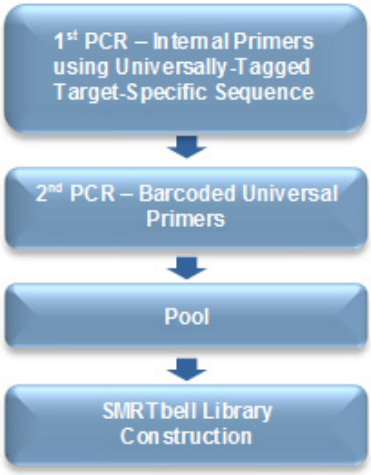
Procedure & Checklist - Preparing SMRTbell™ Libraries using PacBio® Barcoded Universal Primers for Multiplex SMRT® Sequencing

Before You Begin

This document describes methods for generating barcoded PCR products using PacBio Barcoded Universal Primers (BUP) and subsequently constructed to SMRTbell libraries.

The procedure provides recommendations for amplifying targets using primers tailed with two different universal sequences. The amplified products are further amplified using barcoded universal primers. The barcoded PCR products are pooled for SMRTbell library construction and subsequently sequenced on the PacBio system.

The workflow below summarizes this procedure.



To perform this procedure, you must have the following:

- PacBio Barcoded Universal F/R Primers Plate - 96
- PacBio Template Prep Kit
- PacBio DNA/Polymerase Binding Kit
- PacBio AMPure® PB Kit
- PacBio DNA Sequencing Kit
- PacBio SMRT Cells for standard sequencing
- Template-specific Universal Primers (Customer-supplied)
- High Fidelity DNA Polymerase (Thermo Scientific Phusion Hot Start II DNA Polymerase Part Number F-549L is recommended)

Designing Target Specific Primers Tailed with Universal Sequences

Primers can be ordered from any oligo synthesis provider. It is highly recommended to meet the following requirements for designing primers.

1. Add a 5' block (eg., 5' NH4-C6) to ensure that carry over amplicons, from first round PCR, are not ligated to the SMRTbell adapters in subsequent steps.
2. HPLC purified.
3. Use the following primer format:

Primer Type	Universal Sequence	Template-Specific Sequence	Primer to Order
Forward_Internal_PCR_Primer	/5AmMC6/ gcagtcgaacatgtagctgactcaggtcac	FOR EXAMPLE1	/5AmMC6/ gcagtcgaacatgtagctgactcaggtcacFOR EXAMPLE1
Reverse_Internal_PCR_Primer	/5AmMC6/ tggatcacttgtgcaagcatcacatcgtag	FOR EXAMPLE2	/5AmMC6/ tggatcacttgtgcaagcatcacatcgtagFOR EXAMPLE2

Sample Normalization

It is highly recommended to normalize samples going into the second-round PCR step. Additionally, PCR products from the second round PCR should be normalized. Without equivalent concentrations or mass across samples, there is a risk of uneven coverage in the sequence data.

For the second round of PCR:

1. Samples should first be loaded on an agarose gel for visual inspection. If samples show excess dimers or non-specific products, a purification step is necessary. If the non-specific products are not removed, re-optimization may be necessary. However, if by visual inspection, samples show clean profiles, samples may be added to the second round of PCR in equivalent volumes.
2. The amount of PCR product for use in second round of PCR should be equimolar across samples. This requires sample purification followed by concentration measurements using a Qubit system or picogreen. quantitation assay.
3. Samples may be purified using column-based or AMPure PB bead purification steps to remove primer dimers, short non- specific products or excess dNTPs present in the samples.

Pooling after the second round of PCR:

After the second round of PCR, pool samples in equimolar amounts or according to visual inspection of PCR samples on an agarose gel.

1. For best results, samples may be purified with AMPure PB bead or column purification steps, followed by quantification using a Qubit system or picogreen quantitation assay. Equimolar amounts of samples can then be pooled for SMRTbell library preparation. This is recommended for pooling PCR products of varying insert lengths.
2. Another option is to pool, by visual inspection, on an agarose gel. If samples are of equivalent size (+/-10% of each other) and they have equivalent intensity on an agarose gel, it is acceptable to pool equal volumes of the PCR products. If some samples look weak on the agarose gel, then > 2X or more may be necessary to bepooled.

First Round of PCR: Recommendations for Target Specific Primers Tailed with Universal Sequence

The procedure below was optimized using high fidelity Phusion Hot Start II for PCR. Other high fidelity polymerases may be used. We recommend optimizations before proceeding.

1. Prepare the following reaction per sample.

Component	Stock Concentration	Final Concentration	1X
HPLC Water			13.75
PHusion Buffer HF	5X	1X	5
dNTP	2 mM	0.2 mM	2.5
DNA	1 ng/µL[a]		1
Universal sequence tagged Primer 1[b] and Primer 2[b]	2 µM each	0.2 µM	2.5
Phusion HF	2 U/µL		0.25
Total Volume			25

a. General guideline: Optimization is recommended to determine the appropriate amount of template.
b. Five prime end must be blocked (e.g., 5' NH4-C6)

2. Mix gently by tapping the tube. Quick spin the tube.
3. Perform amplification using the following cycling parameters. Amplification depends on the sample and the target specific primers. Optimization is highly recommended.

Step	Temperature	Time
1	98ºC	30 seconds[a]
2	98ºC[b]	15 seconds
3	N[c]	15 seconds
4	72ºC	X min[d]
5	Repeat steps 2 to 4 (20 cycles total)[e]	-
6	72ºC	7 minutes
7	4ºC	Hold

a. Initial denaturation is dependent on template complexity (30 seconds – 3 minutes).
b. Temperature is dependent on the polymerase.
c. N in step 3 is defined by the user depending on Primers 1 and 2.
d. X in step 4 is defined by the user depending on the insert size.
e. The number of cycles in step 5 can be increased to 20-25 cycles for visual inspection on an agarose gel.

4. After amplification, perform visual inspection of the PCR products on an agarose gel.
5. Proceed to the second round of PCR.

Second Round PCR: Recommendations for Target Specific Primers Tailed with Universal Sequence

The procedure below was optimized using high fidelity Phusion Hot Start II for PCR. Other high fidelity polymerases may be used. We recommend to perform optimizations before proceeding.

For even coverage in the sequence data, sample normalization is highly recommended.

1. Prepare the following reaction per sample:

Component	Stock Concentration	Final Concentration	1X
HPLC Water			13.75
PHusion Buffer HF	5X	1X	5
dNTP	2 mM	0.2 mM	2.5
Round 1 PCR	1 ng/µL[a]		1
BUP Primers[b]	2 µM each	0.2 µM each	2.5
Phusion HF	2 U/µL	0.02 U/µL	0.25
Total Volume			25

a. General guideline: Optimization is recommended to determine the appropriate amount of round 1 PCR product.
b. Barcoded Universal Forward and Reverse primers from the 96-well barcode plate.

2. Mix gently by tapping the tube. Quick spin the tube.
3. Perform amplification using the following cycling parameters. Amplification depends on the sample and the target specific primers. Optimization is highly recommended.

Step	Temperature	Time
1	98ºC	30 seconds
2	98ºC	15 seconds
3	64ºC[a]	15 seconds
4	72ºC	X min[b]
5	Repeat steps 2 to 4 (20 cycles total)[c]	-
6	72ºC	7 minutes
7	4ºC	Hold

a. Recommended for Barcoded Universal Primers.
b. Defined by the user depending on the insert size.
c. The number of cycles can be increased to 20-25 cycles for visual inspection on an agarose gel.

4. After amplification, perform visual inspection of the PCR products on an agarose gel.
5. Proceed to pooling. See pooling tips on page 2.
6. Depending on the number of samples and the yield per well, 1-5 µg of DNA can be recovered. Proceed to SMRTbell library construction starting with DNA Damage Repair.

STEP	✓	Purify DNA	Notes
1		Add the **appropriate** volume of AMPure PB beads to the pooled PCR product.	
2		Mix the bead/DNA solution thoroughly by tapping the tube.	
3		Quickly spin down the tube 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.	
5		Spin down the tube 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 the 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 **37 µL** Elution Buffer. Mix until homogeneous, then vortex for 1 minute at 2000 rpm.	
14		Measure concentration using Qubit system or picogreen quantitation assay.	
15		The eluted DNA can be stored overnight at 4ºC or at -20ºC for longer duration.	

SMRTbell Library Construction

The amount of DNA required in this step depends on the number of SMRT Cells required for a project. The table below summarizes the total DNA input guidelines for SMRTbell library construction.

Library Size	Total Input DNA Requirement
< 1 kb	250 ng
1 - 3 kb	500 ng
3 - 10 kb	1 - 5 µg

Repair DNA Damage

Use the following table to repair any DNA damage.

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

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA	–		__ µL for 1.0 to 5.0 µg	–		
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		
DNA Damage Repair Mix			2.0 µL			
H_2O	–		__ µL to adjust to 50.0* µL	–		
Total Volume			50.0 µL	–		

*To determine the correct amount of H_2O to add, use your actual DNA amount noted in the Notes column.

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 37ºC for 20 minutes or longer, then return the reaction to 4ºC for 1 minute.

Repair Ends

Use the following table to prepare your reaction then purify the DNA.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (Damage Repaired)	–		50 µL	–		
End Repair Mix		20 X	2.5 µL	1X		
Total Volume			52.5 µL	–		

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25ºC for 5 minutes, then return the reaction to 4ºC.

STEP	✓	Purify DNA	Notes
1		Add the **appropriate** volume of AMPure PB beads to the pooled PCR product.	
2		Mix the bead/DNA solution thoroughly by tapping the tube.	
3		Quickly spin down the tube 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.	
5		Spin down the tube 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 the 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 **30 µL** Elution Buffer. Mix until homogeneous, then vortex for 1 minute at 2000 rpm.	
14		The eluted DNA can be stored overnight at 4ºC or at -20ºC for longer duration.	

Prepare Blunt-Ligation Reaction

Use the following table to prepare your blunt-ligation reaction:

1. In a LoBind microcentrifuge tube (on ice), add the following reagents in the order shown. If preparing a Master Mix, ensure that the adapter is NOT mixed with the ligase prior to introduction of the inserts. Add the adapter to the well with the DNA. All other components, including the ligase, should be added to the Master Mix.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (End Repaired)	–		29.0 µL to 30.0 µL			
Annealed Blunt Adapter (20 µM)		20 µM	1.0 µL	0.5 µM		
Mix before proceeding						
Template Prep Buffer		10 X	4.0 µL	1X		
ATP low		1 mM	2.0 µL	0.05 mM		
Mix before proceeding						
Ligase		30 U/µL	1.0 µL	0.75 U/µL		
H_2O	–	–	__ µL to adjust to 40.0 µL	–		
Total Volume	–	–	40.0 µL	–		

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 25ºC for 15 minutes. At this point, the ligation can be extended up to 24 hours to maximize ligation efficiency or cooled to 4ºC (for storage of up to 24 hours).
5. Incubate at 65ºC for 10 minutes to inactivate the ligase, then return the reaction to 4ºC. You must proceed with adding exonucleases after this step.

Exonuclease Digestion

Add exonuclease to remove failed ligation products.

Reagent	Tube Cap Color	Stock Conc.	✓	Volume
Ligated DNA				40 µL
Mix reaction well by pipetting				
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 pipetting or flicking the tube.
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. You must proceed with purification after this step.

Purify SMRTbell™ Templates

There are 2 purification steps. The AMPure PB bead concentration for both steps depends on the library size. Use the recommended concentrations shown below:

Library Size	X AMPure PB Beads
250 bp	1.8X
500 bp	1.0X
1 -3 kb	0.6X
3 - 10 kb	0.45X

STEP	✓	Purify SMRTbell™ Templates - First Purification	Notes
1		Add the **appropriate** 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 **50 µL** of Elution Buffer. Vortex for 1 minute at 2000 rpm.	

STEP	✓	Purify SMRTbell™ Templates - Second Purification	Notes
1		Add the **appropriate** volume of AMPure PB beads to the **50 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 **8 - 10 µL** of Elution Buffer. – Mix until homogeneous. – Vortex for 1 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 on a Bioanalyzer instrument using a High Sensitivity DNA Analysis Kit, or check quantitation using Qubit dsDNA HS Assay Kit. If there is too little sample, estimate the concentration based on 10% yield of input amount into the damage repair step.	

Control Complex Dilution

You must have the PacBio Control Complex for this step. Dilute the Control Complex according to the volumes and instructions specified in the Calculator.

Anneal and Bind SMRTbell™ Templates

Before adding the primer to the SMRTbell template, the primer must go through a melting step at 80ºC. This avoids exposing the sample to heat. The template and primer mix can then be incubated at 20ºC for 30 minutes. Note that you must have the PacBio DNA/Polymerase Kit and use LoBind microcentrifuge tubes for this step.

For polymerase binding, incubation at 30ºC for 30 minutes is sufficient. Instructions for polymerase binding are provided by the calculator.

For more information about using the Binding Calculator, see the Pacific Biosciences *Template Preparation and Sequencing Guide* and *QRC - Annealing and Binding Recommendations*.

Sequence

To prepare for sequencing on the instrument, refer to the *RS Remote Online Help* system or *Pacific Biosciences Software Getting Started Guide* for more information. Follow the touchscreen UI to start your run. Note that you must have a DNA Sequencing Kit and SMRT® Cells for standard sequencing.