Procedure & Checklist – Preparing SMRTbell™ Libraries using PacBio® Barcoded Adapters for Multiplex SMRT® Sequencing

Before You Begin

This document describes a procedure for multiplexing 5 Mb microbial genomes up to 12-plex and 2 Mb genomes up to 16-plex, with complete genomes assemblies (<10 contigs). The workflow is compatible for both the PacBio RSII and Sequel Systems. Ten kilobase SMRTbell libraries are constructed for each sample through shearing and Exo VII treatment before going through the DNA Damage Repair and End-Repair steps. After End-Repair, barcoded adapters are ligated to each sample. Following ligation, samples are pooled, treated with Exo III and VII, and then put through two 0.45X AMPure® PB bead purification steps. Note that size-selection using a BluePippin™ system is not required. SMRTLink v4.0 is utilized to demultiplex and assemble the genomes after sequencing.

DNA Input Requirements and Pooling

For this procedure, the required total mass of DNA, after pooling, is 1 – 2 µg. Therefore, the required amount of sheared DNA, per microbe, going into Exo VII treatment is 1 µg divided by the number of microbes. For example, in a 12-plex library, 1 µg ÷ 12 microbes = 83 ng of sheared DNA is needed for Exo VII treatment. Table 1 below summarizes the required mass per microbe for the Exo VII treatment. We highly recommend at least 1 µg genomic DNA (gDNA) for shearing using a Covaris® g-TUBE™ device to offset loss during shearing and concentration (approximately 20 - 50% loss). For shearing recommendations using g-TUBEs, see the "Fragment DNA" section.

Multiplex	Input DNA for Shearing Per Microbe	Sheared DNA into Exo VII Treatment*
2-plex	1.0 µg	500 ng
4-plex	1.0 µg	250 ng
6-plex	1.0 µg	167 ng
8-plex	1.0 µg	125 ng
10-plex	1.0 µg	100 ng
12-plex	1.0 µg	83 ng
16-plex	1.0 µg	63 ng

*The amount of sheared DNA required for Exo VII treatment is calculated by 1 µg / number of microbes.

Table 1: DNA input requirements for shearing and equal mass pooling.

Depending on project requirements, up to twelve 5 Mb genomes may be multiplexed in 1 SMRT Cell 1M. For smaller genomes (<2 Mb), sixteen genomes may be multiplexed.

The efficiency of multiplex library construction, to generate equal read representation across samples, depends on pooling and fragment distribution of each sample. Always pool samples with similar fragment distribution as shown in Figure 1. In this example,12 high-quality gDNA sheared using g-TUBEs and subsequently run on a Bioanalyzer® system, showing similar distributions with modes at approximately 10 kb.

There are two approaches for pooling microbial samples for multiplexing:

1. Equal Mass
2. Equal Molar

The easier and preferred method is pooling by mass. This can be easily achieved when samples have similar fragment distributions as shown in Figure 1. It is, therefore, critical to start with high-quality gDNA so that samples can be fragmented to the desired distribution.

If deviation is significant across samples, it may be necessary to generate an equimolar pool. Calculating the molarity of each sample based on the average insert size and sample concentration and then combining the same molar concentrations of each sample to form the pooled sample. Ensure that there is at least 1 µg total mass in the pooled sample. Another option may be to reduce the number of multiplex libraries to offset the deviation.

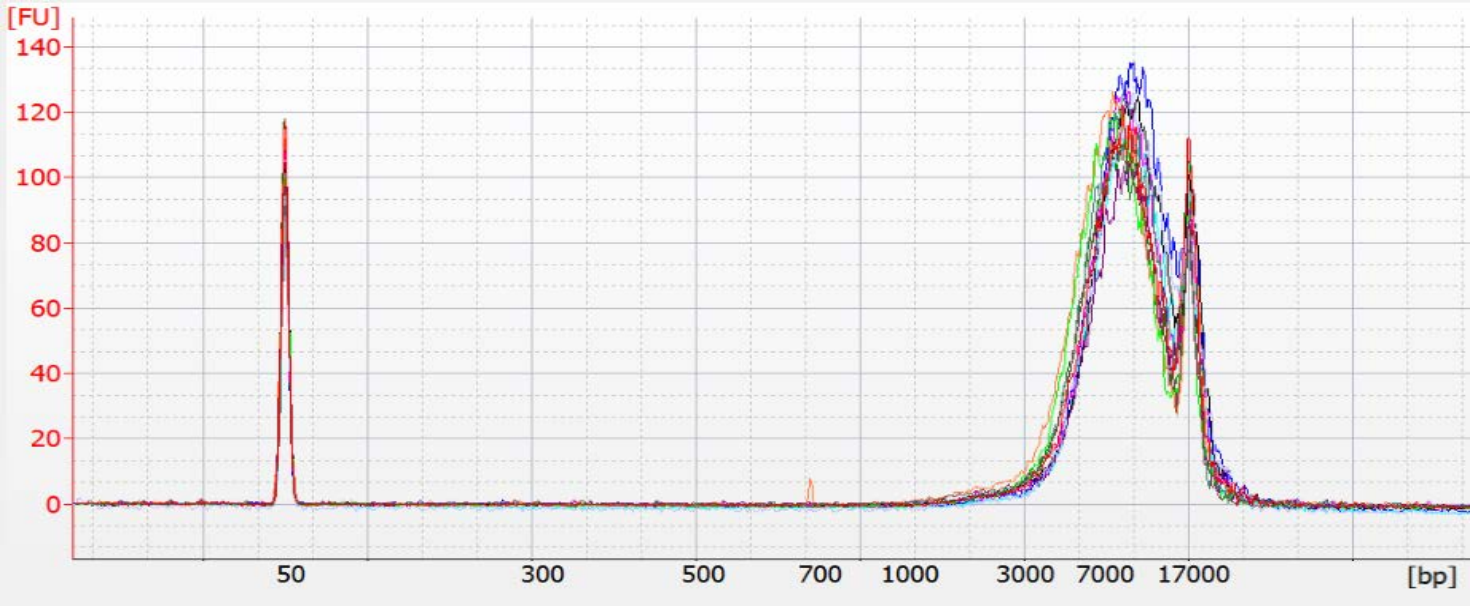

Figure 1: Bioanalyzer system traces of 10 kb shears for a 12-plex library. It is highly recommended to pool samples that have similar shear distribution.

DNA Quantification and Qualification

To minimize bias, samples for one pool should be similar in shear distribution (+/- 10%). Additionally, it is highly recommended to perform accurate quantification using the Bioanalyzer instrument and the Qubit fluorometric quantitation platform.

Workflow

In this procedure, each sample is sheared, quantified and Exo VII treated before going through DNA Damage Repair and End-Repair. After End-Repair, barcoded adapters are ligated to each sample. Following ligation, samples are pooled, treated by an Exo III and VII digestion, and two AMPure® PB bead purifications.

The workflow below summarizes this procedure.

Materials Needed

To perform this procedure, you must have the following:

- SMRTbell Barcoded Adapter Complete Prep Kit - 96
  - Barcoded Adapter Plate - 96
  - SMRTbell Barcoded Adapter Prep Kit
- SMRTbell DNA Damage Repair Kit (PacBio RSII System)
- SMRTbell DNA Damage Repair Kit-SPv3 (Sequel System)
- DNA/Polymerase Binding Kit (Sequel and RSII Systems)
- AMPure® PB Kit
- DNA Sequencing Kit (Sequel and RSII Systems)
- SMRT Cells for standard sequencing (Sequel and RSII Systems)

Barcoded Adapters

To perform this procedure, you must use the recommended barcoded adapters from the PacBio Barcoded Adapter Complete Prep Kit. The following 16 adapters are highly recommended for microbial multiplexing and were chosen based on a highest number of subreads and the best ScoreRatio or separation from other barcodes. In Table 2 below, there are two naming references, but refer to the same barcodes. In the first column, the number convention refers to a naming strategy that recommends barcodes for Sequel. The second column, refers to the original PacBio RSII convention. The third column represents the physical location of the recommended barcodes as shown in Table 3. Please ensure that you utilize the correct barcode fasta file for your analysis. In general, barcode fasta files are located at: http://www.pacb.com/products-and-services/consumables/multiplexing-amplicons/

New Naming Convention	Old Naming Convention	Well location
Master Filename: Sequel_RSII_16_barcodes_v1	**Original Filename: RSII_16_barcodes**	
BC1054	lbc12	D2
BC1093	lbc16	H2
BC1004	lbc25	A4
BC1080	lbc32	H4
BC1100	lbc35	C5
BC1109	lbc39	G5
BC1032	lbc42	B6
BC1063	lbc5	E1
BC1002	lbc51	C7
BC1070	lbc53	E7
BC1115	lbc58	B8
BC1016	lbc78	F10
BC1101	lbc87	G11
BC1055	lbc91	C12
BC1118	lbc92	D12
BC1048	lbc96	H12

Table 2: Recommended barcoded adapters

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ibc1	Ibc9	Ibc17	Ibc25	Ibc33	Ibc41	Ibc49	Ibc57	Ibc65	Ibc73	Ibc81	Ibc89
B	Ibc2	Ibc10	Ibc18	Ibc26	Ibc34	Ibc42	Ibc50	Ibc58	Ibc66	Ibc74	Ibc82	Ibc90
C	Ibc3	Ibc11	Ibc19	Ibc27	Ibc35	Ibc43	Ibc51	Ibc59	Ibc67	Ibc75	Ibc83	Ibc91
D	Ibc4	Ibc12	Ibc20	Ibc28	Ibc36	Ibc44	Ibc52	Ibc60	Ibc68	Ibc76	Ibc84	Ibc92
E	Ibc5	Ibc13	Ibc21	Ibc29	Ibc37	Ibc45	Ibc53	Ibc61	Ibc69	Ibc77	Ibc85	Ibc93
F	Ibc6	Ibc14	Ibc22	Ibc30	Ibc38	Ibc46	Ibc54	Ibc62	Ibc70	Ibc78	Ibc86	Ibc94
G	Ibc7	Ibc15	Ibc23	Ibc31	Ibc39	Ibc47	Ibc55	Ibc63	Ibc71	Ibc79	Ibc87	Ibc95
H	Ibc8	Ibc16	Ibc24	Ibc32	Ibc40	Ibc48	Ibc56	Ibc64	Ibc72	Ibc80	Ibc88	Ibc96

Table 3: Well location of the recommended barcodes in the Barcoded Adapter Plate – 96. The 16 recommended barcoded adapters are found in the Barcoded Adapter Plate – 96. Each well is sufficient for two barcoding reactions.

Fragment DNA using g-TUBE Device

It is recommended to shear the sample to 10 kb shears. The procedure below requires using Covaris g-TUBE device and the Eppendorf MiniSpin Plus Microcentrifuge. Other centrifuges may be used but speeds should be optimized to achieve proper shearing. The most up-to-date guidance on how to use the g-TUBE device, along with recommended centrifuges and centrifugation speeds, can be found in the g-TUBE device user manual available for download from the Covaris website.

Before shearing the DNA sample, be sure to have assessed the quality and purity. Test shears are recommended so that pooled samples have similar fragment distribution. Depending upon the quality of your sample, approximately 20% to 50% sample loss is to be expected as a result of the shearing and concentration process.

STEP	✓	Shear Genomic DNA	Notes
1		Load 1 µg DNA in a volume of 150 µL to top of g-TUBE device and close the cap firmly.	
2		Load g-TUBE device into Eppendorf MiniSpin Plus Microcentrifuge. Spin sample 2 minutes at 7,000 rpm. After 2 minutes, check to confirm all samples has flowed to bottom of tube. If samples still remain at top, continue the first spin (screw-cap up) until all the sample goes through.	
3		Invert the g-TUBE device and spin sample at same speed and duration.	
4		Recover the sample into a new 1.5 mL LoBind microcentrifuge tube.	

STEP	✓	Concentrate DNA	Notes
1		Add **0.45X** volume of AMPure PB magnetic beads. _____ 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 the bead/DNA solution thoroughly.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature. Note that the bead/DNA mixing is critical to yield. After vortexing, the bead/DNA mixture should appear homogenous. We recommend using a VWR vortex mixer with a foam microtube attachment (see the *Guide's* Overview section for part numbers). If using other instrumentation, ensure that the mixing is equally vigorous. Failure to thoroughly mix the DNA with the bead reagent will result in inefficient DNA binding and reduced sample recoveries.	
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. Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8 above.	
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 to 60 seconds.	
13		Elute the DNA off the beads in **20 µL** PacBio Elution Buffer. Mix until homogenous, then vortex for 10 minute at 2000 rpm.	
14		Add the Elution Buffer volume to your beads. – Thoroughly resuspend beads by vortexing for 10 minute at 2000 rpm. If the beads appear over-dried or cracked, let the Elution Buffer sit on the beads for 2 to 3 minutes then vortex again. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads. – Perform concentration measurements. Verify your DNA concentration using a Qubit® quantitation platform. When performing a Qubit system reading, ensure that your sample is within the range of the Qubit kit you are using. For proper concentration calculations, incorporate the dilution factor (used when diluting your sample) to be within range of the Qubit kit and the dilution factor when diluting your sample with the working solution. The latter part of this dilution factor can be calculated automatically by the Qubit system.	
15		Perform qualitative and quantitative analysis using a Bioanalyzer instrument. Note that the Bioanalyzer instrument has different kits in its offering and the appropriate kit, based on insert size, should be used. Dilute the samples appropriately before loading on the Bioanalyzer chip so that the DNA concentration loaded falls well within the detectable minimum and maximum range of the assay. Refer to Agilent Technologies' guides for specific information on the range of the specific kit you might be using. Note that typical yield, at this point of the process (i.e. post-shearing and after one **0.45X** AMPure PB bead purification), is approximately **50%**-**80%**.	
16		The sheared DNA can be stored for up to 24 hours at 4ºC or at -20ºC for longer duration.	
17		Actual recovery per µL and total available sample material: _____	

Exo VII Treatment of DNA

This step improves library quality by removing single-stranded ends from the DNA fragments.

Multiplex	DNA Input per Sample
2-plex	500 ng
4-plex	250 ng
6-plex	167 ng
8-plex	125 ng
10-plex	100 ng
12-plex	83 ng
16-plex	63 ng

1. If fragment distribution for each sample is the same, input the required amount of DNA (using the table above) into the Exo VII treatment reaction. Samples for one pool should be similar in length (+/- 10%) and concentration. **Use equimolar pooling if shear distribution is significant across samples**.

2. In a LoBind tube, prepare a master mix of the following reaction and store in ice. The master mix should be adjusted so that pipetting of each component is greater than 1 µl. Note that the master mix must be used immediately and should not be stored for future use.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Sheared DNA			__ µL			
DNA Damage Repair Buffer		10 X	0.63 µL	0.25 X		
NAD+		100 X	0.06 µL	0.25 X		
ATP high		10 mM	0.63 µL	0.25 mM		
dNTP		10 mM	0.06 µL	0.025 mM		
Exo VII		10 U/µL	0.13 µL	0.05 U/µL		
H_2O			__ µL to adjust to 25.0 µL			
Total Volume			25.0 µL			

3. Mix the reaction well by pipetting or flicking the tube.

4. Spin down contents of tube with a quick spin in a microfuge.

5. Incubate at 37ºC for 15 minutes, then return the reaction to 4ºC.

Repair DNA Damage

1. Dilute DNA Damage Repair Mix. Note that the diluted DNA Damage Repair Mix is one-time use only. The dilution below is sufficient for 16 samples.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA Damage Repair Mix		25 X	4.7 µL	6.5 X		
DNA Damage Repair Buffer		10 X	1.8 µL	1 X		
Molecular biology grade H_2O			11.5 µL			
Total Volume			18.0			

2. Prepare the DNA Damage Repair reaction. Aliquot 25 µL sample into a tube, followed by adding 1.0 µL of the diluted DNA Damage Repair Mix.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Sample (Exo VII treated)			25.0 µL			
DNA Damage Repair Mix		6.5 X	1.0 µL	0.25 X		
Total Volume			26.0 µL			

3. Mix each reaction well by pipetting or flicking the tube.
4. Spin down contents of tube with a quick spin in a microfuge.
5. Incubate at 37ºC for 60 minutes, return the reaction to 4ºC for 1 to 5 minutes.
6. Proceed with purification after this step.

STEP	✓	Purify DNA	Notes
1		Add **0.45X** volume of AMPure PB beads to the DNA Damage Repair reaction.	
2		Mix the bead/DNA solution thoroughly.	
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 shaking in a VWR vortex mixer at 2000 rpm for 10 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 bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above.	
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 to 60 seconds.	
13		Elute the DNA off the beads in **5 µL** Elution Buffer. – Mix until homogeneous. – Vortex for 10 minute at 2000 rpm. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads. **Note: You must elute in 5 µL to ensure that your samples are concentrated enough to go into the next steps of End-Repair and Ligation reactions. While it may be difficult to remove beads, any carry-over AMPure PB beads do not affect subsequent reactions.**	
15		The DNA (damage repaired) can be stored overnight at 4ºC or at -20ºC for longer duration.	
16		Actual recovery per µL and total available sample material: _____	

End-Repair

1. Make a master mix of the following reaction components and store in ice. The master mix should be adjusted so that pipetting of each component is greater than 1 µL.

Reagent	Tube Cap Color	Stock Conc.	For 1X	For '___' Samples + Overage	✓	Notes
Template Prep Buffer	○	10 X	1.0 µL	µL		
ATP high	●	10 mM	1.0 µL	µL		
dNTP	●	10 mM	0.1 µL	µL		
End Repair Mix	●	20 X	0.5 µL	µL		
Total Volume			2.6 µL	µL		

2. Mix by tapping the tube. Do a quick spin down of the tube. Note that the master mix must be used immediately and should not be stored for future use.
3. Transfer 2.6 µL aliquots of the above pre-mix to a 96-well plate or LoBind microcentrifuge tubes.
4. To the 2.6 µL, add sample and barcoded adapter in the order listed (mix after each addition) for a total of 10 µL per reaction. Keep reagents on ice at all times. Spin down to ensure the reactions are in the bottom of the wells or tubes.
   Any untouched adapter barcodes can be stored at -20ºC for future use. See the *User Bulletin - Barcode Plate Mapping* for additional information.

Component	Volume	Final Concentration
Sample	5.0 µL	
Barcoded adapter (20 µM)	2.5 µL	5.0 µM
Pre-Mix	2.6 µL	
Total	10.1 µL	

5. Incubate as follows:

Temperature	Time
25ºC	5 minutes
4ºC	hold

6. After the End-Repair reaction. You must proceed with Ligation Step.

Ligation

1. Prepare the ligase by diluting the Ligase to 10 U/µL with Molecular Biology grade water. Dilute the ligase right before it is added to the reaction. The reaction table below for the diluted ligase is enough for 16 samples. Note: The diluted ligase is for one time use only.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Ligase		30 U/µL	7.0 µL	10 U/µL		
Molecular biology grade H20			14.0 µL			
Total Volume			21.0 µL			

2. To each end-repaired sample, add 1.2 µL of diluted ligase for a total volume of 11.2 µL.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (End Repaired Treated)			10.0 µL			
Ligase		10 U/µL	1.2 µL	1.0 U/µL		
Total Volume			11.2 µL			

3. This step allows ligation at 25ºC for 20 minutes, followed by ligase heat inactivation at 65ºC. The ligation step may be increased up to overnight. The thermal cycler may be used in this step for convenience. Incubate as follows:

Temperature	Time
25ºC	20 minutes
65ºC	10 minutes
4ºC	hold

4. After the Ligation reaction, **pool** the 11.2 µL reactions and measure the total volume.

5. Proceed with purification after this step.

STEP	✓	Purify DNA	Notes
1		Add **1X** volume of AMPure PB beads to the Ligation reaction.	
2		Mix the bead/DNA solution thoroughly.	
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 shaking in a VWR vortex mixer at 2000 rpm for 10 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 bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above.	
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 to 60 seconds.	
13		Elute the DNA off the beads in **36 µL** Elution Buffer. – Mix until homogeneous. – Vortex for 10 minute at 2000 rpm. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads.	

Exo III and VII Digestion

This step improves library quality by removing failed ligation products.

1. In a LoBind microcentrifuge tube, add the following reagents:

Reagent	Tube Cap Color	Stock Conc.	✓	Volume
Ligated DNA				36.0 µL
Exo III		100.0 U/µL		1.0 µL
Exo VII		10.0 U/µL		1.0 µL
Template Prep Buffer		10 X		4.0 µL
Total Volume				42.0 µL

2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37ºC for 1 hour, then return the reaction to 4ºC. Do not exceed the 1-hour incubation time.
4. You must proceed with purification after this step.

STEP	✓	Purify SMRTbell™ Templates - First Purification	Notes
1		Add **0.45X** volume of AMPure PB beads to the exonuclease-treated reaction.	
2		Mix the bead/DNA solution thoroughly.	
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 shaking in a VWR vortex mixer at 2000 rpm for 10 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 bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol and dry the bead pellet. – 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 to 60 seconds.	
13		Elute the DNA off the beads in **100 µL** of Elution Buffer. – Mix until homogeneous. – Vortex for 10 minute at 2000 rpm. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads.	

STEP	✓	Purify SMRTbell™ Templates - Second Purification	Notes
1		Add **0.45X** volume to the **100 µL** of eluted DNA.	
2		Mix the bead/DNA solution thoroughly.	
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 shaking in a VWR vortex mixer at 2000 rpm for 10 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 bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol and dry the bead pellet. – 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 to 60 seconds.	
13		Elute the DNA off the beads in **11 µL** of Elution Buffer. – Mix until homogeneous. – Vortex for 10 minute at 2000 rpm. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads.	
14		Verify your DNA amount and concentration with a HS Qubit fluorometric quantification platform reading. For typical library, final yield, expect 20-25% (based on DNA input after pooling).	
15		Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit.	

Sequel System

Anneal and Bind SMRTbell™ Template

Using the Sequel Binding Calculator, compute the molarity of your library by using an average insert size from the Bioanalyzer instrument.

Anneal Sequencing Primer v3 at a template concentration of 0.833 nM, as directed by the Binding Calculator. Add the appropriate amounts of pre-conditioned primer and 10X Primer Buffer to the SMRTbell template, and incubate at 20ºC for 60 minutes.

Bind the Sequel Polymerase at a concentration of 0.500 nM, for 4 hours at 30ºC. Instructions for polymerase binding are provided by the calculator.

Prepare for MagBead Loading

Optimal loading of microbial multiplexed templates can typically be achieved using an on-plate concentration of 5 pM to 15 pM. We recommend you perform an initial loading titration in this range to determine optimal loading for your sample.

For binding to Magnetic Beads, bind for 1 hour at 4ºC. Follow the Binding Calculator for instructions on preparing samples on the Sequel.

RS II System

Anneal and Bind SMRTbell™ Template

Using the RSII Binding Calculator (v2.3.1.1), compute the molarity of your library by using an average insert size from the Bioanalyzer instrument.

Anneal Sequencing Primer v2 at a template concentration of 0.833 nM, as directed by the Binding Calculator. Add the appropriate amounts of pre-conditioned primer and 10X Primer Buffer to the SMRTbell template, and incubate at 20ºC for 30 minutes.

Bind the P6 Polymerase at a concentration of 0.500 nM, for 30 minutes at 30ºC. Instructions for polymerase binding are provided by the calculator.

Prepare for MagBead Loading

Optimal loading of microbial multiplexed templates using P6 polymerase can typically be achieved using an on-plate concentration of 15 pM to 30 pM. We recommend you perform an initial loading titration in this range to determine optimal loading for your sample.

For binding to Magnetic Beads, bind for 30 minutes at 4ºC. Follow the Binding Calculator for instructions on preparing samples on the RSII.

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