Procedure & Checklist - Multiplexing SMRTbell® Libraries using SMRTbell Express Template Prep Kit 2.0 for Structural Variation Detection

This document describes a procedure for constructing SMRTbell libraries for structural variant detection using the Sequel II System. Two genomic samples can be multiplexed on one SMRT Cell 8M.

High quality genomic DNA (gDNA) is sheared using a Megaruptor instrument, constructed to a SMRTbell library using the SMRTbell Express Template Prep Kit 2.0 and then size-selected. Note that the size distribution of the sheared DNA is critical for generating sufficient unique molecular coverage for structural variant detection.

Size-selection of SMRTbell libraries can be performed using either a BluePippin System or AMPure® PB beads (depending on the desired level of stringency for removing short inserts). The BluePippin System removes short insert SMRTbell libraries <15 kb efficiently and is, therefore, the preferred method for size-selection for Structural Variant multiplexing. Genomic DNA should be sheared so that the size distribution mode is between 20 kb to 40 kb (larger size is preferred). Using this method, short inserts (<15 kb) are removed during the size-selection process using a 15 kb lower cutoff.

To use AMPure PB beads for size-selection, the same sheared gDNA size distribution mode (20 kb - 40 kb) is required; however, it is important to avoid generating fragments <10 kb since AMPure PB beads are effective in removing fragments less than 5 kb only. Therefore, fragments >5 kb are carried through library construction and sequencing, which can result in shorter mean read length and lower unique molecular coverage compared to samples size-selected using the BluePippin System.

Always perform test shears to determine the best parameters to meet the above size distribution mode requirements. Additionally, the response of individual gDNA samples to shearing parameters may differ, so small-scale test shears are always required. If, after shearing, the gDNA sample contains an excess of <10 kb fragments, it is best to use the BluePippin System for size-selection.

For multiplexing, Barcoded Overhang Adapters are required for the ligation step. Any of the barcoded overhang adapters included with the Barcoded Overhang Adapter Kit 8A or Barcoded Overhang Adapter Kit 8B are suitable for use with this procedure.

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
Qubit™ 1X dsDNA HS Assay Kit	Thermo Fisher Scientific Q33230
DNA Shearing	
Megaruptor (shears DNA 20 kb - 40 kb)	Diagenode B06010001
Long Hydropores	Diagenode E07010002
Hydrotubes	Diagenode C30010018
g-TUBE (shears DNA to 20 kb only)	Covaris, 10145
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
Barcoded Overhang Adapter Kit 8A or	Pacific Biosciences 101-628-400
Barcoded Overhang Adapter Kit 8B	Pacific Biosciences 101-628-500
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
Microcentrifuge	Any MLS
Elution Buffer	Pacific Biosciences 101-633-500
Fractionation (If not using AMPure PB Beads for library size-selection)	
BluePippin System	Sage Science BLU0001
0.75% Agarose Cassettes, S1 Marker	Sage Sciences BLF7510

Structural Variant Multiplexing Workflow

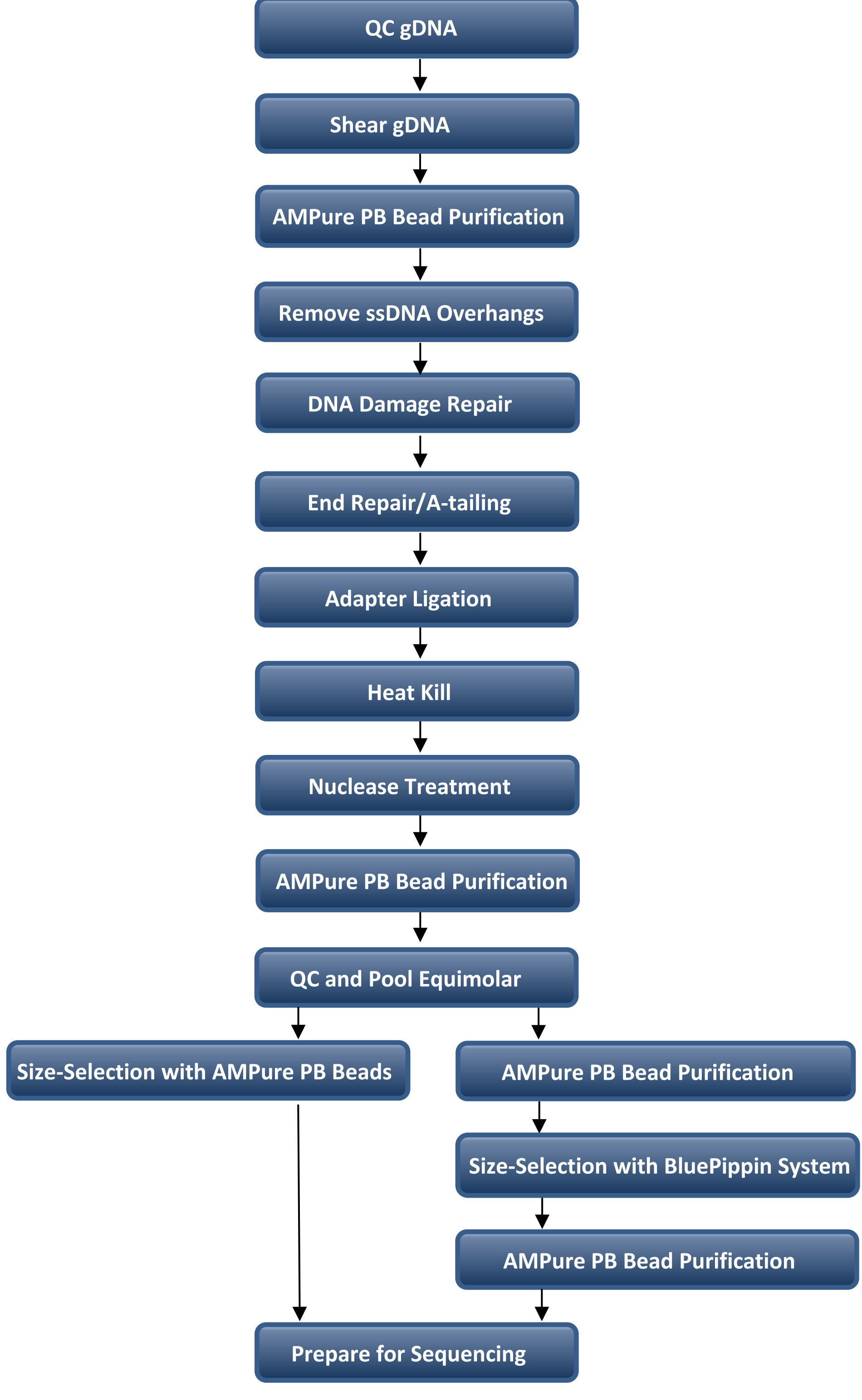

Figure 1: Workflow for multiplexing SMRTbell libraries, for low-fold coverage Structural Variant detection, using SMRTbell Express Template Prep Kit 2.0.

Reagent Handling

Several tubes in the kits (shown in Table 1 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 1: Temperature sensitive reagents.

Evaluate Genomic DNA (gDNA) Quality

This procedure requires high quality, high molecular weight input 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 2 below may be used to evaluate gDNA quality. Links to recommended procedures for each are also provided.

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 2. gDNA quality evaluation methods and procedures.

Figure 2 below 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 are not suitable for use in this procedure.

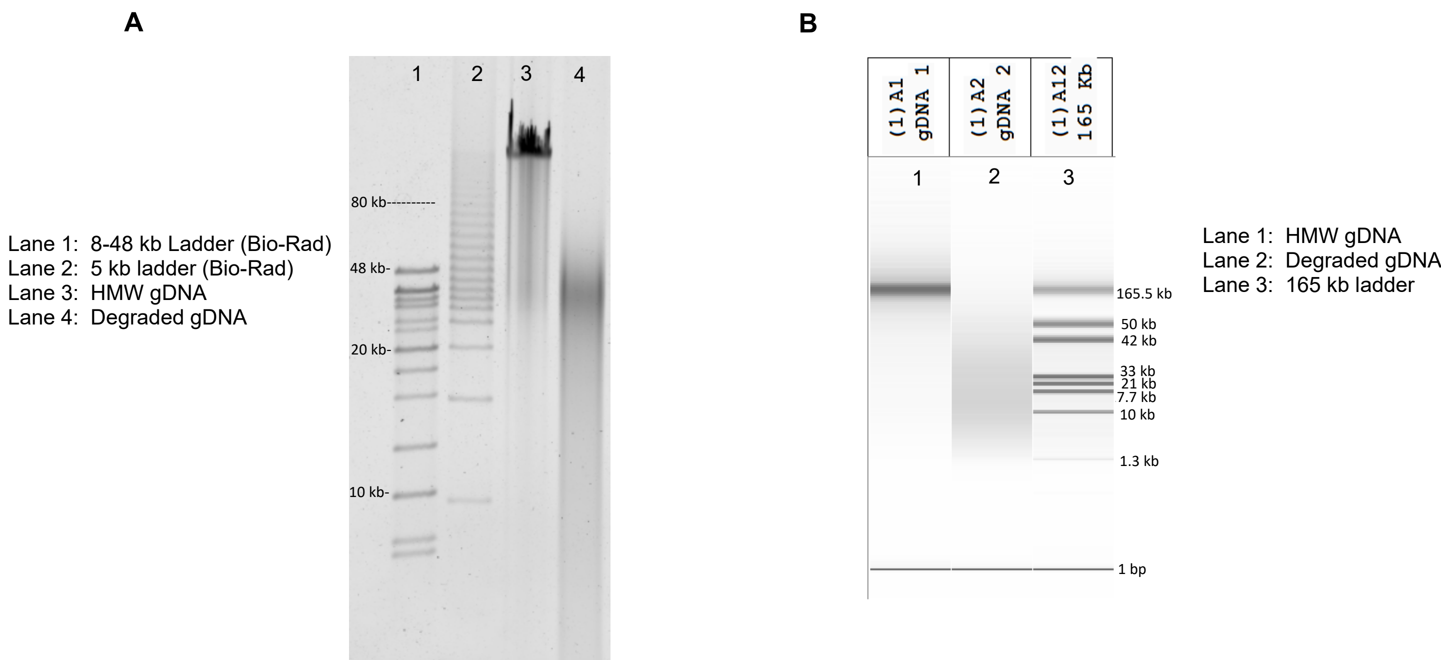

Figure 2: Evaluation of gDNA quality using two sizing characterization systems. A) Bio-Rad CHEF Mapper and B) Agilent Technologies' Femto Pulse.

Shear gDNA

Multiplexing samples for structural variant detection in the Sequel II System requires high molecular weight gDNA sheared to 20 kb - 40 kb and size-selected with the BluePippin System or AMPure PB beads. A distribution mode between 25 kb - 35 kb is recommended. The two size-selection options have different requirements for shearing the DNA.

1) BluePippin System size-selection requires starting with sheared gDNA with a size mode of 20 kb - 40 kb. SMRTbell templates <15 kb are efficiently removed using the *0.75% DF Marker S1 high-pass 15 kb -20 kb* cassette definition. The BluePippin System is the preferred size-selection method.
2) AMPure PB bead size-selection also requires starting with sheared gDNA with the same size distribution mode (20 kb - 40 kb) but **it is important to avoid generating fragments <10 kb**. Having fewer <10 kb fragments in the sheared sample will lead to improved data quality since AMPure PB beads can only remove short inserts <5 kb. A high presence of <10 kb inserts will result in shorter subread lengths, and therefore lower unique molecular yield.

Figure 3 shows an example of an ideal gDNA sample sheared to 20 kb - 40 kb using the Megaruptor System. Figure 4 is an overlay comparison of final SMRTbell libraries size-selected with AMPure PB beads and the BluePippin System.

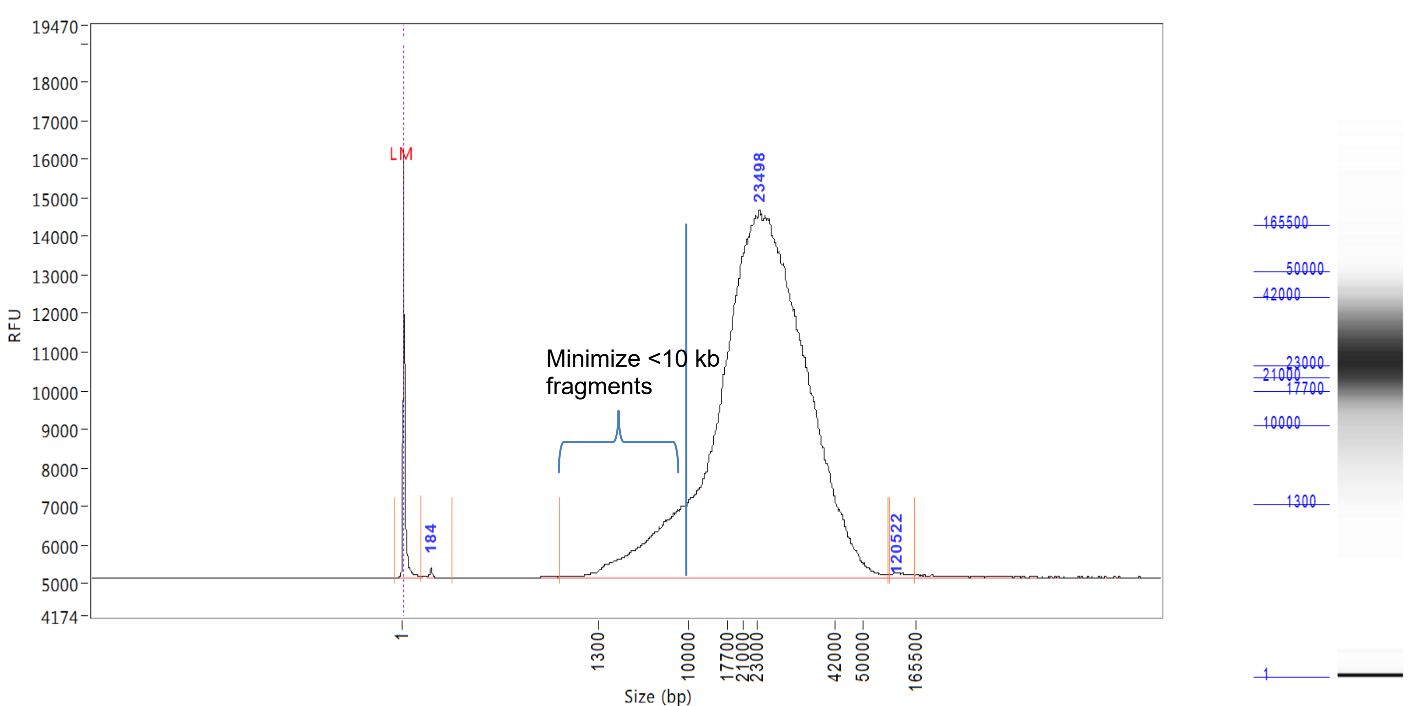

Figure 3: Example of Femto Pulse sizing profile for an ideal sheared gDNA sample suitable for downstream library size selection using either AMPure PB beads or the BluePippin System. With the BluePippin System, short insert SMRTbell templates are removed using a 15 kb cutoff. With AMPure PB beads, only SMRTbell templates below 5 kb can be efficiently removed. Therefore, it is important to optimize gDNA shearing conditions so that fragments <10 kb are minimized as shown in the above electropherogram.

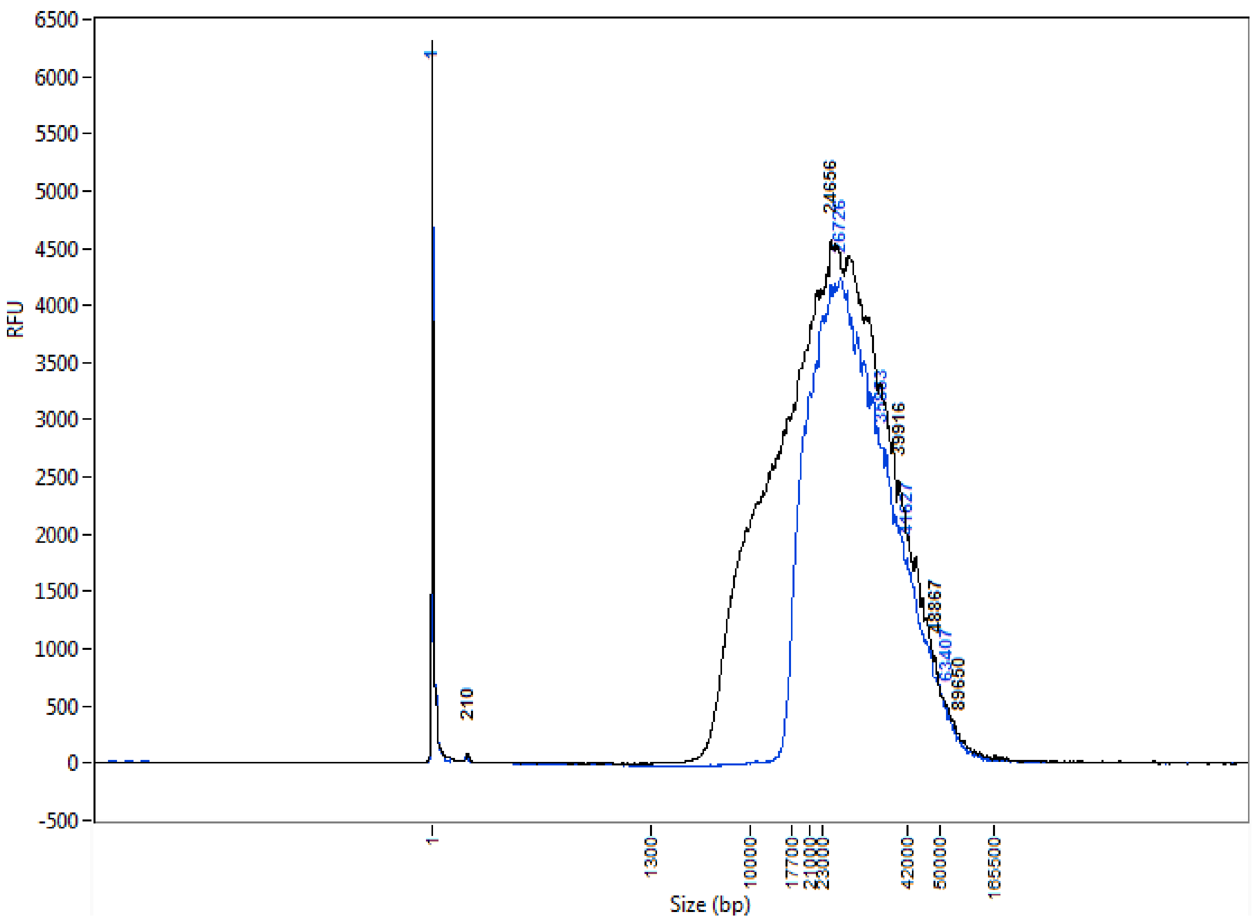

Figure 4: An overlay of Femto Pulse sizing profiles for SMRTbell libraries size-selected using AMPure PB beads (black) and the BluePippin System (Blue). As shown here, the BluePippin System removes short insert SMRTbell templates more efficiently than AMPure PB beads which results in good unique molecular coverage. While using AMPure PB beads is an alternative option for library size selection, ensure that the sheared gDNA sample does not contain excess levels of fragments <10 kb prior to starting SMRTbell library construction.

Shearing Using Diagenode's Megaruptor

The enzymatic reactions in the SMRTbell library construction workflow require 5 µg of sheared gDNA for each individual sample. Since DNA losses are expected during the shearing and concentration steps, be sure to start with sufficient amounts of starting gDNA. We recommend starting with 7 µg of gDNA per sample for shearing.

To shear gDNA using Diagenode's Megaruptor, generally follow the manufacturer's recommendations. It is important to perform small-scale test shears (for example, using a 150 µL volume at a DNA concentration of 15 ng/µL) to evaluate the response of each gDNA sample to shearing parameters.

STEP	✓	Shear DNA	Notes
1		Dilute **5 µg** of high molecular weight gDNA in 1X Elution Buffer to a concentration of 25 ng/µL in a final volume of **200 µL**. Before shearing, remove a 1 µL aliquot (un-sheared sample) for sizing QC.	
2		Shear gDNA with long hydropores using the following recommended settings. Megaruptor 3 — Speed setting 28-29 Megaruptor 1 or 2 — 30 kb - 35 kb setting	
3		Proceed to the next step to concentrate the sheared gDNA using AMPure PB beads. Note that the Megaruptor 1 and Megaruptor 2 systems will dilute DNA samples during shearing, so empirically measure the volume of the sheared gDNA sample before performing AMPure PB bead purification in the next step.	

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 **46 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 2. 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

Each individual sheared sample (up to 5 µg) for multiplexing goes through enzymatic reactions as described below.

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.
Always work in ice.

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.

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 remove single-strand overhangs 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

For multiplexing, use barcoded overhang adapters provided in the PacBio Barcoded Overhang Adapter Kit 8A or 8B. 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		
Barcoded Overhang Adapter		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
Reaction Mix 4		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 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 **15 µL** 1X Elution buffer. Add the 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		Use the remaining 9 µL of 1:10 diluted sample for QC on the Femto Pulse system. Determine the size of the libraries by performing smear analysis.	
16		Proceed to Sample Pooling.	

Sample Pooling

Equal-molar pooling of barcoded libraries is necessary to generate even coverage for each sample.

STEP	✓	Pooling	Notes
1		Use the concentration and average library size* from the Femto Pulse to determine the molarity of each sample.	
2		Use the following equation to determine Molarity: $\frac{\text{concentration (in ng/ µL) X } 10^6}{\text{(650 g/mol x average library size in bp*)}}$ = concentration (in nM)	
3		If you are using the BluePippin for size-selection, go to the next section “Purify SMRTbell Library Using 0.45X AMPure PB Beads”.	
4		If you are using AMPure PB beads for size-selection, go to the Procedure & Checklist – Using AMPure PB Beads for Size-Selection here. – Use 3.1X of 35% AMPure PB beads for size-selection of <5 kb SMRTbell templates – The AMPure PB bead size-selection requires sample concentration **0.5–10 ng/µL.** Make sure to adjust the concentration with Elution Buffer to meet this requirement.	

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		Adjust the volume of the pooled library to 100 **µL** with 1X Elution buffer.	
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 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 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 sizing QC.	

Size Selection Using the BluePippin System

When constructing SMRTbell libraries for structural variant detection, it is beneficial to remove small insert SMRTbell templates by performing size selection with the BluePippin System (which collects fragments above a size cut-off threshold). With the BluePippin System, the BP Start (cut-off) value may be adjusted depending on the size distribution and total available mass of the SMRTbell library. For the latest BluePippin User Manual and guidance on size-selection protocols, please contact Sage Science (www.sagescience.com).

Target lower cut-off threshold (BP Start)	Cassette Definition File	Min. Input DNA per Lane	Marker	BP End (bp)	Run Time
15 kb - 20 kb	0.75% DF Marker S1 high-pass 15 kb – 20 kb	>3.0 µg	S1	50000	5.5 hrs

Table 3. Recommended Blue Pippin Run Setup Protocols for Size Selection.

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 SMRTbell library in a final volume of 30 µL 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. When setting up the run protocol, select the appropriate Cassette Definition File for your sample. b. Using the "Range" selection mode, enter the desired "BPstart" value. A "BP End" value should automatically appear. **Note**: *When using the *'0.75% DF Marker S1 high-pass 15 kb -20 kb* cassette definition file, sample lanes containing **<3 µg** of SMRTbell library material will run faster during electrophoresis. In such cases, PacBio recommends adjusting the BP Start values as follow: **Cassette Definition File** — **If < 3 µg input per lane, use BP Start** 0.75% DF Marker S1 high-pass 15 kb – 20 kb — 12500 for 15 kb cutoff 0.75% DF Marker S1 high-pass 15 kb – 20 kb — 15000 for 20 kb cutoff	
4		To maximize recovery of eluted DNA, wait at least 30 minutes after the run terminates before removing the sample from the elution chamber. a. Collect the eluate into a 1.5 mL DNA LoBind tube. b. Wash the elution well with 40 µL of Sage Science's 0.1% Tween-20 Wash Solution and then add the recovered wash liquid to the eluted sample. Washing the elution well may further increase recovery yields by approximately 10-20%.	

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

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

<table>
<tr><th>STEP</th><th>✓</th><th>AMPure PB Bead Purification</th><th>Notes</th></tr>
<tr><td>1</td><td></td><td>Add 0.50X volume of AMPure PB beads.</td><td></td></tr>
<tr><td>2</td><td></td><td>Mix the bead/DNA solution thoroughly by gently tapping the tube.</td><td></td></tr>
<tr><td>3</td><td></td><td>Quickly spin down the tube (for 1 second) to collect the beads.</td><td></td></tr>
<tr><td>4</td><td></td><td>Allow the DNA to bind to beads by gentle rotation at room temperature for 30 minutes.</td><td></td></tr>
<tr><td>5</td><td></td><td>Quickly spin down the tube (for 1 second) to collect beads.</td><td></td></tr>
<tr><td>6</td><td></td><td>Place the tube in a magnetic bead rack to collect the beads to the side of the tube.</td><td></td></tr>
<tr><td>7</td><td></td><td>Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.</td><td></td></tr>
<tr><td>8</td><td></td><td>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.</td><td></td></tr>
<tr><td>9</td><td></td><td>Repeat step 8.</td><td></td></tr>
<tr><td>10</td><td></td><td>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.</td><td></td></tr>
<tr><td>11</td><td></td><td>Check for any remaining droplets in the tube. If droplets are present, repeat step 10.</td><td></td></tr>
<tr><td>12</td><td></td><td>Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 - 60 seconds.</td><td></td></tr>
<tr><td>13</td><td></td><td>Add 11 µL 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.</td><td></td></tr>
<tr><td>14</td><td></td><td>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.</td><td></td></tr>
<tr><td>15</td><td></td><td>Use the remaining 9 µL of 1:10 diluted sample to evaluate fraction insert size by any of the methods listed in Table 2.</td><td></td></tr>
<tr><td>16</td><td></td><td>Proceed to the next step to prepare for sequencing or store final SMRTbell library at -20C for future use.</td><td></td></tr>
</table>

Anneal and Bind SMRTbell Templates

Follow the SMRT Link Sample Setup v8.0 (or higher) instructions to anneal primer and bind polymerase to the SMRTbell library. For Structural variant multiplexing, Sequencing Primer v2 at 20:1 ratio and Sequel II Binding Kit 2.0 at 10:1 ratio are required. In Sample Setup, select CCS for “Sequencing Mode” to enable a 10:1 polymerase to template ratio.

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.

In Run Design, select “Continuous Long Reads” for sequencing both BluePippin and AMPure PB size-selected Structural Variation samples.

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

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