PacBio Iso-Seq preparation for Sequel II Systems

The Sequel Systems generate long reads that are well-suited for characterizing full-length transcripts produced from high-quality RNA samples. This document describes a method to construct Iso-Seq SMRTbell® libraries for sequencing on both systems allowing detection of full-length transcripts.

I. cDNA synthesis using SMARTer™ PCR cDNA Synthesis Kit

First-Strand cDNA Synthesis

This protocol has been optimized for both total RNA and poly A+ RNA. The minimum amount of starting material for cDNA synthesis is 2 ng of total RNA or 1 ng of poly A+ RNA. However, if your RNA sample is not limiting, we recommend that you start from 1 µg of total RNA or 0.5 µg of poly A+ RNA for cDNA synthesis.

1. For each sample and Control Mouse Liver Total RNA, combine the following reagents in separate 0.5 ml reaction tubes:

1–3.5 µl	**RNA** (1 ng–1 µg of poly A+ RNA or 2 ng–1 µg total RNA)
1 µl	**3' SMART CDS Primer II A** (12 µM)
x µl	**Deionized H2O**
4.5 µl	**Total Volume**

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, then reduce the temperature to 42°C for 2 min.
4. Prepare a Master Mix for all reaction tubes at room temperature by combining the following reagents in the order shown:

2 µl	**5X First-Strand Buffer**
0.25 µl	**DTT** (100 mM)
1 µl	**dNTP Mix** (10 mM)
1 µl	**SMARTer II A Oligonucleotide** (12 µM)
0.25 µl	**RNase Inhibitor**
1 µl	**SMARTScribe Reverse Transcriptase** (100 U)

5.5 µl	**Total Volume added per reaction**

5. 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.
6. Incubate the tubes at 42°C for 90 min.
7. Terminate the reaction by heating the tubes at 70°C for 10 min.
8. Dilute the first-strand reaction product by adding the appropriate volume of TE buffer (10 mM Tris [pH 8.0], 0.1 mM EDTA):
   - Add 40 µl of TE buffer if you used total RNA as the starting material.
   - Add 190 µl of TE buffer if you used more than 0.2 µg of poly A+ RNA as the starting material.
   - Add 90 µl of TE buffer if you used less than 0.2 µg of poly A+ RNA as the starting material.

II. cDNA Amplification by LD PCR

For each sample and control, set up an extra reaction tube to determine the optimal number of PCR cycles. In our experience, each 100 µl reaction typically yields 1–3 µg of ds cDNA after the PCR and purification steps

Table 1. Guidelines for Setting Up PCR Reactions

Total RNA (ng)	Volume of Diluted ss cDNA for PCR	Typical Optimal No. of PCR Cycles
1000	1	18-20
250	4	18-20
50	10	19-21
10	10	21-23
2	10	23-25
Poly A+ RNA (ng)	**Volume of Diluted ss cDNA for PCR**	**Typical Optimal No. of PCR Cycles**
500	2	15-17
100	4	15-17
20	10	16-18
5	10	18-20
1	10	20-22

1. For each reaction, aliquot the appropriate volume (see Table I, above) of each diluted first-strand cDNA into a labeled 0.5 ml reaction tube. If necessary, add deionized H2O to adjust the volume to 10 µl.
2. Prepare a PCR Master Mix for all reactions, plus one additional reaction. Combine the following reagents in the order shown:

74 µl	**Deionized H2O**
10 µl	**10X Advantage 2 PCR Buffer**
2 µl	**50X dNTP Mix** (10 mM)
2 µl	**5' PCR Primer II A** (12 µM)
2 µl	**50X Advantage 2 Polymerase Mix**
90 µl	**Total Volume per reaction**

3. Mix well by vortexing and spin the tube briefly in a microcentrifuge.
4. Aliquot 90 µl of the PCR Master Mix into each tube from Step 1.
5. Cap the tube Commence thermal cycling using the following program:
    - 95°C 1 min
    - X number of cycles (consult Table 1).
    - 95°C 15 sec
    - 95°C 30 sec
    - 95°C 3 min
6. Subject each reaction 8. tube to 15 cycles, then pause the program. Transfer 30 µl from each tube to a second reaction tube labelled "Optimization". Store the "Experimental" tubes at 4°C. Using the Tester PCR tube, determine the optimal number of PCR cycles:
    - Transfer 5 µl from the 15 cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

- Return the Optimization tubes to the thermal cycler. Run three additional cycles (for a total of 18) with the remaining 25 µl of PCR mixture.
- Transfer 5 µl from the 18 cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
- Run three additional cycles (for a total of 21) with the remaining 20 µl of PCR mixture.
- Transfer 5 µl from the 21 cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
- Run three additional cycles (for a total of 24) with the remaining 15 µl of PCR mixture.
- Transfer 5 µl from the 24 cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).
- Run three additional cycles (for a total of 27) with the remaining 10 µl of PCR mixture.

7. Electrophorese each 5 µl aliquot of the PCR reaction alongside 0.1 µg of 1 kb DNA size markers on a 1.2% agarose/EtBr gel in 1X TAE buffer. Determine the optimal number of cycles required for each experimental and control sample.
8. Retrieve the 15 cycle Experimental PCR tubes from 4°C, return them to the thermal cycler, and subject them to additional cycles, if necessary, until you reach the optimal number.
9. When the cycling is completed, analyse a 5 µl sample of each PCR product alongside 0.1 µg of 1 kb DNA size markers on a 1.2% agarose/EtBr gel in 1X TAE buffer. Compare your results to Figure 1 to confirm that your reactions were successful.
10. Add 2 µl of 0.5 M EDTA to each tube to terminate the reaction.

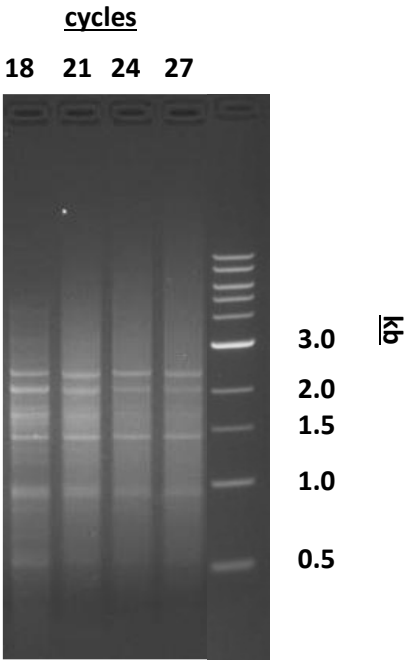

Figure 1. cDNA amplification by LD PCR

III. Purification of Amplified cDNA

1. Add 1X AMPure PB beads to the amplified cDNA.
2. Mix by tapping the LoBind tube until the sample is homogeneous.
3. Incubate at room temperature for 10 minutes.
4. Place on magnetic rack until solution clears. Remove and discard supernatant.
5. With the tube still on magnet, add 200 µL freshly prepared 70% ethanol to the tube containing beads plus DNA.
6. Remove and discard 70% ethanol.
7. Repeat steps 5 to 6 for total of two washes with 70% ethanol.
8. Let beads air dry for 1 minute.
9. Add 27 µL EB and remove the tube from the magnet. Mix by tapping the tube until the sample is homogeneous. Then incubate at room temperature for 2 minutes.

10. Place back on magnet. When the solution clears, remove 25 µL supernatant into new 1.5 mL LoBind tube.
11. Determine concentration using Qubit device or similar quantification assay.
12. Run 1 µL of sample on Agilent DNA 12000 chip according to manufacturer's instructions.
13. The captured cDNA is now ready for SMRTbell library construction.

IV. Repair DNA damage

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

X µl	**cDNA for 500 ng**
7 µl	**DNA Prep Buffer**
0.6 µl	**NAD**
2 µl	**DNA Damage Repair Mix v2**
Up to 57 µl	**H_2O**
57 µl	**Total Volume per reaction**

2. Pipette mix 10 times. It is important to mix well. Perform a quick spin to collect all liquid from the sides of the tube.
3. Place in a thermocycler and run the following program:
    - 37°C 30 min
    - Hold at 4°C

V. End Repair/A-Tailing

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

57 µl	**Reaction Mix from previous step**
3 µl	**End Prep Mix**
60 µl	**Total Volume per reaction**

2. Pipette mix 10 times. It is important to mix well. Perform a quick spin to collect all liquid from the sides of the tube.
3. Place in a thermocycler and run the following program:
    - 20°C 30 min
    - 65°C 20 min
    - Hold at 4°C

VI. Overhang Adapter Ligation

1. Add the following directly to reaction mix from previous step:

60 µl	**Reaction Mix from Previous Step**
3 µl	**Overhang Adapter v3**
30 µl	**Ligation Mix**
1 µl	**Ligation Enhancer**
1 µl	**Ligation Additive**
95 µl	**Total Volume per reaction**

2. Pipette mix 10 times. It is important to mix well. Perform a quick spin to collect all liquid from the sides of the tube.
3. Place in a thermocycler and run the following program:
    - 20°C 60 min
    - Hold at 4°C

VII. Purification of cDNA

1. Add 1X AMPure PB beads to the amplified cDNA.
2. Mix by tapping the LoBind tube until the sample is homogeneous.
3. Incubate at room temperature for 10 minutes.
4. Place on magnetic rack until solution clears. Remove and discard supernatant.
5. With the tube still on magnet, add 200 µL freshly prepared 70% ethanol to the tube containing beads plus DNA.
6. Remove and discard 70% ethanol.

7. Repeat steps 5 to 6 for total of two washes with 70% ethanol.
8. Remove ethanol.
9. Check for any remaining droplets in the tube. If droplets are present spin down down and place tube back on magnetic rack and pipette of any remaining ethanol.
10. Let tube air dry for 1 min.
11. Add 30 µL EB and remove the tube from the magnet. Mix by tapping the tube until the sample is homogeneous. Then incubate at room temperature for 2 minutes.
12. Place back on magnet. When the solution clears, remove 30 µL supernatant into new 1.5 mL LoBind tube.

VIII. Purification of cDNA library

Perform two rounds of Ampure PB bead clean up in step VII.

Prepare for Sequencing

Follow the SMRT Link Sample Setup v8.0 (or higher) instructions for preparing the sample for sequencing on the Sequel II System.