PACBIO®

Procedure & Checklist - Preparing >30 kb Libraries Using SMRTbell® Express Template Preparation Kit

This document provides recommendations for preparing >30 kb size-selected SMRTbell libraries from 3-5 µg of starting **sheared** genomic DNA.

Only high-quality, high molecular weight gDNA of predominantly >50 kb may be used for producing large insert libraries size selected using this Express Procedure. If a significant portion of the gDNA migrates below ~50 kb, do not proceed with this protocol (see Figure 2). If the sample is not predominantly >50 kb, but predominantly >40 kb, please refer to *Procedure & Checklist - Preparing >15 kb Libraries Using SMRTbell Express Template Preparation Kit*.

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 before size selection are typically >50%.

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
26G Blunt End Needles for needle shearing	SAI Infusion	B26-150
1 mL Luer-Lok Tip Syringe for needle shearing	Becton Dickinson	309628
SMRTbell Library		
SMRTbell® Express Template Prep Kit	PacBio	101-357-000
Size Selection		
BluePippin Size-Selection System	Sage Science	BLU0001
Marker U1 Reagent kit	Sage Science	RUK7510
BluePippin Gel Cassettes	Sage Science	PAC30KB
Tips		
Wide Orifice Tips (Tips LTS W-O 200UL Fltr RT-L200WFLR)	Rainin	17014294

SMRTbell Express Workflow

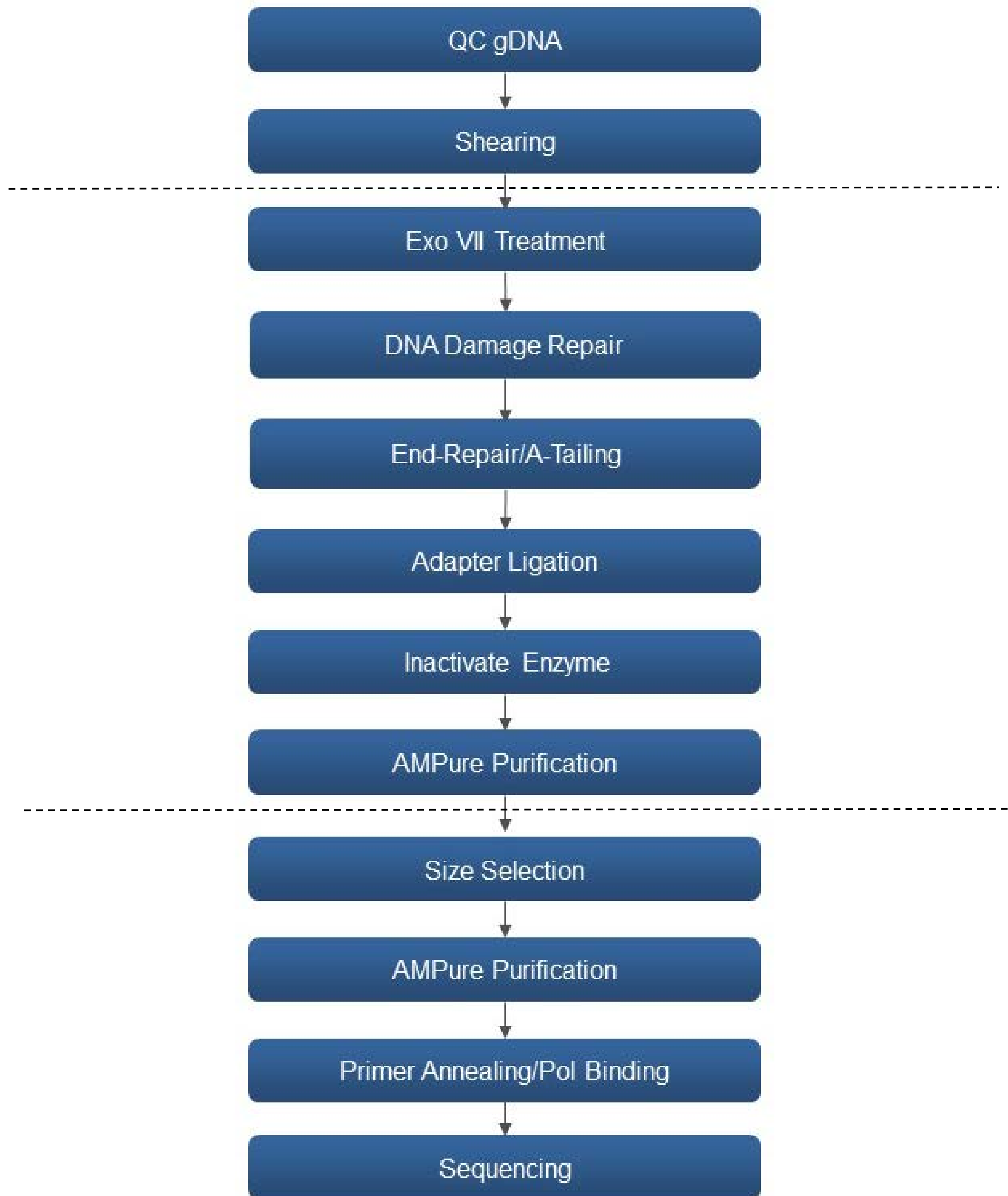

Figure 1: Workflow for Preparing >30 kb SMRTbell Libraries Using the Express Template Preparation Kit

Best Practice Recommendations:

1. Use wide-bore tips for all pipette mixing steps.
2. Never vortex tubes containing high molecular weight DNA.
3. Always follow Qubit best practices:
   - Prepare the Qubit working solution by diluting the Qubit reagent 1:200 in Qubit buffer. Prepare 200 µL of working solution for each standard and sample. Always make new standards for each assay.
   - Set up two 190 µL assay tubes for the standards and one 199 µL assay tube for each sample. Add 10 µL standard (from kit) and 1 µL sample to the respective assay tubes. Both the standard and sample DNAs should be at room temperature.
   - Vortex all tubes for 2 seconds.
   - Incubate the tubes for 2 minutes at room temperature prior to measurement.
4. Always set your heat blocks or thermocyclers to the appropriate temperature for incubations before proceeding with the procedure.

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, you can use the Advanced Analytical Technologies, Inc. FEMTO Pulse (link here) 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.

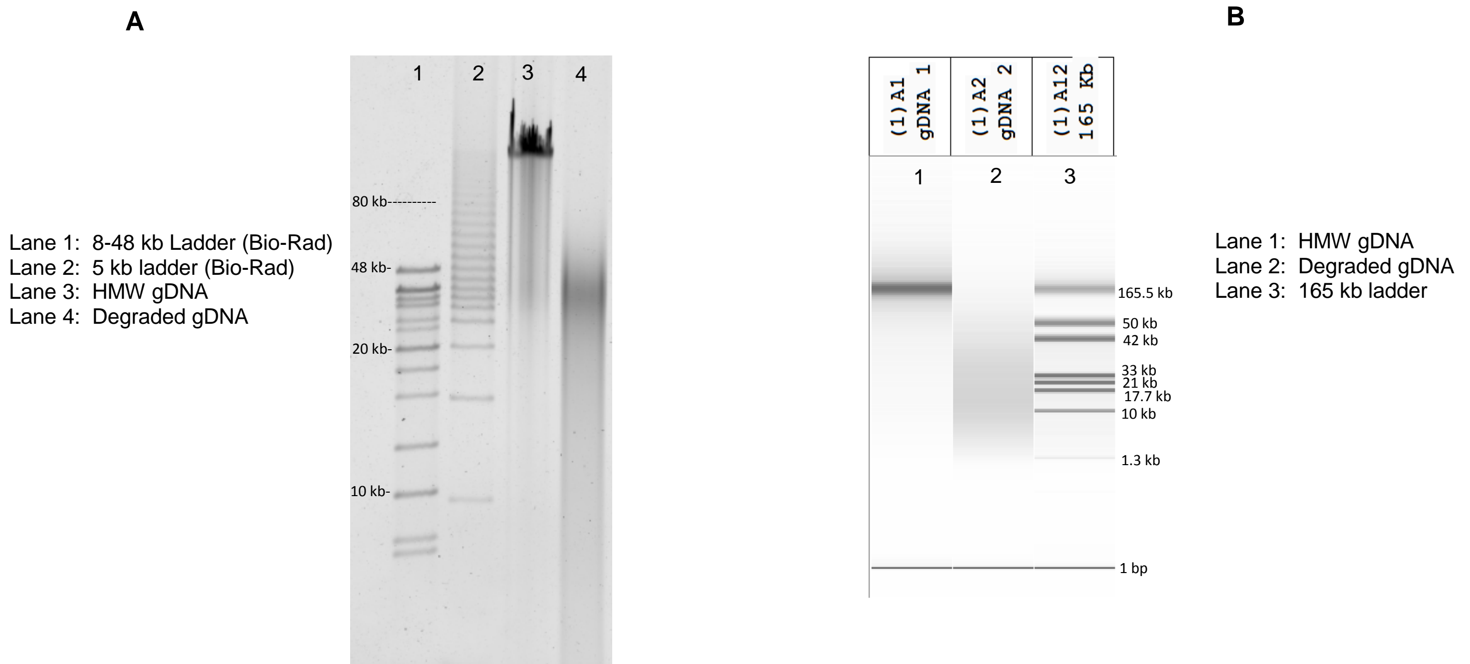

Figure 2: Evaluation of gDNA quality using two systems. A) Bio-Rad CHEF Mapper and B) Advanced Analytical FEMTO pulse. Lanes 3 A and 1B are examples of high quality, high molecular weight genomic DNA. Lanes 4A and 2B are examples of degraded gDNA and should not be used for production of a >30 kb library.

Optimize Shearing Conditions

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 or Needle Shearing. 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 to 60kb in the Megaruptor software; for a >40 kb library, choose a target shear size of 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.

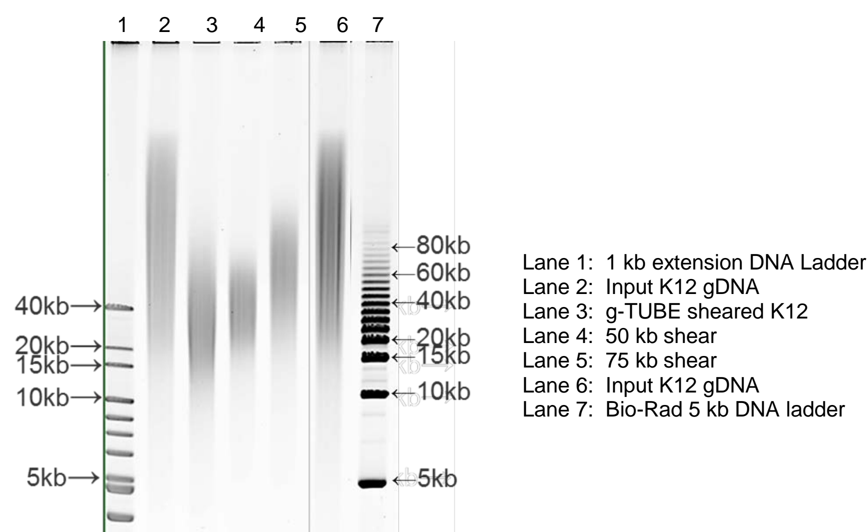

Figure 3: Evaluation of gDNA shears produced by Megaruptor (MR). MR_50kb and MR_75kb are good fragment distributions for a 30 kb size-selection. Sample g-TUBE is oversheared and not appropriate for 30 kb size selection.

Shearing using 26G Needles

Before performing needle shearing, please view a short movie demonstrating how to shear using needles.
Adjust the gDNA concentration to approximately 250 ng/µL with Elution Buffer (EB). If initial DNA concentration is less than 250 ng/µL, concentrate gDNA using AMPure® PB Beads.

1. Perform test shears by preparing a 50 µL volume sample in a 1.5 ml LoBind tube. Remove a 1 µL aliquot (un-sheared sample) for use as control when run on pulsed-field gel electrophoresis gel.
2. Aspirate the entire volume and pass sample through a 26G needle five times, then remove a second 1 µL aliquot (5x sample).
3. Pass the sample through the needle five more times and remove a third 1 µL aliquot (10x sample).
4. Finally, pass the sample through the needle ten more times and remove a fourth 1 µL aliquot (20x sample).
5. Evaluate the distribution of the resulting sheared gDNA by running the un-sheared, 5x, 10x, and 20x samples on a pulsed-field gel as described above.

Typical results are shown in Figure 3 for a bacterial gDNA sample sheared as described. Both the 5x and 20x shears were used to prepare >30 kb libraries, with good overall yields and sequencing performance.

If the gDNA sample appears under-sheared, decrease DNA concentration (for example, try 125 ng/µL) and/or increase the number of passes through the needle until you achieve a similar distribution of fragmented gDNA. If the gDNA sample is over-sheared, reduce the number of passes through the needle (e.g., try 1x and 2x).

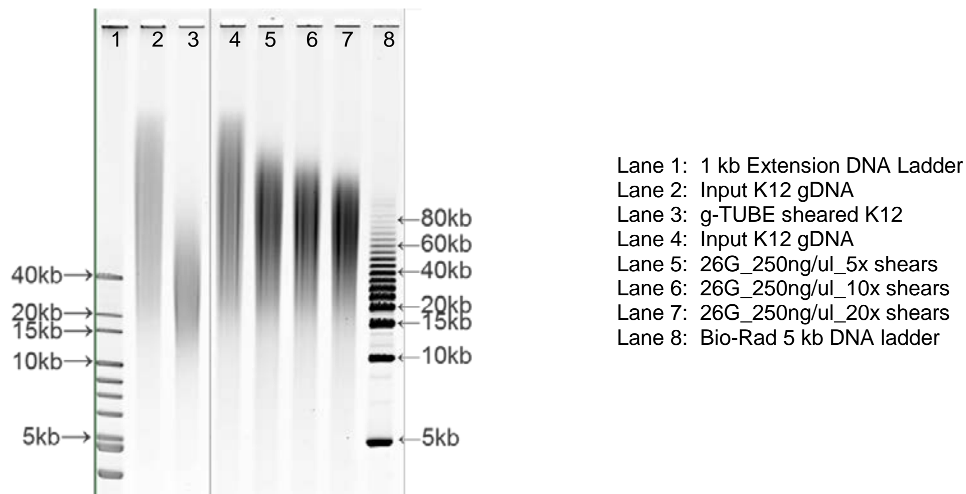

Figure 4: Evaluation of gDNA shears produced by a 26G needles.

Large Scale Shearing

Once you have determined the optimal shearing condition, scale up the shearing process by increasing the volume while maintaining the same concentration used during test shears.

Concentrate DNA Using AMPure PB Beads

STEP	✓	Concentrate DNA	Notes
1		Add **0.60X** volume of AMPure PB magnetic beads to the sheared gDNA _____µL of sample X **0.60X** =________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		Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Incubate the mix on bench top for 5 minutes at room temperature.	
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 beads. 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 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 (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat step 8.	
10		Remove residual 80% ethanol. – Remove tube from magnetic bead rack and spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	

STEP		Concentrate DNA	Notes
12		The volume to use for elution is **48 µL** (the volume to go into Exo VII).	
13		Add the Elution Buffer volume to your beads. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well. – Place at 37°C for 15 minutes to elute the DNA from the beads. – Spin the tube down, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the beads, 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 dsDNA BR Assay kit 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		The sheared DNA can be stored for up to 2 weeks at 4°C or at -20°C for longer duration. Do not freeze/thaw.	
16		Actual recovery per µL and total available sample material: ____	

Exo VII Pre-treatment of DNA

Use the following table to set up a reaction to remove single-stranded ends from 3 - 5 µg of sheared gDNA. If starting with more than 5 µg of sheared gDNA, scale reaction volumes proportionally (i.e., for a mass between 5-10 µg of DNA scale the total volume to 110 µL).

Reagent	Volume	✓	Notes
Sheared DNA (3 µg)	≤ 48.0 µL		
10X DNA Damage Repair Buffer v2	6.0 µL		
Exo VII v2	1.0 µL		
Water	Up to 55 µL		
Total Volume	55.0 µL		

1. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37°C for 15 minutes, then return the reaction to 4°C. Proceed to the next step.

Repair DNA Damage

Use the following table to prepare your reaction.

Reagent	Volume	✓	Notes
DNA (Exo VII treated)	55.0 µL		
DNA Damage Repair Mix	2.0 µL		
Total Volume	57.0 µL		

1. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37°C for 30 minutes, return the reaction to 4°C for 1 to 5 minutes.
4. Proceed to the next step.

Repair Ends/A-Tailing

Use the following table to prepare your reaction.

Reagent	Volume	✓	Notes
DNA (Damage Repaired)	57.0 µL		
End Repair Mix v2	2.0 µL		
End Repair Additive	1.0 µL		
Total Volume	60 µL		

1. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 20°C for 5 minutes
4. Incubate at 65°C for 30 minutes and return the reaction to 4°C. Proceed to the next step.

Adapter Ligation

Use the following table to prepare your reaction, adding the components below in the order listed.

Reagent	Volume	✓	Notes
DNA (End-Repaired)	60.0 µL		
5X Ligation Buffer	22.0 µL		
Water	17.0 µL		
Overhang Adapter v3	5.0 µL		
Pipette mix 15 times with wide-bore pipette tips			
Ligation Additive	1.0 µL		
Ligase v2	5.0 µL		
Total Volume	110.0 µL		

1. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 20°C for 30 minutes, then return the reaction to 4°C.
4. Proceed to the next step.

Termination Reaction

1. Dilute the Terminase with Termination Buffer.

Reagent	Volume	✓	Notes
Termination Buffer	9.0 µL		
Terminase (stock)	1.0 µL		
Total Volume	10 µL		

2. Use the following table to prepare the Terminase reaction.

Reagent	Volume	✓	Notes
Ligated DNA	110 µL		
Diluted Terminase	2.0 µL		
Total Volume	112 µL		

   a. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well.
   b. Spin down contents of tube with a quick spin in a microfuge.
   c. Incubate at 37°C for 15 minutes, then return the reaction to 4°C. Proceed to the next step.

Purify SMRTbell Templates

STEP	✓	Purify DNA	Notes
1		Add **0.60X** volume of AMPure PB beads to the Terminase-treated DNA.	
2		Mix the bead/DNA solution thoroughly by pipette mixing 15 times with wide-bore pipette tips. It is important to mix well.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Incubate samples on bench top for 5 minutes at room temperature.	
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 beads.	
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 (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat step 8.	
10		Remove residual 80% ethanol. – Remove tube from magnetic bead rack and spin. 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		Immediately add **31 µL** of Elution Buffer volume to your beads. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well. – Elute the DNA by letting the mix incubate at 37C for 15 minutes. This is important to maximize recovery of high molecular weight DNA. – Spin the tube down, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	

13		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.	
14		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.	
15		Proceed with size-selection after AMPure PB Bead purification of libraries. If the library is not ready for size selection, it can be stored for up to 2 weeks at 4°C or at -20°C for longer duration. Do not freeze/thaw.	
16		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 ug 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. 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 "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.
   Note: If using < 3ug per lane, use BP start = 25000 for >30 kb size selection and BP start = 35000 for >40 kb size selection.
   c. Be sure to assign a marker lane. PacBio highly recommends using Lane 4 for the marker.

4. Load samples and be sure to include the U1 marker in Lane 4. Start the run. 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 Library 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 pipette mixing 15 times with wide-bore pipette tips. It is important to mix well.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Incubate the mix on bench top for 5 minutes at room temperature.	
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 beads.	
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 (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat step 8.	
10		Remove residual 80% ethanol. – Remove tube from magnetic bead rack and spin to. 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		Add the **10 µL** Elution Buffer volume to your beads. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well. – Place at 37°C for 15 minutes to elute the DNA from the beads. – Spin the tube down, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	
13		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.	
14		AMPure PB bead purified, size-selected libraries may be stored at -20ºC.	

Anneal and Bind BluePippin Size-Selected SMRTbell Templates

Use SMRT Link Sample Setup for instructions for primer annealing and polymerase binding.
For primer annealing, use **Sequencing Primer v4** (found in the SMRTbell Template Prep Kit). This primer is for diffusion loading only. It is not appropriate for MagBead loading.

Prepare for Sequencing

For the Sequel System, Diffusion loading is appropriate for loading large insert libraries prepared by the SMRTbell Express Template Prep Kit. Recommended on-plate loading concentration is 10pM.

It is highly recommended to purify the complex using AMPure Purification to remove excess primer and polymerase prior to sequencing.

See the *Procedure & Checklist - AMPure PB Purification of Polymerase Bound SMRTbell Complexes for Diffusion Loading*.

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