Procedure & Checklist - Preparing SMRTbell® Libraries using PacBio® Barcoded Universal Primers for Multiplexing Amplicons

Before You Begin

The procedure describes a 2-step PCR for generating up to 96 barcoded amplicons, with the first PCR step requiring internal primers that contain a combination of universal and target-specific sequences. The target-specific primers tailed with universal sequences are designed and supplied by users. In the second PCR step, barcodes are incorporated by using universal sequences tailed with 16-bp PacBio barcode sequences. A ready-to-use reagent kit containing 96 Barcoded Universal Primers (BUP) in a plate format is commercially available from Pacific Biosciences. Once the PCR products are barcoded, they can be pooled into a single tube for SMRTbell library construction with the SMRTbell Express Template Prep Kit 2.0.

Figure 1 below summarizes the amplification workflow.

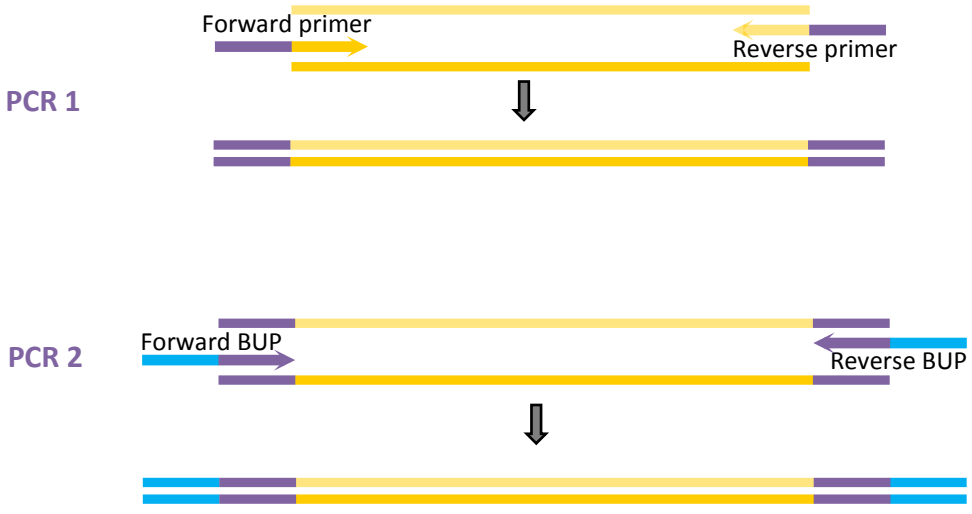

Figure 1: The barcode is incorporated into the PCR amplicon via a two-step tailed PCR approach. The first step is to amplify the region of interest with target-specific primers tailed with Forward and Reverse universal sequences. The second step requires re-amplification of the PCR product with Barcoded Universal Primers. There are 96 Barcoded Universal Primers available from Pacific Biosciences. Purple regions correspond to Universal sequences and Blue regions corresponds to 16-bp Barcode sequences.

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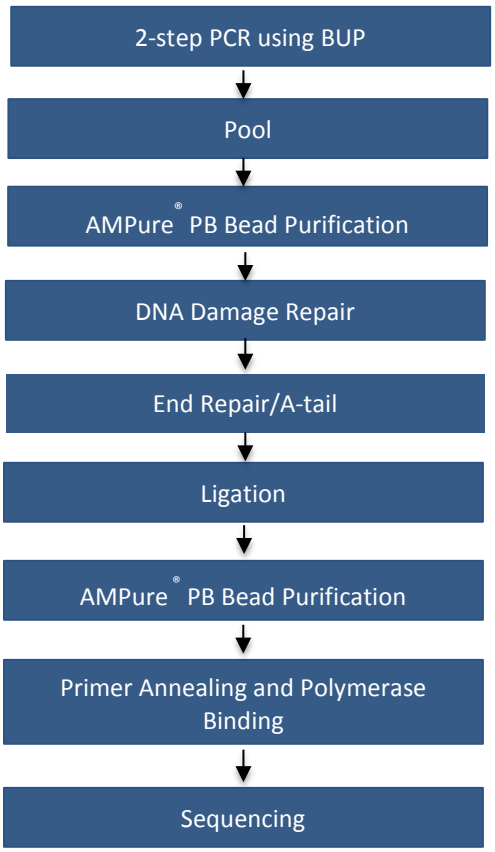

Figure 2: Workflow for preparing multiplexed amplicon libraries using the SMRTbell Express Template Prep Kit 2.0

Required Materials

Item	Where Used	Vendor	Part Number
Target-Specific Primers tailed with F/R Universal Sequences (Customer-supplied)	Amplification (1st-Round)	Oligo Synthesis Company	N/A
PacBio Barcoded Universal Primers	Amplification (2nd-Round)	PacBio	101-629-100
Phusion Hot Start II High Fidelity PCR Master Mix	Amplification	Thermo Scientific	F-565
SMRTbell Express Template Prep 2.0	Library Prep	PacBio	100-938-900
AMPure® PB Kit	Purification	PacBio	100-265-900
8- or 12-Multichannel Pipettor	High Throughput Pipetting	any	Vendor-specific

Table 1. List of Required Materials and Equipment.

Designing Target-Specific Primers Tailed with Universal Sequences

PacBio highly recommends complying with the following requirements when ordering oligos from your oligo synthesis provider:

1. The 5' end of the primer **must** be blocked (eg., 5AmMC6) to prevent amplicons carried over from the first round of PCR from forming SMRTbells during library construction, which can reduce the yield of barcoded reads during sequencing.
2. Oligos are desalted.
3. Use the following primer format:

Primer Type	Universal Sequence	Target-Specific Primer	Primer to Order
Forward_Internal_PCR_Primer	/5AmMC6/ gcagtcgaacatgtagctgactcaggtcac	FOR_EXAMPLE1	/5AmMC6/ gcagtcgaacatgtagctgactcaggtcac FOR_EXAMPLE1
Reverse_Internal_PCR_Primer	/5AmMC6/ tggatcacttgtgcaagcatcacatcgtag	REV_EXAMPLE2	/5AmMC6/ tggatcacttgtgcaagcatcacatcgtag REV_EXAMPLE2

Table 2. Recommended Primer Format for Ordering Oligos

Best Practices for Generating High-Quality PCR Products

Clean, target-specific PCR products are extremely important for obtaining high-quality sequence data. Non-specific products can represent a substantial percentage of the sequencing reads if they are not removed. To minimize the presence of non-specific products, consider the following recommendations for generating high-quality amplicons suitable for SMRTbell library preparation and sequencing.

1. Begin with high-quality nucleic acids and work in a clean environment.
   a. If extracted nucleic acids must be stored, freeze at high concentrations in appropriately-buffered solutions.
   b. To minimize possible contamination and degradation caused by multiple freeze/thaw cycles, sub-aliquot DNA into smaller volumes for storage. For DNA samples, DNAStable® Plus from Biomatrica may be used to help preserve extracted DNA.
   c. Set up PCR reactions in an environment free from sources of non-specific primer and template contaminants; ideally a laminar flow hood, using dedicated pre-PCR pipettor, tips and reagents.
2. Use PCR reagents and conditions for generating target-specific, full length amplicons.
   a. Use the highest fidelity polymerase compatible with your PCR amplification system.
   b. Use desalted or HPLC-purified oligos; damaged bases at the ends of the amplicons cannot be repaired by DNA Damage Repair enzymes.
   c. Optimize PCR conditions to minimize total time spent at high (>65°C) temperatures, particularly during denaturation.
   d. PCR extension time should be long enough to ensure complete extension, taking into consideration the polymerase used and target amplicon size. For mixed samples with similar targets, it is important to complete extension at every step to avoid generating chimeric products in subsequent steps. As a general guideline, use extension times of one minute per 1000 base pairs (e.g. 3 minutes for a 3 kb product).
3. Use the lowest number of cycles required for obtaining adequate yields (ng) of PCR products to proceed with SMRTbell library construction. Avoid over-amplification.
4. If non-specific products are present, optimize PCR conditions or perform AMPure PB Bead-based size selection to enrich for PCR amplicons with the desired target size (see recommendations below).

Recommendations for AMPure PB Purifications

It is highly recommended to purify amplicons before SMRTbell library construction to remove PCR reagents, buffers, primer dimers or short non-specific PCR products. Depending on the amplicon size, the required concentration (volumetric ratio) of AMPure PB beads for purification varies. Use the table below to determine the appropriate concentration of AMPure PB beads to use for your sample.

Amplicon Size	AMPure PB Bead Ratio
250-500 bp	1.8X
500-1000 bp	1.0X
1-3 kb	0.6X
3-10 kb	0.45X
15 kb	0.45X

Table 3. AMPure PB Bead Recommendations Based on Amplicon Size

DNA Input Requirements for Library Construction

When planning your amplification experiments, always consider the total input DNA required for library construction. The table below summarizes the total mass of PCR product required to go into the DNA Damage Repair step.

Amplicon Size	Input DNA Amounts per Pool (ng)
250-500 bp	250 - 500
500-1000 bp	250 - 500
1-3 kb	500 - 1000
3-10 kb	1000 - 2000
15 kb	1500 - 3000

Table 4. Input DNA Recommendations Based on Amplicon Size

If necessary, replicate PCR reactions should be set up to obtain the required amount of DNA product. This approach also minimizes PCR sampling bias for samples containing heterogeneous templates.

Note that if barcoded samples are to be pooled prior to library preparation, the input requirements shown in Table 4 refer to the total pooled mass of DNA, not the mass of individual members of the pool. Samples should be present at equimolar concentrations after pooling (see Best Practices for Equimolar Pooling below).

Best Practices for Equimolar Pooling

For studies targeting a single consensus sequence per sample, amplicons may be multiplexed to leverage the throughput capacity of a single SMRT Cell. However, pooling is generally recommended for amplicons of similar sizes (i.e., within +/-15% of the mean size).

1. Ideally, amplicons should be AMPure PB bead purified prior to multiplexing. See the section “Concentrate PCR products using AMPure PB Beads”.
2. To obtain equal representation of each amplicon in the data, it is important to pool samples in equimolar amounts. To do this, purify with AMPure PB and quantify each amplicon using the Agilent® Bioanalyzer System, Agilent TapeStation, or Advanced Analytical Technologies Fragment Analyzer™ system.
3. Remove non-specific PCR products (contaminating bands) prior to pooling. The presence of non-specific products in the pool will impact sequencing data yield.
   a. If amplicons contain secondary bands that are <1.5 kb, it may be possible to remove them from >3 kb amplicons using AMPure PB bead purification using an appropriate concentration of beads.
   b. If the contaminating bands are close in size to or are larger than the desired amplicon, or are greater than 1.5 kb (i.e, they can’t be removed by AMPure PB purification), size selection using an automated size selection tool or other gel-based method may be necessary.
   c. If removal of contaminating bands is not possible, we recommend re-optimization of the amplification reaction using more stringent PCR conditions.
   d. Always determine the concentration of the amplicon **target band** or **peak only**, and use this value to calculate the mass or volume of the amplicon sample to be used during pooling.
   e. If presence of contaminating bands is determined to be acceptable (i.e., their presence has minimum impact on the sequencing yield of the desired target), you may choose to include the amplicon in the sample pool. In such cases, however, it may be necessary to increase the relative input amounts of such amplicons (with non-specific products) during pooling in order to achieve adequate sequencing data yields for each amplicon in the sample pool.
   f. Always determine the concentration of the **target band** or **peak**.
4. For higher multiplexing (eg. 96-plex), purifying and quantifying each individual PCR product may be difficult or impractical. A QC method that may work well is to load samples on an agarose gel to view the PCR products prior to pooling. This QC method may work well if PCR conditions are fully optimized to generate clean specific PCR products consistently.
   a. PCR products that show the same band intensity on a gel may be pooled by volume or mass. To do this, include control fragments of known concentrations when loading samples to perform agarose gel electrophoresis. The pooled samples must meet the minimum input requirements listed on Table 4.
   b. For samples that show weak signals on a gel, increase the volume or mass used during pooling.

First-Round PCR Using Target Specific Primers Tailed with a Universal Sequence

The procedure below was optimized using high fidelity Phusion Hot Start II for PCR. Other high-fidelity polymerases may be used. PCR optimizations are highly recommended before proceeding with the second-round PCR step.

1. Prepare a master mix by including 25% overage to account for pipetting errors.

   Note: Use the master mix set-up below for experiments requiring amplifications of the same target region (same locus) on different samples. For experiments requiring **different** target regions (different loci), individual PCR reactions using locus-specific universal sequence-tailed primers must be performed by following the 1X reaction in the table below.

Component	Stock Concentration	Final Concentration	1X	N Plex
Nuclease-Free Water			9.5	N x 9.5 x 1.25
Phusion HSII High Fidelity PCR Master Mix	2X	1X	12.0	N x 12 x 1.25
Universal sequence-tailed Primer 1[a] and Primer 2[a]	2 µM each	0.2 µM	2.5	N x 2.5 x 1.25
Total Volume			24.0	

a. 5' end must be blocked (e.g., 5AmMC6)

2. Mix gently by tapping the tube. Quick spin the tube.
3. Transfer 24 µL of the master mix to appropriate PCR tubes.
4. Add 1 µL DNA (1 ng/µL) to the PCR mix. Optimization is highly recommended to determine the appropriate amount of DNA template to use.
5. Mix gently by tapping the tubes. Quick spin the tubes.
6. Perform amplification using the following cycling parameters. Success of the amplification depends on the specific sample and primers used; therefore, optimizations are 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. 1 min should be allowed for every 1 kb of the target product.
e. The number of cycles in step 5 can be increased to 20-25 cycles for visual inspection on an agarose gel.

7. After amplification, perform visual inspection of the PCR products on agarose gel.
8. Proceed to the second-round of PCR.

Second-Round PCR Using Barcoded Universal Primers

The procedure below was optimized using high fidelity Phusion Hot Start II for PCR. Other high-fidelity polymerases may be used. Optimizations are highly recommended before proceeding with SMRTbell library construction.

1. Prepare a master mix by including 25% overage to account for pipetting errors. The BUP primers are not added in the master mix. They are added to each tube in step 4 below.

Component	Stock Concentration	Final Concentration	1X	N Plex[a]
Nuclease-free Water			10.75	N x 10.75 x 1.25
Phusion HSII High Fidelity PCR Master Mix	2X	1X	10.75	N x 10.75 x 1.25
Total Volume			21.5	

a. Include 25% overage in the Master Mix to account for pipetting errors.

2. Mix gently by tapping the tube. Quick spin the tube.
3. Transfer 21.5 µL of the master mix to appropriate PCR tubes.
4. Add 2.5 µL of the Barcoded Universal Primer to each tube.
5. Add 1 µL of Round 1 PCR product (1ng/µL) to the PCR mix. Optimization is highly recommended to determine the appropriate amount of round 1 PCR product to use.
6. Mix gently by tapping the tubes. Quick spin the tubes.
7. Perform amplification using the following cycling parameters. Success of the amplification depends on the specific sample and primers used, therefore, optimizations are 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.

8. After amplification, perform visual inspection of the PCR products on an agarose gel.
9. Proceed to pooling.

Sample Pooling

STEP	✓	Sample Pooling	Notes
1		Refer to Table 4 on page 4 for minimum and maximum DNA input of the pooled sample for library construction. The input requirements depend on average insert size.	
2		Pool samples equimolar. See “Best Practices for Equimolar Pooling” for recommendations for pooling samples.	

Concentrate Pooled PCR Amplicons Using AMPure PB Beads

Refer to Table 3 on page 4 for recommendations for AMPure purification per amplicon size. If the total volume of the pooled sample is <100 µL, bring the volume to a minimum volume of 100 µL with 1X Elution Buffer.

STEP	✓	Concentrate DNA	Notes
1		Add the appropriate volume of AMPure PB beads to the pooled amplicons. The required volume depends on amplicon size. Refer to Table 3. 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. 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 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 to use for elution is **48 µL** (the volume to go into “DNA Damage Repair”) plus 1 µL for QC – see step 14 below).	

13		Add the Elution Buffer volume to your beads. Pipette mix 15 times. 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 the dsDNA HS Assay kit according to the manufacturer's recommendations.	
15		Actual recovery per µL and total available sample material: ____	

SMRTbell Library Construction

The amount of DNA required in this step depends on the size of the amplicons. See Table 4 for input requirements.

DNA Damage Repair

1. Prepare the following reaction.

Reagent (Reaction Mix 1)	Tube Cap Color	Volume	✓	Notes
DNA Prep Buffer		7.0 µL		
Pooled and Purified PCR Product		47.0 µL		
NAD		1.0 µL		
DNA Damage Repair Mix v2		2.0 µL		
Total Volume		57.0 µL		

2. Pipette mix 10 times. It is important to mix well.
3. Spin down the contents of the tube with a quick spin in a microfuge.
4. Incubate at 37°C for 30 minutes, then return the reaction to 4°C. Proceed to the next step.

End-Repair/A-tailing

1. Prepare the following reaction.

Reagent (Reaction Mix 2)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 1		57.0 µL		
End Prep Mix		3.0 µL		
Total Volume		60.0 µL		

2. Pipette mix 10 times. It is important to mix well.
3. Spin down the contents of the tube with a quick spin in a microfuge.
4. Incubate at 20°C for 30 minutes.
5. Incubate at 65°C for 30 minutes, then return the reaction to 4°C. Proceed to the next step.

Adapter Ligation

1. Prepare the following reaction, adding the components below in the order listed.

Reagent (Reaction Mix 3)	Tube Cap Color	Volume	✓	Notes
Reaction Mix 2		60.0 µL		
Overhang Adapter v3		5.0 µL		
Ligation Mix		30.0 µL		
Ligation Additive		1.0 µL		
Ligation Enhancer		1.0 µL		
Total Volume		97.0 µL		

1. Pipette mix 10 times. It is important to mix well.
2. Spin down the contents of the tube with a quick spin in a microfuge.
3. Incubate at 20°C for 60 minutes, then return the reaction to 4°C. Proceed to the next step.

Purification of SMRTbell Templates

Bring to a volume of 100 µL with 1X EB before AMPure PB bead purification. For AMPure PB bead concentration guidelines, refer to Table 3.

STEP	✓	Purify DNA	Notes
1		Add the appropriate volume of AMPure PB beads to the pooled amplicons. The required volume depends on amplicon size. Refer to Table 3. 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		Mix the bead/DNA solution thoroughly by pipette mixing 15 times. 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 a 1.5 mL DNA LoBind 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 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		Immediately add **20 µL** of Elution Buffer volume to your beads. Pipette mix 15 times. It is important to mix well. – Elute the DNA by letting the mix incubate at 37 ºC 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		Actual recovered DNA SMRTbell concentration (ng/µl): _____ Total recovered DNA SMRTbell amount (ng): _____	

Anneal and Bind SMRTbell Library 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 Express Template Prep Kit 2.0).

Prepare for Sequencing

For the Sequel System, Diffusion loading is recommended for loading amplicon libraries prepared by the SMRTbell Express Template Prep Kit 2.0.
Follow the Sample Setup in SMRT Link for preparing your sample for sequencing.

For detailed recommendations for sequencing of specific library insert size ranges, refer to the Quick Reference Card – Diffusion Loading and Pre-Extension Time Recommendations for the Sequel System here.

Revision History (Description)	Version	Date
BETA DRAFT	BETA	May 2019
Initial Release	1	June 2019