffer for each Blue Pippin lane.	
2		Bring the Loading Solution to room temperature, and then add **10 µL** of the Loading Solution to the **30 µL** DNA sample. For loading multiple lanes with the same sample, scale the volumes proportionally. The Loading Solution is viscous, so pipet slowly to ensure complete transfer into the DNA sample. a. Pipette mix using wide-bore pipette tips to mix. b. Spin briefly to collect the contents at the bottom of the tube.	
3		Follow the manufacturer's recommendations to set up a run protocol. a. Select the **"0.75% DF Marker S1 3-10 kb Improved Recovery"** Cassette Definition File for your sample. b. Using the "Range" selection mode, enter a desired "BPstart" value of 9000 and a BP End value of 13000. **Note**: Sample lanes containing **<3 µg** of SMRTbell library material will run faster during electrophoresis. In such cases, PacBio recommends adjusting the BP Start value to 8000 bp and BP End value to 12000 bp. We do not recommend runing lanes with **<2 µg** of SMRTbell library material. c. Be sure to assign a marker lane.	
4		Load S1 marker and samples into the BluePippin gel cassette and start the run. Run time is approximately 4.5 hours.	
5		**Collect the 9-13 kb fraction.** To maximize recovery of eluted DNA, wait at least 20 minutes after the run terminates before removing the sample from the elution chamber. a. Collect the eluate containing the 9-13 kb fraction into a 2.0 mL DNA LoBind tube. b. Wash the elution well with 40 µL of Sage Science's Electrophoresis Buffer and add the recovered wash liquid to the eluted sample. A second wash may further increase recovery yields. c. Refill the elution chamber with 40 µL Electrophoresis Buffer and re-seal the chamber. Close the lid of the BluePippin.	

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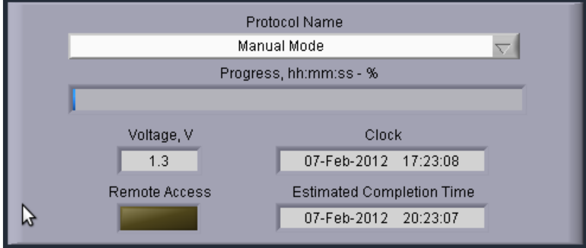
<tr><td>6</td><td></td><td>
Collect the >15 kb fraction. Use Manual Mode to collect a second fraction.

a. From the Main Tab, select “Manual Mode” from the top of the “Protocol Name” drop down menu.



b. Click “Start” on the controller.

c. Click “Elute” box for each sample lane. Box will turn orange and current will be detected.

d. When elution timer reaches 30 minutes, click “Idle” box for each sample lane. Idle box will become white and current will drop.

e. Collect second eluate as described in Step 5 above, including washes. Typical insert size for this manually eluted fraction is ~15kb.
</td><td></td></tr>
<tr><td>7</td><td></td><td>Proceed to the 0.5X AMPure PB Bead purification step, or store fractions at -20°C for future use.</td><td></td></tr>
</table>

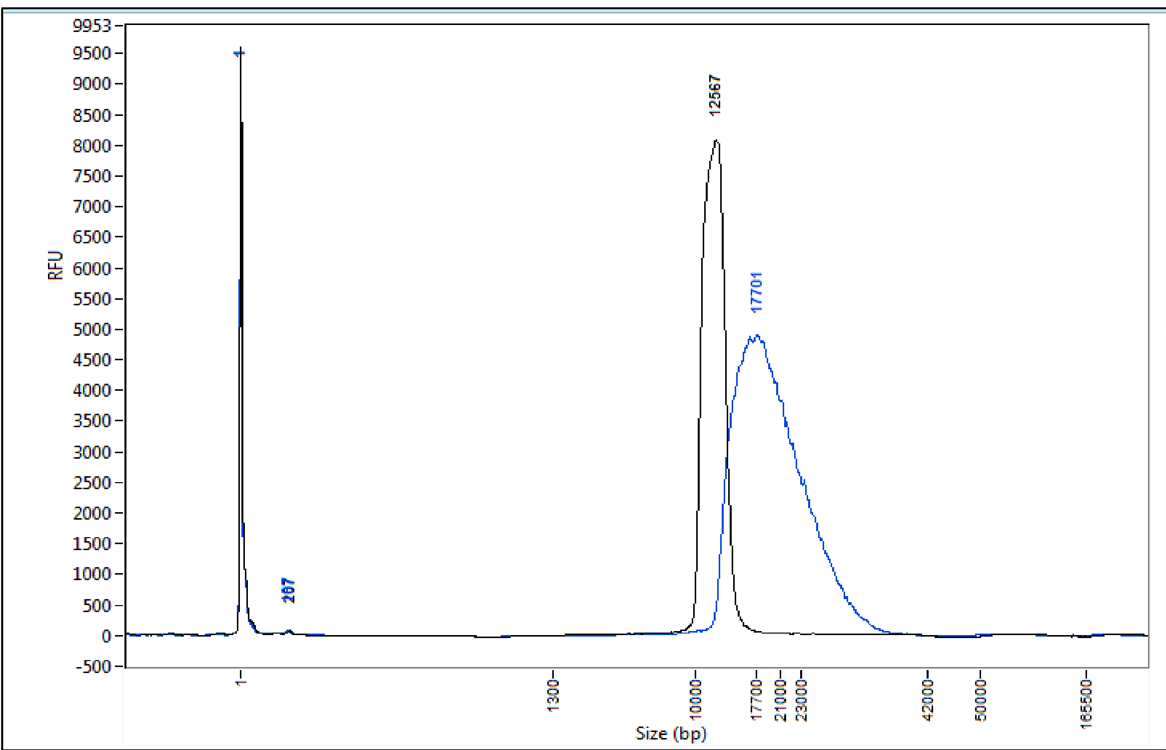


Figure 7: Example of a SMRTbell library fractionated using Sage Science’s BluePippin System. Two fractions are collected with the workflow, 9-13 kb and >15 kb.

Purify SageELF or BluePippin Size-Selected HiFi Library Fractions with 0.50X AMPure® PB Beads

PacBio highly recommends using 2 mL Lo-Bind tubes for this step.

STEP	✓	AMPure PB Bead Purification	Notes
1		Add **0.50X** volume of AMPure PB beads.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by gentle rotation at room temperature for 30 minutes.	
5		Quickly 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 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% 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 80% ethanol to fill the tube. Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat step 8.	
10		Remove residual 80% ethanol. – Remove tube from magnetic bead rack and quickly spin to pellet beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 80% 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 - 60 seconds.	
13		Elute in **11 µL** Elution buffer. Add the Pacific Biosciences® Elution Buffer volume to the beads. Tap the tube with finger to mix until beads are uniformly re-suspended. – Elute the DNA by gentle rotation at room temperature for 10-15 minutes. – Quickly spin down the tube to pellet beads, then place the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new DNA Lo-Bind tube. – Discard the beads.	
14		Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the Qubit dsDNA HS Assay kit according to the manufacturer's recommendations.	
15		Use the remaining 9 µL of 1:10 diluted sample to evaluate fraction insert size by any of the methods listed in Table 4.	
16		Proceed to the next step to prepare for sequencing, or store final SMRTbell fractions at -20C for future use.	

Anneal and Bind SMRTbell Templates

For the SageELF system, we recommend pooling and sequencing fractions 15 – 20 kb. For the BluePippin system, we recommend sequencing the >15 kb fraction. Follow the SMRT Link Sample Setup v8.0 (or higher) instructions to anneal primer and bind polymerase to each purified HiFi SMRTbell library fraction. To anneal HiFi Express TPK 2.0 libraries for sequencing, use Sequencing Primer v2. Sequencing Primer v2 (PN 101-847-900) can be purchased separately from PacBio.

Prepare for Sequencing

Follow the SMRT Link Sample Setup v8.0 (or higher) instructions for preparing the sample for sequencing on the Sequel II System.

For detailed recommendations for sequencing of specific library insert size ranges, refer to the Quick Reference Card – Diffusion Loading and Pre-Extension Time Recommendations for the Sequel II System here.

Revision History (Description)	Version	Date
Initial release.	01	September 2019
Internal revision with no content change (not uploaded to website).	02	December 2019
On page 1, changed “HiFi reads” to just “Reads”. On page 12, under Repair DNA Damage, corrected “remove single strand overhangs” to “repair DNA damage”. On page 13, corrected “remove single strand overhangs” to “adapter ligation”.	03	January 2020

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