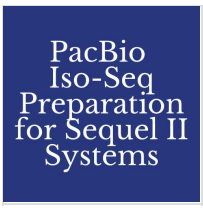

Oct 20, 2022

PacBio Iso-Seq Preparation for Sequel II Systems

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ABSTRACT

The Sequel Systems generate long reads that are well-suited for characterizing fulllength 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.
This protocol describes how to perform PacBio targeted Iso-Seq

ATTACHMENTS

PacBio_Iso-Seq.pdf

DOI

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PROTOCOL CITATION

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KEYWORDS

PacBio Iso-Seq preparation, cDNA synthesis, cDNA amplification, LD PCR, amplified cDNA purification, cDNA purification, ASAPCRN

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OWNERSHIP HISTORY

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PROTOCOL INTEGER ID

47283

MATERIALS TEXT

- SMARTer™ PCR cDNA Synthesis Kit
- RNA
- 3' Smart CDS Primer II A
- Deionized H2O
- 5X First-Strand Buffer
- DTT
- dNTP Mix
- SMARTer II A Oligonucleotide
- RNase Inhibitor
- SMARTScribe Reverse Transcriptase
- TE Buffer
- 10X Advantage 2 PCR Buffer
- 50X dNTP Mix
- 5' PCR Primer II A
- 50X Advantage 2 Polymerase Mix
- 1.2% agarose/EtBr gel in 1X TAE buffer
- 0.5 M EDTA
- 1X AMPure PB beads
- LoBind tube
- 70% ethanol
- cDNA
- DNA Prep Buffer
- NAD
- DNA Damage Repair Mix v2
- H2O
- Overhang Adapter v3
- Ligation Mix
- Ligation Enhancer
- Ligation Additive

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

cDNA synthesis using SMARTer™ PCR cDNA Synthesis Kit First-Strand cDNA Synthesis

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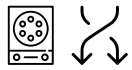
1

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.

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

A	B
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**

3 

Mix contents and spin the tubes briefly in a microcentrifuge.

4 

5m

Incubate the tubes at **72 °C** in a hot-lid thermal cycler for **00:03:00** , then reduce the temperature to **42 °C** for **00:02:00** .

5 Prepare a Master Mix for all reaction tubes at room temperature by combining the following reagents in the order shown:

A	B
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**

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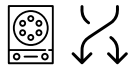
6 

Aliquot **5.5 µL 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.

7 1h 30m

Incubate the tubes at **42 °C** for **01:30:00** .

8 10m

Terminate the reaction by heating the tubes at **70 °C** for **00:10:00** .

9 Dilute the first-strand reaction product by adding the appropriate volume of TE buffer (**10 millimolar (mM) Tris [pH 8.0]** , **0.1 millimolar (mM) EDTA**):

9.1 Add **40 µL TE buffer** if you used total RNA as the starting material.

9.2 Add **190 µL TE buffer** if you used more than **0.2 µg poly A+ RNA** as the starting material.

9.3 Add **90 µL TE buffer** if you used less than **0.2 µg poly A+ RNA** as the starting material.

cDNA Amplification by LD PCR

10 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.

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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

Table 1. Guidelines for Setting Up PCR Reactions

11 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** .

12 Prepare a PCR Master Mix for all reactions, plus one additional reaction. Combine the following reagents in the order shown:

A	B
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**

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13 Mix well by vortexing and spin the tube briefly in a microcentrifuge.

14 Aliquot **90 µL PCR Master Mix** into each tube from Step 11.

15 4m 45s

Cap the tube. Commence thermal cycling using the following program:

- **95 °C** **00:01:00**
- X number of cycles (consult Table 1).
- **95 °C** **00:00:15**
- **95 °C** **00:00:30**
- **95 °C** **00:03:00**

16 Subject each tube from step 9 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:

16.1 Transfer **5 µL** from the 15 cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

16.2 Return the Optimization tubes to the thermal cycler. Run three additional cycles (for a total of 18) with the remaining **25 µL PCR mixture** .

16.3 Transfer **5 µL** from the 18 cycle PCR reaction tube to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

16.4

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Run three additional cycles (for a total of 21) with the remaining **20 µL PCR mixture** .

16.5 Transfer **5 µL** from the 21 cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

16.6

Run three additional cycles (for a total of 24) with the remaining **15 µL PCR mixture** .

16.7 Transfer **5 µL** from the 24 cycle PCR to a clean microcentrifuge tube (for agarose/EtBr gel analysis).

16.8

Run three additional cycles (for a total of 27) with the remaining **10 µL PCR mixture** .

17 Electrophorese each **5 µL** aliquot of the PCR reaction alongside **0.1 µg 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.

18 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.

19 When the cycling is completed, analyse a **5 µL sample of each PCR product** alongside **0.1 µg 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.

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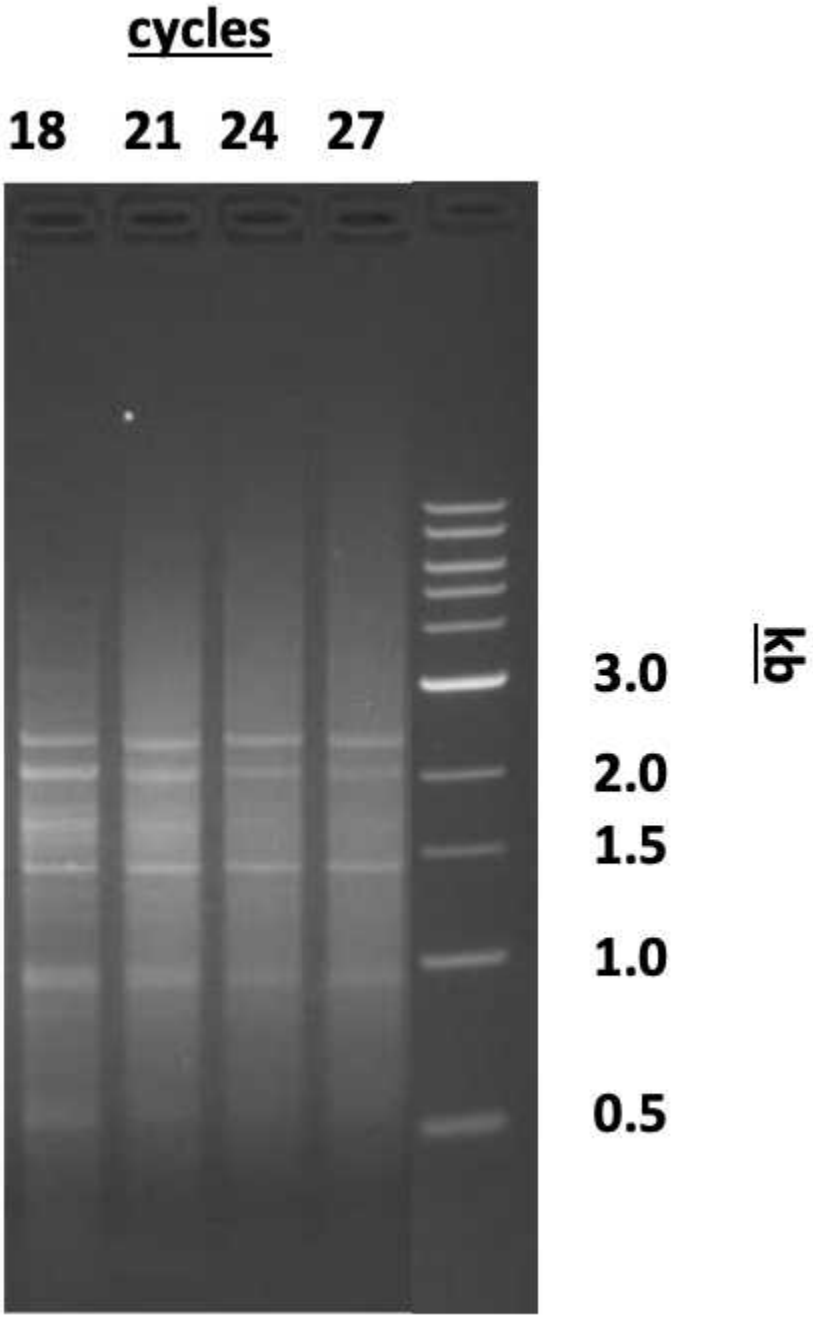

Figure 1. cDNA amplification by LD PCR

20 Add **2 µL 0.5 M EDTA** to each tube to terminate the reaction.

Purification of Amplified cDNA

21 Add 1X AMPure PB beads to the amplified cDNA.

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22 

Mix by tapping the LoBind tube until the sample is homogeneous.

23 

10m

Incubate at **Room temperature** for **00:10:00** .

24 Place on magnetic rack until solution clears. Remove and discard supernatant.

25 

With the tube still on magnet, add **200 µL freshly prepared 70% ethanol** to the tube containing beads plus DNA (1/2).

26 

Remove and discard 70% ethanol (1/2).

27 

With the tube still on magnet, add **200 µL freshly prepared 70% ethanol** to the tube containing beads plus DNA (2/2).

28 

Remove and discard 70% ethanol (2/2).

29 Let beads air dry for **00:01:00** .

1m

2m

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30 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 **00:02:00** .

31 Place back on magnet. When the solution clears, remove **25 µL supernatant** into new 1.5 mL LoBind tube.

32 Determine concentration using Qubit device or similar quantification assay.

33 Run **1 µL sample** on Agilent DNA 12000 chip according to manufacturer's instructions.

34 The captured cDNA is now ready for SMRTbell library construction.

Repair DNA Damage

35 In a LoBind microcentrifuge tube, add the following reagents:

A	B
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	H2O
57 µl	**Total Volume per reaction**

36 Pipette mix 10 times. It is important to mix well. Perform a quick spin to collect all liquid from the sides of the tube.

37 30m

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Place in a thermocycler and run the following program:

- **37 °C** **00:30:00**
- Hold at **4 °C**

End Repair/A-Tailing

38 In a LoBind microcentrifuge tube, add the following reagents:

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

39 

Pipette mix 10 times. It is important to mix well. Perform a quick spin to collect all liquid from the sides of the tube.

40 

50m

Place in a thermocycler and run the following program:

- **20 °C** **00:30:00**
- **65 °C** **00:20:00**
- Hold at **4 °C**

Overhang Adapter Ligation

41 Add the following directly to reaction mix from previous step:

A	B
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**

42 

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Pipette mix 10 times. It is important to mix well. Perform a quick spin to collect all liquid from the sides of the tube.

43  1h

Place in a thermocycler and run the following program:

- **20 °C** **01:00:00**
- Hold at **4 °C**

Purification of cDNA

44 Add 1X AMPure PB beads to the amplified cDNA.

45 

Mix by tapping the LoBind tube until the sample is homogeneous.

46  10m

Incubate at **Room temperature** for **00:10:00** .

47 Place on magnetic rack until solution clears. Remove and discard supernatant.

48 

With the tube still on magnet, add **200 µL freshly prepared 70% ethanol** to the tube containing beads plus DNA (1/2).

49 

Remove and discard 70% ethanol (1/2).

50 

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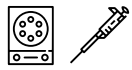
With the tube still on magnet, add **200 µL freshly prepared 70% ethanol** to the tube containing beads plus DNA (2/2).

51 

Remove and discard 70% ethanol (2/2).

52 Remove ethanol.

53 

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.

54 Let tube air dry for **00:01:00** . 1m

55  2m

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 **00:02:00** .

56 Place back on magnet. When the solution clears, remove **30 µL supernatant** into new 1.5 mL LoBind tube.

Purification of cDNA library

57 Perform two rounds of Ampure PB bead clean up in the "Purification of cDNA" section.

Prepare for Sequencing

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

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