Procedure & Checklist – Preparing 10 kb Library Using SMRTbell® Express Template Prep Kit 2.0 for Metagenomics Shotgun Sequencing

Before You Begin

This procedure describes preparing 10 kb libraries using SMRTbell Express Template Prep Kit 2.0 for metagenomics shotgun sequencing. The size distribution of the starting genomic DNA is critical for shearing and we recommend working with samples where the majority of the DNA is greater than 15 kb.

PacBio Instrument	Amount of Input gDNA Required for Shearing	Target Library Insert Size (Mode)
Sequel® System (1 Sample)	1500 ng	10 kb – 12 kb
Sequel II or Sequel IIe System (Up to 4 Multiplexed Samples)	1500 ng for 1 Sample or >750 ng per sample for Multiplexed Samples	10 kb – 12 kb

Required Materials

Item	Vendor and Part Number
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
dsDNA HS Assay Kit	Life Technologies Q32854
DNA Shearing (one of the following)	
Megaruptor	Diagenode B06010001
Long Hydropores	Diagenode E07010002
Hydrotubes	Diagenode C30010018
g-TUBE	Covaris 520079
Microcentrifuge	Any MLS
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
Sequel® II/IIe System: SMRTbell® Barcoded Adapter Plate 3.0 Sequel® System: Barcoded Overhang Adapter Kit 8A or 8B	Pacific Biosciences 102-009-200 Pacific Biosciences 101-628-400 or 101-628-500
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
Elution Buffer	Pacific Biosciences 101-633-500

Workflow

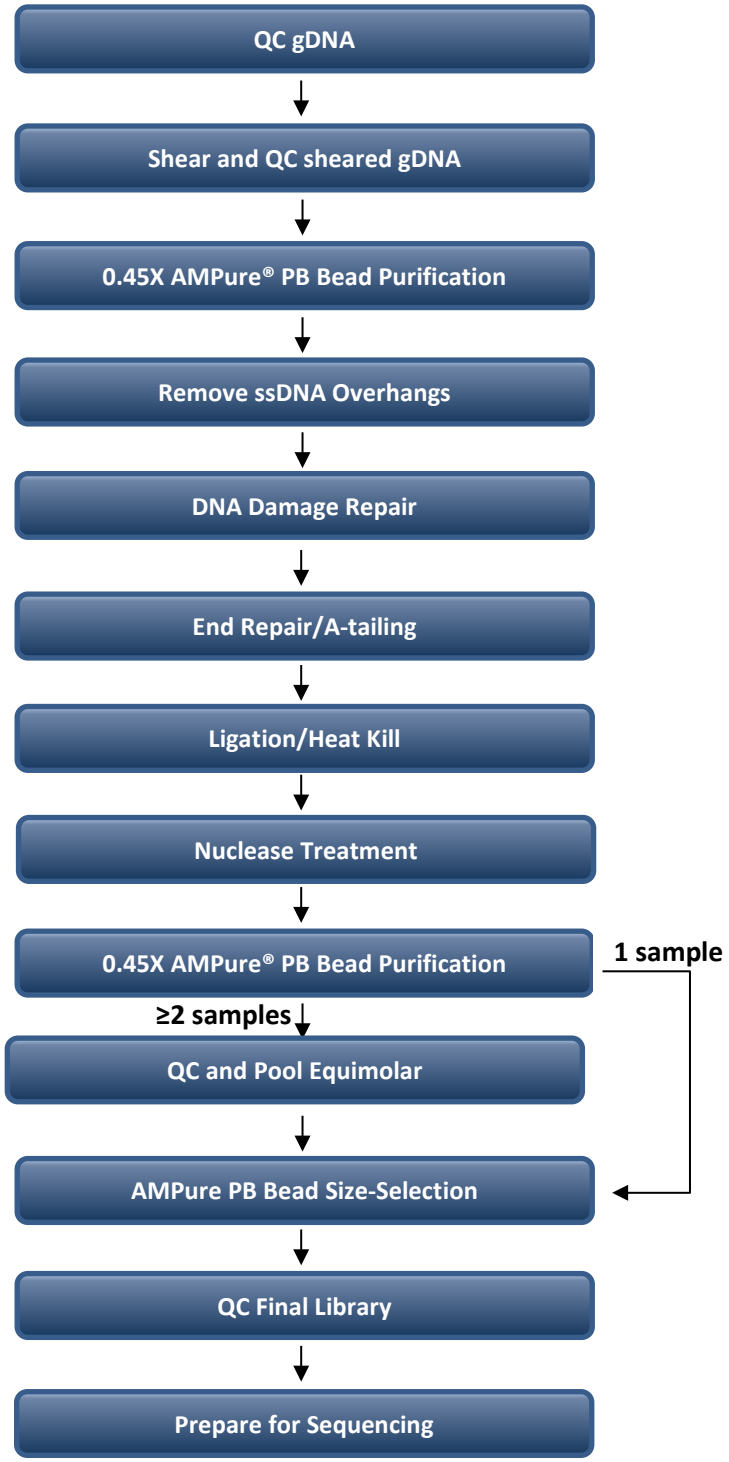


Figure 1: Workflow for preparing SMRTbell libraries using SMRTbell Express Template Prep Kit 2.0 for metagenomics shotgun sequencing on the Sequel and Sequel II Systems.

Reagent Handling

Several tubes in the kits (shown in Table 1 below) are sensitive to temperature and vortexing. We recommend to:

- Never leave tubes 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 or Barcoded Overhang Adapter	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 Quality

We recommend using a Pulsed Field Gel Electrophoresis system for assessing gDNA integrity. 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 or Product Note
Bio-Rad® CHEF Mapper® XA Pulsed Field Electrophoresis System	Procedure & Checklist - Using the BIO-RAD® CHEF Mapper® XA Pulsed Field Electrophoresis System
Agilent Femto Pulse System	Product Note: Fast, high-resolution DNA sizing with the Agilent Femto Pulse System
Sage Science Pippin Pulse	Procedure & Checklist - Using the Sage Science Pippin Pulse Electrophoresis Power Supply System

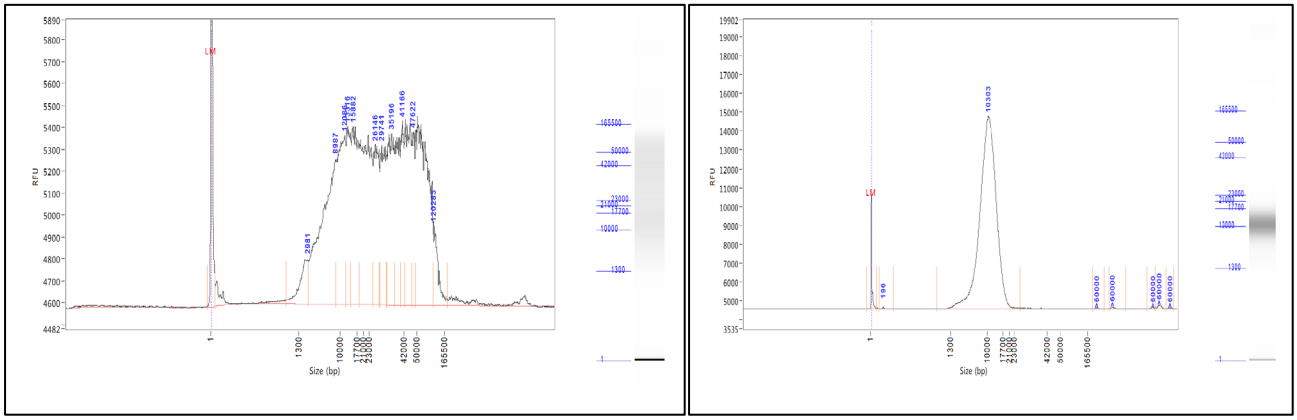
Table 2. gDNA Quality Evaluation Methods and Procedures.

Metagenomics samples often contain impurities that may affect subsequent enzymatic reactions. Before proceeding with library construction, determine DNA purity by measuring the A260/280 and A260/230 ratios using a spectrophotometer instrument (e.g., NanoDrop). If ratios are lower than the expected values (ideally A260/280 = ~1.8, A260/230 = 2.0 – 2.2 for pure DNA), performing a 0.45X AMPure PB bead purification is necessary to remove contaminants.

For accurate quantification, always measure DNA concentration using the Qubit dsDNA HS Assay system.

Megaruptor System for Shearing

PacBio highly recommends using the Megaruptor System for shearing gDNA because of its ability to generate a narrow shear size distribution. Below is a procedure for shearing gDNA to a target mode size of approximately 10 kb (see example in Figure 1 below) using Diagenode's Megaruptor 1 System. For high quality gDNA samples, the typical recovery yield post-shearing and concentration is approximately 70%. For sequencing one sample on one SMRT® Cell 1M/ SMRT Cell 8M, 1.5 µg of starting input gDNA is required for shearing. For multiplexing 2-4 samples for sequencing on one SMRT Cell 8M using the Sequel II System, >750 ng per sample is required for shearing.



A) Unsheared Genomic DNA **B) 10 kb Sheared DNA**

Figure 1: Example Femto Pulse sizing QC analyses of a mock community MSA 1003 DNA sample before and after shearing. A) Unsheared genomic DNA. B) Genomic DNA sheared to a 10 kb mode size using a Megaruptor 1 System

To shear gDNA using Diagenode's Megaruptor System, follow the manufacturer's general operating instructions and the specific recommendations listed in the table below.

IMPORTANT: Because the response of individual gDNA samples to recommended shearing parameters may differ, small scale test shears are highly recommended (for example, starting with 200 µL at a DNA concentration of 1 ng/µL or higher, performed according to the instructions below)**.** Once you have determined the optimal shearing conditions, you can scale up the input gDNA amount to the desired mass.

STEP	✓	Shear DNA	Notes
1		Dilute the gDNA in 1X Elution Buffer to a volume of 200 µL. DNA concentration can be as low as 1 ng/ µL. However, it is important that the DNA concentration not exceed 50 ng/µL during shearing or else the hydropore may become clogged. Before shearing, remove a 1 µL aliquot (un-sheared sample) for sizing QC.	
2		Shear the gDNA using long hydropores and a Megaruptor software Target Fragment Size setting of either 10 kb or 15 kb (choose the appropriate setting based on what you have determined as optimal during the small-scale test shear to achieve a target mode size of 10 - 12 kb for your specific sample). The Megaruptor run time is approximately 15-20 minutes per sample.	
3		Proceed to the next step to concentrate and purify the sheared gDNA sample using AMPure PB beads. Note that the Megaruptor System dilutes DNA samples during shearing, so measure the final total recovered volume of the sheared gDNA sample before starting the AMPure PB bead purification step.	

Covaris g-TUBE for Shearing

Alternatively, gDNA can be sheared using a Covaris g-TUBE device. To shear gDNA using g-TUBEs, we recommend following the procedure below (which varies from the Covaris instructions) to achieve a target sheared mode size of 10 - 12 kb. We recommend performing test shears to determine the optimal conditions for a particular DNA sample. For example, one can use 150 ng of input genomic DNA in a total volume of 150 µL for performing shearing optimizations.

For high-quality gDNA, the typical recovery yield post-shearing and concentration is approximately 70%. For sequencing one sample on one SMRT Cell 1M/ SMRT Cell 8M, , 1.5 µg of starting input gDNA is required for shearing. For multiplexing 2-4 samples for sequencing on one SMRT Cell 8M using the Sequel II System, >750 ng per sample is required for shearing.

STEP	✓	Shear DNA	Notes
1		Dilute **1500 ng of** gDNA in 1X Elution Buffer to a total volume of **150 µL**. For <1500 ng of gDNA, dilute the sample with 1X Elution Buffer to a total volume of 150 ul.	
2		Transfer gDNA to a g-TUBE and centrifuge at 2000 x g (4600 rpm in the Eppendorf MiniSpin Plus) for 2 minutes.	
3		Repeat spins until the entire gDNA sample has passed through the orifice. This may take 2-3 spins.	
4		Invert the g-TUBE and centrifuge at 4600 rpm in the Eppendorf MiniSpin Plus for 2 minutes.	
5		Repeat spins until the entire gDNA sample has passed through the orifice. This may take 2-3 spins.	
6		Transfer the sheared gDNA to a fresh 1.5 mL Lo-bind microfuge tube.	
7		Proceed to the “Concentrate DNA using AMPure PB Beads” section to concentrate the sample.	

Concentrate sheared gDNA using AMPure PB Beads

If necessary, bring all sheared gDNA samples to a minimum volume of 100 µL with 1X Elution Buffer.

STEP	✓	Concentrate DNA	Notes
1		Add **0.45X** volume of AMPure PB beads to the sheared gDNA sample. Note that the beads must be brought to room temperature before use 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 a 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 the 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 and allow the beads to separate. – 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 of Elution Buffer to use for elution is **46 µL** (45 µL are required to go into the "Remove Single-Stranded DNA Overhangs" step; and 1 µL is used for QC).	
13		Add the Elution Buffer volume to the 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 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 the Qubit 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 by pulsed field gel electrophoresis.	
15		The size distribution mode of the sheared gDNA must be between 10-12 kb to help ensure a sufficient yield of the final SMRTbell library. 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.	

Prepare SMRTbell Library Using Express Template Prep Kit 2.0

Each individual sheared gDNA sample is taken through the enzymatic reactions described on Pages 10 – 13 below. The reaction volumes and conditions below can support up to 5 µg of sheared gDNA per sample.

Note that wide-bore pipette tips are required when constructing SMRTbell libraries for metagenomics shotgun sequencing.

Remove Single-Strand Overhangs

Always work on ice.

Follow the steps below for removal of single-strand overhangs:

1. Dilute the stock DNA Prep Additive according to the table below.

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. For each sample to be processed, prepare the following reaction.

Reagent (Reaction Mix 1)	Tube Cap Color	Volume	✓	Notes
DNA Prep Buffer		7.0 µL		
Sheared DNA		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

For each sample to be processed, use the following table to prepare your reaction.

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

For each sample to be processed, use the following table to prepare your reaction.

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 each sample to be processed, use the following table to prepare your reaction, adding the components below in the order listed.

Reagent (Reaction Mix 4)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 3		60.0 µL		
Overhang Adapter v3 (or Barcoded Overhang Adapter if preparing a multiplexed library)		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 incubated 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

Follow the instructions below to set up a reaction to remove damaged or non-intact SMRTbell templates after the adapter ligation step.

1. Prepare an Enzyme Cleanup Master Mix according to the table below. The reagent volumes shown in the table support one ligated sample; scale the reagent volumes accordingly for more samples (e.g., for processing 4 ligated samples, prepare a total of 32.0 µL of Enzyme Cleanup Master Mix). The master mix can be stored at -20C for up to 4 months for future use.

Reagent (Enzyme Cleanup Master Mix)	Tube Cap Color	Volume	✓	Notes
Enzyme A		4.0 µL		
Enzyme B		1.0 µL		
Enzyme C		1.0 µL		
Enzyme D		2.0 µL		
Total Volume		8.0 µL		

2. For each ligated SMRTbell library sample to be processed, add 8.0 µL of Enzyme Cleanup Master Mix (for ligated samples containing ≤500 ng of DNA, see Note below):

Reagent (Reaction Mix 5)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 4		97.0 µL		
Enzyme Cleanup Master Mix		8.0 µL		
Total Volume		105.0 µL		

Note: For ligated samples containing ≤500 ng of DNA, the amount of Enzyme Cleanup Master Mix used for digestion can be reduced. Use the following reaction volumes if the input DNA amount is ≤500 ng.

Reagent (Reaction Mix 5)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 4		97.0 µL		
Enzyme Cleanup Master Mix		4.0 µL		
Total Volume		101.0 µL		

3. Mix the reaction well by pipetting up and down 10 times with wide-orifice pipette tips. It is important to mix well.
4. Incubate at 37°C for 1 hour.
5. 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 sheared gDNA Note that the beads must be brought to room temperature before use 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 a 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.	
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 and allow the beads to separate. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		The volume of Elution Buffer to use for elution is **31 µL**.	

13		Add the Elution Buffer volume to the 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 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 the Qubit 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		**For non-multiplexed SMRTbell libraries for the Sequel and Sequel II Systems**, proceed to "Removal of <3 kb SMRTbell Templates using Diluted AMPure PB Beads". For SMRTbell libraries to be used for multiplexing on the Sequel II System, proceed to the next section ("Sample Pooling").	

Sample Pooling

Equimolar pooling is necessary for multiplexing 2-4 samples for metagenomics shotgun sequencing on the Sequel II System.

STEP	✓	Pooling	Notes
1		Use the Qubit dsDNA concentration and average library size determined from the Femto Pulse (or other systems) to determine the molarity of each barcoded SMRTbell library sample to be pooled.	
2		Use the following equation to determine the Molarity of each barcoded SMRTbell library sample: $\frac{\text{DNA concentration (in ng/ µL)} \times 10^6}{\text{(660 g/mol x average library size in bp*)}} = \text{DNA concentration (in nM)}$ *Use average size as determined by performing a smear analysis.	
3		Pool the barcoded SMRTbell libraries at an equal molar concentration	
4		Proceed to the next section "Removal of <3 kb SMRTbell Templates using Diluted AMPure PB Beads".	

Removal of <3 kb SMRTbell Templates using Diluted AMPure® PB Beads

This AMPure PB bead purification step removes SMRTbell templates <3 kb. AMPure PB beads are diluted to 35% (volume/volume) with Elution Buffer. For effective size-selection, the DNA concentration of the SMRTbell library to be size-selected must be 0.5-10 ng/µL. Use of higher concentrations (>15-100 ng/µL) decreases the efficiency of reducing short insert SMRTbell templates. Adjust the sample concentration so that the DNA concentration is within this range.

The final AMPure PB bead concentration is critical to the success of this procedure. Therefore, accurate pipetting is of utmost importance to achieve a final 35% (v/v) AMPure PB bead in Elution Buffer.

Dilute AMPure PB Beads with Elution Buffer

The final AMPure PB bead concentration is critical to the success of this procedure. Therefore, accurate pipetting is of utmost importance to achieve a final 35% (v/v) AMPure PB bead working solution in Elution Buffer.

Reagent	Volume	✓	Notes
Elution Buffer	6.5 mL		
AMPure PB Beads (stock reagent)	3.5 mL		
Total Volume	10.0 mL		

1. Bring the AMPure PB bead stock to room temperature.
2. Vortex the stock solution for 30 seconds to mix well.
3. Using a P1000 pipette, transfer 6.5 mL of Elution Buffer into a 15 mL conical tube.
4. Add 3.5 mL of the stock AMPure PB beads to the Elution Buffer. When pipetting the viscous AMPure PB bead solution, pipette slowly to ensure that the volume aspirated is as precise as possible. Large residual AMPure PB beads adhering outside of the tip should be removed prior to adding to the Elution Buffer.
5. Vortex the diluted AMPure PB beads solution for 30 seconds to mix well before use. This solution may be stored at 4°C for 2 months for future use.

STEP	✓	Purify SMRTbell Templates	Notes
1		Before starting with the procedure, adjust the DNA concentration (if necessary) with Elution Buffer so that the final concentration is between 0.5 – 10 ng/µL. The most optimal concentration is 10 ng/µL.	
2		Measure the total sample volume accurately with a pipettor.	
3		To remove <3 kb SMRTbell templates, use 3.7X of diluted AMPure PB beads to the sample. **It is critical to mix precise volumes of the sample and diluted AMPure PB beads to achieve successful size-selection.**	
4		Mix the bead/DNA solution thoroughly by pipette mixing 15 times with wide-bore pipette tips. For larger volumes, use a bigger tip. It is important to mix well.	
5		Quickly spin down the tube (for 1 second) to collect the beads.	
6		Incubate the mix on a bench top for 15 minutes at room temperature.	
7		Spin down the tube (for 1 second) to collect beads.	
8		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
9		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the beads.	
10		Wash beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. – 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.	
11		Repeat step 10.	
12		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. Beads will be on the side of the tube. – Pipette off any remaining 80% ethanol.	
13		Check for any remaining droplets in the tube. If droplets are present, repeat step 11.	
14		Add **10 µL** of Elution Buffer volume to the beads. When adding 10 µL of EB, dispense the volume directly to the beads. Do not let the beads dry. Pipette mix 15 times with wide-bore pipette tips. – 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.	
15		Verify total mass 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 Qubit dsDNA HS Assay kit according to the manufacturer's recommendations. – Use the remaining 9 µL of of 1:10 diluted sample for DNA sizing QC.	
16		Purified SMRTbell libraries may be stored at -20ºC.	

Prepare for Sequencing

Follow the instructions in SMRT® Link Sample Setup v10.2 or higher. Select “Shotgun Metagenomic Profiling or Assembly” from application dropdown.

Revision History (Description)	Version	Date
Initial release.	01	May 2019
This procedure is updated for Express Template Prep Kit 2.0.	02	December 2019
Updated for SMRTbell Barcoded Adapter Plate 3.0.	03	November 2021