Procedure & Checklist – Iso-Seq™ Express Template Preparation for Sequel® and Sequel II Systems

Before You Begin

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 up to 10 kb.

Depending on project goals, cDNA samples may be pooled and sequenced, simultaneously, in a single run. To multiplex, use barcoded forward and reverse primers (NEBNext Single Cell cDNA PCR Primer and Iso-Seq Express cDNA PCR Primer) to amplify samples. Once the cDNA samples are barcoded, they are pooled and constructed into a SMRTbell library as a "single" sample. There are 12 pairs of barcoded primers supported by Pacific Biosciences and they are listed in Appendix 2. Primers may be ordered from any oligo synthesis company.

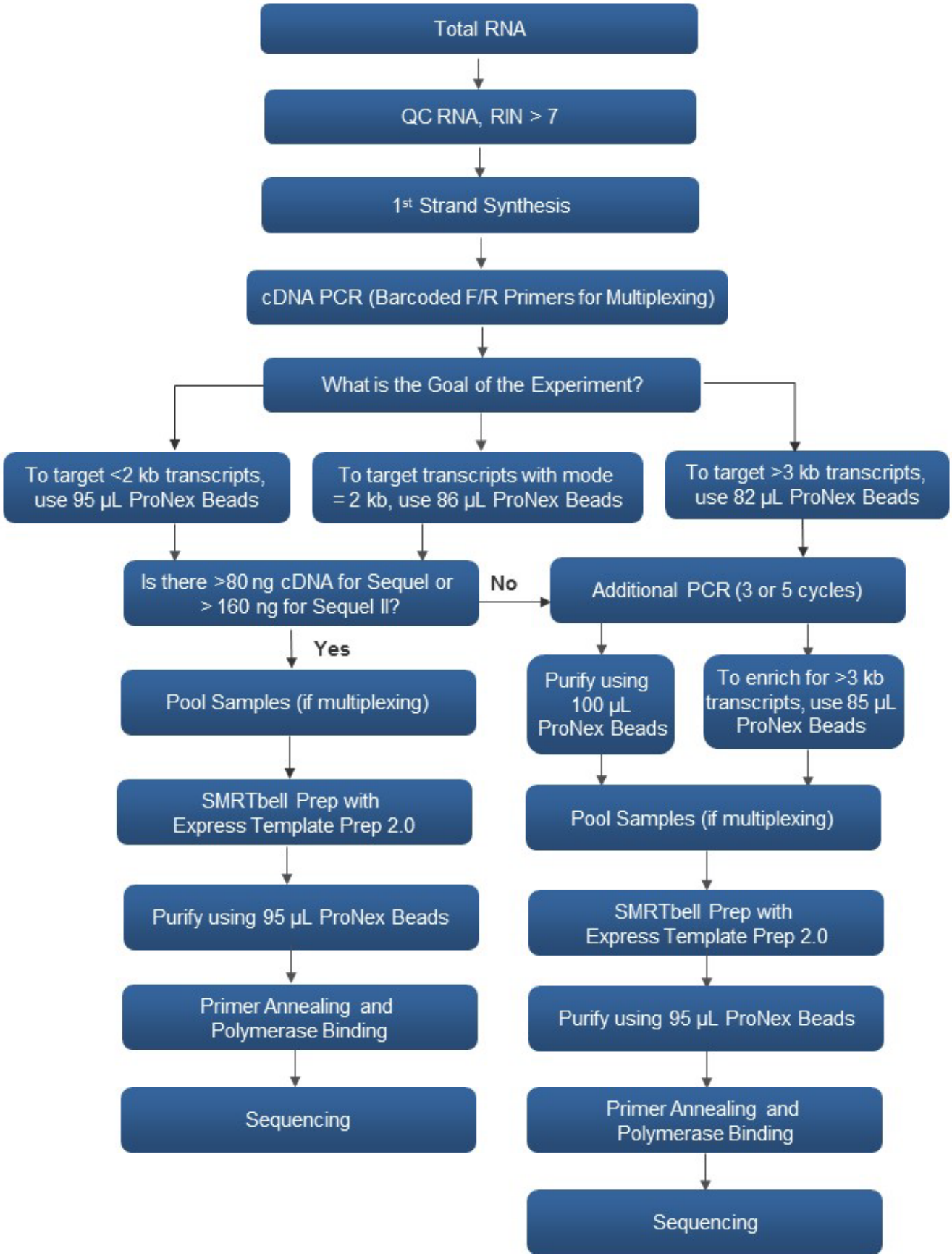
Materials and Kits Needed

Item	Vendor
TempAssure PCR 8-tube strips - 0.2 ml PCR 8-tube FLEX-FREE strip, attached flat caps are recommended OR 0.2 ml 8-Tube PCR Strips without Caps TBS0201 0.2 ml & Domed PCR Tube 8-Cap Strips TCS0801	USA Scientific, Inc. – Catalog No. 1402-4708 (recommended) Bio-Rad
HDPE 8 place Magnetic Separation Rack for 0.2 ml PCR Tubes (recommended) OR Magnetic Separator	V&P Scientific Inc. – Catalog No. VP772F4-1 (International and Dosmestic) Fisher Scientific – Catalog No. NC0988547 (Domestic only) Permagen Labware – Catalog No. MSR812
8-channel pipettes for processing multiple samples (200 µL & 20 µL)	Any MLS
Thermal Cycler that is 100 µL and 8-tube strip compatible	Any MLS
ProNex® Beads (for size selection)	Promega - Catalog numbers: NG2001 - 10mL, NG2002 - 125mL, NG2003 - 500mL
Qubit® dsDNA HS Assay Kit	Invitrogen
Qubit™ Fluorometer	Invitrogen
HS DNA Kit	Agilent
Bioanalyzer Instrument	Agilent
SMRTbell Express Template Prep Kit 2.0	PacBio
NEBNext® Single Cell/Low Input cDNA Synthesis & Amplification Module*	NEB Catalog No.: E6421S for 24 reactions or E6421L for 96 reactions
NEBNext® High-Fidelity 2X PCR Master Mix (for additional PCR reactions)	NEB M0541S
Elution Buffer (50 mL)	PacBio PN 101-633-500
Iso-Seq Express Oligo Kit**	PacBio PN 101-737-500
Ethanol	Any MLS

*This kit contains PCR reagents for 24 reactions. For additional PCR reactions, PacBio recommends the NEBNext® High-Fidelity 2X PCR Master Mix kit.

**For multiplexing, both NEBNext Single Cell cDNA PCR Primer and Iso-Seq Express cDNA PCR Primer must be barcoded. See Appendix 2 for sequences that can be ordered from any oligo synthesis company.

Iso-Seq Express 2.0 Workflow



PN 101-763-800 Version 02 (October 2019)

Recommended Best Practices

- A RIN (RNA integrity number) ≥7.0 (ideally ≥8.0) is sufficient for the Iso-Seq protocol. Samples with a RIN <7.0 can be processed, but the risk of significant underperformance or even failure is greatly increased.
- It is critical to accurately pipette ProNex beads because small changes in volume can significantly alter the size distribution of your sample.
- Equilibrate the Pronex Beads at room temperature for 30 – 60 mins prior to use.
- Using multi-channel pipettes greatly enhances the ease of processing more than 1 sample.

Planning your Iso-Seq Experiments

The entire workflow from cDNA synthesis to SMRTbell library preparation takes approximately 8 hours to complete. Plan your experiments so that the entire workflow can be completed within an 8-hour day. If a stop is necessary, it is safe to pause after PCR and after SMRTbell library construction.

Preparing cDNA from RNA Samples

Before starting your reactions, remove the following reagents from the NEBNext® Single Cell/Low Input cDNA Synthesis & Amplification Module and Iso-Seq Express Oligo Kit.

1. Briefly centrifuge NEBNext Single cell RT enzyme mix, then place on ice.
2. Thaw the following components at room temperature, mix, briefly centrifuge and place on ice:

Component	✓
NEBNext Single Cell RT Primer Mix	
NEBNext Single Cell RT Buffer	
NEBNext Single Cell cDNA PCR Master Mix	
NEBNext Single Cell cDNA PCR Primer	
Nuclease-Free Water	
Iso-Seq Express Template Switching Oligo (Found in Iso-Seq Express Oligo Kit)	
Iso-Seq Express cDNA PCR Primer (Found in Iso-Seq Express Oligo Kit)	

3. Thaw the NEBNext Cell Lysis Buffer at room temperature, mix, briefly centrifuge, and leave at room temperature. If the NEBNext Cell Lysis Buffer appears cloudy after thawing, incubate briefly at 37°C.

Primer Annealing for First-Strand Synthesis

1. For each sample to be processed, add the following components to a single PCR tube of an 8-tube strip on ice:

Reaction Mix 1	Volume	✓	Notes
Total RNA (300 ng)	≤7 µL		
NEBNext Single Cell RT Primer Mix	2 µL		
Nuclease-free Water (NEB)	Up to 9 µL		
Total Volume	9 µL		

2. Gently vortex by performing two 2-second pulses and then perform a quick spin to collect all liquid from the sides of the tube.
3. Place in a thermocycler and run the following program (lid 80°C):
    - 5 minutes at 70°C
    - Hold at 4°C

Reverse Transcription and Template Switching Reaction

1. On ice, add the following components in the order listed, to make Reaction Mix 2. Prepare enough Master Mix for all reactions, plus 10% of the total reaction mix volume.

Reaction Mix 2	Volume	✓	Notes
NEBNext Single Cell RT Buffer (vortex briefly before use)	5 µL		
Nuclease-free Water (NEB)	3 µL		
NEBNext Single Cell RT Enzyme Mix	2 µL		
Total Volume added per reaction	10 µL		

2. Gently vortex by performing two 2-second pulses and then perform a quick spin to collect all liquid from the sides of the tube.
4. Add 10 µL of from Reaction Mix 2 to the 9 µL from Reaction Mix 1 for a total volume of 19 µL. Gently vortex by performing two 2-second pulses and then perform a quick spin to collect all liquid from the sides of the tube.
5. Place in a thermocycler at 42°C with the lid at 52°C for 75 minutes then hold at 4°C. Go to the next step immediately.
6. On ice, add 1 µL of Iso-Seq Express Template Switching Oligo to the 19 µL reaction for a total volume of 20 µL. Gently vortex by performing two 2-second pulses and then perform a quick spin to collect all liquid from the sides of the tube.
7. Place in a thermocycler at 42°C with the lid at 52°C for 15 minutes then hold at 4°C.

STEP	✓	Purification with ProNex Beads	Notes
1		Pronex Beads must be brought to room temperature for 30 to 60 mins prior to use. Add **30 µL** of EB to the 20 µL of the Reverse Transcription and Template Switching reaction for a total volume of 50 µL.	
2		Add **50 µL** of ProNex beads for a total volume of 100 µL and gently pipette mix 10 times.	
3		Incubate on bench for 5 minutes.	
4		Place on a magnet stand and wait until supernatant is clear. Use a P200 pipetter to remove the supernatant.	
5		While on magnet, wash two times with 200 µL of freshly prepared 80% ethanol. After removal of second wash of 200 µL of ethanol, spin the tube strip briefly, return to magnetic stand and remove residual ethanol with a P20 pipetter. Do not let the beads to dry out.	
6		Remove the tube strip from the magnetic stand. **Immediately** add 46 µL of EB and pipette mix 10 times to resuspend. Do not let the beads to dry out. Quick spin to collect all liquid from the sides of the tube. Place at 37°C for 5 minutes to elute the DNA from the beads.	
7		Place the tube on the magnetic stand to separate the beads from the supernatant. When the supernatant is clear, transfer 45.5 µL of eluted Reverse Transcription and Template Switching reaction to a new tube and set it aside in ice until ready to use.	

cDNA Amplification

1. On ice, prepare Reaction Mix 3 by adding the following components in the order listed. Prepare enough Reaction Mix 3 master mix for all reactions, plus 10% of the total reaction mix volume.

Reaction Mix 3	Volume	✓	Notes
NEBNext Single Cell cDNA PCR Master Mix	50 µL		
NEBNext Single Cell cDNA PCR Primer*	2 µL		
Iso-Seq Express cDNA PCR Primer*	2 µL		
NEBNext Cell Lysis Buffer	0.5 µL		
Total Volume	54.5 µL		

*12 µM Barcoded Primers, if multiplexing.

2. Add 54.5 µL of Reaction Mix 3 to the 45.5 µL of eluted Reverse Transcription and Template Switching reaction (from step 7 in the previous section) for a total volume of 100 µL.
3. Gently vortex by performing two 2-second pulses and then perform a quick spin to collect all liquid from the sides of the tube.
4. Place in a thermocycler and run the following program (lid 105°C):

PCR Program	
45 seconds at 98°C	**1 cycle**
10 seconds at 98°C	**12 cycles**
15 seconds at 62°C	
3 minutes at 72°C	
5 minutes at 72°C	**1 cycle**
Hold at 4°C	

Purification of Amplified cDNA

In this section, which method to use to purify the amplified cDNA, depends on the goal of the experiment and distribution of transcripts.

Workflow	Goal of Experiment	ProNex Bead Volume
Standard	Sample is composed primarily of transcripts centered ~2 kb	86 µL
Short Transcripts	Sample is composed primarily of transcripts <2 kb, there is a desire to sequence transcripts primarily <2 kb, or degraded samples with low RIN numbers	95 µL
Long Transcripts	To obtain material enriched for longer transcripts >3 kb	82 µL

<table>
<tr><th>STEP</th><th>✓</th><th>Purification with ProNex Beads</th><th>Notes</th></tr>
<tr><td>1</td><td></td><td>Add the chosen volume of resuspended, room temperature ProNex beads to the cDNA sample. Pipette mix 10 times. Perform a quick spin to collect all liquid from the sides of the tube.</td><td></td></tr>
<tr><td>2</td><td></td><td>Incubate the mix on bench top for 5 minutes at room temperature.</td><td></td></tr>
<tr><td>3</td><td></td><td>Place the tube on a magnetic stand to separate the beads from the supernatant. Use a P200 pipetter to remove the supernatant.</td><td></td></tr>
<tr><td>4</td><td></td><td>Wash 2 times with 200 µL of freshly prepared 80% ethanol. After removal of the second wash of 200 µL of ethanol, spin the tube strip, return to the magnetic stand and remove the residual ethanol with P20 pipetter. Do not let the beads to dry out.</td><td></td></tr>
<tr><td>5</td><td></td><td>Remove the tube from the magnetic separator. Immediately add 47 µL of EB and pipette mix to resuspend. Quick spin to collect liquid from the sides of the tube. Place at room temperature for 5 minutes to elute the DNA from the beads.</td><td></td></tr>
<tr><td>6</td><td></td><td>Place the tube on a magnetic stand to separate the beads from the supernatant. Transfer the eluted DNA samples to a new tube.</td><td></td></tr>
<tr><td>7</td><td></td><td>Use 1 µL of sample to quantify with Qubit dsDNA HS kit. You must have the required mass of purified cDNA to proceed with library construction. See guidelines below:
<table>
<tr><th>Instrument</th><th>1 sample</th><th>Multiplex</th><th>Recommendation for Samples with Low Yield</th></tr>
<tr><td>Sequel</td><td>80-500</td><td>80-500*</td><td>Go to Appendix 1 if total mass is <80 ng (<1.75 ng/µL)</td></tr>
<tr><td>Sequel II</td><td>160-500</td><td>160-500*</td><td>Go to Appendix 1 if total mass is <160 ng (<3.5 ng/µL)</td></tr>
</table>
Over-amplification can result in sub-optimal data. For high-yield samples with concentrations >40 ng/µL, optimal libraries may be obtained by repeating cDNA generation with less RNA input or by decreasing the number of PCR cycles.</td><td></td></tr>
<tr><td>8</td><td></td><td>Optional: Dilute 1 µL of sample to 1.5 ng/µL and run 1 µL on an Agilent Bioanalyzer using a High Sensitivity DNA kit. Examining the amplified cDNA on a Bioanalyzer prior to PacBio library construction is an excellent quality control step to ensure that the amplified cDNA material has the expected size distribution.</td><td></td></tr>
</table>

Sample Pooling (Skip If Not Multiplexing)

Equal molar pooling of barcoded cDNA samples is necessary to generate good representation of samples that are being multiplexed.

1. Use the concentration and average library size* from the Bioanalyzer trace to determine the molarity of each sample. Use the following equation to determine Molarity:

$$\frac{\text{concentration in ng/ µL X } 10^6}{\text{(660 g/mol x average library size in bp*)}} = \text{concentration in nM}$$

*To determine the average library size, select the region of interest by defining the start at 200 bp and the end point at 9500 bp of the smear (on a High Sensitivity DNA kit).

2. Pool equal molar quantities of the barcoded cDNA.
   - Use the maximum total combined mass possible without exceeding 500 ng in 47.4 µL.
   - The total **combined** mass must be >80 ng for Sequel and >160 ng for Sequel II to proceed to DNA Damage Repair.
   - If the volume required to achieve the minimum mass of the pooled cDNA exceeds 47.4 µL, concentrate the pooled cDNA by performing a 1X volume of ProNex beads and elute it in 48 µL. To account for potential losses during concentration at this step, start with ≥100 ng for Sequel and ≥200 ng for Sequel II.
3. The pooled cDNA can now be constructed into a SMRTbell library as a single sample. Proceed to the DNA Damage Repair step below.

DNA Damage Repair

IMPORTANT: Use the maximum available cDNA without exceeding 500 ng for this step.

1. For each sample to be processed, add the following components to a single PCR tube:

Reaction Mix 4	Tube Cap Color	Volume	✓	Notes
DNA Prep Buffer		7 µL		
Purified, Amplified cDNA*		≤47.4 µL		
NAD		0.6 µL		
DNA Damage Repair Mix v2		2 µL		
H_2O		Up to 57 µL		
Total Volume		57 µL		

*Sequel System: 80-500 ng
*Sequel II System: 160-500 ng

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:
   - 30 minutes at 37°C
   - Hold at 4°C

End Repair/A-Tailing

1. With the reaction on ice, add 3 µL End Prep Enzyme Mix directly to Reaction Mix 4:

Reaction Mix 5	Tube Cap Color	Volume	✓	Notes
Reaction Mix 4		57 µL		
End Prep Mix		3 µL		
Total Volume		60 µL		

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:
   - 30 minutes at 20°C
   - 30 minutes at 65°C
   - Hold at 4°C

Overhang Adapter Ligation

1. Add the following components, in the order listed, directly to Reaction Mix 5:

Reaction Mix 6	Tube Cap Color	Volume	✓	Notes
Reaction Mix 5		60 µL		
Overhang Adapter v3		3 µL		
Ligation Mix		30 µL		
Ligation Enhancer		1 µL		
Ligation Additive		1 µL		
Total Volume		95 µL		

2. Pipette mix 10 times. The ligation master mix is viscous making it imperative 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:
   - 60 minutes at 20°C
   - Hold at 4°C

Cleanup cDNA SMRTbell Libraries

STEP	✓	Purification with ProNex Beads	Notes
1		Add **95 µL** of resuspended, room-temperature ProNex beads to the 95 µL Reaction Mix 6. Pipette mix 10 times. Perform a quick spin to collect all liquid from the sides of the tube.	
2		Incubate sample on bench top for 5 minutes at room temperature.	
3		Place the tube on a magnetic stand to separate the beads from the supernatant. Use a P200 pipettor to remove supernatant.	
4		Wash 2 times with 200 µL of freshly prepared 80% ethanol. After removal of the second wash of 200 µL of ethanol, spin the tube strip briefly, return to magnetic stand and remove residual ethanol with a P20 pipetter. Do not let the beads to dry out.	
5		Remove the tube from the magnetic stand. **Immediately** add 12 µL of EB and pipette mix to resuspend. Perform a quick spin to collect all liquid from the sides of the tube. Place at room temperature for 5 minutes to elute the DNA from the beads.	
6		Place the tube on a magnetic stand to separate the beads from the supernatant. Transfer the eluted DNA samples to a new tube.	
7		Use 1 µL of sample to quantify with Qubit dsDNA HS kit.	
8		Dilute 1 µL of sample to 1.5 ng/µL and run 1 µL on an Agilent Bioanalyzer using a High Sensitivity DNA kit. Determine the final size of the Iso-Seq SMRTbell library.	

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.

For detailed recommendations for sequencing of specific library insert size ranges, refer to the Quick Reference Card – Diffusion Loading and Pre-Extension Time Recommendations for the Sequel II System here or Reference Card – Diffusion Loading and Pre-Extension Time Recommendations for the Sequel System here.

Appendix 1 – Recommendations for Additional cDNA Amplification by PCR for Samples with a Lower Yield or to Enrich for Longer Transcripts

The Sequel and Sequel II Systems require different amounts (ng) of cDNA for SMRTbell library construction. The Sequel System requires >80 ng of DNA, while the Sequel II System requires >160 ng DNA.

If there is not enough DNA to proceed with library construction, this section describes a workflow for enriching cDNA by PCR. Alternatively, if you want to enrich for longer transcripts (>3 kb) additional cDNA amplification is required, the amount of ProNex beads for purification can be modified as described in step 1 of the Purification table below.

1. On ice, prepare the following reaction. Combine in the order shown.

PCR Amplification Reaction	Volume	✓	Notes
NEBNext Single Cell cDNA PCR Master Mix OR NEBNext High-Fidelity 2X PCR Master Mix*	50 µL		
NEBNext Single Cell cDNA PCR Primer**	2 µL		
Iso-Seq Express cDNA PCR Primer**	2 µL		
NEBNext Cell Lysis Buffer	0.5 µL		
Purified, Amplified cDNA	45.5 µL		
Total Volume	100 µL		

*PCR Master Mix ordered separately (see Materials and Kits Needed)

**12 µM Barcoded Primers if multiplexing

2. Gently vortex by performing two 2-second pulses and then quick spin to collect liquid from the sides of the tube.
3. Place in a thermocycler and run the following program (lid 105°C):

PCR Program	
45 seconds at 98°C	**1 cycle**
10 seconds at 98°C	**N* cycles (see below)**
15 seconds at 62°C	
3 minutes at 72°C	
5 minutes at 72°C	**1 cycle**
Hold at 4°C	

*The recommended number of cycles depends on the instrument and available cDNA.
Use the following guidelines to determine the number of cycles.

Instrument	Additional # of Cycles	Condition
Sequel System	3	If total mass <80 ng (<1.75 ng/µL)
Sequel II System	3	If total mass >32-160 ng (≥0.70-1.74 ng/ µL)
	5	If total mass ≤32 ng (<0.7 ng/ µL)

STEP	✓	Purification	Notes
1		For low yield reamplified samples: add **100 µL** of resuspended, room-temperature ProNex beads to the amplified cDNA. Pipette mix 10 times. Quick spin to collect liquid from the sides of the tube and proceed to step 2. OR For enriching for longer transcripts: add **85 µL** of resuspended, room-temperature ProNex beads to the amplified cDNA. Pipette mix 10 times. Quick spin to collect liquid from the sides of the tube and proceed to step 2.	
2		Incubate sample on bench top for 5 minutes at room temperature.	
3		Place the tube on a magnetic stand to separate the beads from the supernatant. Use a P200 pipettor to remove supernatant.	
4		Wash 2 times with 200 µL of freshly prepared 80% ethanol. After removal of second wash of 200 µL of ethanol, spin the tube strip, return to magnetic stand and remove residual ethanol with a P20. Do not let the beads to dry out.	
5		Remove the tube from the magnetic stand. **Immediately** add 50 µL of EB and pipette mix to resuspend. Quick spin to collect liquid from the sides of the tube. Incubate at room temperature for 5 minutes to elute the DNA from the beads.	
6		Place the tube on a magnetic stand to separate the beads from the supernatant. Transfer the eluted DNA samples to a new tube.	
7		Use 1 µL of sample to quantify with Qubit dsDNA HS kit.	
8		**Optional**: Dilute 1 µL of sample to 1.5 ng/µL and run 1 µL on an Agilent Bioanalyzer using a High Sensitivity DNA kit. Although this step is optional, examining the amplified cDNA on a Bioanalyzer prior to PacBio library construction is an excellent quality control step to ensure that the amplified cDNA material has the expected size distribution.	
9		Return to “DNA Damage Repair, End Repair, and A-Tailing” section.	

Appendix 2 – Recommended Barcoded NEBNext Single Cell cDNA PCR Primer and Iso-Seq Express cDNA PCR Primer Sequences

Barcoded forward and reverse primers may be ordered from any oligo synthesis company. The oligos must be diluted to 12 µM concentration for use in the "cDNA Amplification" section. Use 10 mM Tris, 0.1 mM EDTA for diluting oligos.

Name	Sequence	Scale	Purification
bc1001-F	CACATATCAGAGTGCGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1001-R	CACATATCAGAGTGCGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1002-F	ACACACAGACTGTGAGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1002-R	ACACACAGACTGTGAGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1003-F	ACACATCTCGTGAGAGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1003-R	ACACATCTCGTGAGAGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1004-F	CACGCACACACGCGCGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1004-R	CACGCACACACGCGCGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1005-F	CACTCGACTCTCGCGTGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1005-R	CACTCGACTCTCGCGTAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1006-F	CATATATATCAGCTGTGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1006-R	CATATATATCAGCTGTAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1008-F	ACAGTCGAGCGCTGCGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1008-R	ACAGTCGAGCGCTGCGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1012-F	ACACTAGATCGCGTGTGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1012-R	ACACTAGATCGCGTGTAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1018-F	TCACGTGCTCACTGTGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1018-R	TCACGTGCTCACTGTGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1019-F	ACACACTCTATCAGATGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1019-R	ACACACTCTATCAGATAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1020-F	CACGACACGACGATGTGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1020-R	CACGACACGACGATGTAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1023-F	CAGAGAGATATCTCTGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1023-R	CAGAGAGATATCTCTGAAGCAGTGGTATCAACGCAGAGT	25nm	STD

Revision History (Description)	Version	Date
Initial release.	01	June 2019
Updated to remove old Sequencing information on page 10 and instead refer to the Quick Reference Cards which contain the latest recommendations.	02	October 2019