Procedure & Checklist - Amplicon Template Preparation and Sequencing

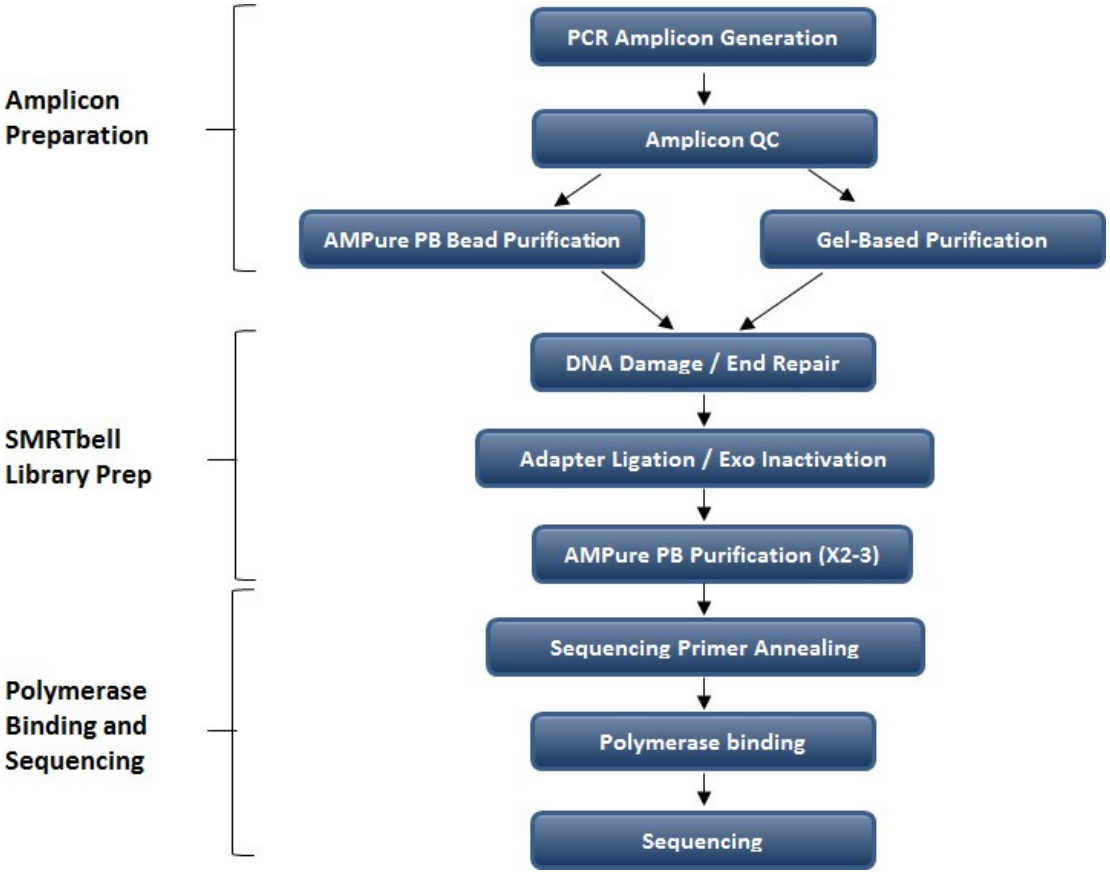
Before You Begin

To perform this procedure, you must have the following PacBio® products:

- SMRTbell™ Template Prep Kit
- DNA/Polymerase Binding Kit
- MagBead Kit for amplicons ≥1 kb
- DNA Sequencing Reagent
- DNA Internal Control Complex
- SMRT® Cells
- AMPure® PB beads

The PacBio System can be used to generate highly accurate sequences from amplicons ranging from several hundred bases to 10 kb or larger. Unlike sheared genomic DNA (gDNA), which is comprised of DNA fragments spanning a range of lengths, PCR products from one reaction are typically the same or similar lengths. This document describes methods for preparing PCR-amplified DNA for sequencing on the PacBio System.

General Workflow for Amplicon Sample Preparation and Sequencing



Generating High-Quality PCR Products

SMRT Sequencing requires high-quality, double-stranded DNA as input. This is true not only for native gDNA, but DNA generated by PCR and RT-PCR as well. Many protocols and reagents are available for PCR, and the resulting amplification products can vary significantly. Below are several recommendations for generating high-quality amplicons suitable for SMRTbell library prep and sequencing.

- Begin with high-quality nucleic acids
  - Ideally, extract nucleic acids just before use as template in amplification reactions. This is particularly important for RNA used in RT-PCR amplification.
  - If extracted nucleic acids must be stored, freeze at high concentrations in appropriately-buffered solutions. To minimize degradation and possible contamination, sub-aliquot extracts into smaller volumes for storage. For DNA samples, DNAStable® Plus from Biomatrica may be used to help preserve extracted DNA.
  - If damage of input DNA is suspected, treat with New England Biolabs PreCR® Repair Mix prior to amplification, or use DNA Damage Repair reagents from a SMRTbell Template Prep Kit.
- Use PCR reagents and conditions likely to generate clean, undamaged, and non-chimeric amplicons
  - Use the highest fidelity polymerase compatible with your amplification system.
  - Use only high-quality primers; damaged bases at the ends of the amplicons cannot be repaired by DNA Damage Repair enzymes.
  - Optimize PCR conditions to minimize total time spent at high (>65°C) temperatures, particularly during denaturation.
  - 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.
  - Use the lowest number of cycles required for obtaining adequate product.
- When planning amplification, keep in mind the input required for purified amplicons *going into the DNA Damage Repair step,* following any size selection and AMPure PB bead purification steps:

Insert Size Range	Input DNA Amount
100 bp to 750 bp	250 ng
750 bp to 10 kb	500 ng

If necessary, replicate PCR reactions should be used to obtain the required amount of product. This also minimizes PCR sampling bias for samples containing heterogeneous templates. Nested or “iterative” PCR is not recommended. Refer to the following Iso-Seq™ procedures for specific cDNA library preparations. Note that all PacBio documentation can be found here: http://www.pacb.com/support/documentation/

- Procedure & Checklist – Isoform Sequencing (Iso-Seq™) Analysis using the Clontech® SMARTer® PCR cDNA Synthesis Kit and SageELF™ Size Selection System
- Procedure & Checklist – Isoform Sequencing (Iso-Seq™) Analysis using the Clontech® SMARTer® PCR cDNA Synthesis Kit and the BluePippin™ Size Selection System
- Procedure & Checklist – Isoform Sequencing (Iso-Seq™) Analysis using the Clontech® SMARTer® PCR cDNA Synthesis Kit and No Size Selection
- Full-length cDNA Target Sequence Capture Using SeqCap® EZ Libraries
- User Bulletin – Guidelines for Preparing cDNA Libraries for Isoform Sequencing (Iso-Seq™ Analysis)
- Unsupported Protocol – Barcoding Samples for Isoform Sequencing (Iso-Seq™ Analysis)

Optional: Multiplexing of Amplicons in Sample Preparation and Sequencing

For studies targeting a single consensus sequence per sample, amplicons may be multiplexed to utilize the complete capacity of a SMRT Cell. However, pooling is generally recommended for amplicons of similar sizes, ie, +/-15% of the mean size. If minor-variant detection within a given sample is a goal, we recommend starting with one sample per SMRT Cell.

PacBio has designed a set of 16-base barcodes for optimal discrimination with SMRT Sequencing. Barcodes may be incorporated into SMRTbell libraries through the use of either barcoded PCR primers or barcoded adapters. Protocols and reagents for both of these options are available:

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

Reagents include 96-barcoded adapters and 96-barcoded universal primers. Additionally, a Shared Protocol for multiplexed sequencing is available, including templates for ordering amplicon-specific barcoded PCR primers and barcoded adapters:

- Unsupported Protocol – Guidelines for Using PacBio® Barcodes for SMRT® Sequencing

Note that if barcoded samples are to be pooled prior to library prep, the input requirements in the table above refers to the total pooled mass of DNA, not the mass of individual members of the pool. Samples should be present at equimolar concentrations, as described below.

Pooling Barcoded Amplicons into a Single SMRTbell™ Library

1. If amplicons have been prepared with barcoded PCR primers, pooling may be done prior to library prep. Using the total input requirements from page 2, calculate the mass of DNA needed to obtain equimolar representation of each sample in the final pool. As mentioned above, pooling is only recommended for amplicons of similar size, +/-15% of the average size.
2. Ideally amplicons should be AMPure purified prior to multiplexing. For high-throughput operations, purifying every reaction prior to pooling may not be practical. However, in order to obtain adequate representation of each sample in the data, it is important to individually quantitate each amplicon to determine how much to add to the pool. Estimate or determine the concentration of the **target band or peak** from the qualitative assessment on an Agilent® 2100 Bioanalyzer System, Agilent 2200 TapeStation, Advanced Analytical Technologies Fragment Analyzer™ system, or agarose gel electrophoresis. When using agarose gel electrophoresis, be sure to include a control fragment at several known concentrations.
3. Note that if unpurified amplicons will be pooled, we recommend preparing a pool with double the input requirement mass to allow for inaccuracies in quantitation as well as loss during initial AMPure clean-up.
4. If off-target products are present, purify as described below. Purification may be done after pooling if all undesired fragments can all be removed by the same method. **To determine the amount of each sample to pool, quantitate the target amplicons only**, as described below. For pools requiring gel purification, increase the input amount by an additional 2- to 3-fold.

Purity and Sizing of Amplicon Samples

It is important to check the size and purity of amplicon samples before preparing SMRTbell libraries for sequencing. Visualize an aliquot of each PCR reaction using an Agilent 2100 Bioanalyzer System, Agilent 2200 TapeStation, Advanced Analytical Technologies Fragment Analyzer, or manual agarose gel electrophoresis, with appropriate markers or ladders. **If off-target products are present, they must be removed prior to library preparation, as described on the following pages. If not removed prior to library prep, shorter contaminants may represent a substantial percentage of the sequencing reads.**

If Required: Methods for Isolating Target Amplicons from Undesired PCR Products

If amplification reactions contain secondary bands that are <1.5 kb, and are both considerably shorter and less abundant than the desired product, it may be possible to remove them using AMPure PB purification at the appropriate concentration, as described on pages 5-6. For substantial levels of contaminating products, several rounds of AMPure PB bead purification may be required.

For most cases, particularly if the contaminating bands are quite close in size or larger than the desired amplicon, or for any contaminants >1.5 kb, a gel-based method is strongly recommended (see below).

Note: recovery of a given target using a gel-based extraction method is typically <50% of the mass of the target. To insure adequate recovery of DNA following extraction, increase the input amount in the table on page 2 by 2-3X.

Gel-based Size Selection

Contaminants >1.5 kb, or any contaminants with similar or larger size than the desired product, require gel purification. We recommend the use of an automated gel electrophoresis system such as the BluePippin™ or SageELF™ systems from Sage Science, which have reduced risk of sample cross contamination. For relevant protocols, visit the documentation section of our website.

Size selection may also be performed by manual excision from an agarose gel. Recommendations for manual gel size selection are provided on the following page.

Materials

- 1X TAE Buffer
- 0.8% Seakem LE or Seakem Gold LE Agarose gel
  - Note: adjust gel concentration as appropriate; use 1% or higher for amplicons < 1 kb
- SYBR® Safe Gel Dye / SYBR® Gold Nucleic Acid Gel Stain
- Loading buffer and dye of your choice
- NEB Quick Load 2-Log DNA Ladder (0.1 – 10 kb)
- Gel electrophoresis apparatus
- Razor blades
- Gloves
- Blue light box
- Qiagen® Gel Extraction Kit
- Eppendorf® LoBind Microcentrifuge Tubes or equivalent, 1.5 and 2.0 mL

Note: We highly recommend using fresh reagents for each gel run, including gel buffer, agarose gel, gloves, and tips. Running multiple samples in the same run is discouraged as it may lead to cross contamination.

Tips for Running the Gel

1. Make or pour fresh 1X **TAE** buffer for your run and agarose gel.
2. Make a new 0.8% agarose gel.
3. Add the appropriate amount of dye for your gel.
   a. For example: For SYBR Safe DNA Gel Stain, 1 µL is added to a 10 mL solution, so for a 100 mL gel, use 10 µL of SYBR dye.

b. **Do not use ethidium bromide with a UV lamp for visualization during size selection. This combination will cause irreparable damage to the DNA, resulting in sub-optimal read lengths on the PacBio System.** Instead, we recommend using SYBR®Safe DNA gel stain with a non-UV blue light.

4. If it is necessary to load multiple samples on the same gel, we suggest skipping wells between different samples, and flanking the sample on either side with the mass ladder to perform gel cuts more precisely.
   a. Note: This method is not sufficient to prevent all cross contamination, and should not be used if the goal of the project is to detect minor variants within a sample. In that case, physical barriers between samples are required, or a single gel per sample should be used.
   b. Load 500 ng of sample per lane, or an amount appropriate for gel and comb used. Run gel at 4.2 V/cm for approximately an hour.
   c. Measure the distance between the two electrodes in cm and multiply by 4.2 to determine the appropriate running voltage for your gel.

Tips for Cutting the Gel

5. As noted above, **do not use ethidium bromide or UV light to visualize banding**.
6. Avoid running the razor along the gel when cutting; instead cut the gel by pressing down on the razor evenly.
7. Important: Use a new razor blade for each cut to prevent contamination between size fractions.
8. When picking up the gel slice, use a small bore pipette and lift the sample up and out to minimize handling.

Gel Extraction

Recover size-selected DNA from the gel with standard gel purification kits such as QIAquick® or QIAEX® II kits from Qiagen. Elute your sample in 37-40 µL of 10 mM Tris pH 8, or equivalent.

Gel extraction efficiency is proportional to the size of the gel cuts. The bigger the gel slices, the longer the extraction time. Avoid large gel slices.

DNA Input Requirements

Check recovery of purified amplicon to insure adequate input for library prep. The table on page 2 shows inputs for sample *going into the DNA Damage Repair step*.

We recommend Qubit® or Nanodrop® instruments for quantitation of AMPure-purified amplicons. If the Agilent Bioanalyzer or Tape Station instrument is used for quantitation, **include all peaks or sizes where DNA is present in determining ng/µL**. Note that this is different from the quantitation for pooling amplicons.

If recovery is *below* the required input amount, consider running additional replicates of the primary PCR. If this is not possible, there are several other options for sequencing samples which do not meet the above input requirements:

1. If the project includes multiple amplicons < 5 kb in length, consider using Barcoded Adapters. Note that, reads must include a barcode from the distal adapter (i.e., they must include at least 1 full pass). Protocols for barcoded adapters are listed on page 2.
2. For single samples (if there is at least 200 ng) this protocol may be used, but library yield is likely to be lower. However, it is normally adequate for at least 1 SMRT Cell. Use an estimate of 10% library yield to determine whether the desired number of SMRT Cells can be run.
3. For single samples with 50-200 ng, consider using *Procedure & Checklist - 10 to 20 kb Template Preparation and Sequencing with Low (100 ng) Input DNA*. Skip shearing steps and begin with the **Repair DNA Damage** step.
4. For 1-2 kb amplicons with only 5-50 ng available, consider using the *Procedure & Checklist - Very Low (10 ng) Input 2 kb Template Preparation and Sequencing with Carrier DNA*.

If none of the above is possible, a secondary amplification of the primary PCR product can be attempted. However, this increases the risk of PCR bias, and only the absolute minimum number of cycles to generate enough material should be used. A second gel purification may be required.

Alternative Protocol for Amplicons ≤ 250 bp

Amplicons ≤250 bp may be prepared using an *Unsupported Protocol - 250 bp Amplicon Library Preparation and Sequencing***,** which includes A-tailing and ligating to an overhang adapter. Please inquire regarding reagents.

AMPure® PB Bead Purification

Purify individual amplicons using appropriate volumes of AMPure PB beads. In addition to removing PCR reagents and exchanging buffers, this step will remove primer dimers, and is essential prior to SMRTbell library preparation. Use the table below to determine the appropriate concentration of AMPure PB beads.

Insert Size Range	Volume of AMPure PB Beads
100 bp to 300 bp	1.8X*
301 bp to 750 bp	1.0X
750 bp to 3 kb	0.6X
3 kb to 10 kb	0.45X

* "X" denotes the volume of AMPure PB beads to use relative to the sample volume

Tips for AMPure PB bead purifications

- Pipette a minimum of 15 µL of beads per reaction to assure the specified ratio of beads:amplicon is attained. This may require increasing the sample volume with PacBio Elution Buffer. For example, purification of a 5 kb amplicon in a PCR reaction volume of 20 µL would require 0.45X volume of AMPure PB beads, which is 9 µL. To assure the correct final concentration of beads is obtained, double the volume: increase the sample volume to 40 µL and add 18 µL of beads.
- Bring beads to room temperature prior to mixing with the sample. 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.
- Carry out purifications in Eppendorf LoBind tubes or equivalent.

Recovery of clean samples from AMPure PB Bead purification should be between 80-100%; however, yields will be lower for samples with smaller contaminating fragments.

STEP	✓	Purify DNA	Notes
1		Determine the sample volume. Add the appropriate volume of AMPure PB beads to the samples using the table on page 6.	
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 relevant PacBio Guide 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 bead 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 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.	

STEP		Purify 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		Add **37 to 40 µL** PacBio Elution Buffer volume to the beads. – Mix until homogenous. – Vortex for 1 to 2 minutes at 2000 rpm. – Spin the tube down to pellet the beads, then place the tube rack on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads.	
14		Perform quantitative analysis using a Qubit or Nanodrop platform.	

Repair DNA Damage

Use the following table to repair any DNA damage.

1. In a LoBind microcentrifuge tube, add the following reagents. For more than 1 sample, prepare a pre-mix of DNA Damage Repair Buffer, NAD+, ATP high, dNTP and add 11 µL of pre-mix per sample.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
ds DNA	-		Up to 37 µL	-		
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	-		

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin down tube contents with a quick spin in a microfuge.
4. Incubate at 37ºC for 60 minutes or longer (up to 150 minutes), then return reaction to 4ºC for 1 minute.

Repair Ends

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

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

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

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin down tube contents with a quick spin in a microfuge.
4. Incubate at 25ºC for 5 minutes, then return the reaction to 4ºC. Do not incubate the End Repair reaction longer than 5 minutes. Proceed directly to the next step.

STEP	✓	Purify DNA	Notes
1		Determine the sample volume. Add the appropriate volume of AMPure PB beads to the samples using the table on page 6.	
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		Add **31 to 33 µL** PacBio Elution Buffer volume to the beads: – Mix until homogenous. – Vortex for 1 to 2 minutes at 2000 rpm. – Spin the tube down to pellet the beads, then place the tube rack on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads.	
14		Optional: Verify your DNA amount and concentration using a Nanodrop or Qubit quantitation platform, as appropriate. Actual recovery per µL and total available sample material: _____ Note that typical yield, at this point of the process (following End-Repair and one AMPure PB bead purification), is approximately **80-100%** of the total starting material going into the Damage Repair reaction.	
15		The End-Repaired 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. In a LoBind microcentrifuge tube (on ice), add the following reagents in the order shown.

- If preparing a Master Mix, combine all components, including ligase but NOT the adapter. Add the adapter directly to the DNA.
- The adapter concentration below is appropriate for the input amounts from the table on page 2. If a higher amount of input DNA is being used, adjust the adapter concentration accordingly (to minimize double-inserted SMRTbell templates). A 30Xto 50X molar excess isrecommended.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (End Repaired)	-		31.0 µL			
Blunt Adapter (20 µM)		20 µM	2.0 µL	1.0 µM		
Mix before proceeding						
Template Prep Buffer		10X	4.0 µL	1X		
ATP Lo		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 µL	-		

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down tube contents with a quick spin in a microfuge.
3. Incubate at 25ºC for 15 minutes up to 24 hours (overnight).
4. Incubate at 65ºC for 10 minutes to inactivate the ligase, then return the reaction to 4ºC.

Add exonuclease to remove failed ligation products.

Reagent	Tube Cap Color	Stock Conc.		Volume
Ligated DNA				40 µL
ExoIII		100.0 U/µL		0.5 µL
ExoVII		10.0 U/µL		0.5 µL
Total Volume	-	-		41 µL

1. Mix the reaction well by pipetting or flicking the tube.
2. Spin down tube contents with a quick spin in a microfuge.
3. Incubate at 37ºC for 1 hour, then return the reaction to 4ºC. You must proceed with purification after this step.

Purify SMRTbell™ Templates

There are 2 or 3 purification steps using the appropriate volume of AMPure PB beads for each step using the table on page 6. Note that 3 AMPure PB bead purifications may be required to remove all adapter dimers. For 3 purifications, repeat the protocol below for the second purification. Then proceed to the next page for the final purification.

STEP	✓	Purify SMRTbell Templates - First or Second Purification	Notes
1		Add the appropriate volume of AMPure PB beads to the exonuclease-treated reaction. (For detailed instructions on AMPure PB bead purification, see the table on page 6).	
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. – Mix until homogenous. – Vortex for 1 to 2 minutes at 2000 rpm. – Spin the tube down to pellet the beads, then place the tube rack on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads.	
14		The eluted DNA in **50 µL** Elution Buffer should be taken into the second AMPure PB bead purification step.	

STEP	✓	Purify SMRTbell Templates - Final Purification	Notes
1		Add the appropriate volume of AMPure PB beads to the **50 µL** of eluted DNA from the first AMPure PB bead purification step above. For the appropriate amount of AMPure PB bead volumes to use, see the table on page 6.	
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 **10 µL** of Elution Buffer. – Mix until homogenous. – Vortex for 1 to 2 minutes at 2000 rpm. – Spin the tube down to pellet the beads, then place the tube rack on the magnetic bead rack. – Carefully collect the eluted sample. – Discard the beads.	
14		Verify your DNA amount and concentration with either a Nanodrop or Qubit quantitation platform reading. For general library yield expect 20% total yield from End Repair input. If your yield concentration is below 12 ng/µL, use the Qubit system for quantitation. – To estimate your final concentration: (____ng of DNA going into End Repair X 0.2) /___of Elution Buffer =____ng/µL	
15		Perform qualitative analysis using a Bioanalyzer instrument. Note that typical DNA yield, at this point of the process can range from approximately **15-50%** of the total starting material going into the DNA Damage Repair reaction (depending on the purity and quality of input DNA.	

Anneal and Bind SMRTbell™ Templates

The Pacific Biosciences DNA/Polymerase Kit is required for this step. Use the Binding Calculator to determine conditions for annealing the sequencing primer and binding polymerase to SMRTbell templates, with the modifications below for the P6-C4 sequencing chemistry.

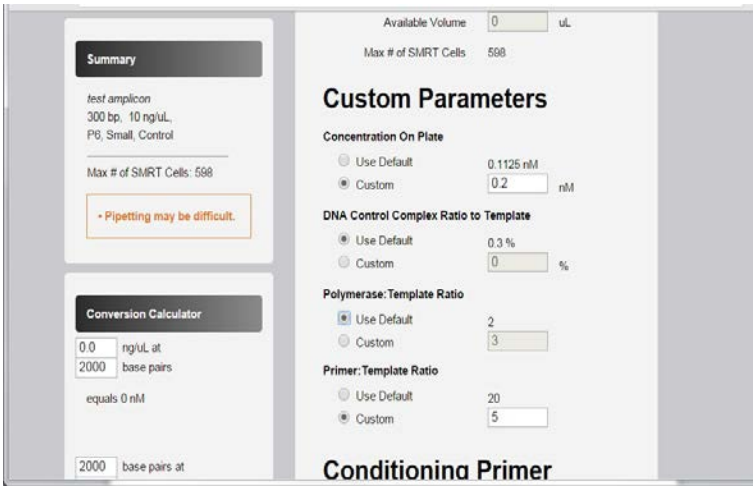
The on-plate loading concentrations listed here are generally higher than the Binding Calculator recommendations, which were largely determined with sheared templates containing some smaller fragments. A range is provided since the optimal concentration for a given size range varies, depending on several factors, including application or project (target yield) and the relative sizes and abundances of contaminating amplicons. For projects with multiple SMRT Cells,

We recommend performing loading titrations to determine the appropriate loading concentration. For more information, refer to *Quick Reference Card – Diffusion Loading and Movie Time Recommendations for the Sequel System*.

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

Insert Size Range	100 bp - 300 bp	301 bp - 999 bp	1 kb - 5 kb	5 kb - 10 kb
Run Protocol	Standard (Diffusion)	Standard (Diffusion)	MagBead OCPW or MagBead Standard	MagBead OCPW or MagBead Standard
Stage Start	No	No	1 kb - 3 kb (No) 3 kb - 5 kb (Yes)	Yes
Primer:Template Ratio	5 *(custom)*	5 *(custom)*	20 (standard)	20 (standard)
Polymerase:Template Ratio	2 (standard) or 3 *(custom)*	2 (standard) or 3 *(custom)*	10 (standard)	10 (standard)

Custom parameters must be manually entered, as shown below for a 300 bp insert:



Optional MagBead conditions for 500-999 bp amplicons: The MagBead OCPW protocol may be used with amplicons >500 bp. As the Calculator will not allow inserts <1000 bp with MagBead protocols, instead enter 1000 bp as the insert size into the calculator. Use standard MagBead parameters for a 1000 bp library (10:1 polymerase:template ratio, 20:1 primer:template ratios), *except increase on-plate loading concentration using custom parameters to 0.020-0.050 nM, with higher concentrations for shorter amplicons*.

Data collection time

The table below may be used to help determine the appropriate data collection time for your library. In general, increasing the number of passes increases accuracy. However, coverage beyond 60 passes of the same molecule may not significantly improve accuracy. Consult with an experienced bioinformatician for more information on the optimal number of passes for your application. Note: Gray numbers reflect conditions currently not recommended for most applications as they result in either too few passes or excessive passes that increase file size and processing time but may not significantly improve accuracy.

Movie Time	30	45	60	90	120	180	240	360
Bases/ Run[1]	3750	5625	7500	11250	15000	22500	30000	45000
Insert Size	Minimum number of passes for movie-limited reads[2]							
100	38	56	75	113	150	225	300	450
300	13	19	25	38	50	75	100	150
1000	4	6	8	11	15	23	30	45
5000	0.8	1.1	1.5	2.3	3.0	4.5	6	9
10000	0.4	0.6	0.8	1.1	1.5	2.3	3.0	4.5

[1] Theoretical minimum read length for a movie-limited read

[2] Based on 125 bases/min, or 2.08 bases/sec, to include slow or paused polymerases

For more information about using the Calculator, see the appropriate Pacific Biosciences guide.

DNA Control Complex Dilution

ThePacific Biosciences DNA Control Complex is required for this step. Dilute the DNA Control Complex according to the volumes and instructions specified in the Calculator.

Sequence

To prepare for sequencing on the instrument, refer to the *Online Help* system or the relevant Pacific Biosciences 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.

Revision History (Description)	Version	Date
Removed loading specifics and referenced “Quick Reference Card – Diffusion Loading and Movie Time Recommendations for the Sequel System” for more information.	03	February 2018