Procedure & Checklist - Preparing SMRTbell® Libraries for HiFi Long Read Sequencing on Sequel® and Sequel II Systems

This document describes a method for constructing SMRTbell libraries suitable for generating high accuracy long reads on the Sequel Systems. This procedure requires 15 µg of high molecular weight genomic DNA (gDNA). DNA is sheared to a mode of 15 kb using Diagenode's Megaruptor. SMRTbell libraries are prepared from sheared gDNA using PacBio's SMRTbell Template Prep Kit 1.0. SMRTbell libraries are then size-fractionated using Sage Science's SageELF. The fractions suitable for sequencing have insert sizes from 10 kb to 15 kb.

Required Materials

Item	Vendor
gDNA QC (one of the following) CHEF Mapper XA Pippin Pulse FEMTO Pulse	 Bio-Rad 170-3670 Sage Science PP10200 Agilent Technologies, Inc. (formerly Advanced Analytical Technologies, P-0003-0817)
DNA Quantitation Qubit 3.0 Fluorometer dsDNA HS Assay Kit	 Life Technologies Q33216 Life Technologies Q32854
DNA Shearing Megaruptor Long Hydropores Hydrotubes	 Diagenode B06010001 Diagenode E07010002 Diagenode C30010018
SMRTbell Library Preparation SMRTbell Template Prep Kit 1.0 AMPure PB Beads Rotator 100% Ethanol, Molecular Biology Grade 2.0 mL DNA Lo-Bind Tubes Thermomixers	 Pacific Biosciences 100-259-100 Pacific Biosciences 100-265-900 Any MLS Any MLS Eppendorf 022431048 Any MLS
Fractionation SageELF System 0.75% Agarose Cassettes	 Sage Science ELF0001 Sage Science ELD7510

Workflow

Figure 1: Workflow for preparing SMRTbell libraries for high accuracy long read sequencing.

Evaluate Genomic DNA (gDNA) Quality

This procedure requires high quality, high molecular weight gDNA with a mode size of >40 kb as determined by pulsed field gel or capillary electrophoresis. Any of the three commercially available systems listed in Table 2 below may be used to evaluate gDNA quality. Links to recommended procedures for each are provided. Figure 2 gives examples of gDNA quality assessment using Bio-Rad's CHEF Mapper (2A) and Agilent's FEMTO Pulse (2B). Lanes A3 and B1 contain high quality gDNA suitable for use in this procedure. Lanes A4 and B2 show degraded gDNA 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. FEMTO Pulse	Agilent Technologies Website
Sage Science Pippin Pulse	Procedure & Checklist - Using the Sage Science Pippin Pulse Electrophoresis Power Supply System

Table 2. gDNA Quality Evaluation Methods and Procedures.

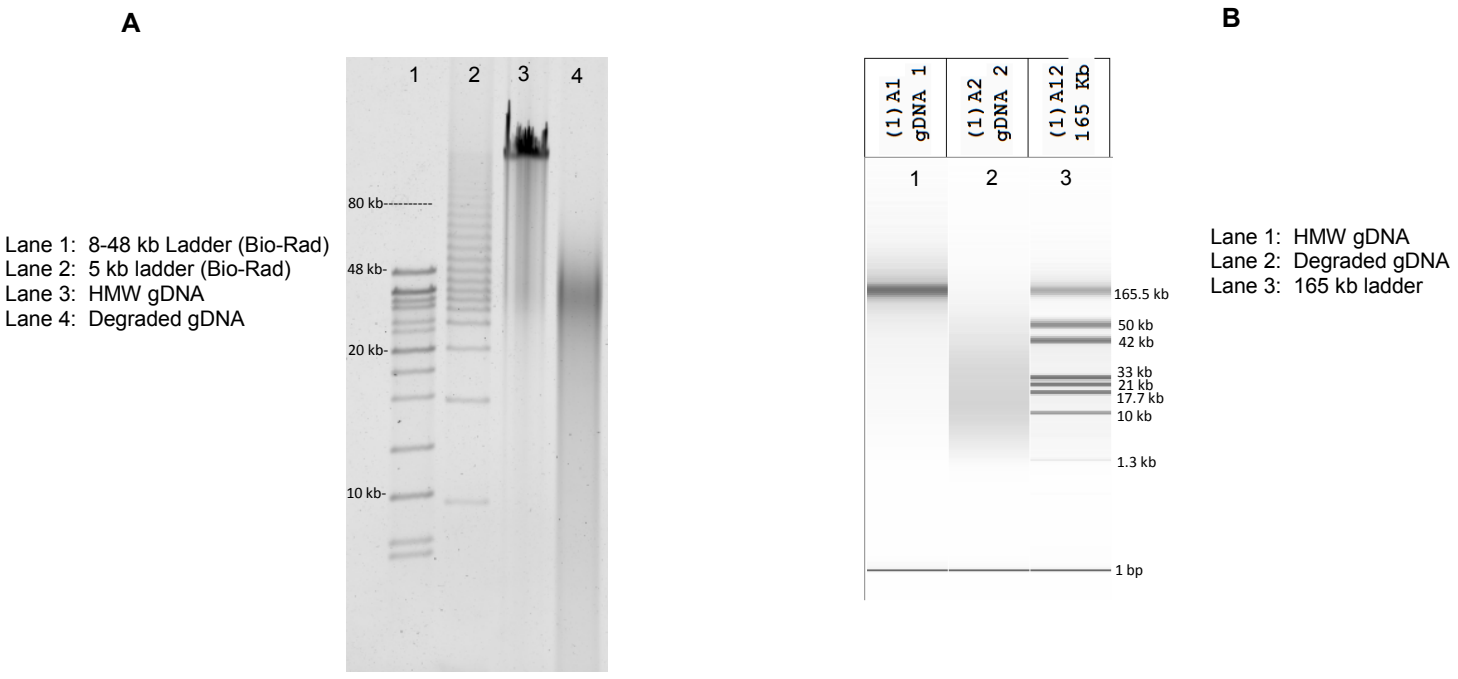

Figure 2: Evaluation of gDNA quality using two systems. A) Bio-Rad CHEF Mapper and B) Agilent FEMTO Pulse.

Shear gDNA

Below is a procedure for shearing gDNA to a mode size of approximately 12 kb (see example in Figure 3 below) using Diagenode's Megaruptor. For high quality gDNA, typical yields of sheared and concentrated DNA are ≥60%. Because 10 µg of sheared gDNA is needed for the subsequent enzymatic steps, we recommend you start the shearing procedure with 15 µg input gDNA.

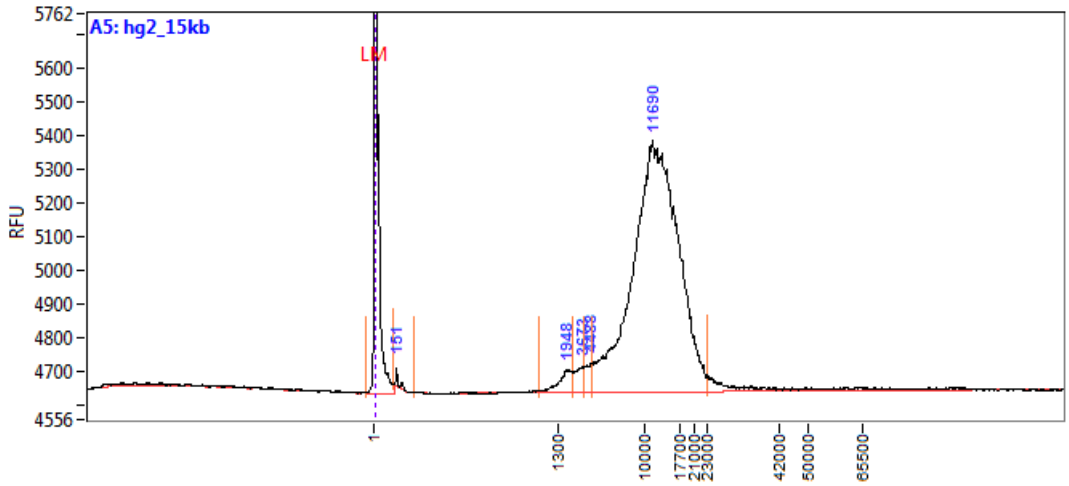

Figure 3: Example of human gDNA sheared to 12 kb using 15 kb shearing mode on the Megaruptor and evaluated on FEMTO Pulse.

To shear gDNA using Diagenode's Megaruptor, generally follow the manufacturer's recommendations.

NOTE: Because the response of individual gDNA samples to recommended shearing parameters may differ, small scale test shears are highly recommended (for example, 50 µL at a concentration of 50 ng/µL, performed according to the instructions below). Under- or over-shearing gDNA will result in low yields of final, size-fractionated library.

STEP		Shear DNA	Notes
1		Dilute**15 µg** high molecular weight gDNA in 1x Elution Buffer to a concentration of 50 ng/µL in a volume of 300 µL. It is important not to exceed this DNA concentration during shearing or the hydropore may become clogged. Before shearing, remove a 1 µL aliquot (un-sheared sample) for QC.	
2		Shear gDNA using long hydropores and a target size of 15 kb in the Megaruptor software. Run time is approximately 15-20 minutes.	
3		Proceed to the next step to concentrate your sheared gDNA using AMPure PB beads. Note that the Megaruptor dilutes DNA during shearing, so measure the volume of your sheared gDNA sample before AMPure PB bead purification.	

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 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		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 spin briefly. 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.	

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 - 60 seconds.	
13		Elute in **71 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. – Spin the tube down, then place the tube back on the magnetic bead rack. – Let beads separate fully. – 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 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. The mode size of sheared gDNA must be ~12 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

The following enzymatic steps for SMRTbell preparation require **10 µg sheared** gDNA. For more than 10 µg sheared gDNA, scale all enzymatic reaction volumes proportionally. We recommend using 2.0 mL DNA Lo-Bind tubes for all SMRTbell library preparation steps. Work on ice at all times.

Exo VII Pre-treatment of DNA to Digest DNA Ends

Use the following table to prepare the reaction.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Sheared DNA (10 µg)	–		70.0 µL	–		
DNA Damage Repair Buffer		10 X	10 µL	1 X		
NAD+		100 X	1 µL	1 X		
ATP high		10 mM	10 µL	1 mM		
dNTP		10 mM	3 µL	0.3 mM		
Exo VII		10 U/µL	2 µL	0.2 U/µL		
Total Volume			96.0 µL	–		

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37°C for 15 minutes, then return the reaction to 4°C.
4. Proceed to the next step.

Repair DNA Damage

Use the following table to prepare the reaction.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (Exo VII treated)	–		96.0 µL	–		
DNA Damage Repair Mix		25 X	4.0 µL	1X		
Total Volume			100.0 µL	–		

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37°C for 60 minutes, return the reaction to 4°C for 1 to 5 minutes.
4. Proceed to the next step.

End-Repair

Use the following table to prepare the reaction.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (Damage Repaired)	–		100.0 µL	–		
End Repair Mix		20 X	5.0 µL	1X		
Total Volume			105.0 µL	–		

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25°C for 15 minutes, return the reaction to 4°C.
4. Proceed to the next step.

Purify DNA 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 End-Repair 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		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 spin briefly. 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 **46 µL** 1x Elution buffer. Add Elution Buffer 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. – Spin down the tube, then place the tube back on the magnetic bead rack. – Let beads separate fully. – Without disturbing the bead pellet, transfer supernatant to a new 2.0 mL DNA Lo-Bind tube. – Discard the beads.	

Prepare Blunt-Ligation Reaction

Use the following table to prepare the ligase reaction, adding the components below in the order listed. Be sure to mix DNA and adapter BEFORE adding ligase.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (End Repaired)	–		46.0 µL			
Blunt Adapter (20 µM)		20 µM	20.0 µL	5 µM		
Finger tap to mix before proceeding						
Template Prep Buffer		10 X	8.0 µL	1X		
ATP low		1 mM	4.0 µL	0.05 mM		
Finger tap to mix before proceeding						
Ligase		30 U/µL	2.0 µL	0.75 U/µL		
Total Volume	–	–	80.0 µL	–		

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25°C overnight.
4. Incubate at 65°C for 10 minutes to inactivate the ligase, then return the reaction to 4°C.
5. Proceed to the next step.

Exo III/VII Digestion to Remove Failed Ligation Products

Use the following table to prepare the reaction.

Reagent	Tube Cap Color	Stock Conc.	Volume	✓	Notes
Ligated DNA			80 µL		
Exo III		100.0 U/µL	2.0 µL		
Exo VII		10.0 U/µL	2.0 µL		
Total Volume			84 µL		

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37°C for 1 hour, then return the reaction to 4°C.
4. You must immediately proceed with AMPure PB bead purification after this step.

Purify SMRTbell Templates with 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 Exonuclease-treated DNA.	
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		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 spin briefly. 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		For up to 10 µg input size-selected library, elute in **31 µL** Elution buffer. – Add Elution Buffer 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. – Spin the tube down, then place the magnetic bead rack. – Let beads separate fully. – 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 Fractionation or store the sample at -20ºC for future use.	

Size-fractionation of SMRTbell libraries

Optimal insert sizes for high accuracy long read sequencing on Sequel Systems are approximately 10-15 kb. The procedure below describes size selection of 10-15 kb SMRTbell libraries using Sage Science's SageELF system. Typical size-selection yields are 15-30% and are highly dependent on the distribution of the starting SMRTbell library. Figure 4 shows an example of three fractions from the SageELF with insert sizes of 10 kb, 11 kb, and 13 kb, all of which are suitable for high accuracy long read sequencing.

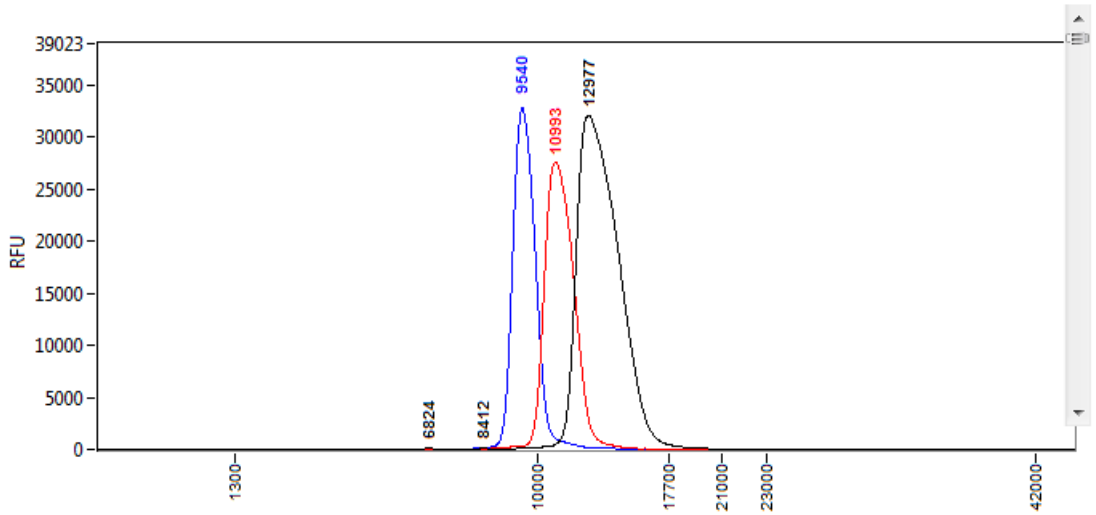

Figure 4: Example of 10 kb, 11 kb and 13 kb human SMRTbell library fractionated in the SageELF system.

Fractionation Using the SageELF System

STEP	✓	Running the SageELF System	Notes
1		Follow the SageELF manufacturing 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 each elution well and re-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 "moat" on both sides of the cassette, that connect the electrode reservoirs, are full. Add additional electrophoresis buffer to completely fill up the moat. – Remove 1 mL of buffer from the reservoirs to ensure the proper buffer level for the run (see User's Manual for more information). – Close the lid and perform a Current Test.	
4		Prepare samples for loading. – Prepare **30 µL** tube with up to ~5 µg of library. – 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 **3400** 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. – Rinse each well by adding **30 µL** of fresh Elution Buffer into the empty elution well. Rinse by pipetting up and down 10 times 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 hsDNA kit. Measure 1 uL 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**, select the region of interest by defining the start and end points of the fractions. The Bioanalyzer system may be used for sizing fractions <13 kb. The fractions suitable for HiFi sequencing have insert sizes from 10 kb to 15 kb. See Figure 4.	
12		Proceed to the next step with fractions of the desired size or store fractions at -20°C for future use.	

Purify SMRTbell 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		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 spin briefly. 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 Elution Buffer 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. – Spin down the tube, then place the magnetic bead rack. – Let beads separate fully. – 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 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 2.	
16		Proceed to the next step to prepare for sequencing or store final SMRTbell fractions at -20ºC for future use.	

Anneal and Bind SMRTbell Templates

We highly recommend treating the three SageELF fractions individually. The sequencing primer to use is Sequencing Primer v2 found in Template Prep Kit 1.0. Follow the SMRT Link Sample Setup instructions to anneal primer and bind polymerase to each fraction.

Sequencing

PacBio recommends performing loading titrations to determine the appropriate loading concentrations for both systems.

	Sequel	Sequel II
Loading Method	Diffusion	Diffusion
Movie time	20 hrs	30 hrs
Pre-extension time	8 hrs	2 hrs
On-plate loading concentration	10 - 40 pM*	25 - 75 pM*
Sequencing Kit	Sequel Sequencing Plate 3.0	Sequel II Sequencing Kit 1.0

* Sample quality, size, and binding efficiency may affect loading concentrations

Revision History (Description)	Version	Date
Initial release.	01	April 2019