Procedure and Checklist - Isoform Sequencing (Iso-Seq™ Analysis) Using the Clontech SMARTer cDNA Synthesis Kit and SageELF™ Size-selection System

Before You Begin

The long read lengths of the PacBio® System are well-suited for characterizing full-length transcript isoforms produced from high-quality RNA samples. This document describes methods for generating size-selected cDNA libraries using the SageELF System for isoform sequencing. The SageELF system is an option for sizing double-stranded cDNA samples for sequencing in the PacBio RS II.

To perform this procedure, you must have reviewed the *User Bulletin - Guidelines for Preparing cDNA Libraries for Isoform Sequencing (Iso-Seq™ Analysis)*. Below are the four available procedures for specific project requirements.

Procedure	Size Selection	Target Size	Required Size Selection Equipment
Procedure & Checklist - Isoform Sequencing (Iso-Seq™ Analysis) Using the Clontech SMARTer PCR cDNA Synthesis Kit with No Size Selection	No	1.0 kb - 1.5 kb Full-length Transcripts	None
Procedure & Checklist - Isoform Sequencing (Iso-Seq™ Analysis) Using the Clontech SMARTer PCR cDNA Synthesis Kit and Manual Agarose-gel Size Selection	Yes	1.0 kb - 6 kb Full-length Transcripts	Agarose Gel Electrophoresis Chamber and Agarose
Procedure & Checklist - Isoform Sequencing (Iso-Seq™ Analysis) Using the Clontech SMARTer PCR cDNA Synthesis Kit and the BluePippin Size-Selection System	Yes	1.0 kb - 10 kb Full-length Transcripts	BluePippin System
Procedure & Checklist - Isoform Sequencing (Iso-Seq™ Analysis) using the Clontech® SMARTer® PCR cDNA Synthesis Kit and SageELF™ Size Selection System	Yes	1.0 kb - 10 kb Full-length Transcripts	SageELF System

This procedure describes cDNA fractionization using the SageELF system.

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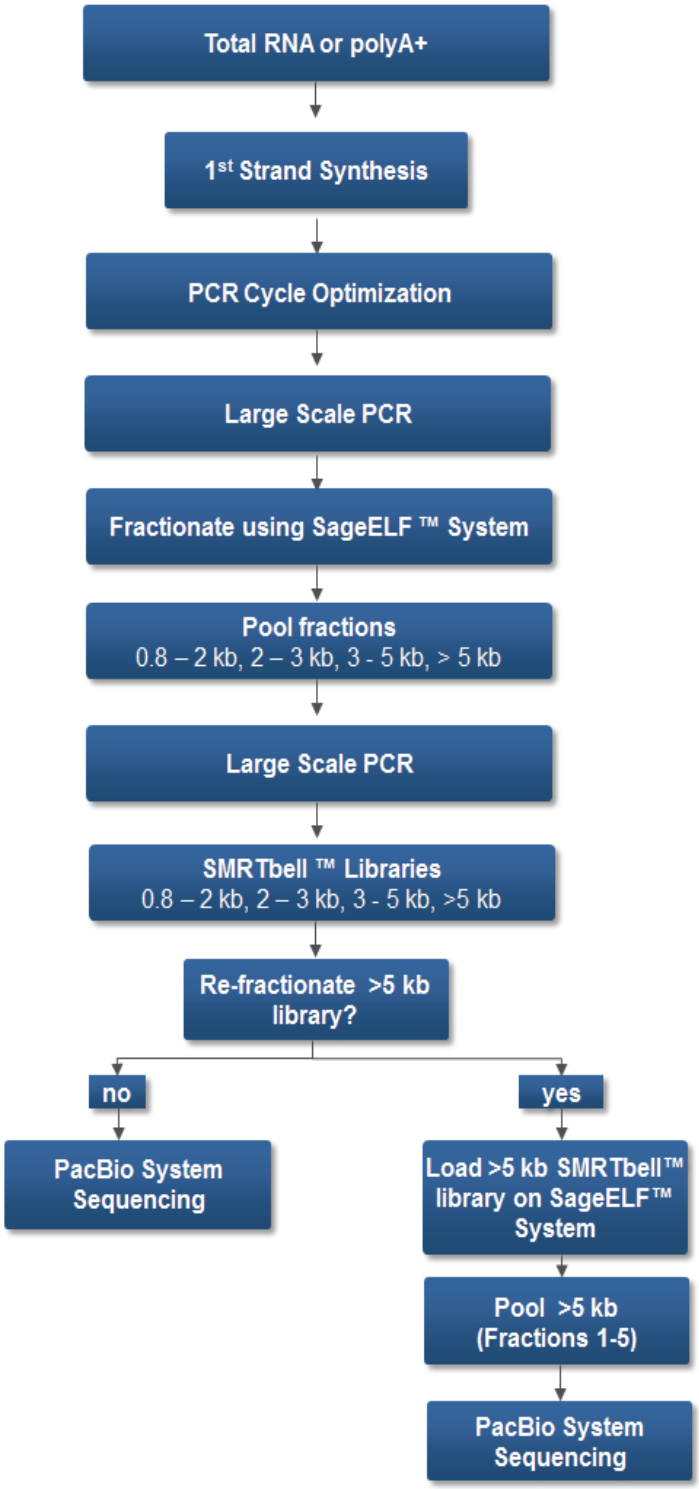
Overview Workflow of Using the SageELF System



Materials and Kits Needed

Item	Vendor
SMARTer PCR cDNA Synthesis Kit	Clontech (634925 or 634926)
KAPA™ HiFi PCR Kit	Kapa Biosystems (KK2101 or KK2102)
Additional 5' PCR Primer IIA	Any Oligo Synthesis Vendor
1.2% FlashGel® system or 0.80% Agarose Gels	Lonza Any MLS
FlashGel DNA Marker (100 bp - 4 kb)	Lonza
Qubit® dsDNA BR Assay Kit	Invitrogen
DNA 7500 Kit	Agilent
SageELF™ system with Software v0.57 or later PacBio SMRTbell cassette definition set "0.75% DF 2 – 6kb Marker S1" 0.75% Agarose, 1kb-18kb" Loading Solution DNA Marker Electrophoresis Buffer	Sage Science
Template Prep Kit DNA/Polymerase Binding Kit DNA Sequencing Kit AMPure® PB Beads	Pacific Biosciences

Preparing cDNA from RNA Samples

First-Strand Synthesis

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.

- Before proceeding with the first-strand synthesis, determine if one primer annealing and first-strand reaction is enough to proceed to the Test Amplification and Large-Scale PCR steps (see dilution table on step 7).
- Perform additional annealing and first-strand synthesis reactions, if necessary. If starting with total RNA, we recommend setting up three separate reactions of first-strand synthesis to ensure there is enough diluted first-strand product for the Test Amplification and Large-Scale PCR steps. If starting with polyA+ RNA, one first-strand reaction is sufficient.

1. For each sample and Control Mouse Liver Total RNA, combine the reagents below in separate PCR tubes. For polyA+ RNA, the minimum is 1 ng; total RNA requires 2 ng.

   Do not change the size (volumes) of any of the reactions. All components have been optimized for the volumes specified. If using > 1 µg RNA, split the sample into multiple reactions.

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

2. Mix contents and spin the tubes briefly in a microcentrifuge.
3. Incubate the tubes at 72°C in a hot-lid thermal cycler for 3 min; slow ramp to 42°C at 0.1°C/sec let sit for 2 minutes.

   During this incubations step, prepare a Master Mix for all reaction tubes, at room temperature, by combining the following reagents in the order shown. It is important to 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 II A Oligonucleotide (12 µM)	1 µL		
RNase Inhibitor	0.25 µL		
SMARTScribe Reverse Transcriptase (100 U) - add before use	1 µL		
Total Volume added per reaction	5.5 µL		

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 the appropriate volume of PacBio Elution Buffer (EB):

Input Sample	Volume of EB added
Total RNA (2 ng - 1 µg)	40 µL
PolyA+ RNA, > 0.2 µg	190 µL
PolyA+ RNA, < 0.2 µg	90 µL

8. If multiple reactions were performed with the same RNA samples, pool the diluted first-strand reactions together before the amplification steps.

Large-Scale PCR

It is highly recommended to perform cycle optimization to determine the optimal number of cycles (while minimizing artifacts during large-scale amplification) for large-scale PCR.

PCR Cycle Optimization

Test Amplification

In this section, perform test amplifications to determine the best number of cycles required for the sample. Collect a total of 5 x 5 µl aliquots from each recommended cycle below.

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

Reagent	Volume	✓	Notes
KAPA HiFi Fidelity Buffer (5X)	10 µL		
Diluted first-strand cDNA from step 7 above	10 µL		
KAPA dNTP Mix (10 mM)	1.5 µL		
5' PCR Primer II A (12 µM)	3.2 µL		
Nuclease-free water	24.3 µL		
KAPA HiFi Enzyme (1U/µL)	1 µL		
Total Volume	50 µL		

2. Cycle the reaction with the following conditions (using a heated lid):
   - Initial denaturation:
     – 95°C for 2 minutes
   - 8 cycles at the following temperatures and times:
     – 98°C for 20 seconds
     – 65°C for 15 seconds
     – 72°C for 4 minutes
   - Final extension:
     – 72°C for 5 minutes
3. After the initial **8** cycles, remove 5 µL of the reaction and transfer it to a tube labeled "8."
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 20 seconds
     – 65°C for 15 seconds
     – 72°C, for 4 minutes
   - Final extension:
     – 72°C for 5 minutes
5. Remove 5 µL again and transfer to a tube labeled "10."
6. Repeat steps 4-5 for 12, 14 and 16 cycles.

Note that the number of cycles is dependent on the sample, and may be changed for particular samples. Therefore, it may be necessary to adjust the cycle PCR optimization starts.

7. Load the 5 aliquots on an Agarose gel or Bioanalyzer® instrument to view distribution of the ds cDNA.

Large-Scale PCR for Size Selection on the SageELF™ System

Use the cycle number (as determined in the PCR Cycle Optimization step) to generate ds cDNA for size selection on the SageELF System.

1. Set up 8 X 50 µL PCR reactions.
2. Make a master mix by adding the following reagents:

Reagent	Volume	✓	Notes
KAPA HiFi Fidelity Buffer (5X)	80 µL		
Diluted first-strand cDNA Synthesis	80 µL		
KAPA dNTP Mix (10 mM)	12 µL		
5' PCR Primer II A (12 µM)	25.6 µL		
Nuclease-free water	194.4 µL		
KAPA HiFi Enzyme (1U/µL)	8 µL		
Total Volume	400 µL		

3. Transfer 50 µL aliquots into 8 PCR tubes and perform PCR using the cycle number determined during the optimization step. Cycle the reaction with the following conditions (using a heated lid):
   - Initial denaturation:
     – 95°C for 2 minutes
   - ***n*** cycles (optimal cycle determined in the optimization step) at the following temperatures and times:
     – 98°C for 20 seconds
     – 65°C for 15 seconds
     – 72°C for 4 minutes
   - Final extension:
     – 72°C for 5 minutes
4. Pool the eight PCR reactions and perform a 1X AMPure® PB bead purification step.

Purifying the Large-Scale PCR Products

STEP		Purify the Pooled PCR Products	Notes
1		Add **1X** volume of AMPure® PB magnetic 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 tube.	
7		With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above for a total of 2 ethanol washes.	
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 the tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **30 µL** of Elution Buffer. Vortex for 10 minutes at 2000 rpm.	
14		Determine concentration using a Qubit system or another double-strand-specific quantitation system.	

Size Selection using the SageELF™ System

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 5 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. – In 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 PacBio Elution Buffer into the empty elution well. Rinse by pipetting up and down several times, and collect the rinse into the same tube.	
9		For concentration measurements, Qubit is highly recommended	
10		Check the sizes of all 12 fractions by loading on a Bioanalyzer system.	
11		The 12 fractions can be used directly for the second large-scale PCR step or can be stored at - 20°C for future use.	
12		Before proceeding to large-scale PCR, pool fractions as recommended below: Pool #1 0.8 kb - 2.0kb (fractions 9 -1 2) Pool #2 2 kb - 3 kb (fractions 7 - 8) Pool #3 3 kb - 5 kb (fractions 5 - 6) Pool #4 > 5 kb (fractions 1 - 4) When pooling, mix fractions in equimolar quantities. (This is why accurate quantitation using the Qubit system is essential).	

Large-Scale PCR for SMRTbell™ Library Preparation

The mass of the pooled cDNA fractions is not sufficient for SMRTbell library construction. This section outlines the procedure to generate more double stranded cDNA by performing a second large scale PCR of the pooled fractions.

1. Prepare 4 X 50 µL PCR reactions for each pooled fraction: 0.8 kb – 2 kb, 2 kb – 3 kb, 3 kb – 5 kb, and > 5 kb.
2. For each pooled fraction, prepare the following reagents to an appropriately sized PCR tube:

Reagent	Volume	✓	Notes
KAPA HiFi Fidelity Buffer (5X)	40 µL		
Pooled cDNA	40* µL		
KAPA dNTP Mix (10 mM)	6.0 µL		
5' PCR Primer II A (12 µM)	12.8 µL to 1 µM final concentration		
Nuclease-free water	Adjust volume to 200 µL		
KAPA HiFi Enzyme (1U/µL)	4 µL		
Total Volume	200 µL		

*For > 5 kb fractions, it may be necessary to add more of the pooled cDNA. Then, adjust reaction accordingly.

3. Aliquot 50 µL into 4 PCR tubes and perform PCR using the cycle number and extension parameters below.

Size Desired	Extension Time	Number of Cycles
0.8 kb - 2 kb	1 min 30 sec	8 cycles
2 kb - 3 kb	1 min 45 sec	9 cycles
3 kb - 5 kb	3 min 30 sec	12 cycles
> 5 kb	5 min 30 sec	15 cycles

4. Cycle the reaction with the following conditions (using a heated lid):
   - Initial denaturation:
     – 95°C for 2 minutes
   - ***n*** cycles at the following temperatures and times:
     – 98°C for 20 seconds
     – 65°C for 15 seconds
     – 72°C for X minutes (for this step, see extension times in table above)
   - Final extension:
     – 72°C for 5 minutes

Purifying the Large-Scale PCR Products

After PCR, pool the reactions for each size fraction into a 1.5 mL tube and purify with 1X AMPure PB beads. (Optional: Before pooling the replicates, load on an Agarose Gel to check amplification efficiency.)

STEP		Pooling and Purification	Notes
1		Add **1X** 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. 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 tube.	
7		With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above for a total of 2 ethanol washes.	
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 the tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in Elution Buffer. We recommend eluting in volume, one tenth of the initial PCR Master Mix volume (e.g., for 400 µL of PCR, 40 µL of Elution Buffer). Mix sample until homogeneous, then vortex for 10 minutes at 2000 rpm.	
14		Spin down the tube (for 1 second) to collect beads.	
15		Place the tube in a magnetic bead rack to collect the beads to the side of the tube. Carefully pipette eluted DNA into a LoBind microcentrifuge tube.	
16		Check concentration of samples using a Qubit system.	

cDNA SMRTbell™ Template Preparation and Sequencing

Repair DNA Damage

In general, 1 – 1.5 µg of cDNA is recommended for each sample going into SMRTbell template preparation. For size-selected samples, the input amount depends on the size.

Note that short insert SMRTbell templates may be present in the > 5 kb fraction, and will load preferentially during sequencing. Their removal may be necessary to maximize full-length cDNA greater than 5 kb. This can be achieved by running the > 5 kb SMRTbell library on a SageELF system.

If preparing larger amounts of DNA, scale the following reaction volumes accordingly.

Fraction	Input Requirement
0.8 kb - 2 kb	Up to 500 ng
2 kb - 3 kb	Up to 1 µg
3 kb - 5 kb	Up to 1.5 µg
> 5 kb	Up to 5 µg

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

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Amplified ds cDNA	-		__ µL for 0.5 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
DNA (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		For all insert sizes, 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 to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol and dry the bead pellet. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **30 µL** Elution Buffer. Mix until homogenous, then vortex for 10 minutes at 2000 rpm.	
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 7500 Kit. Note that typical yield at this point of the process (following End-Repair and one 1X AMPure PB bead purification) is approximately between 80-100% of the total starting material.	
16		The End-Repaired DNA can be stored overnight at 4ºC or (or -20ºC for longer).	
17		Actual recovery per µL and total available sample material:	

Prepare Blunt Ligation Reaction

Note: It is important to maintain an optimal ratio of adapter to insert molecules. If a larger amount of library is being prepared than what is indicated in the table below, scale the ligation reaction volumes appropriately.

Fraction	Input Requirement
0.8 kb - 2 kb	Up to 500 ng
2 kb - 3 kb	Up to 1 µg
3 kb - 5 kb	Up to 1.5 µg
> 5 kb	Up to 5 µg

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
ds cDNA (End Repaired)	-		29.0 µL to 30.0 µL			
Annealed 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 DNA				40 µL
Mix reaction well by pipetting				
ExoIII		100.0 U/µL		1.0 µL
ExoVII		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		For all insert sizes, 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 to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol and dry the bead pellet. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **50 µL** of Elution Buffer. Vortex for 10 minute at 2000 rpm.	
14		The eluted DNA in **50 µL** Elution Buffer should be taken into the second and final AMPure bead purification step.	

STEP	✓	Purify SMRTbell™ Templates - Second Purification	Notes
1		For all insert sizes, 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 to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above.	
10		Remove residual 70% ethanol and dry the bead pellet. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **10 µL** of Elution Buffer. Vortex for 10 minute at 2000 rpm.	
14		Verify your DNA amount and concentration with either a Nanodrop or Qubit quantitation platform reading. For general library yield expect 20% total yield from the Damage Repair input. If your yield concentration is below 12 ng/µL, use the Qubit system for quantitation. To estimate your final concentration: (____ng of DNA going into Damage Repair X 0.2) / of Elution Buffer = ng/µL	
15		Perform qualitative and quantitative analysis using a Bioanalyzer instrument. Note that typical DNA yield, at this point of the process, following blunt ligation, exonuclease treatment and two AMPure PB bead purifications is between approximately **15-25%** of the total starting material going into the ligation reaction.	

Optional Second SageELF™ Size Selection for the > 5 kb SMRTbell™ library to Remove Contaminating Short SMRTbell Templates (~2 kb)

Short contaminating SMRTbell templates, that may be present in the >5 kb fraction, will preferentially load resulting in reduced representation of >5 kb full-length cDNAs. Removal may be necessary to increase yield of > 5 kb cDNA.

This optional step further removes short contaminating SMRTbell templates.

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. – 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 5 µg of cDNA. – Add **10 µL** of Sage Science's Marker75. 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.	

STEP		Running the SageELF System	Notes
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. – In 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 PacBio Elution Buffer into the empty elution well. Rinse by pipetting up and down several times, and collect the rinse into the same tube.	
9		For concentration measurements, Qubit is highly recommended.	
10		Check the sizes of fractions 1 - 5 by loading on a Bioanalyzer system.	
11		Fractions 1 – 5 can be pooled and sequenced as one sample. The majority of reads should be greater than 5Kb. Otherwise, fractions 1 - 3 can be pooled to represent cDNAs greater than 7 kb and fractions 4 - 5 can be pooled for cDNAs between 5 kb – 7 kb.	
12		Perform one final round of 1X AMPure PB bead purification.	

One round of 1X AMPure PB bead purification is sufficient in this step.

STEP	✓	Purify SMRTbell™ Templates - Final Purification	Notes
1		For all insert sizes, 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 to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8 above for a total of 2 ethanol washes.	
10		Remove residual 70% ethanol and dry the bead pellet. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **10 µL** of Elution Buffer. Vortex for 10 minute at 2000 rpm.	
14		Verify your DNA amount and concentration with either a Nanodrop or Qubit quantitation platform reading. For general library yield expect 20% total yield from the Damage Repair input. If your yield concentration is below 12 ng/µL, use the Qubit system for quantitation. To estimate your final concentration: (____ng of DNA going into Damage Repair X 0.2) / of Elution Buffer = ng/µL	
15		Perform qualitative analysis on all SMRTbell libraries (5 fractions) using a Bioanalyzer instrument. Run 20 - 50 ng on a 12000 DNA Chip. Use the average size as determined by the region function for the Binding Calculator.Typical yield is approximately **15-30%**.	

Anneal and Bind BluePippin™ Size-Selected SMRTbell™ Templates

To anneal sequencing primer and bind polymerase to SMRTbell templates, see the Calculator. For more information about using the calculator, see the *Pacific Biosciences Template Preparation and Sequencing Guide*.

Table 1: Loading Recommendations for P6-C4 Chemistry (MagBead Standard Protocol)

Fraction	On-Plate Concentration
0.8 kb - 2 kb	15 pM
2 kb - 3 kb	25 pM
3 kb - 5 kb	50 pM
> 5 kb	62.5 pM

Sequence

MagBead loading is suggested for all fractions. We recommend performing loading titrations to determine an appropriate loading concentration.

The Calculator provides recommended sample concentrations for binding polymerase/template complexes to MagBeads, and for loading complexes on PacBio systems. For information on how to prepare and sequence using MagBeads, see the *Pacific Biosciences Procedure & Checklist - Preparing MagBeads for Sequencing.*