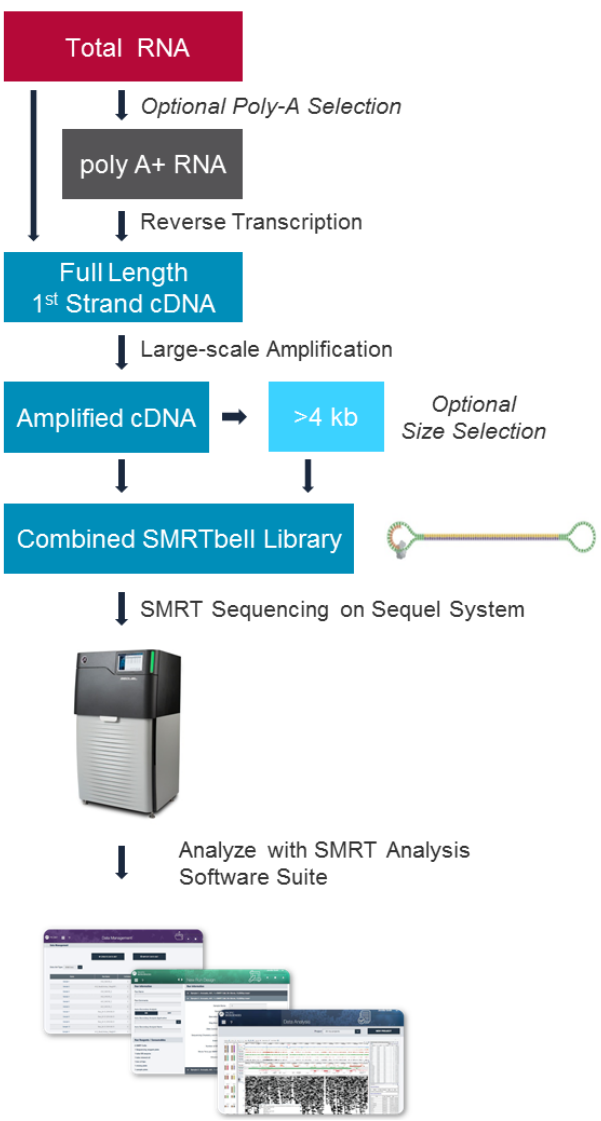
Procedure & Checklist - Iso-Seq™ Template Preparation for Sequel™ Systems

Before You Begin

The long read lengths of the PacBio® System are well-suited for characterizing full-length transcripts produced from high-quality RNA samples. This document describes methods for generating full-length cDNA Iso-Seq template libraries for Iso-Seq analysis.

Once double-stranded cDNA is prepared, the PacBio Template Prep Kit is used to generate SMRTbell™ libraries. The SMRTbell templates are then sequenced on the PacBio Sequel System.

This procedure allows detection of full-length transcripts up to 4 kb (without doing size-selection). To increase the sequencing yield of >4 kb transcripts, consider size-selection using BluePippin, SageELF, or Agarose Gels.



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	Any Oligo Synthesis Vendor
1.2% FlashGel® system or 0.80% Agarose Gels	Lonza (5702
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
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 and performing size- selection, 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.

For each Total RNA sample, combine the reagents below in separate PCR tubes.
For poly A+ RNA, the minimum is 1 ng; total RNA requires 2 ng. We recommend using 1µg of total RNA per reaction for optimized results in the following steps.
Do not change the 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		

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. 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		

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 the appropriate volume of PacBio Elution Buffer (EB):

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

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

PCR Cycle Optimization

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.

Test Amplification – we highly recommend performing the below test amplification procedure in order to determine the best number of cycles required for the sample to minimize artifacts during large-scale amplification.

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 II A (12 µM)	1 µL		
Nuclease-free water	24 µL (sub-total)		
PrimeSTAR GXL DNA Polymerase (1.25 U/µL)	1 µL		
Total Volume	50 µL		

2. Cycle the reaction with the following conditions (using a heated lid):
   - 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, 1 µg input of total RNA requires 10 to 12 cycles of PCR amplification. When input total RNA is <1 µg, the cycle number might need to be increased.
7. Load the 3 aliquots on an Agarose gel or Lonza flash Gel to view distribution of the ds cDNA.

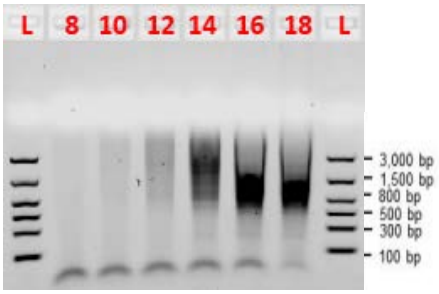


Figure 1 Analysis for optimizing PCR cycles: This sample is Human Brain Total RNA from Biochain. 1ug of starting RNA was used to set up cDNA synthesis. Gel electrophoresis was performed on a 1.2% Lonza® FlashGel. Lane L: Lonza FlashGel Ladder (100 bp – 3 kb). The numbers above the other lanes indicate cycle number. One reaction of 50 µL PCR was set up to obtain this series cycle of optimization. In this example, PCR with 10 cycles was determined to be optimal for large-scale amplification. Cycles 8 and 10 look similar in banding and size, however cycle 10 has more products than 8 cycles. In cycles 12 and above, the cDNA smears show over- amplification.

Large-Scale PCR

Use the cycle number (as determined in the PCR Cycle Optimization step) to generate ds cDNA

1. Set up 16 X 50 µL PCR reactions. (Set up 24 X 50 µL PCR reactions if size-selection is required)
2. Make a master mix by adding the following reagents:

Reagent	Volume	✓	Notes
5X PrimeSTAR GXL Buffer	160 µL		
Diluted first-strand cDNA Synthesis	160 µL		
dNTP Mix (2.5mM each)	64 µL		
5’ PCR Primer II A (12 µM)	16 µL		
Nuclease-free water	384 µL (to 392 µL sub-total)		
PrimeSTAR GXL DNA Polymerase (1.25 U/µL)	16 µL		
Total Volume	800 µL		

3. Transfer 50 µL aliquots into 16 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) - see step 2 page 5:
   - 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 step requires two tubes of pooled PCR products that are processed in parallel. The first pool is purified using 1X AMPure PB beads and the second pool is purified using 0.40X AMPure PB beads.

STEP		Purify the Pooled PCR Products - First Purification	Notes
1		Pool 8 x 50 PCR reactions and add **1X** volume of AMPure® PB magnetic beads. Label the tube appropriately.	
2		Pool another 8 x 50 PCR reactions and add **0.40X** volume of AMPure PB magnetic beads in a separate 1.5mL LoBind tubes. Label the tube appropriately.	
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. Do not pellet 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 both tubes in a magnetic bead rack until the beads collect to the side of the tube.	
8		With the tubes still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet.	
9		Wash beads with freshly prepared 70% ethanol.	
10		Repeat step 8 above for a total of 2 ethanol washes.	
11		Remove residual 70% ethanol and dry the bead pellet. – 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 10.	
13		Remove the tubes from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds.	
14		Elute the DNA off the beads in **100 µL** of Elution Buffer. Vortex for 10 minutes at 2000 rpm and then place the tubes on the magnetic rack to elute supernatant off the beads.	

STEP		Purify the Pooled PCR Products - Second Purification	Notes
1		Perform a second AMPure PB bead purification for both pools. The first pool undergoes a **1X** AMPure PB bead purification and the second pool undergoes a **0.40X** AMPure PB bead purification.	
2		Process both tubes in parallel by mixing the bead/DNA solution thoroughly.	
3		Quickly spin down the tubes (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	
5		Spin down both tubes (for 1 second) to collect beads.	
6		Place both tubes in a magnetic bead rack until the beads collect to the side of the	
7		With the tubes 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.	
11		– Check for any remaining droplets in the tubes. If droplets are present, repeat step 10.	
12		Remove the tubes 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 **21 µL** of Elution Buffer. Vortex for 10 minutes at 2000 rpm and then place the tubes on the magnetic rack to elute supernatant off the beads.	
14		Using 1µL of DNA for qubit quantification, run the Bioanalyzer instrument to check the size distribution.	
15		Pool the two purified samples in equimolar concentration. Now you should have one tube of PCR product with 1-5 µg of Amp cDNA for SMRTbell library preparation.	

cDNA SMRTbell™ Template Preparation and Sequencing

Repair DNA Damage

In general, 1 – 5 µg of cDNA is recommended for each sample going into SMRTbell template preparation.

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		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		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
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				
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 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	
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. If size selection is required (> 4 kb), elute in **30 µL** Elution Buffer instead.	
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		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	
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 -30 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.	
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.	

Anneal and Bind SMRTbell™ Templates

To anneal sequencing primer and bind polymerase to SMRTbell templates, see the Binding Calculator.

Sequence

MagBead loading is suggested for Iso-Seq libraries. We recommend performing loading titrations to determine an appropriate loading concentration.

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

Sequencing recommendations:

- Loading: 40-50 pM on-plate concentrations to hit up to 50% P1
- Collection time: 360 – 600 minutes
- Pre-extension: 120 minutes
- Spin-Columns: Not necessary

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