

# Procedure & Checklist - Isoform Sequencing (Iso-Seq™) using the Clontech® SMARTer® PCR cDNA Synthesis Kit and No Size Selection

## 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 libraries (using the BluePippin™ System) for isoform sequencing.

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

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

Procedure	Size Selection	Target Size	Required Size Selection Equipment
Procedure & Checklist - Isoform Sequencing 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 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 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™) 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 described in this document is for isoform sequencing without size selection.

## Materials and Kits Needed

Item	Vendor
SMARTer PCR cDNA Synthesis Kit	Clontech (cat#s 634925 or 634926)
KAPA™ HiFi PCR Kit	Kapa Biosystems (PNs 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	Life Technologies
DNA 7500 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 is required, 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		

- Mix contents and spin the tubes briefly in a microcentrifuge.
- 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.

- 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.
- Incubate the tubes at 42°C for 90 minutes.
- Terminate the reaction by heating the tubes at 70°C for 10 min.
- 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

- If multiple reactions were performed with the same RNA sample, 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 the large-scale amplification step) 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		
<b>Total Volume</b>	<b>50 µL</b>		

2. Cycle the reaction with the following conditions (using a heated lid):

- Initial denaturation:
  - 95°C for 2 minutes
- 10 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 **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 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 “12.”

6. Repeat steps 4-5 for 14, 16, and 18 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 five aliquots on an Agarose gel or Bioanalyzer<sup>®</sup> instrument to view distribution of the ds cDNA. See *UB - Guidelines for Preparing cDNA Libraries for Isoform Sequencing (Iso-Seq™)*.

## Large-Scale PCR

Use the cycle number (as determined in the PCR Cycle Optimization step) to generate large-scale double-stranded DNA for SMRTbell library preparation.

1. 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		
<b>Total Volume</b>	<b>400 µL</b>		

2. 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
    - 74°C for 4 minutes
  - Final extension:
    - 72°C for 5 minutes
3. Pool the eight PCR reactions together and perform a 1X AMPure® PB bead purification step.

STEP	Purify the Pooled PCR Products	Notes
1	Add <b>1X</b> 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 <b>step 8</b> above for a total of 2 ethanol washes.	

STEP	Purify the Pooled PCR Products	Notes
10	Remove residual 70% ethanol and dry the bead pellet. <ul style="list-style-type: none"> <li>– 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.</li> <li>– Place the tube back on magnetic bead rack.</li> <li>– Pipette off any remaining 70% ethanol.</li> </ul>	
11	Check for remaining droplets in the tube. If droplets are present, repeat <a href="#">step 10</a> .	
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 <b>40 µL</b> of Elution Buffer. Vortex for 10 minutes at 2000 rpm.	
14	Determine concentration using a Qubit system or another double-strand-specific quantitation system.	

The time required to extract DNA from gels depends on the size of the gel cuts. Bigger gel slices require longer extraction time and bigger volumes that may result in lower recovery. Avoid large gel slices if possible.

## cDNA SMRTbell™ Template Preparation and Sequencing

### Repair DNA Damage

For no size-selected cDNA SMRTbell library, 500 ng of cDNA is recommended. If preparing larger amounts of DNA, scale the reaction volumes appropriately.

Note that this procedure generates sufficient cDNA for no size selection or size selection. If you are interested in sequencing long transcripts (> 3 kb), we recommend performing size-selection. Refer to the Pacific Biosciences *Procedure & Checklist - Isoform Sequencing Using the Clontech SMARTer PCR cDNA Synthesis Kit and Manual Agarose-gel Size Selection* and *Procedure & Checklist - Isoform Sequencing Using the Clontech SMARTer PCR cDNA Synthesis Kit and the BluePippin Size-Selection System*.


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 500 ng	–		
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 <sub>2</sub> O	–		___ µL to adjust to 50.0 µL	–		
Total Volume			50.0 µL	–		

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

## Repair Ends

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

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	–		

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

STEP	✓	Purify DNA	Notes
1		For all insert sizes, add <b>1X</b> 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 <a href="#">step 8</a> above.	
10		Remove residual 70% ethanol and dry the bead pellet. <ul style="list-style-type: none"> <li>– 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.</li> <li>– Place the tube back on magnetic bead rack.</li> <li>– Pipette off any remaining 70% ethanol.</li> </ul>	
11		Check for any remaining droplets in the tube. If droplets are present, repeat <a href="#">step 10</a> .	
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 <b>30 µL</b> 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.	





STEP	✓	Purify DNA	Notes
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 4°C or (or -20°C for longer).	
17		Actual recovery per $\mu\text{L}$ 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 (> 500 ng), scale the ligation reaction volumes appropriately.

Use the following table to prepare your blunt ligation reaction:



- In a LoBind microcentrifuge tube (on ice), add the following reagents in the order shown. Note that you can add water to achieve the desired DNA volume. 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 DNA (End Repaired)	–		29.0 $\mu\text{L}$ to 30.0 $\mu\text{L}$			
<b>Annealed</b> Blunt Adapter (20 $\mu\text{M}$ )		20 $\mu\text{M}$	1.0 $\mu\text{L}$	0.5 $\mu\text{M}$		
Mix before proceeding						
Template Prep Buffer		10 X	4.0 $\mu\text{L}$	1X		
ATP low		1 mM	2.0 $\mu\text{L}$	0.05 mM		
Mix before proceeding						
Ligase		30 U/ $\mu\text{L}$	1.0 $\mu\text{L}$	0.75 U/ $\mu\text{L}$		
H <sub>2</sub> O	–	–	__ $\mu\text{L}$ to adjust to 40.0 $\mu\text{L}$	–		
Total Volume	–	–	40.0 $\mu\text{L}$	–		

- Mix the reaction well by pipetting or flicking the tube.
- Spin down contents of tube with a quick spin in a microfuge.
- Incubate at 25°C for 15 minutes. At this point, the ligation can be extended up to 24 hours or cooled to 4°C (for storage of up to 24 hours).
- Incubate at 65°C for 10 minutes to inactivate the ligase, then return the reaction to 4°C. 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 37°C for 1 hour, then return the reaction to 4°C. You must proceed with purification after this step.

## Purify SMRTbell™ Templates

STEP	✓	Purify SMRTbell™ Templates - First Purification	Notes
1		For all insert sizes, add <b>1X</b> 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 <a href="#">step 8</a> above.	
10		Remove residual 70% ethanol and dry the bead pellet. <ul style="list-style-type: none"> <li>– 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.</li> <li>– Place the tube back on magnetic bead rack.</li> <li>– Pipette off any remaining 70% ethanol.</li> </ul>	
11		Check for any remaining droplets in the tube. If droplets are present, repeat <a href="#">step 10</a> .	
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 <b>50 µL</b> of Elution Buffer. Vortex for 10 minute at 2000 rpm.	
14		The eluted DNA in <b>50 µL</b> 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 <b>1X</b> 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 <a href="#">step 8</a> above.	
10		Remove residual 70% ethanol and dry the bead pellet. <ul style="list-style-type: none"> <li>– 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.</li> <li>– Place the tube back on magnetic bead rack.</li> <li>– Pipette off any remaining 70% ethanol.</li> </ul>	
11		Check for any remaining droplets in the tube. If droplets are present, repeat <a href="#">step 10</a> .	
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 <b>10 µL</b> 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 <b>15-25%</b> 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 Calculator. For more information about using the calculator, see the *Pacific Biosciences Template Preparation and Sequencing Guide*.

## Sequence

MagBead loading is suggested for all three fractions and stage start is not recommended for these runs. We recommend performing loading titrations to determine 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*.