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

This procedure describes the construction of HiFi SMRTbell libraries for *de novo* assembly and variant detection applications using the SMRTbell Express Template Prep Kit 2.0 and recommended HiFi sequencing conditions using PacBio's new Sequel® II Binding Kit 2.2. A minimum input amount of 5 µg of high-molecular weight genomic DNA is recommended for generating HiFi library yields sufficient for running multiple SMRT® Cells on the Sequel II or Sequel IIe System (Sequel II Systems). Note that final HiFi library construction yields will be dependent on the specific size-selection method employed.

We recommend fragmenting the gDNA so that the target size distribution mode is between 15 kb - 18 kb. To reduce the presence of fragments >30 kb, PacBio recommends a 2-cycle shearing method on the Megaruptor 3 system. Generally, a narrower fragment size distribution results in more uniform and higher-quality HiFi data. Details regarding DNA shearing conditions (e.g., buffers and DNA sample concentration) are described in the "DNA Requirements for Shearing" section.

Required Equipment	Vendor	Throughput	Run Time
Femto Pulse	Agilent Technologies	Process up to 11 samples per run Batch process up to 88 samples	85 mins
Megaruptor 3	Diagenode	Shear up to 8 samples at a time	40 mins (for 1 cycle of shearing)
PippinHT	Sage Science	Maximum of 20 samples per instrument run	2 hrs
BluePippin	Sage Science	Maximum of 4 samples per instrument run	4.5 hrs
SageELF	Sage Science	Maximum of 2 samples per instrument run	4.5 hrs

Table 1: Recommended equipment for HiFi SMRTbell library construction for *de novo* assembly and variant detection applications.

Required Materials

DNA Sizing	
Femto Pulse	Agilent Technologies, Inc. P-0003-0817
DNA Quantitation	
Qubit™ Fluorometer	ThermoFisher Scientific Q33238
Qubit 1X dsDNA HS Assay Kit	ThermoFisher Scientific Q33230
DNA Shearing	
Megaruptor 3 System	Diagenode B06010003
Megaruptor 3 Shearing Kit	Diagenode E07010003
SMRTbell Library Preparation	
SMRTbell® Express Template Prep Kit 2.0	PacBio 100-938-900
AMPure® PB Beads	PacBio 100-265-900
SMRTbell® Enzyme Clean Up Kit 2.0 (New*)	PacBio 101-932-600
Sequencing Primer v5 (New*)	PacBio 102-067-400
100% Ethanol, Molecular Biology Grade	Any MLS
Wide Orifice Tips (Tips LTS W-O 200UL Fltr RT-L200WFLR)	Rainin 30389241
Lo-Bind 0.2 mL tube strips	USA Scientific, TempAssure 1402-4708
Multi-channel Pipette	Rainin, 17013810
Magnetic separation rack	V&P Scientific, Inc, VP 772F4-1
Thermal Cycler that is 100 µL and 8-tube strip compatible	Any MLS
Size-selection (One of the following systems)	
PippinHT System	Sage Science HTP0001
0.75% Agarose Gel Cassettes, Marker 75E	Sage Science HPE7510
BluePippin System	Sage Science BLU0001
0.75% Agarose Cassettes, Marker S1	Sage Sciences BLF7510
SageELF System	Sage Science ELF0001
0.75% Agarose Cassettes	Sage Science ELD7510
Sequencing	
Sequel® II Binding Kit 2.2 (New*)	PacBio 101-894-200
Sequel® II Sequencing Kit 2.0	PacBio 101-820-200
SMRT® Cell 8M Tray	PacBio 101-389-001

* To obtain a copy of the previous version of this Procedure & Checklist that specifies use of SMRTbell Enzyme Clean Up Kit (PN 101-746-400) and Sequencing Primer v2 (PN 101-847-900), contact support@pacb.com.

HiFi Library Construction Workflow

PacBio recommends that gDNA samples be resuspended in an appropriate buffer (e.g., Qiagen Elution Buffer) before proceeding with DNA shearing.

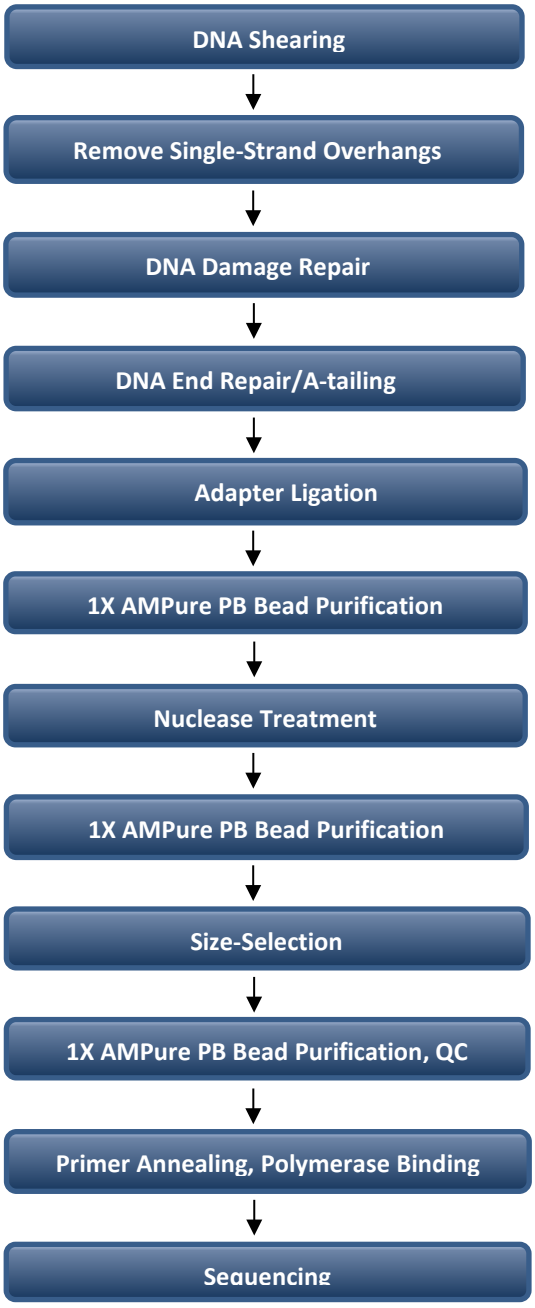


Figure 1: Workflow for preparing HiFi libraries using the SMRTbell Express Template Prep Kit 2.0.

Reagent Handling

Several reagents in the SMRTbell Express Template Prep Kit 2.0 (shown in Table 2 below) are sensitive to temperature and vortexing. We recommend to:

- Never leave reagents at room temperature.
- Always work on ice 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
SMRTbell Enzyme Clean Up Mix	Nuclease Treatment
SMRTbell Enzyme Cleanup Buffer 2.0	Nuclease Treatment

Table 2: Temperature sensitive reagents

Genomic DNA (gDNA) Quality Evaluation

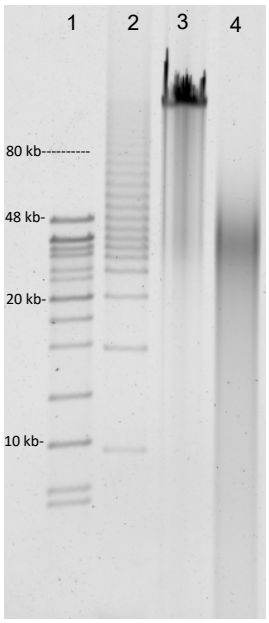
This procedure requires high-quality, high-molecular weight input gDNA with a majority of the DNA fragments >50 kb as determined by pulsed-field gel or capillary electrophoresis. Any of the three commercially available systems listed in Table 4 below may be used to evaluate gDNA quality, but the Femto Pulse system is highly recommended for high-throughput library construction due to its ability to rapidly process multiple samples in a single run using very low amounts (<1 ng) of DNA per sample. Links to recommended procedures for each system are also provided in the table. Examples of gDNA quality assessment using Bio-Rad's CHEF Mapper (2A) and Agilent Technologies' Femto Pulse (2B) are shown in Figure 2. Lanes A3 and B1 correspond to high-quality gDNA samples that are suitable for HiFi library construction using this procedure. Lanes A4 and B2 show degraded gDNA samples that not suitable for use in this procedure.

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

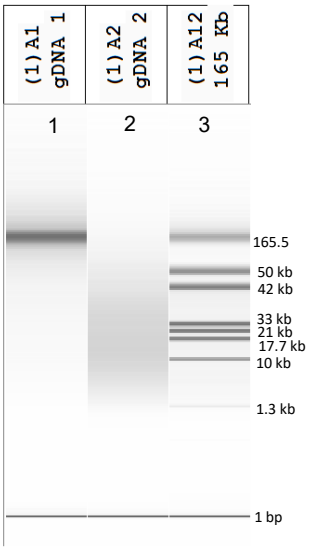
Table 3. gDNA Quality Evaluation Methods and Procedures.

A

Lane 1: 8 kb - 48 kb Ladder (Bio-Rad)
Lane 2: 5 kb ladder (Bio-Rad)
Lane 3: HMW gDNA
Lane 4: Degraded gDNA



B



Lane 1: HMW gDNA
Lane 2: Degraded gDNA
Lane 3: 165 kb ladder

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

DNA Requirements for Shearing

Before shearing, ensure that the genomic DNA is in an appropriate buffer **(e.g., Qiagen Elution Buffer, 10 mM Tris-Cl, pH 8.5 or PacBio EB buffer)**. If you are unsure of the buffer composition or if the gDNA is not in Elution Buffer, perform a 1X AMPure PB bead purification followed by elution with Elution Buffer or an equivalent low salt buffer (i.e., 10 mM Tris-Cl, pH 8.5- 9.0).

PacBio highly recommends Diagenode's Megaruptor 3 system for shearing gDNA. The Megaruptor 3 system allows up to 8 gDNA samples to be processed simultaneously with a consistent fragment size distribution across multiple hydropore-syringes. Furthermore, the Megaruptor 3 system generates a narrower size distribution than the g-TUBE device (Covaris).

Shearing Using Diagenode's Megaruptor 3 System

To maximize HiFi yield per SMRT Cell, PacBio recommends fragmenting the gDNA to a size distribution mode between 15 kb – 18 kb for human whole genome sequencing. Libraries with a size distribution mode larger than 20 kb are not recommended for HiFi sequencing. Recommended library insert size distributions to use for different WGS applications are summarized in Table 4 below.

Application	Recommended Library Insert Size
Human Variant Detection	15 – 18 kb
Human *de Novo*	15 – 18 kb
Plant/Animal *de Novo*	15 – 20 kb

Table 4: Library size recommendations for Human variant detection and *de novo* assembly.

To shear gDNA on the Megaruptor 3 system, use a two-cycle shear method, which requires running a second round of shearing immediately following the first fragmentation step in the same hydropore-syringe. The recommended concentration is 83.3 ng/µL (5 µg of input DNA in 60 µL Elution Buffer).

The DNA shearing guidelines below have been tested by PacBio on the Megaruptor 3 system only. The response of individual gDNA samples to the shearing recommendations described below may differ; therefore, performing a small-scale test shear is highly recommended, including the Megaruptor 3 system.

For the Megaruptor and Megaruptor 2 systems, shearing optimization is necessary before proceeding with this Procedure & Checklist. The shearing procedure described in the **"**Shearing Using Diagenode's Megaruptor 3 system" section below is not compatible with the Megaruptor or Megaruptor 2 systems. For Megaruptor and Megaruptor 2 systems, follow Diagenode's DNA shearing recommendations described in their manual. For additional guidance, contact Technical Support or your local FAS.

The g-TUBE device generates a broader DNA fragment size-distribution compared to the Megaruptor 3 system. Note that HiFi read quality and overall HiFi data yield may be reduced due to the residual presence of large DNA fragments generated by g-TUBEs. For additional guidance, contact Technical Support or your local FAS.

STEP	✓	Shear DNA	Notes
1		Dilute **5 µg** of high-molecular weight gDNA in 1X Elution Buffer to a concentration of 83.3 ng/µL in a final volume of **60 µL**.	
2		Shear the gDNA sample with a disposable shearing syringe using the following recommended settings for the Megaruptor 3 system software. See Figure 3 for examples of human DNA samples sheared using the following 2-cycle shear method below (use the same hydropore-syringe device for both shearing cycles). Shearing Cycle — Megaruptor 3 Speed Setting Cycle 1 — 31* Cycle 2 — 32* *Adjust speed setting parameters if the size distribution mode is larger than 20 kb. Always perform test shears before scaling up for production workflows. Recover the sheared DNA into a Lo-Bind tube strip. The typical sample recovery volume range is **45.0** µL - **53.0** µL. Note: If the input gDNA sizing QC indicates the sample is already fragmented, 1 cycle of shearing may be sufficient.	
3		Proceed to the “Remove Single-strand Overhangs” section.	

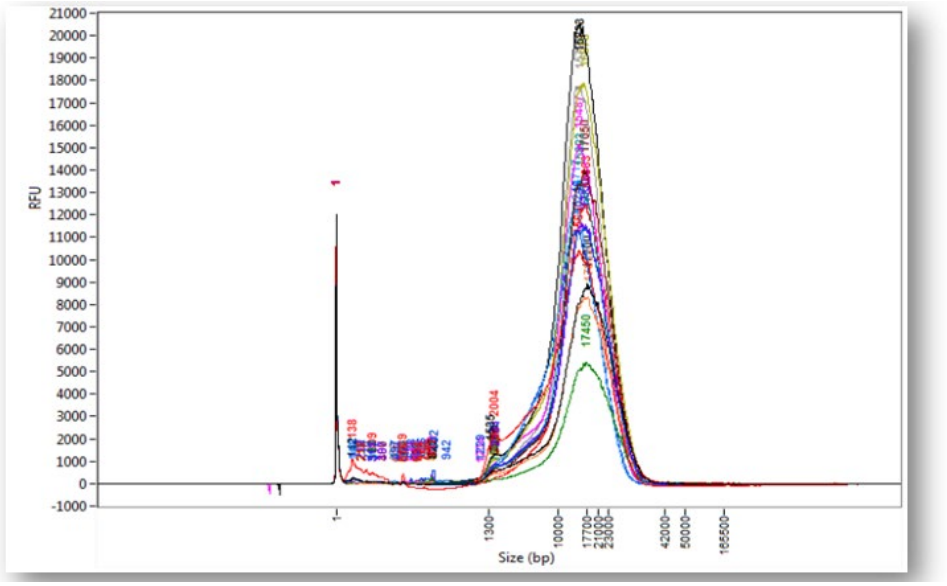


Figure 3: Examples of human genomic DNA samples sheared to a target 15 kb - 18 kb size distribution mode using a 2-cycle shear method on the Megaruptor 3 system.

Prepare SMRTbell Libraries

Always work on ice throughout the library construction process. To process multiple samples at a time, the following equipment are required:

- Lo-Bind tube strips
- Multi-channel pipette
- Wide-bore tips
- Magnetic rack compatible with tube strips
- Thermocycler compatible with tube strips

Remove Single-Strand Overhangs

The sample volume recovered from the Megaruptor 3 system after shearing is used directly in the single-strand overhang digestion step. Before proceeding, ensure that the sheared DNA is in Elution Buffer or an equivalent low salt buffer (i.e., 10 mM Tris-Cl, pH 8.5- 9.0). In this step, DNA Prep Additive is diluted first followed by digestion. Scale up the reaction volumes for digestion if working with multiple samples.

1. Prepare the DNA Prep Additive. The DNA Prep Additive is diluted with Enzyme Dilution Buffer to a total volume of **5** µL. This amount is sufficient for processing 1 to 4 samples. The volume may not be sufficient for 5 samples due to pipetting errors. We recommend scaling up the dilution volume based on the number of samples to be processed (example: prepare 2X volume for 8 samples and 4X volume for 16 samples).

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

Reagent	Tube Cap Color	1 to 4 Samples	Up to 8 Samples	Notes
Enzyme Dilution Buffer		4.0 µL	8.0 µL	
DNA Prep Additive		1.0 µL	2.0 µL	
Total Volume		5.0 µL	10.0 µL	

2. Prepare the digestion by following the reaction table below. For multiple samples, prepare a master mix, followed by addition of **10.0** µL master mix to each sheared DNA sample.

Reagent (Reaction Mix 1)	Tube Cap Color	1 Sample	N Samples	Notes
DNA Prep Buffer		7.0 µL	7.0 x N x 1.2*	
NAD		1.0 µL	1.0 x N x 1.2*	
Diluted DNA Prep Additive (see step 1)		1.0 µL	1.0 x N x 1.2*	
DNA Prep Enzyme		1.0 µL	1.0 x N x 1.2*	
Total Volume		10.0 µL		

*includes 20% overage for pipetting errors

3. Add **10.0** µL of the above master mix to the tube-strips containing **45.0** µL **- 53.0** µL of sheared DNA. The total volume in this step is **55.0** µL **- 63.0** µL.
4. Using a multi-channel pipette, mix the reaction wells by pipetting up and down 10 times with wide-orifice pipette tips.
5. Spin down the contents of the tube strips with a quick spin in a microfuge.
6. Incubate at 37°C for 15 minutes, then return the reaction to 4°C.
7. Proceed to the next step.

Repair DNA Damage

To each Reaction Mix 1, add **2.0** µL of DNA Damage Repair Mix v2.

Reagent (Reaction Mix 2)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 1		55.0 µL - 63.0 µL		
DNA Damage Repair Mix v2		2.0 µL		
Total Volume		57.0 µL - 65.0 µL		

1. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips.
2. Spin down the contents of the tube strips 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

To each Reaction Mix 2, add **3.0** µL of End Prep Mix.

Reagent (Reaction Mix 3)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 2		57.0 µL - 65.0 µL		
End Prep Mix		3.0 µL		
Total Volume		60.0 µL - 68.0µL		

1. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips.
2. Spin down the contents of the tube strips 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

In this step, **5.0** µL of Overhang Adapter is added to each Reaction Mix 3 (from the previous step). Then, **32.0** µL of the ligase master mix is added to each Reaction Mix 3/Adapter Mix for incubation. Always work on ice.

1. To each Reaction Mix 3, add **5.0** µL of Overhang Adapter.

Reagent	Tube Cap Color	Volume	✓	Notes
Reaction Mix 3		60.0 µL - 68.0 µL		
Overhang Adapter v3		5.0 µL		
Total Volume		65.0 µL - 73.0 µL		

2. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips. Leave the tube strips on ice.

3. Prepare a Master Mix containing Ligation Enhancer, Ligation Additive and Ligation Mix using the table below:

Reagent (Reaction Mix 4)	Tube Cap Color	1 sample	N Samples	✓	Notes
Ligation Mix		30.0 µL	30.0 x N x 1.2*		
Ligation Additive		1.0 µL	1.0 x N x 1.2*		
Ligation Enhancer		1.0 µL	1.0 x N x 1.2*		
Total Volume		32.0 µL			

*includes 20% overage for pipetting errors

4. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips. It is important to mix well.
5. To the Reaction Mix 3/Adapter Mix, add **32.0** µL of the Ligase Master Mix. The total volume in this step is **97.0** µL - **105.0** µL.
6. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips. It is important to mix well.
7. Incubate at 20°C for 1 hour. Optional: The Ligation reaction may also be left at 20°C overnight.
8. Proceed to the next step.

Purify SMRTbell Library Using 1.0X AMPure® PB Beads

STEP	✓	AMPure PB Bead Purification	Notes
1		Add **1.0X** volume of AMPure PB beads to the ligation reaction.	
2		With a multi-channel pipette, mix by pipetting 10X – 15X with wide bore tips.	
3		Allow the DNA to bind to beads on the bench top at room temperature for 20 minutes.	
4		Quickly spin down the tube strip (for 1 second) to collect beads.	
5		Place the tube strip in a magnetic rack to collect the beads to the side.	
6		Slowly pipette off the cleared supernatant. Avoid disturbing the beads. Optional: save the supernatant in another tube strip in case of poor DNA recovery.	
7		Wash the 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. – Fill the tubes with 200 µL of 80% ethanol. Slowly dispense the 80% ethanol on the side of the tubes. Ensure the bead pellet is covered with ethanol solution. – After 30 seconds, pipette and remove the 80% ethanol.	
8		Repeat step 7.	
9		Remove residual 80% ethanol. – Remove tube strip from the magnetic rack and quickly spin. Both beads and any residual 80% ethanol will be at the bottom of each tube. – Place the tube strip back on magnetic rack. – Pipette off any remaining 80% ethanol.	
10		Remove the tube strip from the magnetic rack.	
11		Add **15.0** µL Elution Buffer to elute the DNA. Pipette mix 10X – 15X until beads are uniformly re-suspended – Elute the DNA at 37°C for 15 minutes. – Quickly spin. – Let beads separate fully in the magnetic rack. Then without disturbing the beads, transfer supernatant to a new DNA Lo-Bind tube strip.	
12		Optional: Take 1.0 µL of eluted sample and measure the DNA concentration using the dsDNA HS Assay Kit with a Qubit fluorometer. The same sample aliquot used to measure DNA concentration on the Qubit system may be used for DNA sizing QC on the Femto Pulse system. Dilute the sample to 250 pg/ µL with the Femto Pulse Dilution Buffer (0.25X TE).	
13		Proceed to the Nuclease Treatment section below.	

Nuclease Treatment of SMRTbell Library

To each library sample, add the nuclease mix to remove damaged SMRTbell templates.

1. Prepare a Master Mix of the Enzyme Cleanup Mix and Buffer.

Reagent (Reaction Mix 5)	Tube Cap Color	1 sample	N samples	✓	Notes
SMRTbell Enzyme Clean Up Mix		7.0 µL	7.0 x N x 1.2*		
SMRTbell Enzyme Clean Up Buffer 2.0		7.0 µL	7.0 x N x 1.2*		
Molecular Biology Grade Water		41.0 µL	41.0 x N x 1.2*		
Total Volume		55.0 µL			

*includes 20% overage for pipetting errors

2. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips. It is important to mix well.
3. Spin down the contents of the tube strips with a quick spin in a microfuge.
4. To each **15.0** µL of sample, add **55.0** µL of Nuclease Master Mix. The total reaction volume at this step is 70.0 µL.
5. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips. It is important to mix well.
6. Incubate at 37°C for 30 mins and store on ice immediately.
7. Spin down the contents of tube strips with a quick spin in a microfuge.
8. Proceed directly to the AMPure PB bead purification step below **immediately**. Do not store samples at this stage. Do not let samples sit for long periods of time. Always work on ice.

Purify SMRTbell Library Using 1.0X AMPure® PB Beads

STEP	✓	AMPure PB Bead Purification	Notes
1		Bring the volume of each sample to **100** µL with Elution Buffer. Add **1.0X** volume of AMPure PB beads to the nuclease-treated ligation reaction.	
2		With a multi-channel pipette, mix by pipetting 10X - 15X with wide bore tips.	
3		Allow the DNA to bind to beads on the bench top at room temperature for 20 minutes.	
4		Quickly spin down the tube strip (for 1 second) to collect beads.	
5		Place the tube strip in a magnetic rack to collect the beads to the side.	
6		Slowly pipette off the cleared supernatant. Avoid disturbing the beads. Optional: save the supernatant in another tube strip in case of poor DNA recovery.	
7		Wash the 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. – Fill the tubes with 200 µL of 80% ethanol. Slowly dispense the 80% ethanol on the side of the tubes. Ensure the bead pellet is covered with ethanol solution. – After 30 seconds, pipette and remove the 80% ethanol.	
8		Repeat step 7.	
9		Remove residual 80% ethanol. – Remove tube strip from the magnetic rack and quickly spin. Both beads and any residual 80% ethanol will be at the bottom of each tube. – Place the tube strip back on magnetic rack. – Pipette off any remaining 80% ethanol.	
10		Remove the tube strip from the magnetic rack.	
11		Add **21.0** µL Elution Buffer to elute the DNA. – Pipette mix 10X – 15X until beads are uniformly re-suspend. – Elute the DNA at 37°C for 15 minutes. – Quickly spin. – Let beads separate fully in the magnetic rack. Then without disturbing the beads, transfer supernatant to a new DNA Lo-Bind tube strip.	
12		Take 1.0 µL of eluted sample and measure the DNA concentration using the dsDNA HS Assay Kit with a Qubit fluorometer. The same sample aliquot used to measure DNA concentration on the Qubit system may be used for DNA sizing QC on the Femto Pulse system. Dilute the sample to 250 pg/ µL with the Femto Pulse Dilution Buffer (0.25X TE).	
13		Proceed to size-selection or store the samples at -20°C for future use.	

Size Selection of SMRTbell Libraries

For high-throughput whole genome sequencing applications, PacBio highly recommends the PippinHT system (Sage Science) for size-selection of SMRTbell libraries for HiFi sequencing. Typical recovery yields are 35% - 50% and are highly dependent on the size distribution of the starting SMRTbell library.

Size Selection Using the PippinHT System

Verify that your PippinHT system software is up to date and follow the procedure below to remove SMRTbell templates <10 kb using the PippinHT system. For the latest PippinHT System Operations Manual and guidance on size-selection protocols, contact Sage Science (www.sagescience.com).

STEP	✓	PippinHT Size Selection	Notes
1		Prepare **~1.5 µg** of SMRTbell libraries in a final volume of **20.0 µL** of Elution Buffer for each PippinHT lane.	
2		Bring the Loading Solution to room temperature, and then add **5.0 µL** of the Loading Solution to the **20.0 µL** DNA sample. For loading multiple lanes with the same sample, scale up the volumes proportionally. The Loading Solution is viscous, so pipet slowly to ensure complete transfer into the DNA sample. – Pipette mix using wide-bore pipette tips to mix. – Spin briefly to collect the contents at the bottom of the tube.	
3		Follow the manufacturer's recommendations to set up a run protocol. – Select the **"6-10kb High Pass Marker 75E"** Cassette Definition File. – Using the "Range" selection mode, enter a desired "BPstart" value of 10000 and a BP End value of 50000. Be sure to assign a marker lane.	
4		Load the 75E marker and samples into the gel cassette and start the run. Run time is approximately 1 hr 15 mins.	
5		To maximize recovery of eluted DNA, wait at least 45 minutes after the run terminates before removing the sample from the elution chamber. – Wash the elution well with 30 µL PippinHT 0.1% Tween20 (supplied with the cassettes). Wait 1 minute and add the recovered wash liquid to the eluted sample. – Wash the elution well with 30 µL of Elution Buffer and add the recovered wash liquid to the eluted sample. – The total volume after the pooling recovered washes with the original eluted sample is ~ 90 µL.	
6		Proceed to the AMPure PB bead purification step.	

Size Selection Using the BluePippin System

Sage Science's BluePippin system may also be used for size-selection of HiFi SMRTbell libraries. Verify that your BluePippin system software is up to date and follow the procedure below to remove SMRTbell templates <10 kb using the BluePippin system. Typical recovery yields are highly dependent on the size distribution of the starting SMRTbell library. For the latest BluePippin system User Manual and guidance on size-selection protocols, contact Sage Science (www.sagescience.com).

STEP	✓	BluePippin Size Selection	Notes
1		Prepare **~1.5 µg** of SMRTbell library in a final volume of **30 µL** of Elution Buffer for each BluePippin lane.	
2		Bring the Loading Solution to room temperature, and then add **10 µL** of the Loading Solution to the **30 µL** DNA sample. The Loading Solution is viscous, so pipet slowly to ensure complete transfer into the DNA sample. – Pipette mix using wide-bore pipette tips to mix. – Spin briefly to collect the contents at the bottom of the tube.	
3		Follow the manufacturer's recommendations to set up a run protocol. – Select the **"0.75%DF Marker S1 High-Pass 6-10kb vs3**" Cassette Definition File. – Using the "Range" selection mode, enter a desired "BPstart" value of 10000 and a "BP End" value of 50000. – 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		To maximize recovery of eluted DNA, wait at least 45 minutes after the run terminates before removing the sample from the elution chamber. – Collect the eluate (~40 µL) into a 1.5 mL DNA LoBind tube or 0.2 mL PCR tube strips. • Wash the elution well with 40 µL BluePippin 0.1% Tween20 (supplied with the cassettes). • Wash the elution well with 40 µL of Elution Buffer and add the recovered wash liquid to the eluted sample. The total volume after the pooling recovered washes with the original eluted sample is ~ 120 µL.	
6		Proceed to the AMPure PB Bead purification step.	

Size Selection Using the SageELF System

Sage Science's SageELF system may also be used to fractionate SMRTbell libraries for HiFi whole genome sequencing applications. Verify that your SageELF system software is up to date and follow the size selection procedure below. For the latest SageELF User Manual and guidance on size-selection protocols, contact Sage Science (www.sagescience.com).

STEP	✓	SageELF Size Selection	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 >1 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**.	
9		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.	
10		Pool together fractions that have an average library size 10 – 20 kb.	
11		Proceed to the AMPure PB Bead purification step.	

Purify Size-Selected HiFi Library Fractions with 1.0X AMPure® PB Beads

STEP	✓	AMPure PB Bead Purification	Notes
1		For samples with volumes <100 µL, bring to 100 µL with 1X Elution Buffer (EB) before performing AMPure PB bead purification. Add **1.0X** volume of AMPure PB beads to the size-selected libraries.	
2		With a multi-channel pipette, mix by pipetting 10X – 15X with wide bore tips.	
3		Allow the DNA to bind to beads on the bench top at room temperature for 20 minutes.	
4		Quickly spin down the tube strip (for 1 second) to collect beads.	
5		Place the tube strip in a magnetic bead rack to collect the beads to the side.	
6		Slowly pipette off the cleared supernatant. Avoid disturbing the beads. Optional: save the supernatant in another tube strip in case of poor DNA recovery.	
7		Wash the 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. – Fill the tubes with 200 µL of 80% ethanol. Slowly dispense the 80% ethanol on the side of the tubes. Ensure the bead pellet is covered with ethanol solution. – After 30 seconds, pipette and remove the 80% ethanol	
8		Repeat step 7.	
9		Remove residual 80% ethanol. – Remove tube strip from the magnetic rack and quickly spin. Both beads and any residual 80% ethanol will be at the bottom of each tube. – Place the tube strip back on magnetic rack. – Pipette off any remaining 80% ethanol.	
10		Remove the tube strip from the magnetic rack.	
11		Add **11.0 µL** Elution Buffer to elute the DNA. Pipette mix 10X – 15X until beads are uniformly re-suspend. – Elute the DNA at 37°C for 15 minutes. – Quickly spin. – Let beads separate fully in the magnetic rack. Then without disturbing the beads, transfer supernatant to a new DNA Lo-Bind tube strip.	
12		Take 1.0 µL of eluted sample and measure the DNA concentration using the dsDNA HS Assay Kit with a Qubit fluorometer. The same sample aliquot used to measure DNA concentration on the Qubit system may be used for DNA sizing QC on the Femto Pulse system. Dilute the sample to 250 pg/µL with the Femto Pulse Dilution Buffer (0.25X TE).	
13		Proceed to the next step or store the final SMRTbell libraries at -20ºC for future use.	

Sequencing Preparation

See Quick Reference Card - Loading and Pre-Extension Recommendations for Sequel II/IIe Systems.

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
Updated for SMRTbell Enzyme Clean Up Kit 2.0 and Sequencing Primer v5.	04	April 2021
Removed SMRT Link Sample Setup and Run Design tables. Added reference to QRC.	05	August 2021

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