Customer Collaboration – Iso-Seq® Express Capture Using IDT xGen® Lockdown® Probes

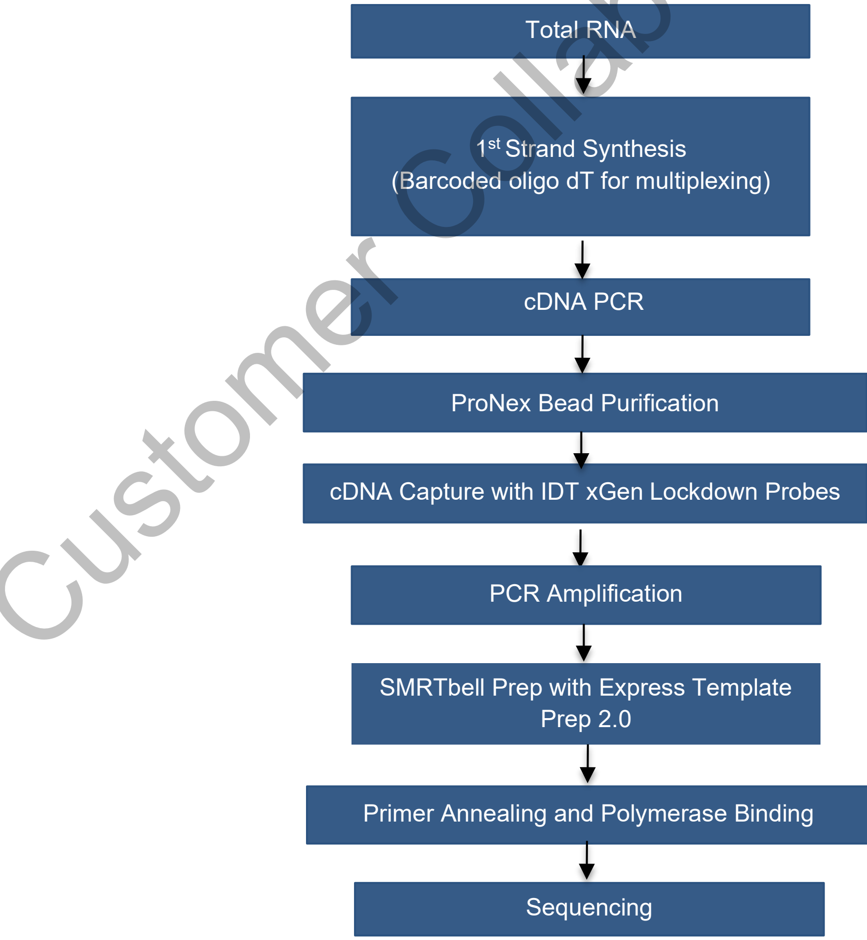
Before You Begin

This document describes the process for capturing cDNA prepared with the NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module and pulled-down using xGen Lockdown Probes/Panels from IDT.

Workflow

The workflow includes the following:

1. Preparing the cDNA library using the NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module.
2. Capturing cDNA with the IDT xGen Lockdown Probes (biotinylated probes).
3. Constructing SMRTbell® libraries with Express Template Prep Kit 2.0
4. Sequencing using the PacBio System.



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Materials Needed

Item	Vendor
cDNA Library	
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
dNTP Mix (25 mM each)	Thermo Fisher Scientific R1121
Elution Buffer	PacBio PN 101-633-500
Ethanol	Any MLS
IsoSeq Express Oligo Kit	PacBio PN 101-737-500
Qubit dsDNA HS Assay Kit	Any MLS
Qubit Fluorometer	Invitrogen
HS DNA Kit	Invitrogen
Bioanalyzer Instrument	Agilent
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
Target Capture	
xGen RT-primer block	IDT
xGen TSO block	IDT
xGen Hybridization and Wash Kit (16 or 96 reaction)	IDT
xGen Lockdown Panels/Probes (target probes)	IDT
SMRTbell Library Construction and Sequencing	
SMRTbell Express Template Prep Kit 2.0	PacBio
DNA/Polymerase Binding Kit	PacBio
DNA Sequencing Kit	PacBio
ProNex® Beads (for size selection and purification)	Promega - Catalog numbers: NG2001 - 10mL, NG2002 - 125mL, NG2003 - 500mL

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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.

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Planning your Iso-Seq Experiments

The generation of amplified cDNA for capture takes approximately 4 hours, the capture and amplification takes approximately 8 hours, and the SMRTbell library preparation takes about 4 hours.

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 or barcoded RT Primers (See Appendix 3)	
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)	≤5.4 µL		
NEBNext Single Cell RT Primer Mix or 12 µM barcoded RT Primer * 25 mM each dNTP (Thermo)**	2 µL 1.6 µL		
Nuclease-free Water (NEB)	Up to 9 µL		
Total Volume	9 µL		

*12 µM Barcoded Primers if multiplexing. See Appendix 3.

** Extra dNTP is required in the reaction if multiplexing, do not add if NEB Single Cell RT Primer Mix is used.

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.

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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.	

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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		

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	**14 cycles**
15 seconds at 62°C	
3 minutes at 72°C	
5 minutes at 72°C	**1 cycle**
Hold at 4°C	

STEP	✓	Purification with ProNex Beads	Notes
1		Add **86 µL** 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. Note: If the average expected target transcript size is <2kb, add 95 µL of ProNex beads to the cDNA sample.	
2		Incubate the mix 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 pipetter to remove the 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, return to the magnetic stand and remove the residual ethanol with P20 pipetter. Do not let the beads to dry out.	
5		Remove the tube from the magnetic separator. **Immediately** add 50 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.	
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. You must have the required mass of purified cDNA to proceed with hybridization. Refer to Appendix 1 for guidelines if total mass is <500ng (<10ng/µL). 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.	
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. 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.	

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Sample Pooling (Skip If Not Multiplexing)

Based on the Qubit results, pool equal mass of barcoded cDNA to a total of 500ng per capture reaction.

If the average transcriptome size is significantly different across libraries, 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:

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

2. Pool equal molar quantities of the barcoded cDNA. Use the maximum total combined mass possible without exceeding 500 ng.

STEP	✓	Prepare the Hybridization Sample	Notes
		In this section, you will need the following: • xGen Asym TSO block (IDT), see appendix 4 • xGen RT-primer block (IDT), see appendix 4 • 2X Hybridization Buffer contained in xGen Lockdown Hybridization and Wash Kit • Hybridization Buffer Enhancer in xGen Lockdown Hybridization and Wash Kit • xGen Lockdown Panels/Probes (target probes)	
1		Together with 7.5 µL of Cot DNA, concentrate a total of 500ng cDNA (up to 24 plex) in a 1.5 mL LoBind tube.	
2		Add 1.8X volume of ProNex beads to the cDNA pool with Cot DNA. Gently pipette mix 10 times and incubate for 10 min at room temperature. Prepare hybridization mix to be used in Step 5 and set aside.	
3		Place on a magnet stand and wait until supernatant is clear. Use a P200 pipetter to remove the supernatant.	
4		While on magnet stand, wash two times with 200 µL of freshly prepared 80% ethanol. After removal of second wash, 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		Immediately add the hybridization reaction mix to elute the cDNA:	
6		Gently pipette mix 10 times and incubate for 5 min at room temperature.	
7		Place the tube on the magnetic stand to separate the beads from the supernatant.	
8		When the supernatant is clear, transfer 17 µL of the supernatant to a new 0.2 mL PCR tube. Briefly centrifuge. Make sure the tubes are tightly sealed to prevent evaporation.	
9		Place the sample tube in the thermal cycler and start the hybridization program:	
10		During the incubation, prepare 1X working buffers and beads for capture.	

Step 5 hybridization reaction mix:

Component	Volume
2X Hybridization Buffer	9.5 µL
Hybridization Buffer Enhancer	3 µL
xGen Asym TSO block	1 µL
xGen RT-primer-barcode block	1 µL
1X xGen Lockdown Panels/Probes	4.5 µL

Step 9 hybridization program:

HYB program (lid set at 100°C)	
95°C	30 sec
65°C	4hr
65°C	Hold

*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).

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Prepare capture reagents

1. To resuspend xGen blocking oligos, see this Resuspension protocol
2. To resuspend xGen Lockdown Probes and Panels, refer to this xGen resuspension protocol For additional support regarding resuspension of Lockdown Probes pools, visit www.idtdna.com/xgen ► xGen Lockdown Probes► Support tab ►expand Number of Reactions and Resuspension Volume.

STEP	✓	Preparing Beads for Capture	Notes
		In this section, you will need the following: • Wash buffers contained in the xGen Lockdown Hybridization and Wash Kit • Dynabeads M-270 Streptavidin	
1		Prepare Wash Buffers: a. Prepare 1X working solutions of the buffers listed in the below table. The total volume of 1X buffer in the table is for a single experiment. Scale up accordingly when multiple samples are required. Ensure that 10X Wash Buffer I is in solution before use. If it is cloudy, incubate at 65°C heat block to allow resuspension.	

Buffer Stock	Stock Conc.	Vol. Buffer	Vol. Water	Total Volume*	Final Conc.
Wash Buffer I (tube 1)	10X	28 µL	252 µL	280 µL	1X
Wash Buffer II (tube 2)	10X	16 µL	144 µL	160 µL	1X
Wash Buffer III (tube 3)	10X	16 µL	144 µL	160 µL	1X
Stringent Wash Buffer (tube S)	10X	32 µL	288 µL	320 µL	1X
Bead Wash Buffer	2X	160 µL	160 µL	320 µL	1X

*Store working solutions at room temperature (+15 to +25°C) for up to 4 weeks. The volumes in this table are calculated for a single capture; scale up accordingly if multiple hybridization reactions will be processed.

b. Preheat the following wash buffers to +65°C in a heat block or water bath:
- 110 µL of 1X Wash Buffer I (Tube1)
- 320 µL of 1X Stringent Wash Buffer (Tube S), aliquot into 2 tubes (160 µL each)

STEP	✓	Preparing Beads for Capture	Notes
2		Prepare the capture beads: a. Allow the Dynabeads M-270 Streptavidin to warm to room temperature for 30 minutes prior to use. b. Mix the beads thoroughly by vortexing for 15 seconds. c. For a single sample, aliquot 50 µL beads into a 0.2 mL PCR tube. Scale up volume for multiple samples. d. Add 100 µL of 1X Bead Wash Buffer per capture, pipette the mix 10 times. e. Place the PCR tube in a magnetic rack. When the supernatant is clear, remove and discard the supernatant being careful not to disturb the beads. Any remaining traces of liquid will be removed with subsequent wash steps. Note: Allow the Dynabeads to settle for at least 1 minute before removing the supernatant. f. Remove the tube from the magnetic rack and perform the following wash: i) Add 100 µL of 1X Bead Wash Buffer, pipette 10 times to mix ii) Place the PCR tube on the magnetic rack, allowing the beads to fully separate from the supernatant iii) Carefully remove and discard the clear supernatant g. Repeat steps f for a total of two washes. h. Resuspend the beads in 17 µL per capture of Bead Resuspension Mix. For multiple samples, scale up accordingly.	

Bead Resuspension Mix Component	Volume per reaction
xGen 2X Hybridization Buffer	8.5 µL
xGen Hybridization Buffer Enhancer	2.7 µL
Nuclease-Free Water	5.8 µL

i. Aliquot 17 µL of resuspended beads in each capture reaction, pipette 10 times to mix.

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STEP	✓	Binding cDNA to Beads and Wash	Notes
1		Bind cDNA to the capture beads: Incubate in a thermocycler set to +65°C for 45 minutes (heated lid set to +70°C, it is important to reduce the lid temperature from previous step). Hand mix every 10-12 min by gently tapping the tube to keep the beads in suspension.	
2		Wash the captured cDNA: a. Pre-heat 1X Wash Buffer (tube 1) and 1X Stringent Wash Buffer (Tube S) to 65°C b. After 45 minutes of incubation, remove the tube from the 65°C thermomixer and immediately add 100 µL pre-heated 1X Wash Buffer I (Tube 1). c. Mix thoroughly by pipetting up and down until the sample is homogeneous. d. Place the PCR tube in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. e. Remove the tube from the magnetic rack and add 150 µL of 1X Stringent Wash Buffer (TubeS) heated to +65°C. Pipette mix 10 times. Work quickly so that the temperature does not drop below +65°C. f. Incubate at +65°C for 5 minutes. g. Repeat steps d - f for a total of two washes using 1X Stringent Wash Buffer (TubeS) heated to +65°C. h. Place the tubes in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. i. Add 150 µL of room temperature 1X Wash Buffer I (Tube1) and pipette mix 10 times. Quick spin. j. Place the tube in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. k. Add 150 µL of room temperature 1X Wash Buffer II (Tube2) and pipette mix 10 times. Quick Spin. l. Place the tube in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. m. Add 150 µL of room temperature 1X Wash Buffer III (Tube 3) and pipette mix 10 times. Quick Spin. n. Place the tubes in the magnetic rack to collect the beads to the side of the tube. Remove and discard the liquid once clear. o. Remove the tubes from the magnetic rack and add 46 µL of EB to each tube of bead-bound captured sample. Pipette mix 10 times p. Proceed immediately to the next step or store the beads plus captured samples at -15 to -25°C. It is not necessary to separate the beads from the eluted DNA. The bead/sample mix can be added to the PCR reaction directly.	

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STEP	✓	Amplification of Captured DNA Sample	Notes
		In this section, you will need the following: • NEB High-Fidelity 2X PCR Master Mix • NEBNext Single Cell cDNA PCR Primer • Iso-Seq Express cDNA PCR Primer	
		PacBio recommends using NEBNext High-Fidelity 2X PCR Master Mix or NEBNext Single Cell cDNA PCR Master Mix for post capture amplification. a. Assemble the following PCR reaction:	

Component	Volume
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
Captured Library	45.5 μL
Total	100 μL

b. Amplify using the following PCR protocol:

Step	Temp	Time
1	98°C	45 seconds
2	98°C	10 seconds
3	62°C	15 seconds
4	72°C	3 minutes
	Repeat steps 2-4 for a total of 12 cycles *	
5	72°C	5 minutes
6	4°C	Hold

* 12 cycles is the recommended starting point, selecting the cycle of PCR is extremely dependent on capture panel size and transcript expression level. Over-amplification is sub-optimal.

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STEP	✓	Post Amplification Clean Up	Notes
1		Add **100 µL (1X)** ProNex beads to the PCR reaction.	
2		Mix by pipetting until the sample is homogeneous.	
3		Incubate at room temperature for 5 minutes.	
4		Place on magnetic rack until solution clears. Remove and discard supernatant.	
6		With the tube still on magnet, add 200 µL freshly prepared 80% ethanol to the tube containing beads plus DNA.	
7		Remove and discard 80% ethanol.	
8		Repeat steps 5 to 6 for total of two washes with 80% ethanol.	
9		Spin the tube down, return to magnetic stand and remove residual ethanol with a P20. Do not let the beads to dry out.	
10		Immediately add 50 µL EB and remove the tube from the magnet. Pipette mix to resuspend. Then incubate at room temperature for 5 minutes.	
11		Place the tube on a magnetic stand to separate the beads from the supernatant. Transfer the eluted DNA samples to a new tube.	
12		Use 1 µL of sample to quantify with Qubit dsDNA HS kit. You must have the required mass of purified captured cDNA to proceed with library construction. See guidelines below:	

Instrument	Required mass (ng)	Recommendation for Samples with Low Yield
Sequel	80-500	Go to Appendix 2 if total mass is <80 ng (<1.75 ng/µL)
Sequel II	160-500	Go to Appendix 2 if total mass is <160 ng (<3.5 ng/µL)

STEP	✓	Post Amplification Clean Up	Notes
13		**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.	
14		The captured cDNA is now ready for SMRTbell library construction.	

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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

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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 (1X)** 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.	

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Prepare for Sequencing

Use Sequencing Primer v4 for both systems.

Sequel System Sample Setup/Calculator

Options	Recommendations
SMRT Link v8.0 Sample Setup	Follow instructions in Sample Setup.
SMRT Link v7.0 Sample Setup	Follow instructions in Sample Setup.

Sequel II System Sample Setup/Calculator

Options	Recommendations
SMRT Link v9.0 Sample Setup	Follow instructions in Sample Setup.
SMRT Link v8.0 Sample Setup	Follow instructions in Sample Setup.
Excel Calculator	Sample Setup Calculator

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	50 - 100 pM

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

If there is not enough DNA to proceed with hybridization, 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 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	**3-5* cycles**
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 available cDNA. 3-5cycles is generally sufficient to meet 500ng input requirement, adjust if necessary. Keep in mind that over-amplification is sub-optimal.

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STEP	✓	Purification	Notes
1		For low yield reamplified samples: add **100 µL (1X)** 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 “Prepare the Hybridization Sample” section.	

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Appendix 2 – Recommendations for Additional cDNA Amplification by PCR for Samples with a Lower Post Capture 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.

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	50 µL		
NEBNext Single Cell cDNA PCR	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

1. Gently vortex by performing two 2-second pulses and then quick spin to collect liquid from the sides of the tube.
2. 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. Combination of target panel size and gene expression level could result in very low recovery of captured cDNA. More cycles than indicated in the table might be required.

Instrument	Additional # of Cycles	Condition
Sequel System	3	If total mass <80 ng (<1.75 ng/µL)
	5	If total mass <10ng (<0.2ng/ 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)
	7	If total mass <10 ng (<0.2ng/ µL)

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STEP	✓	Purification	Notes
1		Add **100 µL (1X)** 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.	

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Appendix 3 – Recommended Barcoded RT Primer Sequences

Barcoded primers may be ordered from any oligo synthesis company. The oligos must be diluted to 12 µM concentration for use in the "Primer Annealing for First-Strand Synthesis" section. Use 10 mM Tris, 0.1 mM EDTA for diluting oligos.

Name	Sequence	Scale	Purifi-cation
dT_bc1002_PB	AAGCAGTGGTATCAACGCAGAGTACACACACAGACTGTGAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1003_PB	AAGCAGTGGTATCAACGCAGAGTACACACATCTCGTGAGAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1004_PB	AAGCAGTGGTATCAACGCAGAGTACCACGCACACACGCGCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1009_PB	AAGCAGTGGTATCAACGCAGAGTACACACACGCGAGACAGATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1010_PB	AAGCAGTGGTATCAACGCAGAGTACACGCGCTATCTCAGAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1012_PB	AAGCAGTGGTATCAACGCAGAGTACACACTAGATCGCGTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1013_PB	AAGCAGTGGTATCAACGCAGAGTACCTCTCGCATACGCGAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1015_PB	AAGCAGTGGTATCAACGCAGAGTACCGCATGACACGTGTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1017_PB	AAGCAGTGGTATCAACGCAGAGTACCACACGCGCGCTATATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1018_PB	AAGCAGTGGTATCAACGCAGAGTACTCACGTGCTCACTGTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1022_PB	AAGCAGTGGTATCAACGCAGAGTACCACTCACGTGTGATATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1024_PB	AAGCAGTGGTATCAACGCAGAGTACCATGTAGAGCAGAGAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1027_PB	AAGCAGTGGTATCAACGCAGAGTACCTCACACTCTCTCACATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1030_PB	AAGCAGTGGTATCAACGCAGAGTACTCTCTCTATCGCGCTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1031_PB	AAGCAGTGGTATCAACGCAGAGTACGATGTCTGAGTGTGTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1032_PB	AAGCAGTGGTATCAACGCAGAGTACGAGACTAGAGATAGTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1035_PB	AAGCAGTGGTATCAACGCAGAGTACGCGCGCGCACTCTCTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1036_PB	AAGCAGTGGTATCAACGCAGAGTACGAGACACGTCGCACACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1037_PB	AAGCAGTGGTATCAACGCAGAGTACACACATATCGCACTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1038_PB	AAGCAGTGGTATCAACGCAGAGTACGTGTGTCTCGATGCGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1039_PB	AAGCAGTGGTATCAACGCAGAGTACCGCACACATAGATACATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1044_PB	AAGCAGTGGTATCAACGCAGAGTACGCTGAGACGACGCGCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1047_PB	AAGCAGTGGTATCAACGCAGAGTACTGTCATGTGTACACACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE
dT_bc1048_PB	AAGCAGTGGTATCAACGCAGAGTACGTGTGCACTCACACTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	100nm	PAGE

Appendix 4 – Recommended Blocking Oligo Sequences

Contact IDT for custom order of the xGen blockers.

To resuspend xGen blocking oligos, refer to the [Resuspension protocol: xGen Standard and Universal Blocking Oligos]

Name	Sequence	Scale
xGen_PB_TSO_BLOCK	GCAATGAAGTCGCAGGGTTGGG/3SpC3/	25 nmole xGen Blocking Oligo
xGen_PB_RT_BLOCK	AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT/3SpC3/	25 nmole xGen Blocking Oligo

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