Procedure & Checklist - Iso-Seq™ Template Preparation for Sequel® Systems

Before You Begin

The Sequel System generates long reads that are well-suited for characterizing full-length transcripts produced from high-quality RNA samples. This document describes two methods to construct Iso-Seq SMRTbell® libraries with and without size selection allowing detection of full-length transcripts

Section 1 of this document describes the construction of a non-size selected Iso-Seq library. This procedure allows detection of full-length transcripts up to 4 kb (without doing size-selection). Using the Clontech SMARTer PCR cDNA Synthesis Kit, RNA is synthesized to cDNA and subsequently amplified to generate double-stranded cDNA. Without size selection, the cDNA is constructed to a SMRTbell library for sequencing.

Section 2 describes a combined procedure to construct and pool a non-size selected Iso-Seq library with a size selected Iso-Seq library for sequencing on the Sequel System. By incorporating a size selection step using BluePippin™ or SageELF™, this procedure enables one to increase the sequencing yield of >4 kb transcripts. Using the Clontech SMARTer PCR cDNA Synthesis Kit, RNA is synthesized to cDNA and subsequently amplified to generate double-stranded cDNA. One portion of the amplified cDNA product is used directly to construct a non-size selected SMRTbell library. In parallel, a second portion of the amplified cDNA product is first size selected using either BluePippin or SageELF, and then the size-fractionated cDNA is used to construct a size-selected SMRTbell library. Finally, the non-size selected SMRTbell library and the size-selected SMRTbell library are separately annealed and bound, and then pooled together for sequencing on the Sequel System.

Users should proceed with following either Section 1 or Section 2 of this document to construct the desired type of Iso-Seq library that is most appropriate for their experimental design. PacBio recommends performing a no-size selection experiment first to better understand diversity of transcripts in your samples.

Sections	Procedure	Target Size	Size Selection
1	No Size-Selection Iso-Seq Template Preparation for Sequel Systems	< 4kb	No
2	Iso-Seq Template Preparation for Sequel Systems with Size selection	For enriching >4kb transcripts	Yes

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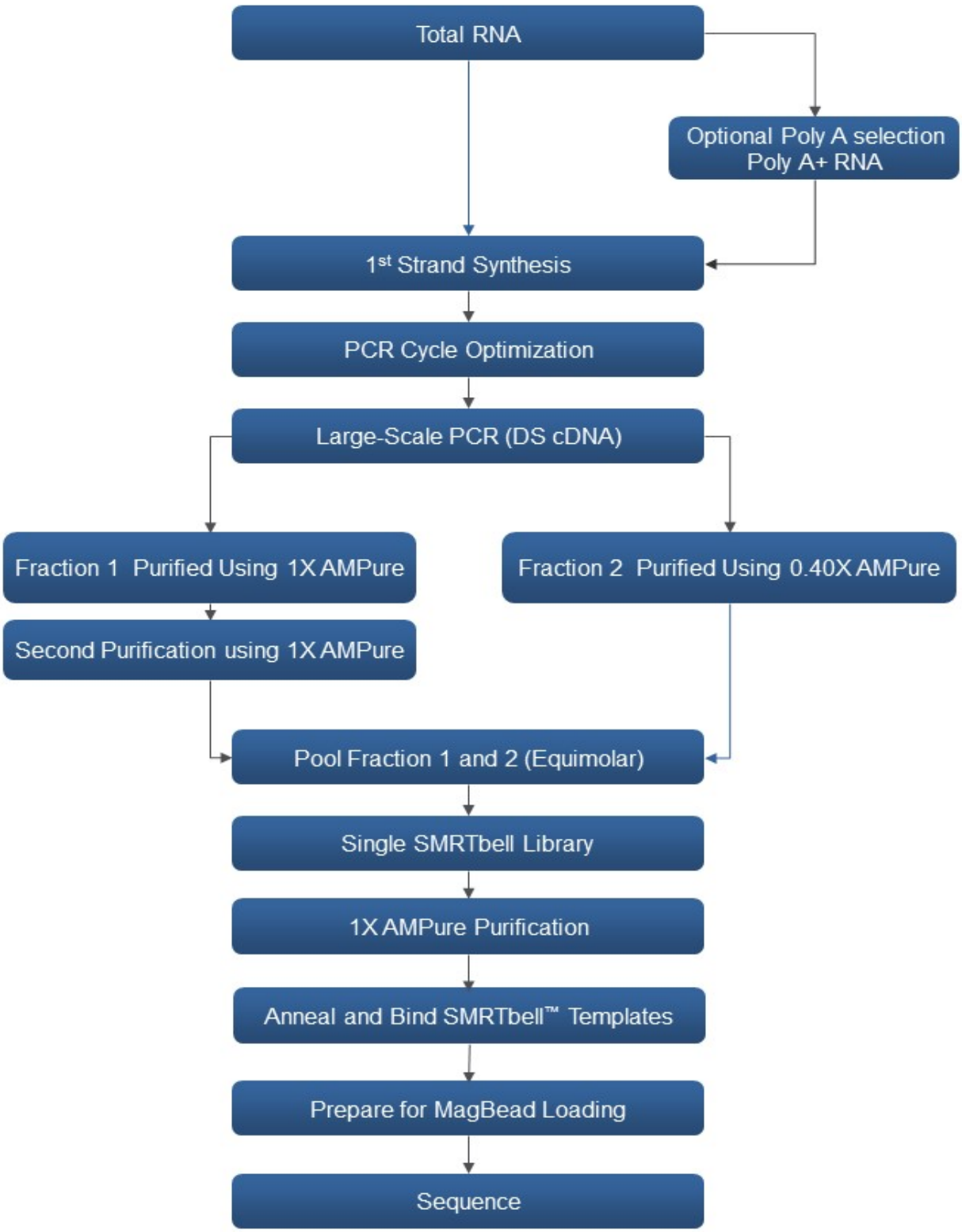
Materials and Kits Needed

Item	Vendor
SMARTer PCR cDNA Synthesis Kit	Clontech (634925 or 634926)
PrimeSTAR GXL DNA Polymerase	Clontech (R050A or R050B)
Additional 5' PCR Primer IIA 5' AAG CAG TGG TAT CAA CGC AGA GTA C 3'	Any Oligo Synthesis Vendor
1.2% FlashGel® system or 0.80% Agarose Gels	Lonza (57023) or (57029) or Any MLS
FlashGel DNA Marker (100bp – 3 kb or 100 bp - 4 kb)	Lonza
Qubit® dsDNA BR Assay Kit or HS Assay Kit	Invitrogen
DNA 12000 Kit or HS DNA Kit	Agilent
SMRTbell Template Prep Kit Sequel Binding Kit Sequel Sequencing Kit AMPure® PB Beads	Pacific Biosciences
Optional for Size Selection: ● Blue Pippin System and Consumables: o BluePippin system with Software v5.90 or later o PacBio SMRTbell cassette definition set "0.75% DF 2 –6kb Marker S1" o 0.75% Dye-Free Agarose Gel Cassettes o Loading Solution o S1 Marker o Electrophoresis Buffer ● SageELF System and Consumables: o SageELF system with Software v0.57 or later o PacBio SMRTbell cassette definition set "0.75% Agarose, 1 kb-18 kb" o 0.75% Dye-Free Agarose Gel Cassettes o Loading Solution o DNA Marker	Sage Science

Section 1: No Size-Selection Iso-Seq Template Preparation for Sequel Systems



Preparing cDNA from RNA Samples

RNA Input Requirements

First strand cDNA synthesis employs the Clontech SMARTer PCR cDNA Synthesis Kit. The CDS Primer IIA is first annealed to the polyA+ tail of transcripts, followed by first-strand synthesis with SMARTScribe™ Reverse Transcriptase. The first-strand product is diluted with Elution Buffer (EB) to an appropriate volume and subsequently used for large-scale PCR.

If starting with Total RNA, prepare two or more first-strand cDNA synthesis reactions.
Consult your local Field Applications Scientist (FAS) for recommendations if starting with poly A+ RNA.

First-Strand Synthesis

This procedure has been optimized using 800 to1000 ng of total RNA as input for each cDNA synthesis reaction. Note that as low as 2 ng of total RNA may be used in each cDNA synthesis reaction – however, the resulting amount of cDNA product will not be sufficient to proceed with this procedure. In this case, PacBio recommends constructing an Iso-Seq library using a 1X AMPure PB bead purification method. Please contact your local Field Applications Scientist for recommendations on how to proceed with low input total RNA samples.

For each sample, combine the reagents below. When using >1 µg of Total RNA as input, do not scale up the reaction volumes. Instead, perform multiple reactions separately (with each reaction in a 4.5 µL Total Volume).

Reagent	Volume	✓	Notes
Total RNA (800 - 1000 ng)	1 - 3.5 µL		
3' SMART® CDS Primer IIA (12 µM)	1 µL		
Nuclease-Free Water	X		
Total Volume	4.5 µL		

1. Mix contents and spin the tubes briefly in a microcentrifuge.
2. Incubate the tubes at 72°C in a hot-lid thermal cycler for 3 min; slowly ramp to 42°C at 0.1°C/sec, then let sit for 2 minutes.
   - During this step, prepare a Master Mix for all reaction tubes, at room temperature, by combining the following reagents in the order shown. Important: go immediately into step 4 after step 3. However, add the reverse transcriptase to the master mix just prior to use.
   - Mix well by pipetting and spin the tube briefly in a microcentrifuge.

Reagent	Volume	✓	Notes
5X First-Strand Buffer	2 µL		
DTT (100 mM)	0.25 µL		
dNTP (10 mM)	1 µL		
SMARTer IIA Oligonucleotide (12 µM)	1 µL		
RNase Inhibitor	0.25 µL		
SMARTScribe Reverse Transcriptase (100 U) - add immediately before use	1 µL		
Total Volume added per reaction	5.5 µL		

3. Place the master mix at 42°C for 1 min to bring it up to temperature and proceed immediately to step 4.
4. Aliquot 5.5 µL of the Master Mix into each reaction tube. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.
5. Incubate the tubes at 42°C for 90 minutes.
6. Terminate the reaction by heating the tubes at 70°C for 10 min.
7. Dilute the first-strand reaction product by adding 90 µL of PacBio Elution Buffer (EB):

Input Sample	Volume of EB Added
Total RNA (2 ng - 1 µg)	90 µL

8. Pool the diluted first-strand reactions for large-scale amplification.
9. Proceed to PCR cycle optimization

PCR Cycle Optimization

RNA samples from different sources behave differently during amplification. PacBio highly recommends performing cycle number optimizations to minimize PCR bias (prevent under or over-amplification). For 800 ng – 1000 ng input total RNA into 1st strand synthesis, the optimal number of cycles is typically 10-12. If input total RNA is less than 800 ng, it is highly recommended to perform cycle optimizations to determine the best condition for large scale PCR.

1. Add the following reagents to an appropriately sized PCR tube:

Reagent	Volume	✓	Notes
5X PrimeSTAR GXL buffer	10 µL		
Diluted first-strand cDNA from step 7 above	10 µL		
dNTP Mix (2.5 mM each)	4 µL		
5’ PCR Primer IIA (12 µM)	1 µL		
Nuclease-free water	24 µL		
PrimeSTAR GXL DNA Polymerase (1.25 U/µL)	1 µL		
Total Volume	50 µL		

2. Cycle the reaction with the following conditions (using the default heated lid setting):
   - Initial denaturation:
     - 98°C for 30s
   - 10 cycles at the following temperatures and times:
     - 98°C for 10 seconds
     - 65°C for 15 seconds
     - 68°C for 10 minutes
   - Final extension:
     - 68°C for 5 minutes
3. After the initial 10 cycles, remove 5 µL of the reaction and transfer it to a tube labeled “10.”
4. Return the remaining 45 µL PCR reaction to the thermocycler and run two cycles of the above amplification conditions.
   - 2 cycles at the following temperatures and times:
     - 98°C for 10 seconds
     - 65°C for 15 seconds
     - 68°C for 10 minutes
   - Final extension:
     - 68°C for 5 minutes

5. Remove 5 µL again and transfer to a tube labeled “12.”
6. Repeat steps 4-5 for 14 cycles.

   Note that the number of cycles is dependent on the sample input. Typically, 800-1000 ng input of total RNA requires 10 to 12 cycles of PCR amplification. Input total RNA of <800 ng may require more cycles.

7. Load the 3 aliquots on a 1% Agarose gel or Lonza flash Gel.

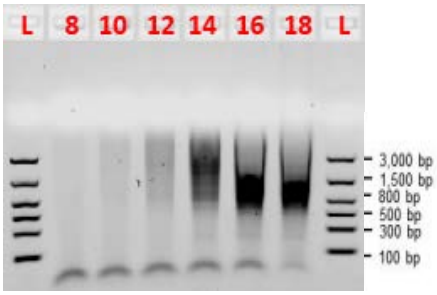


Figure 1. This sample is Human Brain Total RNA from Biochain. One (1) µg Total RNA was used for 1st strand synthesis. With 1 ug input, the optimal number of PCR cycles is 10-12 cycles. To demonstrate cDNA distribution, PCR aliquots (8, 10, 12, 14, 16, 18) collected during PCR optimization were run on agarose electrophoresis (1.2% Lonza® FlashGel, Lonza FlashGel Ladder (100 bp – 3 kb). The numbers above lanes indicate cycle number. In this example, 10 cycles were determined to be optimal for large-scale amplification. Smear distribution from 8 and 10 cycles look similar, however 10 cycles shows more products than 8 cycles. Cycles above 12 show signs of over-amplification which will result in biased sequencing representation.

Large-Scale PCR

Use the cycle number (as determined in the PCR Cycle Optimization step) to generate a sufficient amount of double-stranded cDNA product for SMRTbell library construction.

1. Set up 16 X 50 µL PCR reactions using the diluted first-strand cDNA as input.
2. Make a master mix by adding the following reagents:

Reagent	Volume (1 rxn)	Volume (16 rxns)	✓	Notes
5X PrimeSTAR GXL Buffer	10 µL	160 µL		
Diluted first-strand cDNA	10 µL	160 µL		
dNTP Mix (2.5 mM each)	4 µL	64 µL		
5' PCR Primer IIA (12 µM)	1 µL	16 µL		
Nuclease-free water	24 µL	384 µL		
PrimeSTAR GXL DNA Polymerase (1.25 U/µL)	1 µL	16 µL		
Total Volume	50 µL	800 µL		

3. Transfer 50 µL aliquots into 16 PCR tubes and perform PCR using the optimal cycle number determined from the optimization step. Cycle the reaction with the following conditions (using a heated lid):
   - Initial denaturation:
     - 98°C for 30 seconds
   - **N** cycles (optimal cycle determined in the optimization step) at the following temperatures and times:
     - 98°C for 10 seconds
     - 65°C for 15 seconds
     - 68°C for 10 minutes
   - Final extension:
     - 68°C for 5 minutes

AMPure® PB Bead Purification of Large-Scale PCR Products

This procedure requires the use of AMPure purification methods, 1X and 0.40X. Fraction 1 (pool of 6 tubes x 50 µL) is purified twice using 1X AMPure PB beads. Fraction 2 (pool of 10 tubes x 50 µL) is purified once using 0.40X AMPure PB beads.

STEP		1X and 0.40X AMPure PB Bead Purification	Notes
1		Pool 6 x 50 µL PCR reactions and add **1X** volume of AMPure PB magnetic beads. This is **Fraction 1**.	
2		Pool 10 x 50 µL PCR reactions and add **0.40X** volume of AMPure PB magnetic beads in a separate 1.5mL LoBind tube. This is **Fraction 2**.	
3		Process both tubes in parallel by mixing the bead/DNA solution thoroughly.	
4		Quickly spin down the tubes (for 1 second) to collect the beads.	
5		Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature.	
6		Spin down both tubes (for 1 second) to collect beads.	
7		Place the tubes in a magnetic bead rack until the beads collect to the side of the tubes and the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.	
8		With the tubes still on the magnetic bead rack, slowly pipette off cleared supernatant and save in other tubes. 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.	
9		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 tubes from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tubes (1.5 mL for 1.5 mL tubes or 2 mL for 2 mL tubes). Slowly dispense the 70% ethanol against the side of the tubes opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
10		Repeat step 9.	
11		Remove residual 70% ethanol. – Remove tubes from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tubes. – Place the tubes back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
12		Check for any remaining droplets in the tubes. If droplets are present, repeat step 11.	
13		Remove the tubes from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 - 60 seconds.	

<table>
<tr>
<td>14</td>
<td></td>
<td>Add the Elution Buffer volume (see table below) to your beads. Tap the tubes with finger to mix until beads are uniformly re-suspended. Do not pipet to mix.

Elute the DNA by letting the mix stand at room temperature for 2 minutes
Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.
Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube.
Discard the beads.

<table>
<tr><th>Fractions</th><th>Elution Buffer Volume</th></tr>
<tr><td>Fraction 1 (1X AMPure)</td><td>100 µL</td></tr>
<tr><td>Fraction 2 (0.40X AMPure)</td><td>22 µL</td></tr>
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<tr>
<td>15</td>
<td></td>
<td>Fraction 1 requires a second round of AMPure PB bead purification. Proceed directly to the next section (‘Second Purification’).
Fraction 2 does not require a second AMPure PB bead purification. Set this tube aside on ice and measure the DNA concentration along with Fraction 1 after the second 1X AMPure PB bead purification for Fraction 1 is completed.</td>
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STEP		Second Purification	Notes
1		Perform a second round of AMPure PB bead purification for **Fraction 1** (now in 100 µL of EB) using **1X** volume of AMPure PB magnetic beads	
2		Quickly spin down the tube (for 1 second) to collect the beads.	
3		Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature.	
4		Spin down both tube (for 1 second) to collect beads.	
5		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.	
6		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.	
7		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. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
8		Repeat step 7.	
9		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
10		Check for any remaining droplets in the tube. If droplets are present, repeat step 9.	
11		Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 - 60 seconds.	
12		Add **22 µL** of Elution Buffer volume to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	

13		Verify the DNA amount and concentration of **Fraction 1** and **Fraction 2** using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit dsDNA BR Assay kit and the dsDNA HS Assay kit according to the manufacturer's recommendations.	
14		Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit. To determine the **average library size**, select the region of interest by defining the start and end points of the smear.	

Pooling Fraction 1 (1X) and Fraction 2 (0.40X) for SMRTbell Template Preparation

The two purified fractions are pooled for library construction.

STEP		Pooling	Notes
1		Based on sample information from the Qubit and BioAnalyzer, determine the molarity of the two fractions, which can be calculated by the following equation: $\frac{\text{concentration in ng/uL} \times 10^6}{(660 \text{ g/mol} \times \text{average library size in bp*})} = \text{concentration in nM}$ *To determine the average library size, select the region of interest by defining the start and end points of the smear.	
2		Pool equal molar quantities of the two fractions in a clean LoBind microcentrifuge tube. The total combined mass must be at least 1 µg.	
3		Proceed to **SMRTbell Template Preparation** below. You need at least 1 µg of pooled cDNA for library construction.	

SMRTbell Template Preparation

Repair DNA Damage

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

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Pooled cDNA	-		___ µL for 1 to 5 µg	-		
DNA Damage Repair Buffer		10 X	5.0 µL	1 X		
NAD+		100 X	0.5 µL	1 X		
ATP high		10 mM	5.0 µL	1 mM		
dNTP		10 mM	0.5 µL	0.1 mM		
DNA Damage Repair Mix			2.0 µL			
H_2O	-		___ µL to adjust to 50.0 µL	-		
Total Volume			50.0 µL	-		

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

Repair Ends

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

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Pooled cDNA (Damage Repaired)	-		50.0 µL	-		
End Repair Mix		20 X	2.5 µL	1X		
Total Volume			52.5 µL	-		

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

STEP	✓	Purify DNA	Notes
1		Add **1X** volume of AMPure PB beads.	
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 until the beads collect to the side of the tubes 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 other tubes. 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 rack. – Use a sufficient volume of 70% ethanol to fill the tubes (1.5 mL for 1.5 mL tubes or 2 mL for 2 mL tubes). Slowly dispense the 70% ethanol against the side of the tubes opposite the beads. – 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. – 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 tubes. – Place the tubes 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 **32 µL** of Elution Buffer volume to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	
14		Optional: Verify your DNA amount and concentration using a Nanodrop or Qubit quantitation platform, as appropriate.	
15		Optional: Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit.	
16		The End-Repaired DNA can be stored overnight at 4ºC or (or -20ºC for longer).	
17		Enter your actual recovery per µL and total available sample material: _____	

Prepare Blunt Ligation Reaction

Use the following table to prepare your blunt ligation reaction:

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

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Pooled cDNA (End Repaired)	-		31.0 µL			
Blunt Adapter (20 µM)		20 µM	2.0 µL	1 µM		
Mix before proceeding						
Template Prep Buffer		10 X	4.0 µL	1X		
ATP low		1 mM	2.0 µL	0.05 mM		
Mix before proceeding						
Ligase		30 U/µL	1.0 µL	0.75 U/µL		
H_2O	-	-	__ µL to adjust to 40.0 µL	-		
Total Volume	-	-	40.0 µL	-		

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

Add Exonuclease to Remove Failed Ligation Products

Reagent	Tube Cap Color	Stock Conc.	✓	Volume
Ligated cDNA				40 µL
Mix reaction well by pipetting				
Exo III		100.0 U/µL		1.0 µL
Exo VII		10.0 U/µL		1.0 µL
Total Volume				42 µL

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

Purify SMRTbell Templates

STEP	✓	Purify SMRTbell Templates - First Purification	Notes
1		Add **1X** volume of AMPure PB beads.	
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 until the beads collect to the side of the tubes 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 other tubes. 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 rack. – Use a sufficient volume of 70% ethanol to fill the tubes (1.5 mL for 1.5 mL tubes or 2 mL for 2 mL tubes). Slowly dispense the 70% ethanol against the side of the tubes opposite the beads. – 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. – 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 tubes. – Place the tubes 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 **50 µL** of Elution Buffer volume to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	

STEP	✓	Purify SMRTbell Templates - Second Purification	Notes
1		Add **1X** volume of AMPure PB beads.	
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 until the beads collect to the side of the tubes 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 other tubes. 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 rack. – Use a sufficient volume of 70% ethanol to fill the tubes (1.5 mL for 1.5 mL tubes or 2 mL for 2 mL tubes). Slowly dispense the 70% ethanol against the side of the tubes opposite the beads. – 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. – 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 tubes. – Place the tubes 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 **10 µL** of Elution Buffer volume to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	

14		Verify the DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit dsDNA BR Assay kit and the dsDNA HS Assay kit according to the manufacturer's recommendations.	
15		Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit. To determine the **average library size**, select the region of interest by defining the start and end points of the smear.	

Anneal and Bind Non-Size Selected SMRTbell Templates

Follow the SMRT Link Sample Setup instructions for primer annealing and polymerase binding conditions to prepare your non-size selected Iso-Seq library for sequencing.

Sequence

MagBead loading is recommended for Iso-Seq libraries prepared using this procedure. PacBio recommends performing loading titrations to determine an appropriate loading concentration.

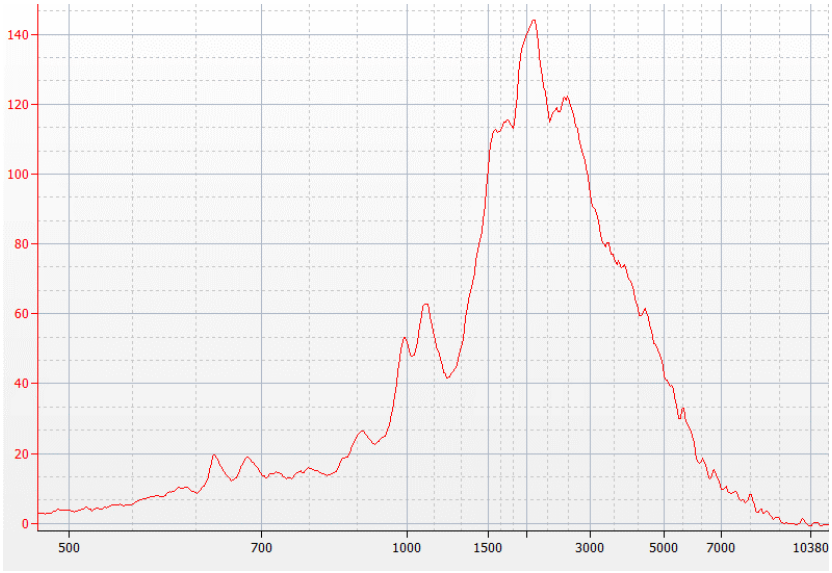
Sequencing recommendations:

- Loading: >40 pM on-plate concentration, Target P1 ≥50%
  (Performing loading titrations to determine the appropriate loading concentration is recommended.)
- Movie Collection time: 360 – 600 minutes
- Pre-extension time: 120 minutes
- Sample Cleanup: Not required

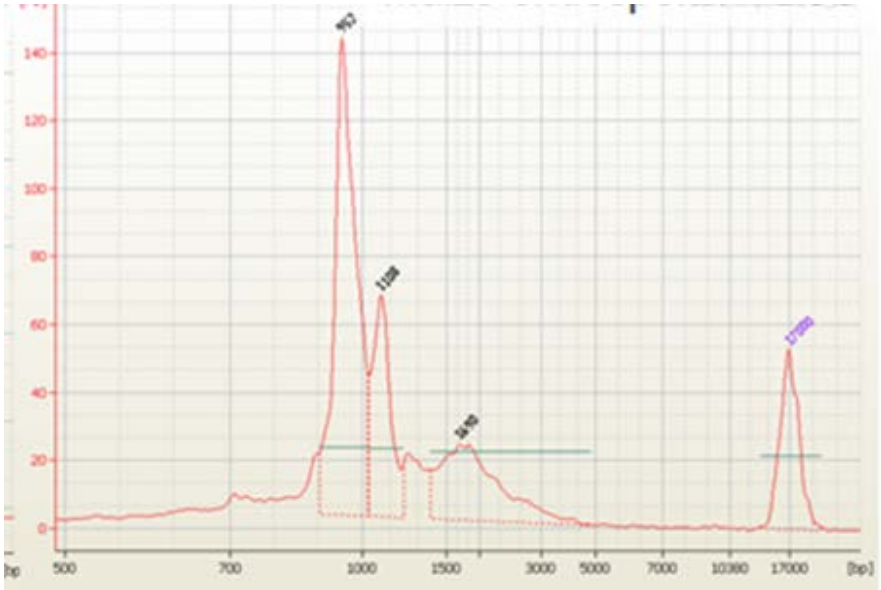
Section 2: Iso-Seq Template Preparation for Sequel Systems with Size Selection

Sample Considerations for Performing Size Selection

To sequence a broad range of transcripts (5 kb -10 kb) using the Sequel System, consider including a size selection step as part of the Iso-Seq library preparation workflow. Amplification and sequencing of full-length transcripts > 5 kb can be achieved depending on the sample type and quality. Sample 1 below is an example of a sample that would benefit from size-selection since the Bioanalyzer sizing QC plot indicates the presence of transcripts between 5 kb to 8 kb. However, Sample 2 will likely *not* benefit from size selection because there are no transcripts >5 kb visible in the QC plot.

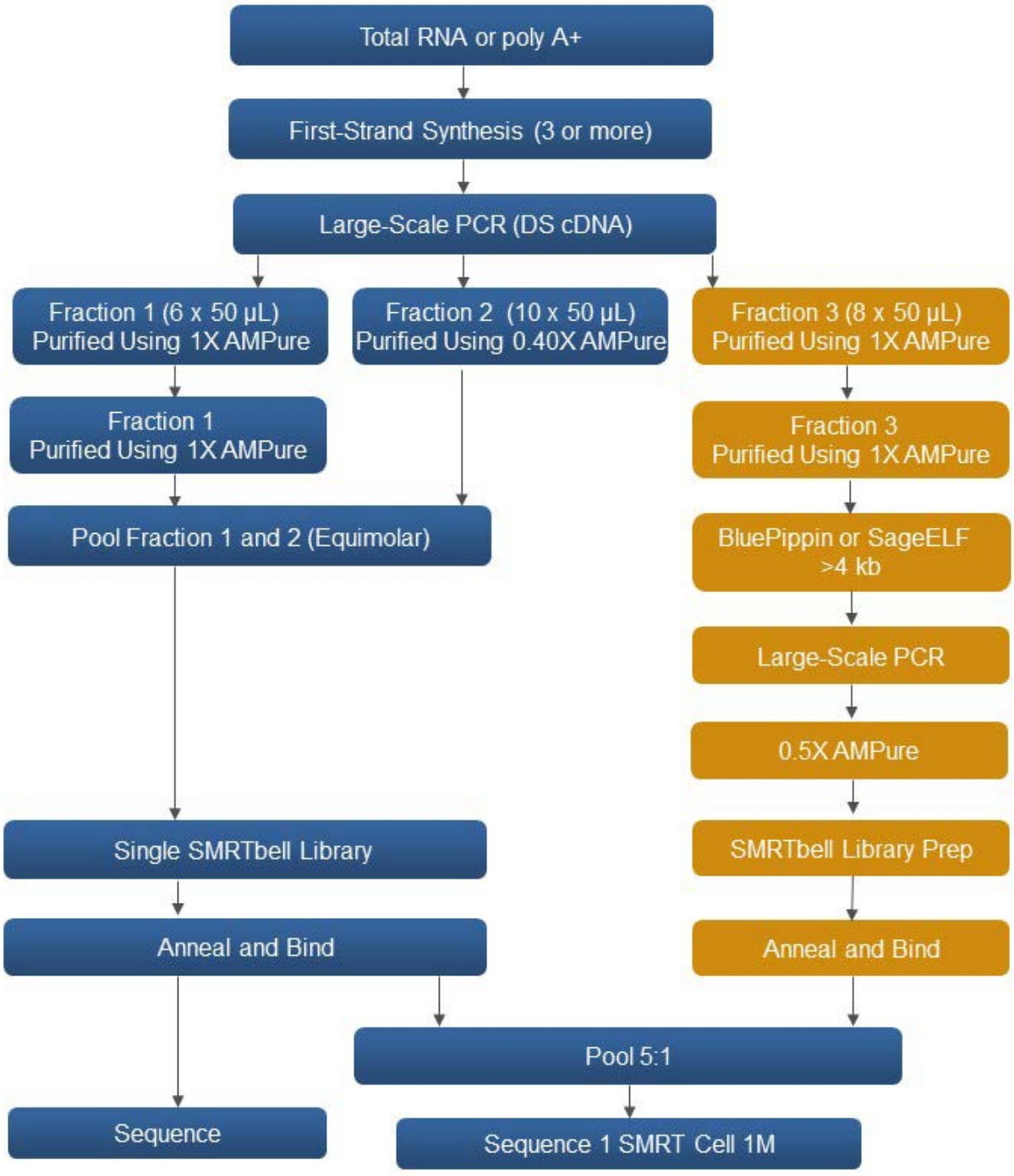


Sample 1



Sample 2

This section of the procedure describes how to construct Iso-Seq libraries using a parallel workflow that incorporates both no-size selection steps and size selection steps (see flow chart below).


Total RNA or poly A+
First-Strand Synthesis (3 or more)
Large-Scale PCR (DS cDNA)
Fraction 1 (6 x 50 µL) Purified Using 1X AMPure
Fraction 2 (10 x 50 µL) Purified Using 0.40X AMPure
Fraction 3 (8 x 50 µL) Purified Using 1X AMPure
Fraction 1 Purified Using 1X AMPure
Fraction 3 Purified Using 1X AMPure
Pool Fraction 1 and 2 (Equimolar)
BluePippin or SageELF >4 kb
Large-Scale PCR
0.5X AMPure
Single SMRTbell Library
SMRTbell Library Prep
Anneal and Bind
Anneal and Bind
Pool 5:1
Sequence
Sequence 1 SMRT Cell 1M

First-Strand Synthesis Requirements

The table below summarizes the total number of first-strand cDNA synthesis reactions required for carrying out parallel non-size selection and size-selection Iso-Seq library construction.

RNA Input Type	Recommended Number of First-Strand Synthesis Reactions
Total RNA	3 or more

Refer to "Preparing cDNA from RNA Samples" in Section 1 for detailed instructions on how to prepare First-strand cDNA synthesis reactions.

For each cDNA synthesis reaction, dilute the first-strand reaction product by adding the appropriate volume of PacBio Elution Buffer (EB) as follows:

Input Sample	Volume of EB Added
Total RNA (2 ng - 1 µg)	90 µL

Large-Scale PCR

1. Set up 24 x 50 µL PCR reactions if 3 first-strand cDNA synthesis reaction with Total RNA were prepared in the previous step above. Scale up the large-scale PCR reaction volumes if additional first-strand cDNA synthesis reactions are needed for your experiment.
2. Make a master mix by adding the following reagents:

Reagent	Volume (1 rxn)	Volume (24 rxns)	✓	Notes
5X PrimeSTAR GXL Buffer	10 µL	240 µL		
Diluted first-strand cDNA	10 µL	240 µL		
dNTP Mix (2.5mM each)	4 µL	96 µL		
5' PCR Primer IIA (12 µM)	1 µL	24 µL		
Nuclease-free water	24 µL	576 µL		
PrimeSTAR GXL DNA Polymerase (1.25 U/µL)	1 µL	24 µL		
Total Volume	50 µL	1200 µL		

3. Transfer 50 µL aliquots into 24 PCR tubes and perform PCR using the optimal cycle number determined from the optimization step. Cycle the reaction with the following conditions (using a heated lid):

- Initial denaturation:
  - 98°C for 30 seconds
- **N** cycles (optimal cycle determined in the optimization step) at the following temperatures and times:
  - 98°C for 10 seconds
  - 65°C for 15 seconds
  - 68°C for 10 minutes
- Final extension:
  - 68°C for 5 minutes

AMPure PB Bead Purification of Large-Scale PCR Products

Fraction 1 (pool of 6 tubes x 50 µL) is purified using 1X AMPure PB beads. **Fraction 2** (pool of 10 tubes x 50 µL) is purified using 0.40X AMPure PB beads. **Fraction 3** (pool of 8 tubes x 50 µL) is purified using 1X AMPure PB beads and will be used for size selection.

STEP		1X and 0.40X AMPure PB Bead Purification	Notes
1		Pool 6 x 50 µL PCR reactions and add **1X** volume of AMPure PB magnetic beads. This is **Fraction 1**.	
2		Pool 10 x 50 µL PCR reactions and add **0.40X** volume of AMPure PB magnetic beads in a separate 1.5mL LoBind tube. This is **Fraction 2**.	
3		Pool 8 x 50 µL PCR reactions and add **1X** volume of AMPure PB magnetic beads in a separate 1.5mL LoBind tube. This is **Fraction 3**.	
4		Process all tubes in parallel by mixing the bead/DNA solution thoroughly.	
5		Quickly spin down the tubes (for 1 second) to collect the beads.	
6		Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature.	
7		Spin down tubes (for 1 second) to collect beads.	
8		Place the tubes in a magnetic bead rack until the beads collect to the side of the tubes and the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.	
9		With the tubes still on the magnetic bead rack, slowly pipette off cleared supernatant and save in other tubes. 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.	
10		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 tubes from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tubes (1.5 mL for 1.5 mL tubes or 2 mL for 2 mL tubes). Slowly dispense the 70% ethanol against the side of the tubes opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
11		Repeat step 10.	
12		Remove residual 70% ethanol. – Remove tubes from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tubes. – Place the tubes back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
13		Check for any remaining droplets in the tubes. If droplets are present, repeat step 12.	

<table>
<tr><td>14</td><td></td><td>Remove the tubes from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 - 60 seconds.</td><td></td></tr>
<tr><td>15</td><td></td><td>Add the Elution Buffer volume (see table below) to your beads. Tap the tubes with finger to mix until beads are uniformly re-suspended. Do not pipet to mix.
– Elute the DNA by letting the mix stand at room temperature for 2 minutes
– Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack.
– Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube.
– Discard the beads.
<table><tr><th>Fractions</th><th>Elution Buffer Volume</th></tr><tr><td>Fraction 1 (1X AMPure)</td><td>100 µL</td></tr><tr><td>Fraction 2 (0.40X AMPure)</td><td>22 µL</td></tr><tr><td>Fraction 3 (1X AMPure)</td><td>100 ul</td></tr></table></td><td></td></tr>
<tr><td>16</td><td></td><td>Fraction 1 and Fraction 3 require a second purification. Proceed directly to the next section (‘Second Purification’).
Fraction 2 does not require a second purification. Set aside in ice and measure concentration along with Fraction 1 and Fraction 3 after the second AMPure PB bead purification.</td><td></td></tr>
</table>

STEP		Second Purification	Notes
1		Perform a second 1X AMPure PB bead purification for **Fraction 1** and **Fraction 3**.	
2		Quickly spin down the tubes (for 1 second) to collect the beads.	
3		Allow the DNA to bind to beads by shaking in a VWR® vortex mixer at 2000 rpm for 10 minutes at room temperature.	
4		Spin down both tubes (for 1 second) to collect beads.	
5		Place the tubes 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.	
6		With the tubes 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.	
7		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 tubes 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. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
8		Repeat step 7.	
9		Remove residual 70% ethanol. – Remove tubes 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.	
10		Check for any remaining droplets in the tube. If droplets are present, repeat step 9.	
11		Remove the tubes from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 - 60 seconds.	
12		Add **22 µL** of Elution Buffer volume to your beads. Tap the tube with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mixes stand at room temperature for 2 minutes – Spin the tubes down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	

13		Verify the DNA amount and concentration of Fractions 1, 2 and 3 using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit dsDNA BR Assay kit and the dsDNA HS Assay kit according to the manufacturer's recommendations.	
14		Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit. To determine the **average library size**, select the region of interest by defining the start and end points of the smear.	
15		Use **Fraction 3** for size selection. Use **Fraction 1** and **Fraction 2** for Pooling in the next section.	

Pooling Fraction 1 (1X) and Fraction 2 (0.40X) for Non-Size Selected SMRTbell Template Preparation:

STEP		Pooling	Notes
1		Based on sample information from the Qubit and BioAnalyzer, determine the molarity of the two fractions, which can be calculated by the following equation: $\frac{\text{concentration in ng/ul X } 10^6}{\text{(660 g/mol x average library size* in bp)}}$ = concentration in nM *To determine the average library size, select the region of interest by defining the start and end points of the smear.	
2		Pool equal molar of the two fractions. The total mass must be at least 1 µg.	
3		The pooled fractions can now be taken directly into SMRTbell Library construction starting with "**Repair DNA Damage**". You need at least 1 µg cDNA for library construction.	

Size Selection Procedure for Fraction 3 (1X AMPure Purified):

The **Fraction 3 (1X)** AMPure PB bead-purified sample is size-selected using a BluePippin System or a SageELF System.

Option 1 - Size Selection of Fraction 3 (1X) using the BluePippin System

For BluePippin, 500 ng – 5000 ng is required.

STEP		Running the BluePippin System	Notes
1		Follow the BluePippin Manual and instructions to calibrate your instrument. A new calibration is recommended before each BluePippin run.	
2		Inspect the gel cassette (using Sage Sciences' BluePippin manual). – Ensure that the buffer wells are full. – Ensure that there is no separation of the gel from the cassette.	
3		Prepare the gel cassette: – Remove all bubbles from the elution buffer chamber by tilting the cassette and tapping it until all air bubbles move into the buffer chamber. – Place the gel cassette in the BluePippin System and carefully remove the plastic seals on the cassette. – Remove the buffer from the elution well and fill with **40 µL** of fresh Electrophoresis Buffer. ● Keep the pipette down the center of the well and avoid creating a vacuum in the well. ● The bottom of the well is okay to touch. ● If the well "bubbles" over when adding the buffer to the well, remove buffer and try again. If the well continues to "bubble" over, then use this well for the S1 marker. The elution well may be damaged and should not be used for sample collection. – Cover the elution wells with a clear adhesive tape – Remove the buffer from the sample well and fill with **70 µL** of fresh Electrophoresis Buffer – Close the lid and perform a Continuity Test.	
4		Prepare samples for loading – >500 ng (up to **5 µg**) is required. – Use Elution Buffer to dilute sample to **30 µL**. – Add **10 µL** of the Loading Solution and vortex to mix well.	
5		Load samples: – Remove **40 µL** of buffer from each well. – Load all **40 µL** of the sample prepared in step 4 into each lane. – Load **40 µL** of S1 Marker in one of the lanes.	

STEP		Running the BluePippin System	Notes
6		Set up the run Protocol: – Click on the “New” button to create a new protocol. – Select the “0.75% DF 2 – 6kb Marker S1” cassette definition file. – Click on the box below “End Run when Elution is Complete.” – Set the lane where the S1 Marker is loaded as the reference “Ref” lane. – Click on the “Range” button and enter the following: Library Size: 5 kb - 10 kb; BP Start: 4500; BP End: 10000	
7		Start the run.	
8		After the run, collect approximately **40 µL** the respective fractions from each lane.	
9		Wash the elution well with 40uL of EB, pipet up and down 10 times, let the buffer sit for 10 minutes before removing from well. Combine the 40uL wash with the 40uL of eluate	

The samples can be stored in - 20°C or used directly in the next step (**‘Large-Scale PCR Post-Size Selection’**). Note that DNA quantification using a Qubit system is not necessary at this point.

Option 2 – Size Selection of Fraction 3 (1X) using the SageELF System

For Sage ELF, 1000 ng - 5000 ng is required.

STEP		Running the SageELF System	Notes
1		Follow the SageELF Manual and instructions to calibrate your instrument. – A new calibration is recommended before each run.	
2		Inspect the gel cassette (using Sage Science's SageELF manual). – Ensure that the buffer wells are full. – Ensure that there is no separation of the gel from the cassette.	
3		Prepare the gel cassette: – While the cassette is sealed, remove all bubbles from the elution buffer chamber by tilting the cassette and tapping it until all air bubbles move into the buffer chamber. – Hold the cassette firmly on the bench top and carefully remove the plastic seals on the cassette. – Remove the buffer from the elution well and fill with **30 µL** of fresh Electrophoresis Buffer. • Keep the pipette down the center of the well and avoid creating a vacuum in the well. • The bottom of the well is okay to touch. • If the well "bubbles" over when adding the buffer to the well, remove buffer and try again. – Cover the elution wells with a clear adhesive tape and verify that it is tightly sealed. – Remove the buffer from the sample well and fill with **70 µL** of fresh Electrophoresis Buffer. Do not touch the sides and bottom of the sample well. – Carefully place the gel cassette in the SageELF System. – Verify that the "moat" on both sides of the cassette, that connect the electrode reservoirs, are full. Add additional electrophoresis buffer to fill up the moat, if necessary. – Close the lid and perform a Current Test.	
4		Prepare samples for loading – Prepare **30 µL** tube with 1 - 5 µg of amplified cDNA. It is highly recommended to start with >3 µg of cDNA. – Add **10 µL** of Sage Science's Marker 75. Mix well and do a quick spin down.	
5		Load samples: – Remove **40 µL** of buffer from the sample well. – Load all **40 µL** of the sample prepared in step 4 into the sample well. – If necessary, top off well with additional Electrophoresis Buffer. Do not overflow the well.	

6		Set up the run Protocol: – In the “Protocol Editor” tab, click on the “New Protocol” button. – Select the “0.75% Dye Free 1-18kb” in the cassette definition menu. – Select “size-based” for separation mode. – Fill in the “Target Value” field and use the slider to select well #10. Enter 1500 bp in the target value. – Save as new protocol. – On the Main screen, clear previous run data, select cassette description, cassette definition and protocol, enter sample ID(s). – Select in the Nest Selector the cartridge that will be run.	
7		Start the run.	
8		Once the run is complete, (approximately 3 hours), collect **30 µL** of the respective fractions from the elution wells. Rinse each well by adding **30 µL** of fresh Elution Buffer into the empty elution well. Rinse by pipetting up and down several times, and collect the rinse into the same tube.	
9		Measure concentration by Qubit.	
10		Check the sizes of all 12 fractions by loading on a Bioanalyzer system using a DNA 12000 kit. To determine the **average library size**, select the region of interest by defining the start and end points of the smears.	
11		This is a safe stopping point. The 12 fractions can be stored at - 20°C for future use.	
12		Pool fractions 1 - 4. This pool will contain >5 kb cDNA. When pooling, mix fractions in equimolar quantities. (Note that this is the reason accurate quantitation using the Qubit system is essential).	

The samples can be stored in - 20°C or used directly in the next step (**‘Large-Scale PCR Post-Size Selection’**). Note that DNA quantification using a Qubit system is not necessary at this point.

Large-Scale PCR Post-Size Selection

The amount of recovered size-selected **Fraction 3 (1X)** sample is not sufficient for SMRTbell library construction. It is necessary to perform additional amplification to enrich for >5 kb cDNA.

1. Set up 6 X 50 µL PCR reactions.
2. Add the following reagents to an appropriately sized PCR tube:

Reagent	Volume (1 rxn)	Volume (6 rxns)	✓	Notes
5X PrimeSTAR GXL Buffer	10 µL	60 µL		
Eluted DNA from size selection	10 µL	60 µL		
dNTP Mix (2.5 mM)	4 µL	24 µL		
5' PCR Primer IIA (12 µM)	1 µL	6 µL		
Nuclease-free water	24 µL	144 µL		
PrimeSTAR GXL DNA Polymerase (1.25U/µL)	1 µL	6 µL		
Total Volume	50 µL	300 µL		

3. Aliquot 50 µL into 6 PCR tubes and perform PCR using the cycle number and extension parameters below.

Size Desired	Extension Time	Number of Cycles
5 kb - 10 kb	10 min	6 -10 *

*The number of cycles depends on your recovery after size selection. If concentration is > 4ng/µL, we recommend 6 cycles. If <4 ng/µL, use 10 cycles.

4. Cycle the reaction with the following conditions (using a heated lid):
   - Initial denaturation:
     - 98°C for 30s
   - ***N (see above recommendation)*** cycles at the following temperatures and times:
     - 98°C for 10 seconds
     - 65°C for 15 seconds
     - 68°C for 10 minutes (for this step, see extension times in table above)
   - Final extension:
     - 68°C for 5 minutes

Purifying the Large-Scale PCR Products

After PCR, pool the PCR reactions into a 1.5 mL tube and purify with 0.5X AMPure PB beads.

STEP		AMPure PB Bead Purification	Notes
1		Add **0.5X** volume of AMPure PB magnetic beads to the amplified cDNA sample.	
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.	
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 tubes 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 other tubes. 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.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tubes. – 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 the tube caps open) for 30 - 60 seconds.	
13		Add the 10-30 ul Elution Buffer volume to your beads. Tap the tubes with finger to mix until beads are uniformly re-suspended. Do not pipet to mix. – Elute the DNA by letting the mix stand at room temperature for 2 minutes – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	
14		Verify the DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer.	
15		Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit. To determine the **average library size**, select the region of interest by defining the start and end points of the smear.	
16		The enriched size selected sample can now be taken into SMRTbell Library construction starting with “Repair DNA Damage”. You need at least 1 µg cDNA for library construction.	

SMRTbell Template Preparation

Prepare a non-size selected SMRTbell library using the (non-size selected) equimolar-pooled **Fraction 1 (1X)** and **Fraction 2 (0.40X)** cDNA samples. In parallel, prepare a second SMRTbell library using the size-selected **Fraction 3 (1X)** cDNA sample. For detailed instructions on how to prepare SMRTbell libraries, see the '**SMRTbell Template Preparation**' procedure in **Section 1**.

After completing the SMRTbell Library construction steps, proceed to '**Anneal and Bind Non-Size Selected and Size-Selected SMRTbell Templates**' below.

Anneal and Bind Non-Size Selected and Size-Selected SMRTbell Templates

Prepare your non-size selected Iso-Seq library and size-selected Iso-Seq library for sequencing by following the SMRT Link Sample Setup instructions for primer annealing and polymerase binding conditions:

1. Perform primer annealing and polymerase binding of the pooled non-size-selected Iso-Seq library.
2. In parallel, perform primer annealing and polymerase binding of the size-selected Iso-Seq library
3. The non-size-selected binding complex and size-selected binding complex can now be pooled together in a 5:1 molar ratio for sequencing on the same Sequel SMRT Cell.
4. [Optional: The non-size selected complex can also be sequenced on its own Sequel SMRT Cell (i.e., without pooling with the size-selected complex) if desired]

Sequence

MagBead loading is recommended for Iso-Seq libraries prepared using this procedure. PacBio recommends performing loading titrations to determine an appropriate loading concentration.

Sequencing recommendations:

- Loading: >40 pM on-plate concentration, Target P1 ≤ 50% (Performing loading titrations to determine the appropriate loading concentration is recommended.)
- Movie Collection time: 360 – 600 minutes
- Pre-extension time: 120 minutes
- Sample Cleanup: Not required

Revision History (Description)	Version	Date
Incorporates (adds in) procedure on constructing and pooling a non-size selected Iso-Seq library with a size selected Iso-Seq library.	03	October 2017
On page 14 (Blunt Ligation Reaction table), sample volume (Pooled cDNA) showed 32 µL. It has been corrected to 31 µL.	04	October 2017
On pages 17 and 31, on-plate concentration changed from 40 – 50 pM to >40 pM. Error fixed in flowchart of page 19: AMPure volume after large-scale PCR (for Fraction 3) changed from 1X to 0.5X AMPure.	05	November 2017