Procedure & Checklist - Preparing >30 kb SMRTbell® Libraries Using Megaruptor® Shearing and BluePippin™ Size-Selection for PacBio RS II and Sequel® Systems

This document provides recommendations for preparing >30 kb size-selected SMRTbell libraries from 5 µg of starting **sheared** genomic DNA (gDNA).

Only high-quality, high molecular weight gDNA may be used for producing >30 kb libraries. To ensure success, gDNA size and integrity must be verified by pulsed field gel electrophoresis (PFGE) before beginning library preparation. In addition, conditions for shearing gDNA to a size that can support producing >30 kb libraries must be determined and verified empirically for each sample.

Overall yields of >30 kb libraries are typically 5-10%. For large genome projects, we recommend starting this procedure with >10 µg of high quality gDNA sample.

Required Materials

Item	Vendor	Part Number
Pulsed Field Gel Electrophoresis		
Pulsed Field Gel Electrophoresis System: CHEF Mapper XA	Bio-Rad	170-3670
Pulsed Field Certified Agarose	Bio-Rad	162-0137
CHEF DNA Size Standard 5 kb	Bio-Rad	170-3624
Invitrogen 1 kb DNA extension ladder	Life Technologies	10511-012
Shearing		
Megaruptor	Diagenode	B06010001
Long Hydropores	Diagenode	E07010002
Hydrotubes	Diagenode	C30010018
SMRTbell Library		
SMRTbell Template Prep Kit 1.0	Pacific Biosciences	100-259-100
SMRTbell Damage Repair Kit	Pacific Biosciences	100-465-900
Ampure PB Beads	Pacific Biosciences	100-265-900
Tube rotator (or equivalent)	VWR	10136-084
Qubit 3.0 Fluorometer and	Life Technologies	Q33216
dsDNA HS Assay Kit or equivalent	Life Technologies	Q32854
Size Selection		
BluePippin Size-Selection System	Sage Science	BLU0001
Marker U1 Reagent kit	Sage Science	RUK7510
BluePippin Gel Cassettes	Sage Science	PAC30KB

Workflow

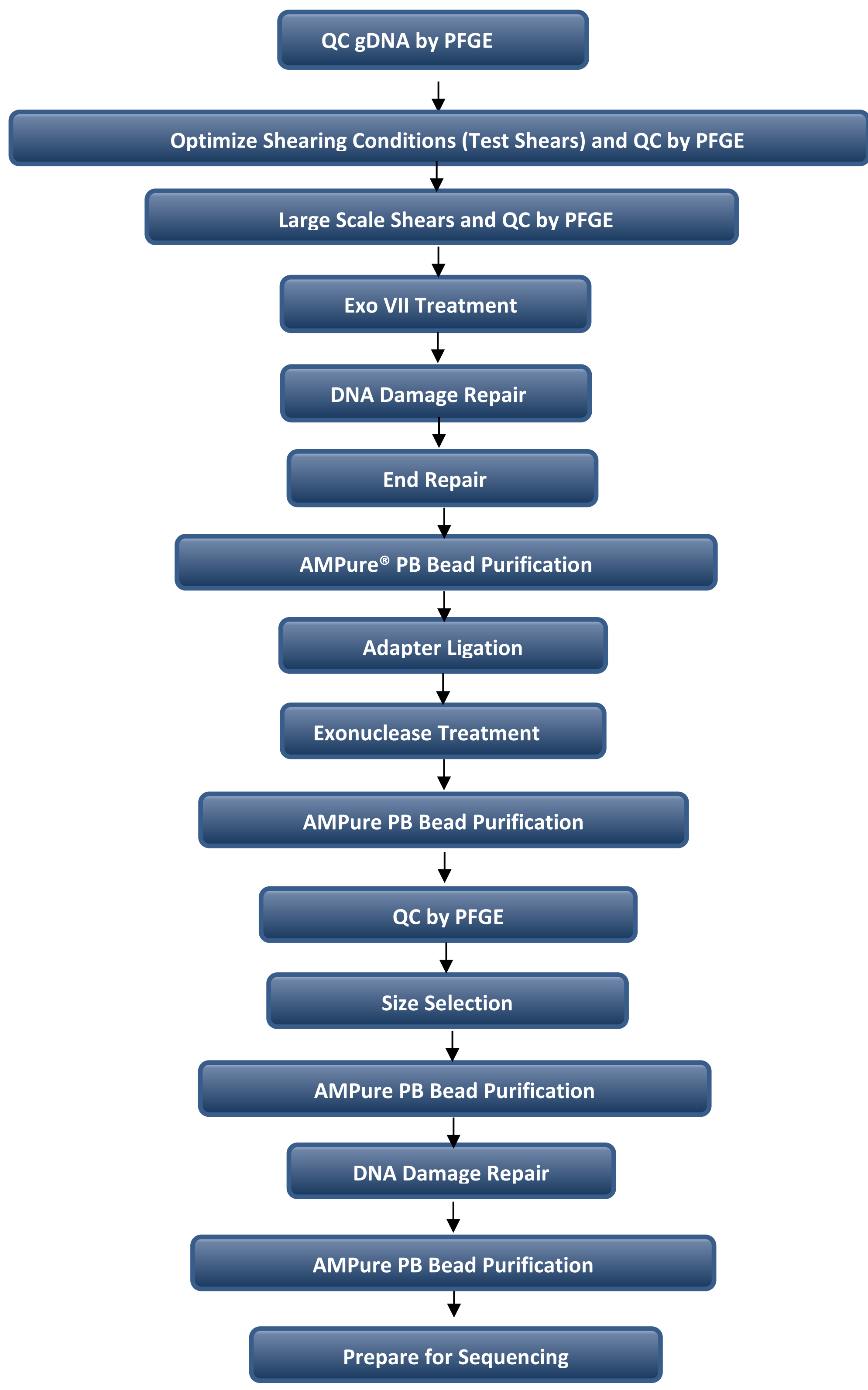


Figure 1: Workflow for preparing >30 kb SMRTbell libraries.

Evaluate Genomic DNA Quality

We highly recommend using Bio-Rad® CHEF Mapper® XA Pulsed Field Electrophoresis system for evaluating gDNA quality. The procedure is available here.

Additionally, there are other commercially available systems capable of resolving DNA fragments and smears up to ~50 kb. Recommendations for using Sage Science's Pippin Pulse Electrophoresis Power Supply are available here. Alternatively, Advanced Analytical Technologies, Inc. FEMTO Pulse is an automated pulsed-field capillary electrophoresis instrument for evaluating the integrity of genomic DNA with a run time of approximately 1.5 hours.

Lane 3 in Figure 2A and Lane 1 in Figure 2B are examples of high molecular weight DNA run on CHEF Mapper and FEMTO Pulse, respectively. If a significant portion of the gDNA migrates below ~50 kb (Lane 4 in Figure 2A and Lane 2 in Figure 2B), do not proceed with this 30 kb size selection procedure. Instead, see the PacBio *Procedure & Checklist >20 kb Template Preparation Using BluePippin™ Size-Selection System (15 - 20 kb Cutoff) for Sequel Systems* for recommendations on how to prepare >6 kb to >15 kb libraries.

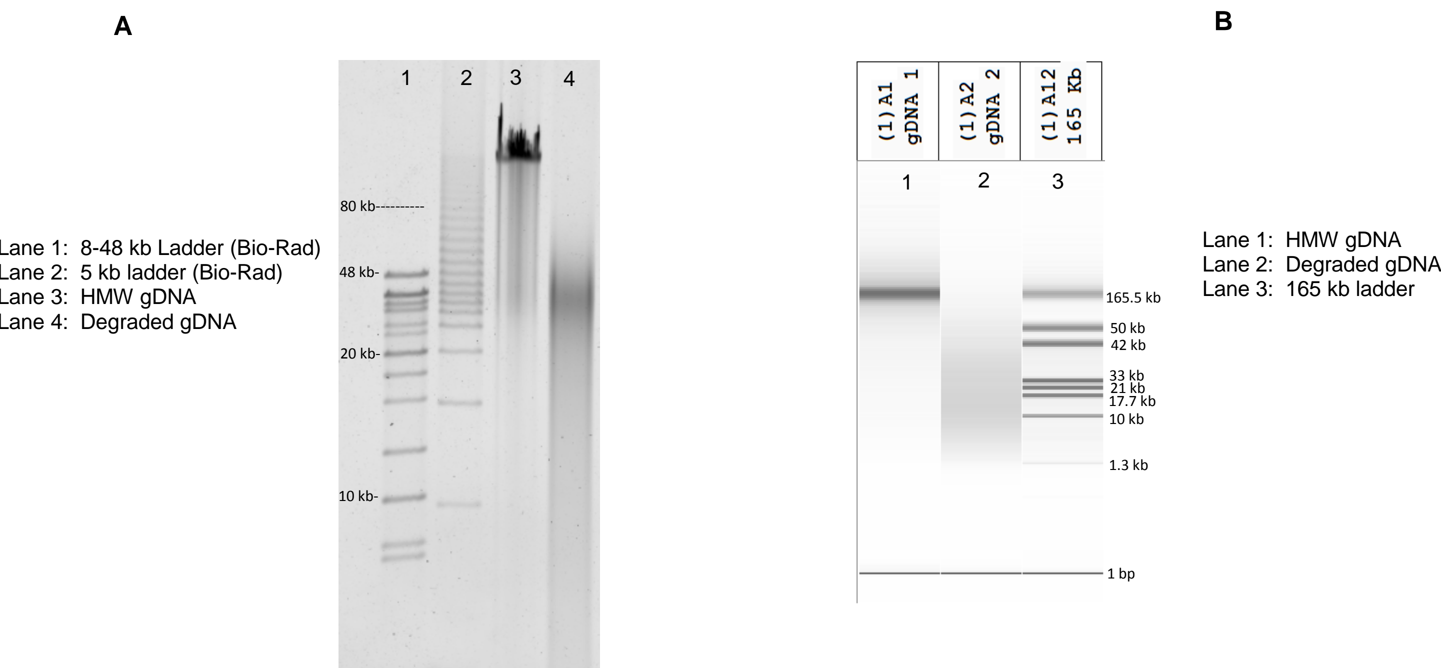


Figure 2: Evaluation of gDNA quality using two systems. A) Bio-Rad CHEF Mapper and B) Advanced Analytical FEMTO pulse. Lanes 3 A and 1B are examples of high quality, high molecular weight genomic DNA. Lanes 4A and 2B are examples of degraded gDNA and should not be used for production of a >30 kb library.

Optimize Shearing Conditions and Shear gDNA

To ensure sufficient yields of final >30 kb libraries, input gDNA must be sheared carefully so that the average size of fragmented DNA remains well above the desired size selection cut-off. The response of individual gDNA samples to recommended shearing parameters may differ and must be determined empirically and evaluated by PFGE. Test shears are highly recommended.

Note that for preparing >30 kb libraries, gDNA may be sheared by using Diagenode's Megaruptor. Here we provide initial starting parameters methods as well as strategies for optimization of gDNA shearing.

Shearing Using Diagenode's Megaruptor

For shearing gDNA using Diagenode's Megaruptor, generally follow the manufacturer's recommendations.

1. Dilute your gDNA in Elution Buffer (EB) to a concentration of 25-50 ng/µL in a volume of 50 µL to 400 µL. It is important not to exceed this DNA concentration during Megaruptor shearing or you may clog the hydropore. Before shearing, remove a 4 µL aliquot (un-sheared sample) for QC.
2. To shear gDNA for preparation of a >30 kb library, choose a target shear size of 50 kb in the Megaruptor software; for a >40 kb library, choose a target shear size of 60 kb to 75 kb. For both of these target shear sizes you must use Long Hydropores, and have the Long Hydropore option selected in the Megaruptor software.
3. Evaluate the distribution of the resulting sheared gDNA by running the un-sheared and sheared samples on a Bio-Rad® CHEF Mapper® XA Pulsed Field Electrophoresis system or AATI FEMTO Pulse. Other systems do not provide good resolution above 50 kb.

Typical results are shown in Figure 3 for a bacterial gDNA sample. The MR_50kb shear was used to prepare a >30 kb library, and the MR_75kb shear was used to prepare a >40 kb library.

If the gDNA sample appears under-sheared, try smaller target shear sizes (for example, 40 kb for a >30 kb library) and/or a lower DNA concentration until you achieve a similar distribution of fragmented gDNA.

If the gDNA sample is over-sheared, try a larger shear size (for example, 75 kb for a >30 kb library). It is also possible to increase or decrease the number of shearing cycles; you can contact Diagenode customer support to enable this option. Note that PacBio has not developed a tested protocol for this.

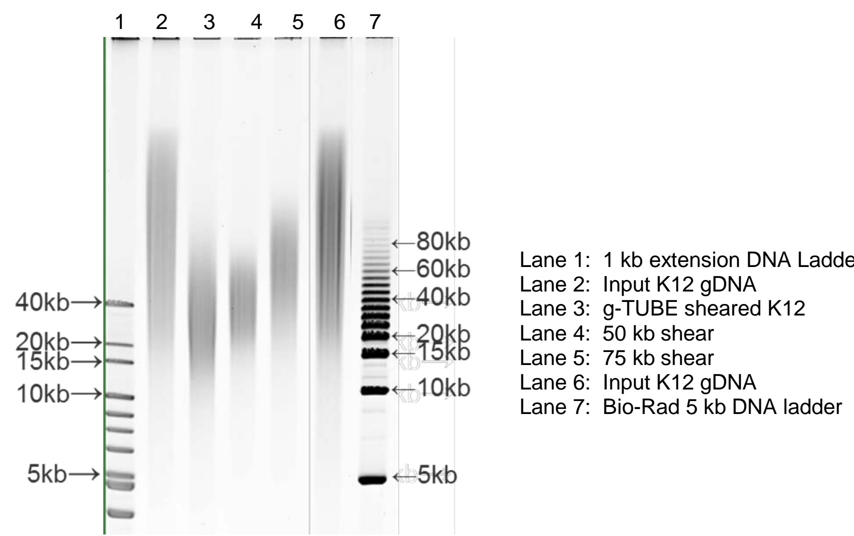


Figure 3: Evaluation of gDNA shears produced by Megaruptor (MR). MR_50kb and MR_75kb are good shears for a 30 kb size selection. Sample g-TUBE is oversheared and not appropriate for 30 kb size selection.

Concentrate DNA Using AMPure® PB Beads

If the concentration of sheared gDNA is less than 140 ng/µL, concentrate the sheared gDNA using AMPure PB Bead purification before proceeding. If the sheared gDNA concentration is greater than 140 ng/uL, adjust the gDNA concentration to 140 ng/uL with EB and proceed directly to ExoVII treatment below.

STEP		Concentrate DNA	Notes
1		Add **0.45X** volume of AMPure PB magnetic beads to the sheared gDNA _____µL of sample X **0.45X** =_______µL of beads 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. Do not pipet to mix.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by gentle end-over-end rotation for 15-20 minutes at room temperature. We recommend using a tube rotator.	
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, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	

STEP		Concentrate DNA	Notes
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 - 60 seconds.	
13		Calculate appropriate volume of Elution Buffer. ____ng X **0.5** / (_____ng/µL) =______µL of Elution Buffer needed The minimum DNA concentration required to proceed to the next step (End-Repair) is **140 ng/µL** with preferred mass of at least **5 µg**.	
14		Add the Pacific Biosciences® Elution Buffer volume (calculated in step 13) to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down 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 1.5 ml Lo-Bind tube. – Discard the beads.	
15		Verify your DNA amount and concentration using a Qubit quantitation platform. – 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 dsDNA BR Assay kit and the dsDNA HS Assay kit according to the manufacturer's recommendations. Yield up to this point should be 80%. The remaining 9 µL of 1:10 diluted sample may be used for QC by pulsed field gel electrophoresis (see example in Figure 4).	
16		The sheared DNA can be stored for up to 24 hours at 4°C or at -20°C for longer duration.	
17		Actual recovery per µL and total available sample material: ____	

ExoVII Pre-treatment of DNA

Use the following table to set up a reaction to remove single-stranded ends from 5 µg of sheared gDNA at 140 ng/µL. If starting with more than 5 µg of sheared gDNA, scale reaction volumes proportionally (i.e., for a mass between 6-10 µg of DNA scale the total volume to 96 µL).

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Sheared DNA (5 µg)	–		36.0 µL	–		
DNA Damage Repair Buffer		10 X	5.0 µL	1 X		
NAD+		100 X	0.5 µL	1 X		
ATP high		10 mM	5.0 µL	1 mM		
dNTP		10 mM	0.5 µL	0.1 mM		
ExoVII		10 U/µL	1.0 µL	0.2 U/µL		
Total Volume			48.0 µL	–		

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

Repair DNA Damage

Use the following table to prepare your reaction. For more than 5 µg input DNA, scale all reaction volumes proportionally.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (ExoVII treated)	–		48.0 µL	–		
DNA Damage Repair Mix		25 X	2.0 µL	1X		
Total Volume			50.0 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.

Repair Ends

Use the following table to prepare your reaction. For more than 5 µg input DNA, scale all reaction volumes proportionally.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (Damage Repaired)	–		50.0 µL	–		
End Repair Mix		20 X	2.5 µL	1X		
Total Volume			52.5 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 5-10 minutes, return the reaction to 4°C. Proceed to the next step.

Purify DNA Using 0.45X AMPure® PB Beads

STEP	✓	Purify DNA	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. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 15-20 minutes at room temperature. We recommend using a tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 - 60 seconds.	
13		For 5 µg of input sheared gDNA, elute in **23 µL** Elution buffer. If you started with more than 5 µg input sheared gDNA, scale volume of EB proportionally (i.e., for 6-10 µg of DNA, elute in 46 µL EB). Add the Pacific Biosciences Elution Buffer volume to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes. – Spin the tube down 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 1.5 ml Lo-Bind tube. – Discard the beads.	
14		Optional: Verify the DNA amount and concentration using a Qubit quantitation platform. – 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		The End-Repaired DNA can be stored overnight at 4°C or at -20°C for longer durations.	
16		Actual recovery per µL and total available sample material: _______	

Prepare Blunt-Ligation Reaction

Use the following table to prepare your reaction, adding the components below in the order listed. Be sure to mix insert gDNA and adapter BEFORE adding ligase. For more than 5 µg input DNA, scale all reaction volumes proportionally.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (End Repaired)	–		23.0 µL			
Blunt Adapter (20 µM)		20 µM	10.0 µL	5 µM		
Finger tap to mix before proceeding						
Template Prep Buffer		10 X	4.0 µL	1X		
ATP low		1 mM	2.0 µL	0.05 mM		
Finger tap to mix before proceeding						
Ligase		30 U/µL	1.0 µL	0.75 U/µL		
Total Volume	–	–	40.0 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.

ExoIII/VII Digestion to Remove Failed Ligation Products

Use the following table to prepare your reaction. For more than 5 µg input DNA, scale all reaction volumes proportionally.

R e	Tube Cap Color	Stock Conc.	Volume	✓	Notes
Ligated DNA			40 µL		
ExoIII		100.0 U/µL	1.0 µL		
ExoVII		10.0 U/µL	1.0 µL		
Total Volume			42 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. You must immediately proceed with AMPure PB bead purification after this step.

Purify SMRTbell Templates with 0.45X AMPure PB Beads

STEP	✓	Purify DNA	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. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 15-20 minutes at room temperature. We recommend using a tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 - 60 seconds.	
13		For up to 10 µg input size-selected library, elute in **31 µL** Elution buffer. If you size select more than 10 µg of SMRTbell library, scale volume proportionally. Add the Pacific Biosciences® Elution Buffer volume to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down 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 1.5 ml Lo-Bind tube. – Discard the beads.	

14		Verify your DNA amount and concentration using a Qubit quantitation platform. – 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. Yield up to this point should be 40-60%. The remaining 9 µL of 1:10 diluted sample may be used for QC by pulsed field gel electrophoresis (see example in Figure 4).	
15		It is highly recommended to perform qualitative and quantitative analysis using Pulse Field Gel Electrophoresis before size selection. This allows to choose appropriate Blue Pippin cut off for size selection. Choosing aggressive BP cutoff without knowing size distribution of SMRTbell Templates might lead to significant sample loss.	
16		Proceed with size-selection after AMPure PB Bead purification of exonuclease-treated libraries. Otherwise, samples may be stored at -20ºC at this point.	
17		Actual recovery per µL and total available sample material: ____________________	

Size-Selection Using the BluePippin™ System

Follow the instructions in the BluePippin User Manual and User Guides (see www.sagescience.com), and the specific recommendations below, for >30 kb or 40 kb size selection of the SMRTbell templates.

Note that you must use BluePippin Software **v6.20** (or higher) and the "0.75%DF Marker U1 high-pass 30-40kb vs3" run protocol for this procedure. Use the U1 marker for this protocol.

1. Prepare up to 5 µg SMRTbell templates in a final volume of 30 µL Elution Buffer for each lane. Size selection using this protocol can be aggressive and if not cautious, recovery may be impacted.

2. Bring the Loading Solution to room temperature, then add 10 µL of the Loading Solution to the 30 µL DNA sample. For multiple lanes, scale volumes proportionally. The Loading Solution is viscous so pipet slowly to ensure complete transfer into the DNA sample.
   a. Mix by gentle pipetting; do not vortex.
   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 "0.75%DF Marker U1 high-pass 30-40 kb vs3" cassette definition file.
   b. Using the "Range" selection mode, enter the desired "BPstart" value of 30000 or 40000 bp. A "BP End" value of 80000 bp should automatically appear. Be sure to assign a marker lane.

   Note: If using < 3ug per lane, use BP start = 25000 for >30 kb size selection and BP start = 35000 for >40 kb size selection.

4. Load samples and start the run. Be sure to include the U1 marker in the correct lane. Typical run times are ~10 hours.

5. 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 elution well with 40 µL of Sage Science's 0.1% Tween-20 Wash Solution, and add wash to eluted sample. Washing the elution well may increase yield 10-20%.

Purify Size-Selected SMRTbell Templates with 1X AMPure PB Beads

STEP	✓	Purify DNA	Notes
1		Add **1X** volume of AMPure PB beads to the size selected library.	
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. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 15-20 minutes at room temperature. We recommend using a tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect thebeads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30-60 seconds.	
13		For up to 5 µg input non-size-selected library gDNA, elute in **38 µL** Elution buffer. If you size-selected more than 5 µg of SMRTbell template, scale volume of EB proportionally (i.e., for up to 10 µg of input DNA, elute in 75 µL EB). Add the Pacific Biosciences® Elution Buffer volume to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down 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 1.5 ml Lo-Bind tube. – Discard the beads.	

14		Optional: Verify your DNA amount and concentration using a Qubit quantitation platform. – Using 1 µL of the purified 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 by pulsed field gel electrophoresis (see example in Figure 4).	
15		AMPure PB bead purified, size-selected libraries may be stored at -20°C.	
16		Actual recovery per µL and total available sample material: ______	

Repair DNA Damage After Size-Selection

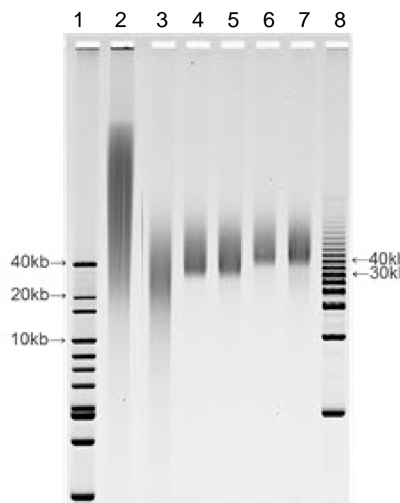
Using the table below, set up a reaction to repair any DNA damage present in SMRTbell templates after >30 kb or >40 kb size-selection. For up to 5 µg of size selected DNA, use a reaction volume of 50 µL. If starting with more than 5 µg of size-selected template, scale reaction volumes proportionally (i.e., for up to 10 µg of size selected DNA use a 100 µL reaction volume).

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Size-selected DNA	–		37 µL for 5.0 µg	–		
DNA Damage Repair Buffer	Red	10 X	5.0 µL	1 X		
NAD+	Red	100 X	0.5 µL	1 X		
ATP high	Red	10 mM	5.0 µL	1 mM		
dNTP	Red	10 mM	0.5 µL	0.1 mM		
DNA Damage Repair Mix	Red	25 X	2.0 µL	1X		
Total Volume			50.0 µL	–		

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

Purify Damage-Repaired, Size-Selected SMRTbell Templates with 1X AMPure PB Beads

STEP	✓	Purify DNA	Notes
1		Add **1X** volume of AMPure PB beads to the DNA Damage-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. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 15-20 minutes at room temperature. We recommend using a tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30-60 seconds.	
13		For up to 5 µg size-selected library, elute in **10 µL** Elution buffer. For more than 5 µg of SMRTbell template, scale volume of EB proportionally (i.e., for up to 10 µg of input DNA, elute in 20 µL EB). Add the Pacific Biosciences Elution Buffer volume to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down 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 1.5 ml Lo-Bind tube. – Discard the beads.	
14		Verify your DNA amount and concentration using a Qubit quantitation platform. – Using 1 µL of the purified 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 by pulsed field gel electrophoresis.	
15		AMPure PB bead purified, size-selected libraries may be stored at -20ºC.	



Lane 1: 1 kb extension DNA Ladder
Lane 2: Input K12 gDNA
Lane 3: SMRTbell library, no size selection
Lane 4: SMRTbell library, 30 kb Size selection cutoff
Lane 5: SMRTbell library, 30 kb Size selection cutoff with DNA Damage Repair post size selection
Lane 6: SMRTbell library, 40 kb Size selection cutoff
Lane 7: SMRTbell library, 40 kb Size selection cutoff with DNA Damage Repair post size selection
Lane 8: Bio-Rad 5 kb DNA ladder

Figure 4: Evaluation of >30 kb and >40 kb libraries using pulsed-field gel electrophoresis (CHEF Mapper).

Anneal and Bind BluePippin™ Size-Selected SMRTbell Templates

Before adding the primer to the SMRTbell template, pre-condition the primer by heating to 80ºC for 2 minutes, then place immediately on ice. (Note that if kept on ice during use, and stored at -20ºC, pre-conditioned primer may be used multiple times without re-heating.)

PacBio RS II System:
For the PacBio RS II System, follow the PacBio RS II Binding Calculator. Anneal 20X sequencing primer at a template concentration of 0.833 nM and incubate at 20ºC for 30 minutes.

Bind 10X P6 polymerase at an annealed template concentration of 0.500 nM (according to the Binding Calculator). For >30 and >40 kb libraries, incubation of the binding reaction at 30ºC for 4 hours may slightly improve loading relative to 30-minute binding reactions.

Sequel System:
For Sequel Systems, follow the SMRT Link Sample Setup instructions. Anneal 10X sequencing primer at a template concentration of 0.833nM and incubate at 20ºC for 60 minutes.

Bind 10X Sequel polymerase at an annealed template concentration of 0.500 nM. For >30 and >40 kb libraries, incubation of the binding reaction at 30ºC for 4 hours is required.

Prepare for Sequencing

PacBio RS II System:

For the PacBio RS II System, MagBead loading is required. Optimal loading of >30 and >40 kb SMRTbell libraries using P6 polymerase can typically be achieved using an on-plate concentration of 225 - 375 pM. We recommend you perform an initial loading titration in this range to determine optimal loading for your sample.

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

Sequel System:

For the Sequel System, both Diffusion and MagBead loading are appropriate for loading large insert libraries. We recommend performing loading titrations to determine the appropriate loading concentration. For more information, refer to *Quick Reference Card – Diffusion Loading and Movie Time Recommendations for the Sequel System*.

Note that it is highly recommended to purify the complex using Clean-up columns to remove excess primers and polymerase prior to sequencing. See the PacBio Procedure & Checklist – Sample Purification Using SMRTbell Clean Up Columns v2 for MagBead Loading and Procedure & Checklist - Sample Purification Using SMRTbell Clean Up Columns v2 for Diffusion Loading

Follow the SMRT Link Sample Setup instructions to anneal, bind and clean-up your samples.

Revision History (Description)	Version	Date
Added Diffusion as a method for loading large insert libraries.	05	October 2017
Removed loading specifics and referenced “Quick Reference Card – Diffusion Loading and Movie Time Recommendations for the Sequel System” for more information.	06	February 2018

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