Procedure & Checklist – Preparing Single-Cell Iso-Seq™ Libraries Using SMRTbell® Express Template Prep Kit 2.0

Before You Begin

The Sequel Systems generate long reads that are well-suited for characterizing full-length transcripts produced from Single-Cell platforms. This document describes a method for constructing Single-Cell Iso-Seq SMRTbell® libraries for sequencing.

Generating Single-Cell Iso-Seq SMRTbell libraries is a two-step process. Initially, the intact RT-PCR product from a typical Single-Cell preparation is reamplified to increase the mass. Then the SMRTbell Express Template Prep Kit 2.0 is used for SMRTbell library preparation.

For best analytical results, we recommend combining matching (i.e., the same exact library) short-read and Iso-Seq datasets. We recommend that the reamplification yield allow for parallel processing of both short-read sequencing and SMRT® Sequencing. The Sequel System requires >80 ng of DNA, while the Sequel II System requires >160 ng DNA. These are target amounts for the reamplification steps for the Iso-Seq Express workflows.

Reamplification is typically achieved by using the PCR primers specific to a Single-Cell platform. If these are not supplied in the quantity required for the both the short read and SMRT Sequencing reamplification, order the oligonucleotides separately. The PCR primer sequences can be typically obtained from the Single-Cell platform provider. An example is provided in the Materials and Kits Needed section below.

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 Domestic) 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® High-Fidelity 2X PCR Master Mix (for additional PCR reactions)	NEB M0541S
Elution Buffer (50 mL)	PacBio PN 101-633-500
Ethanol	Any MLS
Single-Cell library amplification primers for specific platform (primers may be ordered from any oligo synthesis company)	10x Chromium Single Cell 3' Solution V2 and V3: cDNA Forward primer (e.g., PCR Primer 1): 5'- CTACACGACGCTCTTCCGATCT -3' cDNA Reverse primer (e.g., PCR Primer 2): 5'- AAGCAGTGGTATCAACGCAGAGT-3'
Single cell cDNA amplification kit from your single cell vendor†	Any Single-Cell Vendor
Additional Single-Cell library amplification primers for specific platform may be required for parallel reamplification	For example, Drop-Seq: Both Primers are the same sequence for PCR Primer 1 and 2: 5'- AAGCAGTGGTATCAACGCAGAGT -3' [Macosko, et. al., Cell, 2015:pp1202-14] Commercial vendors may have different sequences for their PCR Primers. Please consult your vendor for the primer(s) sequence(s).

† Pacific Biosciences does not sell a kit for carrying out the Single-Cell RNA Sequencing method. Use of these methods may require rights to third-party owned intellectual property.

Recommended Best Practices

- 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 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 re-amplification to SMRTbell library preparation takes approximately 5 hours to complete.

cDNA Re-Amplification

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

Reaction Mix 1	Volume	✓	Notes
NEBNext® High-Fidelity 2X PCR Master Mix	50 µL		
12 µM PCR Primer 1	2 µL		
12 µM PCR Primer 2	2 µL		
Single-Cell cDNA	≤46 µL		
H_2O	Up to 100 µL		
Total Volume	100 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 105°C):

PCR Program	
45 seconds at 98°C	**1 cycle**
10 seconds at 98°C	**6 cycles***
15 seconds at 62°C	
3 minutes at 72°C	
5 minutes at 72°C	**1 cycle**
Hold at 4°C	

* Target yield is approximately 200 ng. The number of cycles will vary significantly across systems, number of cells, etc. 6 cycles is a decent starting point. If > 500 ng is routinely obtained reduce the number of cycles for future experiments. If < 200 ng is routinely obtained increase the number of cycles for future experiments.

Purification of Amplified cDNA

The amount of ProNex beads to use to purify the amplified cDNA depends on the distribution of transcripts produced by the Single-Cell cDNA preparation and the goal of the experiment. The cDNA coming out of Single-Cell preparations tends to have a shorter size distribution than similar libraries prepared from bulk RNA.

Experimental Considerations	ProNex Bead Volume
Single-Cell cDNA sample is 1) composed of transcripts centered ~2 kb or larger or 2) a reduction in shorter transcripts is desired.	86 µL
Single-Cell cDNA sample is composed primarily of transcripts <2 kb or no reduction in shorter transcripts is desired.	95 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>Sample (ng)</th><th>Recommendation for Samples with Low Yield</th></tr>
<tr><td>Sequel</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>Go to Appendix 1 if total mass is <160 ng (<3.5 ng/µL)</td></tr>
</table></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>

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

Use Sequencing Primer v4 for both systems.

Sequel System Sample Setup/Calculator

Options	Recommendations
SMRT Link 7.0 Sample Setup	Follow instructions in Sample Setup.
SMRT Link 6.0 Sample Setup	Swap ProNex beads for AMPure PB beads.

Sequel II System Sample Setup/Calculator

Options	Recommendations
Excel Calculator	Sample Setup Calculator
SMRT Link 7.0 Sample Setup	Not supported

Sequencing

Diffusion loading is recommended for Iso-Seq libraries prepared using this procedure. PacBio recommends performing loading titrations to determine an appropriate loading concentration.

	Sequel System	Sequel II System
Loading Method	Diffusion	Diffusion
Movie time	20 hrs	24 hrs
Pre-extension time	4 hrs	2 hrs
Sample Cleanup	ProNex beads	ProNex beads
On-plate loading concentration	2 - 8 pM	75 - 100 pM

Appendix 1 – Recommendations for Additional cDNA Amplification by PCR for Samples with a Lower Yield

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.

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

PCR Amplification Reaction	Volume	✓	Notes
NEBNext High-Fidelity 2X PCR Master Mix*	50 µL		
12 µM PCR Primer 1	2 µL		
12 µM PCR Primer 2	2 µL		
Purified, Amplified cDNA	46 µL		
Total Volume	100 µL		

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.	

Revision History (Description)	Version	Date
Initial release.	01	January 2020