Amplicon Template Preparation and Sequencing

Before You Begin

This procedure requires 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 in size 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

```
Amplicon Preparation

SMRTbell Library Prep

Polymerase Binding and Sequencing

PCR Amplicon Generation

Amplicon QC

AMPure PB Bead Purification

Gel-Based Purification

DNA Damage / End Repair

Adapter Ligation / Exo Digestion

AMPure PB Purification (X2-3)

Sequencing Primer Annealing

Polymerase binding

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:

<table>
<thead>
<tr>
<th>Insert size range</th>
<th>100 - 750 bp</th>
<th>750 bp – 10 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input DNA amount</td>
<td>250 ng</td>
<td>500 ng</td>
</tr>
</tbody>
</table>

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.

Note: please refer to Iso-Seq™ procedures for details specific to the preparation of cDNA libraries, found here: [http://www.pacificbiosciences.com/support/pubmap/documentation.html](http://www.pacificbiosciences.com/support/pubmap/documentation.html)

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 variants detection within a given sample is a goal, we recommend starting with 1 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, and may be found at [http://www.pacificbiosciences.com/support/pubmap/documentation.html](http://www.pacificbiosciences.com/support/pubmap/documentation.html). 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: [https://pacbio.secure.force.com/Share/Protocol?id=a1q7000000J4m5AAC&strRecordTypeName=Protocol](https://pacbio.secure.force.com/Share/Protocol?id=a1q7000000J4m5AAC&strRecordTypeName=Protocol)
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 the preceding page, 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™, 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. Protocols for size selection with the BluePippin or SageELF Systems may be found on the User Documentation page of the PacBio website: http://www.pacificbiosciences.com/support/pubmap/documentation.html

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 a 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.
   a. 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

1. As noted above, do not use ethidium bromide or UV light to visualize banding.
2. Avoid running the razor along the gel when cutting; instead cut the gel by pressing down on the razor evenly.
3. Important: Use a new razor blade for each cut to prevent contamination between size fractions.
4. 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 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 below 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.

<table>
<thead>
<tr>
<th>Insert size range</th>
<th>100 - 750 bp</th>
<th>750 bp – 10 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input DNA amount</td>
<td>250 ng</td>
<td>500 ng</td>
</tr>
</tbody>
</table>

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

1. If the project includes multiple amplicons < 5 kb in length, consider using Barcoded Adapters. Keep in mind that in order to be utilized, reads must include a barcode from the distal adapter, i.e., they must include at least 1 full pass. Protocols may be found at http://www.pacificbiosciences.com/support/pubmap/documentation.html

2. For single samples, if there is at least 150 ng, this protocol may be used, but library yield is likely to be lower than would be obtained from the recommended input. However, it is usually enough 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 50-100 ng, consider using the 10 kb to 20 kb Template Preparation and Sequencing with Low-Input DNA Shared Protocol. Skip shearing steps and begin with the Repair DNA Damage step. This protocol is available for downloading at the following link: https://pacbio.secure.force.com/Share/Protocol?id=a1q70000000JhEeAAK&strRecordTypeName=Protocol

4. For 1-2 kb amplicons with only 10-50 ng available, consider using the Very Low (10 ng) 2 kb Library Preparation with Carrier DNA Shared Protocol, available here: https://pacbio.secure.force.com/Share/Protocol?id=a1q70000000HjMwAAK&strRecordTypeName=Protocol

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 alternative Shared protocol, 250 bp Amplicon Library Preparation and Sequencing, which includes A-tailing and ligating to an overhang adapter, available here: https://pacbio.secure.force.com/Share/Protocol?id=a1q70000000JhEeAAK&strRecordTypeName=Protocol
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 prep. Use the table below to determine the appropriate concentration of AMPure PB beads for your amplicons size.

<table>
<thead>
<tr>
<th>Insert size range</th>
<th>100 - 300 bp</th>
<th>301 - 750 bp</th>
<th>750 bp - 3 kb</th>
<th>3 - 10 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of AMPure PB Beads</td>
<td>1.8X</td>
<td>1.0X</td>
<td>0.60X</td>
<td>0.45X</td>
</tr>
</tbody>
</table>

NOTE: “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.
<table>
<thead>
<tr>
<th>STEP</th>
<th>AMPure PB Bead Purification Protocol</th>
<th>NOTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Determine the sample volume. Add the appropriate volume of AMPure PB magnetic beads to the sample from the table above.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mix the bead/DNA solution thoroughly</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Quickly spin down the tube (for 1 second) to collect the beads</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Allow the DNA to bind to beads by mixing 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 mixing, 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.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Spin down the tube (for 1 second) to collect the beads</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>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.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>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.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>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.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Repeat step 8 above.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>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.</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Check for any remaining droplets in the tube. If droplets are present, repeat step 10.</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds.</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Add 37-40 μL of Pacific Biosciences® Elution Buffer to the beads to elute the DNA – Mix until homogeneous. – Vortex for 1-2 minutes 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.</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>After AMPure PB bead clean-up, quantitate PCR products using the Qubit or Nanodrop platforms.</td>
<td></td>
</tr>
</tbody>
</table>

### Repair DNA Damage

Use the following table to repair any DNA damage.

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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Tube Cap Color</th>
<th>Stock Conc.</th>
<th>Volume</th>
<th>Final Conc.</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ds DNA</td>
<td></td>
<td></td>
<td>Up to 37 μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA Damage Repair Buffer*</td>
<td></td>
<td>10X</td>
<td>5.0 μL</td>
<td>1X</td>
<td></td>
</tr>
<tr>
<td>NAD+*</td>
<td></td>
<td>100X</td>
<td>0.5 μL</td>
<td>1X</td>
<td></td>
</tr>
<tr>
<td>ATP high*</td>
<td></td>
<td>10 mM</td>
<td>5.0 μL</td>
<td>1 mM</td>
<td></td>
</tr>
<tr>
<td>dNTP*</td>
<td></td>
<td>10 mM</td>
<td>0.5 μL</td>
<td>0.1 mM</td>
<td></td>
</tr>
<tr>
<td>DNA Damage Repair Mix</td>
<td></td>
<td></td>
<td>2.0 μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td></td>
<td>__ μL to adjust to 50.0 μL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total Volume 50.0 μL

* For more than 1 sample, prepare a pre-mix of these reagents; add 11 μl of pre-mix per sample.

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 37°C for 60 minutes or longer, up to 150 minutes, then return the reaction to 4°C for 1 minute.

### Repair Ends

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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Tube Cap Color</th>
<th>Stock Conc.</th>
<th>Volume</th>
<th>Final Conc.</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (Damage Repaired)</td>
<td></td>
<td></td>
<td>50 μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>End Repair Mix</td>
<td></td>
<td>20X</td>
<td>2.5 μL</td>
<td>1X</td>
<td></td>
</tr>
</tbody>
</table>

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 25°C for 5 minutes, return the reaction to 4°C. Do not incubate the End Repair reaction longer than 5 minutes. Proceed directly to the next step.
<table>
<thead>
<tr>
<th>STEP</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Add the appropriate volume of AMPure PB beads to the End-Repair reaction, following the chart below. (For more details, see the Ampure PB Bead Purification section, page 7.)</td>
</tr>
<tr>
<td>2</td>
<td>Mix the bead/DNA solution thoroughly.</td>
</tr>
<tr>
<td>3</td>
<td>Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.</td>
</tr>
<tr>
<td>4</td>
<td>Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.</td>
</tr>
<tr>
<td>5</td>
<td>Spin down the tube (for 1 second) to collect beads.</td>
</tr>
<tr>
<td>6</td>
<td>Place the tube in a magnetic bead rack to collect the beads to the side of the tube.</td>
</tr>
<tr>
<td>7</td>
<td>Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.</td>
</tr>
<tr>
<td>8</td>
<td>Wash beads with freshly prepared 70% ethanol.</td>
</tr>
<tr>
<td>9</td>
<td>Repeat step 8 above.</td>
</tr>
</tbody>
</table>
| 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:  
|      |   - Add 31-33 μL Elution Buffer, mix until homogenous  
|      |   - Vortex for 1-2 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   | 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 4°C, or at -20°C for longer duration. |

<table>
<thead>
<tr>
<th>Insert size range</th>
<th>100 - 300 bp</th>
<th>301 - 750 bp</th>
<th>750 bp - 3 kb</th>
<th>3 - 10 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>X AMPure PB Beads</td>
<td>1.8X</td>
<td>1.0X</td>
<td>0.60X</td>
<td>0.45X</td>
</tr>
</tbody>
</table>
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, combine all components, including ligase, EXCEPT 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-insert SMRTbell templates; a 30-50X molar excess of adapter is recommended.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Tube Cap Color</th>
<th>Stock Conc.</th>
<th>Volume</th>
<th>Final Conc.</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (End Repaired)</td>
<td></td>
<td></td>
<td>31.0 μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annealed Blunt Adapter (20 μM)</td>
<td></td>
<td>20 μM</td>
<td>2.0 μL</td>
<td>1.0 μM</td>
<td>Mix before proceeding</td>
</tr>
<tr>
<td>Template Prep Buffer</td>
<td></td>
<td>10 X</td>
<td>4.0 μL</td>
<td>1X</td>
<td></td>
</tr>
<tr>
<td>ATP low</td>
<td></td>
<td>1 mM</td>
<td>2.0 μL</td>
<td>0.05 mM</td>
<td>Mix before proceeding</td>
</tr>
<tr>
<td>Ligase</td>
<td></td>
<td>30 U/μL</td>
<td>1.0 μL</td>
<td>0.75 U/μL</td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td></td>
<td>___ μL to adjust to 40 μL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td></td>
<td></td>
<td>40.0 μL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 25°C for 15 minutes to 24 hours (overnight).
5. Incubate at 65°C for 10 minutes to inactivate the ligase, then return the reaction to 4°C.

Add Exonucleases to Remove Failed Ligation Products

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Tube Cap Color</th>
<th>Stock Conc.</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligated DNA</td>
<td></td>
<td>40 μL</td>
<td></td>
</tr>
<tr>
<td>ExoIII</td>
<td></td>
<td>100.0 U/μL</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>ExoVII</td>
<td></td>
<td>10.0 U/μL</td>
<td>0.5 μL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td></td>
<td></td>
<td>41.0 μL</td>
</tr>
</tbody>
</table>

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 37°C for 1 hour, then return the reaction to 4°C. 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, see table below.

<table>
<thead>
<tr>
<th>STEP</th>
<th>Purify SMRTbell Templates - First Purification</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Add the appropriate volume of AMPure PB beads to the exonuclease-treated reaction, following the chart below. (For more detailed instructions, see the AMPure PB bead Purification section, page 7.)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mix the bead/DNA solution thoroughly.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Spin down the tube (for 1 second) to collect beads.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Place the tube in a magnetic bead rack to collect the beads to the side of the tube.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Wash beads with freshly prepared 70% ethanol.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Repeat step 8 above.</td>
<td></td>
</tr>
</tbody>
</table>
| 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:  
   - Add 50 μL Elution Buffer, mix until homogenous  
   - Vortex for 1-2 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   | The eluted DNA in 50 μL Elution Buffer should be taken into the second AMPure PB bead purification step. |       |

<table>
<thead>
<tr>
<th>Insert size range</th>
<th>100 - 300 bp</th>
<th>301 - 750 bp</th>
<th>750 bp - 3 kb</th>
<th>3 - 10 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>X AMPure PB Beads</td>
<td>1.8X</td>
<td>1.0X</td>
<td>0.60X</td>
<td>0.45X</td>
</tr>
</tbody>
</table>

Note: 3 AMPure PB bead purifications may be required to remove all adapter dimers. For 3 purifications, repeat the protocol above for the second AMPure purification, then proceed to the next page for the third AMPure PB bead purification.
<table>
<thead>
<tr>
<th>STEP</th>
<th>Purify SMRTbell Templates – Second or Third Purification</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Add the appropriate volume of AMPure PB beads to the 50 μL of eluted DNA from the previous AMPure PB bead purification.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mix the bead/DNA solution thoroughly.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Spin down the tube (for 1 second) to collect beads.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Place the tube in a magnetic bead rack to collect the beads to the side of the tube.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Wash beads with freshly prepared 70% ethanol.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Repeat step 8 above.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Remove residual 70% ethanol and dry the bead pellet.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– 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.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Place the tube back on magnetic bead rack.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Pipette off any remaining 70% ethanol.</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Check for any remaining droplets in the tube. If droplets are present, repeat step 10.</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Elute the DNA off the beads:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Add 10 μL Elution Buffer, mix until homogenous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Vortex for 1-2 minute at 2000 rpm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Spin the tube down to pellet beads</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Place the tube back on the magnetic bead rack and carefully collect the eluted sample</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Carefully collect the eluted sample.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>– Discard the beads.</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Verify your DNA amount and concentration with either a Nanodrop or Qubit quantitation reading. For general library yield expect 20% total yield from the Damage Repair input. If your yield concentration is below 12 ng/μL, use the Qubit for quantitation. To estimate your final concentration:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>__ng of DNA going into Damage Repair X 0.2) / __μL Elution Buffer = ___ng/μL</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Perform qualitative analysis using a Bioanalyzer instrument. Note that typical DNA yield, at this point of the process can range from 15-50% of the total starting material going into the DNA damage repair reaction, depending on the purity and quality of input DNA.</td>
<td></td>
</tr>
</tbody>
</table>
Annealing, Binding, and Sequencing 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, a loading titration with a project-specific sample is recommended to identify the ideal on-plate loading concentration. As a general rule, 30-45% P1 is a good target, with P2 ideally ≤10% and a maximum 20%, with P2<P0

<table>
<thead>
<tr>
<th>Insert size range</th>
<th>100 - 300 bp</th>
<th>301 - 999 bp</th>
<th>1 - 5 kb</th>
<th>5 – 10 kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run Protocol</td>
<td>Standard (Diffusion)</td>
<td>Standard (Diffusion)</td>
<td>MagBead OCPW* or Magbead Standard</td>
<td>MagBead OCPW or MagBead Standard</td>
</tr>
<tr>
<td>On-Plate Loading Concentration (nM)</td>
<td>0.1-0.2 (custom)</td>
<td>0.2-0.45 (custom)</td>
<td>0.010-0.025 (custom)</td>
<td>0.025-0.040 (custom)</td>
</tr>
<tr>
<td>Primer:Template Ratio</td>
<td>5 (custom)</td>
<td>5 (custom)</td>
<td>20 (standard)</td>
<td>20 (standard)</td>
</tr>
<tr>
<td>Polymerase:Template Ratio</td>
<td>2 (standard) or 3 (custom)</td>
<td>2 (standard) or 3 (custom)</td>
<td>10 (standard)</td>
<td>10 (standard)</td>
</tr>
</tbody>
</table>

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 is unlikely to be beneficial. Consult with an experienced bioinformatician for more information on the optimal number of passes for your application.

<table>
<thead>
<tr>
<th>Movie time</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>180</th>
<th>240</th>
<th>360</th>
</tr>
</thead>
<tbody>
<tr>
<td>1Bases/run</td>
<td>3,750</td>
<td>5,625</td>
<td>7,500</td>
<td>11,250</td>
<td>15,000</td>
<td>22,500</td>
<td>30,000</td>
<td>45,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Insert size</th>
<th>Minimum number of passes for movie-limited reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>38 67 56 75 113 150 225 300 450</td>
</tr>
<tr>
<td>300</td>
<td>13 19 25 38 50 75 100 150</td>
</tr>
<tr>
<td>1,000</td>
<td>4 6 8 11 15 23 30 45</td>
</tr>
<tr>
<td>5,000</td>
<td>0.8 1.1 1.5 2.3 3.0 4.5 6 9</td>
</tr>
<tr>
<td>10,000</td>
<td>0.4 0.6 0.8 1.1 1.5 2.3 3.0 4.5</td>
</tr>
</tbody>
</table>

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 Pacific Biosciences Template Preparation and Sequencing Guide.

DNA Control Complex Dilution
The Pacific 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 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.