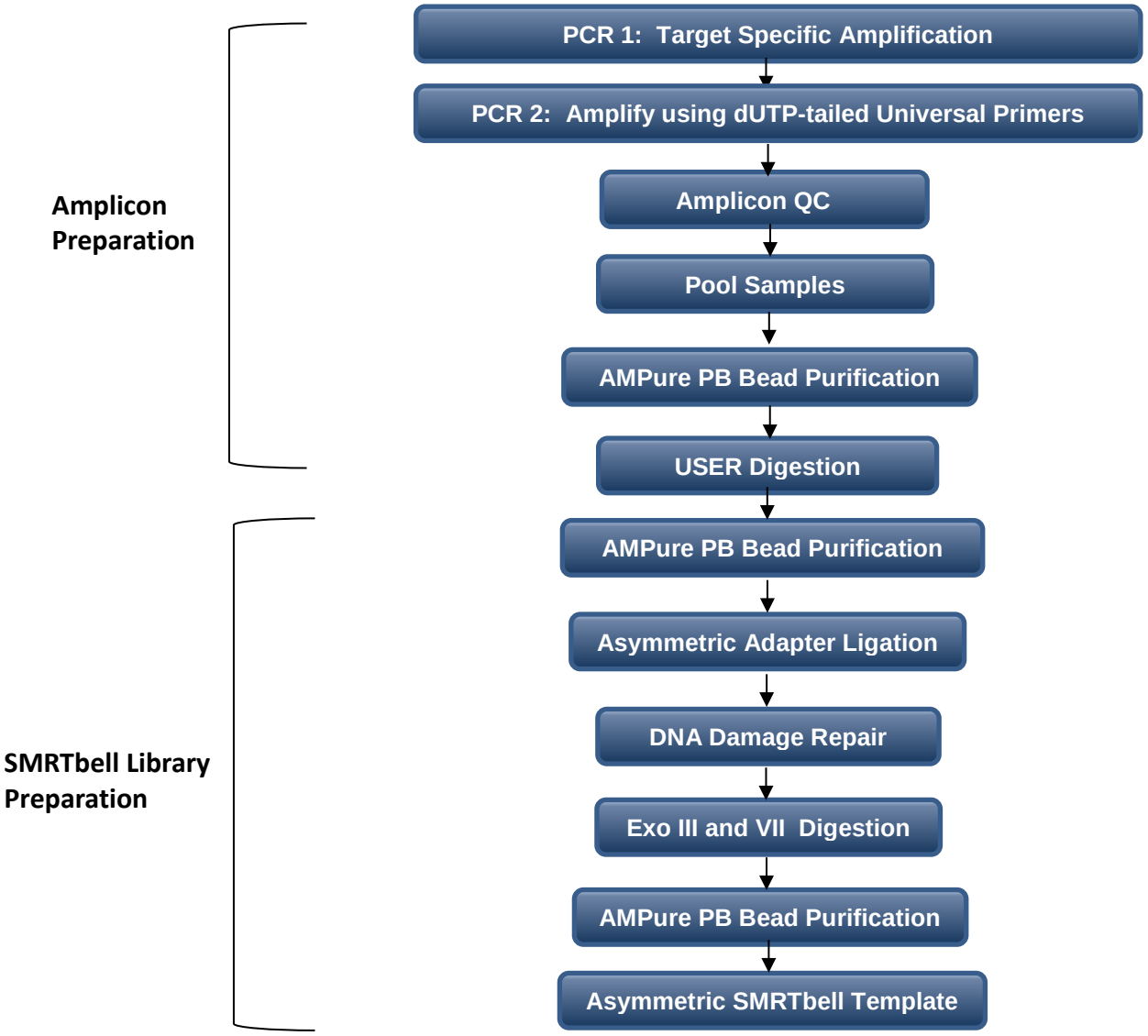
Procedure & Checklist - Preparing Asymmetric SMRTbell™ Templates

Before You Begin

In this procedure, PCR products are generated using two rounds of amplification. The first round uses target specific primers tailed with a universal sequence and the second uses barcoded universal primers tailed with a 4 bp sequence containing dUTP. The PCR product is treated with USER (Uracil-Specific Excision Reagent) Enzyme resulting in 4 bp overhangs differentiated by a 3 bp sequence, followed by ligation with Asymmetric SMRTbell adapters. The resulting SMRTbell templates are annealed to two different primers: the first primer has a 3' poly A tail for binding oligo dT magnetic beads, and the second primer is available for polymerase binding.

General Workflow for Asymmetric Template Preparation



To perform this procedure, you must have the following:

Amplification:

- KAPA HiFi HotStart Uracil+ PCR Kit from Kapa Biosystems (Kit Codes KK2801, KK2802)
- Primers for 1st and 2nd PCR:
  1. Target Specific Primers tailed with universal sequence (see Designing Primers section below)
  2. Forward and Reverse Barcoded Universal Primers tailed with 4 bp containing dUTP (see Designing Primers section below)

Library Construction:

- PacBio® SMRTbell™ Template Prep Kit (100-259-100)
- NEB USER™ Enzyme (Catalog #M5505)
- PacBio SMRTbell Asymmetric Auxiliary TPK (101-080-400) includes asymmetric Adapters with sequencing and capture primers
- PacBio AMPure® PB Beads (100-265-900)

PCR Amplification

Before amplification, review guidelines for generating high-quality PCR products in PacBio's *Procedure and Checklist - Amplicon Template Preparation and Sequencing* (http://www.pacb.com/support/documentation/)

Designing Target Specific Primers Tailed with Universal Sequence for First-Round PCR

Primers can be ordered from any oligo synthesis provider (e.g., https://www.idtdna.com/site/order/menu).

Primers must meet the following:

1. Be desalted.
2. A 5' blocker (eg., 5' NH4-C6) must be added to prevent residual amplicons from first the round PCR from ligation to SMRTbell adapters.

Use the following format when ordering oligos:

Primer	5' Blocking Group* + Universal Sequence	Target-Specific Sequence	Primer to Order
Forward PCR Primer	/5AmMC6/ gcagtcgaacatgtagctgactcaggtcac	FOREXAMPLE1	/5AmMC6/gcagtcgaacatgtagctgactcaggtcac FOREXAMPLE1
Reverse PCR Primer	/5AmMC6/ tggatcacttgtgcaagcatcacatcgtag	FOREXAMPLE2	/5AmMC6/tggatcacttgtgcaagcatcacatcgtag FOREXAMPLE2

Designing dUTP-Tailed Barcoded Universal Primers for Second-Round PCR

Primers can be ordered from any oligo synthesis provider (e.g., https://www.idtdna.com/site/order/menu).

Primers must meet the following:

1. Be desalted.
2. The forward barcoded primer must be tailed with GGGdUTP.
3. The reverse barcoded primer must be tailed with CAGdUTP.

Use the following format when ordering oligos:

Primer	Primer to Order
GGGdUTP-Tailed Barcoded Universal Forward Primer	Example: /GGG/ideoxyU/CACATATCAGAGTGCGgcagtcgaacatgtagctgactcaggtcac
CAGdUTP -Tailed Barcoded Universal Reverse Primer	Example: /CAG/ideoxyU/CGCACTCTGATATGTGtggatcacttgtgcaagcatcacatcgtag

384 Barcodes. Click **here** to obtain the 384 barcode sequences.

Qualitative and Quantitative Assessment of PCR Products

Clean, target-specific PCR products are extremely important for obtaining high-quality sequence information. It is important to perform both quantification and quality checks (for contaminants or impurities) of PCR amplicons before constructing SMRTbell libraries. Presence of short non-specific products will result in less than optimal results.

Some steps that can be taken to ensure high-quality PCR amplicons include:

- Generating high-quality amplicons by ensuring amplification parameters are optimal.
- Using high-quality genomic DNA for PCR.
- Verifying amplicon sizes by running on a Bioanalyzer® system (using the DNA 12000 assay kit), Agilent® 2200 TapeStation, agarose gel electrophoresis, or Flash Gels with appropriate molecular weight markers. Note that primer dimers and non-specific PCR products inflate readings when using Qubit® or Nanodrop® platforms. They must first be removed by purification (see figures 1A and 1B).

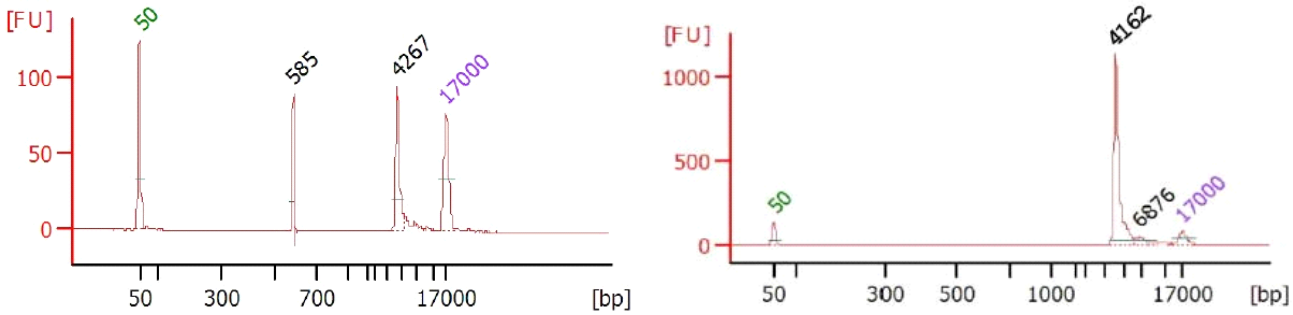


Figure 1: A. Example of a 4 kb HLA PCR product with contaminating non-specific PCR product (585 bp).
B. Using AMPure PB bead purification, the non-specific band can be easily removed.

Remove contaminants and/or non-specific products less than 1 kb by performing AMPure PB bead purification. Depending on the size of the unwanted products, use the following AMPure PB Bead guidelines for purification.

Insert size range	1.5 kb - 3 kb	3 kb - 10 kb
AMPure PB Beads Concentration	0.5X	0.45X

Pooling

To obtain adequate representation of each amplicon in the subsequent sequencing data, it is highly recommended to pool in equimolar concentrations. Pool amplicons of similar size, generally within 15% the size of each other. Ideally, PCR products should be AMPure purified and quantified using a system that provides both size and concentration information (Agilent BioAnalyzer system). The size and concentration information is necessary for calculating the required amount of each sample for equimolar pooling.

As your throughput increases, purifying every reaction prior to pooling may not be practical. You may decide to pool samples by equal-volume instead of equimolar. However, this pooling method may work if your PCR reactions are fully optimized to generate consistent yields across all samples. To QC hundreds of PCR products, you may analyze samples by agarose gel electrophoresis.

DNA Requirements for Asymmetric Library Construction

The amount of PCR products required for library construction depends on the size of the PCR product. Table 1 summarizes input requirements for library construction.

Insert size range	2 - 3 kb	3 - 4 kb	4 - 5 kb	>5 kb
Input DNA amount	2-3 µg	3-4 µg	4-5 µg	5 µg

Table 1: The amount of DNA for ligation depends on the size of the amplicon, a general guideline is 1 µg/kb

If you are constructing a single PCR product, prepare enough PCR products to meet the above input DNA requirements.

For multiplexing, prepare enough PCR products such that when pooled, the total amount of PCR product meets the above input DNA requirements.

First-Round PCR Reaction and Cycling Parameters Recommendations

1. Prepare the following reaction per sample.

Component	Stock Conc	Volume / Reaction	Final Conc
KAPA HiFi HotStart Uracil+ ReadyMix[1]	2X	10 µL	1X
Forward primer[2]	2 µM	3.1 µL	0.31 µM
Reverse primer[2]	2 µM	3.1 µL	0.31 µM
Template DNA		1 ng[3]	
PCR-grade water		Up to 20 µL	
Final volume		20 µL	

[1] KAPA HiFi HotStart Uracil+ ReadyMix contains 2.5 mM $MgCl_2$ (1X). Additional $MgCl_2$ may be added separately if required.
[2] Target specific primer tailed with universal sequence, 5-prime end blocked with NH_4-C6
[3] Optimization is highly recommended

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

Step	Temp	Time	# Cycles
Initial denaturation	94°C	2 min	1
Denaturation	94°C	30 sec	N[2]
Annealing[1]	64°C	30 sec	
Extension	68°C	90 sec / kb	
Final extension	68°C	10 min	1

[1] The optimal annealing temperature for a specific primer pair is likely to be higher than when used in a conventional PCR buffer. If a three-step cycling protocol is used as in the parameters in the table, a 60°C to 64°C / 30 sec annealing is recommended, with an extension temperature in the range of 68°C – 75°C for 60-100 seconds / kb.
[2] Twenty (20) cycles or fewer is recommended. Reactions with low template concentrations may require more cycles in order to obtain the necessary input amount.

4. After amplification, perform visual inspection of the PCR products on an agarose gel, Bioanalyzer® system (using the DNA 12000 assay kit) or Agilent® 2200 TapeStation.
5. Proceed to the second round of PCR.

Second-Round PCR Reaction and Cycling Parameters Recommendations

The procedure below was optimized using KAPA HiFi HotStart Uracil high fidelity for PCR. We recommend performing optimizations before proceeding.

1. Prepare the following reaction per sample:

Component	Stock Conc	Volume / Reaction	Final Conc
KAPA HiFi HotStart Uracil+ ReadyMix[1]	2X	10 µL	1X
GGGdUTP-Tailed Barcoded Universal Forward Primer	2 µM	3.1 µL	0.31 µM
CAGdUTP -Tailed Barcoded Universal Reverse Primer	2 µM	3.1 µL	0.31 µM
Round 1 PCR product		1 ng[2]	
PCR-grade water		Up to 20 µL	
Final volume		20 µL	

[1] KAPA HiFi HotStart Uracil+ ReadyMix contains 2.5 mM MgCl2 (1X). Additional MgCl2 may be added separately if required.
[2] Optimization is highly recommended

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

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Step	Temp	Time	# Cycles
Initial denaturation	94°C	2 min	1
Denaturation	94°C	30 sec	N[2]
Annealing[1]	64°C	30 sec	
Extension	68°C	90 sec / kb	
Final extension	68°C	10 min	1

[1] The optimal annealing temperature for a specific primer pair is likely to be higher than when used in a conventional PCR buffer.
[2] Twenty (20) cycles or fewer is recommended. Reactions with low template concentrations may require more cycles in order to obtain the necessary input amount.

4. After amplification, perform visual inspection of the PCR products on an agarose gel, Bioanalyzer® system (using the DNA 12000 assay kit) or Agilent® 2200 TapeStation.
5. When multiplexing, proceed to pooling. Prepare enough PCR products such that when pooled, the total amount of PCR product meets the input DNA requirements recommended in Table 1.
6. Depending on the number of samples and the yield per well, 1-5 µg of DNA can be recovered.

STEP	✓	Purify DNA	Notes
1		Add the **appropriate** volume of AMPure PB beads to the pooled PCR product.	
2		Mix the bead/DNA solution thoroughly by tapping the tube.	
3		Quickly spin down the tube (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. – Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). – Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol 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 **21 µL** Elution Buffer, mix until homogenous. – Vortex for 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.	
14		Measure concentration using Qubit system or PicoGreen® quantitation assay.	
15		The eluted DNA can be stored overnight at 4ºC or at -20ºC for longer duration.	

Asymmetric SMRTbell™ Template Preparation

The PCR product, or pooled PCR product, is treated with USER (Uracil-Specific Excision Reagent) Enzyme resulting in 4 bp overhangs differentiated by a 3 bp sequence, followed by ligation with Asymmetric SMRTbell adapters.

USER Digest

Use the following table to digest amplicons to generate 3' overhangs.

1. Dilute the USER enzyme 1/10 in 1X CutSmart Buffer; scale up as necessary for multiple samples:

Reagent	Stock Conc	Volume	✓	Notes
DI Water		8.0 µL		
CutSmart Buffer	10 X	1.0 µL		
USER enzyme	1 U/µL	1.0 µL		
TOTAL		10.0 µL		

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

Reagent	Stock Conc	Volume	✓	Notes
From second-round PCR	≥50 ng/µL	20.0 µL		
CutSmart Buffer	10 X	2.5 µL		
Diluted USER enzyme	0.1 U/µL	3.0 µL		
TOTAL		25.5 µL		

3. Mix the reaction well by pipetting or flicking the tube.
4. Spin down contents of tube with a quick spin in a microfuge.
5. Incubate at 37ºC for 15 minutes, 25ºC for 15 minutes, then return the reaction to 4°C for 1 minute.
6. Add an equal volume (25.5 µL) of EB prior to AMPure PB bead purification.

STEP	✓	Purify DNA	Notes
1		Add the **appropriate** volume of AMPure PB beads to the pooled PCR product.	
2		Mix the bead/DNA solution thoroughly by tapping the tube.	
3		Quickly spin down the tube (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. – Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). – Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol 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 **27 µL - 28 µL** Elution Buffer, mix until homogenous. – Vortex for 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.	
14		Optional: Verify your DNA amount and concentration using a Nanodrop or Qubit quantitation platform, as appropriate.	
15		The AMPure-cleaned DNA can be stored overnight at 4ºC, or at -20ºC for longer duration.	

Ligate Asymmetric Adapters

Using the guidelines in Table1, prepare the following ligation reaction. In a LoBind microcentrifuge tube (on ice), add the following reagents in the order shown.

1. Components should be added individually in the order listed.
2. Adapter concentrations (in ligation reaction below) are appropriate for input amounts shown in Table 1.
3. If a higher amount of input DNA is being used, adjust the adapter concentration accordingly. A minimum of >, 32X molar excess of adapter is recommended.

Reagent	Tube Cap Color	Stock Conc.	Volume	✓	Notes
USER digested DNA			25 µL		
Asymmetric Adapter A		20 µM	4.0 µL		
Asymmetric Adapter B		20 µM	4.0 µL		
Template Prep Buffer		10 X	4.0 µL		
Ligase		20 U/µM	1.0 µL		
ATP Low		1mM	2.0 µL		
Total Volume			40.0 µL		

4. Mix the reaction well by pipetting or flicking the tube.
5. Spin down contents of tube with a quick spin in a microfuge
6. Incubate at 25°C for 1 hour or overnight.
7. Incubate at 65°C for 10 minutes to inactivate the ligase, then return the reaction to 4°C.

Repair DNA Damage

Use the following table to repair any DNA damage.

1. To the 40 µL ligation reaction, add the following reagents:

Reagent	Tube Cap Color	Stock Conc.	Volume	✓	Notes
Ligation Reaction (from above)			40 µL		
Template Prep Buffer*		10 X	1.0 µL		
NAD+*		100 X	0.5 µL		
ATP high*		10 mM	5.0 µL		
dNTP*		10 mM	0.5 µL		
DNA Damage Repair Mix*			2.0 µL		
H_2O			1.0 µL		
Total Volume			50.0 µL		

* For more than 1 sample, prepare a pre-mix of these reagents and then add 10 µL of the pre-mix to the 40 µL ligation reaction. Also, note that the Template Prep Buffer is used in the above reaction instead of the DNA Damage Repair Buffer.

2. Mix the reaction well by pipetting or flicking the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 37ºC for 60 minutes or longer, up to 150 minutes, then return the reaction to 4°C for 1 minute. You must proceed with adding exonuclease after this step.

Add Exonucleases to Remove Failed Ligation Products

Reagent	Tube Cap Color	Stock Conc.	Volume	✓	Notes
Repaired Asymmetric SMRTbell			50 µL		
Mix reaction well by pipetting					
Exo III		100.0 U/µL	0.5 µL		
Exo VII		10.0 U/µL	0.5 µL		
Total Volume			51 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 37°C for 60 minutes, then return the reaction to 4°C. You must proceed with purification after this step.

Purify SMRTbell™ Templates

There are 2 purification steps using the appropriate volumes of AMPure PB beads for each step, as shown in the tables below.

STEP	✓	Purify SMRTbell Templates - First Purification	Notes
1		Add the appropriate volume of AMPure PB beads to the exonuclease-treated reaction.	
2		Mix the bead/DNA solution thoroughly.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by shaking in a VWR vortex mixer at 2000 rpm for 10 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol. – Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). – Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol 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 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.	
14		The eluted DNA in **50 µL** Elution Buffer should be taken into the second AMPure PB bead purification step.	

STEP	✓	Purify SMRTbell Templates - Second Purification	Notes
1		Add the appropriate volume of AMPure PB beads to **50 µL** of eluted DNA from the previous AMPure PB bead purification, as with the first purification.	
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. – Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). – Slowly dispense the 70% ethanol against the side of the tube opposite the beads. – Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol 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 **11 µL** Elution Buffer, mix until homogenous. – Vortex for 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.	
14		Verify your DNA amount and concentration with either a Nanodrop or Qubit quantitation reading.	
15		Perform qualitative analysis using a Bioanalyzer instrument. Note that typical DNA yield, at this point of the process, can range from **10-25%** of the total starting material going into the DNA damage repair reaction, depending on the purity and quality of input DNA.	

Proceed to the next step or store SMRTbell templates at -20°C prior to annealing, binding, and sequencing.

Primer Annealing and Polymerase Binding

1. Use the annealing and binding calculator for asymmetric templates
2. Asymmetric templates require two primers.
3. In a Lobind tube, prepare a total 83.3 nM of primer by mixing equimolar amount of the two primers (ie., 41.65 nM each).
4. Condition the primer mix at 80ºC for 2 minutes and hold at 4ºC.
5. Bind conditioned primer mix at 20ºC for 60 minutes.
6. Perform polymerase binding final concentration 0.5nM, 30ºC for 4 hours. See the Binding Calculator for recommendations.

Sequence

To prepare for sequencing on the instrument, refer to the PacBio *Operations Guide – Sequel System*. We suggest the following On-Plate loading concentration for the following amplicon size as a starting point. Use MicroSpin columns to remove excess polymerase prior to loading in the Sequel system.

Amplicon Size	Loading Method	Pre-Extension	On-Plate Concentration
2000 - 3000 bp	Diffusion	Recommended	3.3 pM - 5.2 pM
3001 – 7500 bp	Diffusion	Recommended	6.6 pM – 13.2 pM
7501 – 15000 bp	Diffusion	Consult your FAS	4 pM - 8 pM

Data Collection: 6 – 10 hrs

Pre-Extension Time: Use the following equation to calculate the optimal pre-extension time for the sample:

Pre-extension time (mins) = insert size x 2 / 1.5 bases/second / 60 seconds

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