Preparing Iso-Seq® libraries using SMRTbell® prep kit 3.0

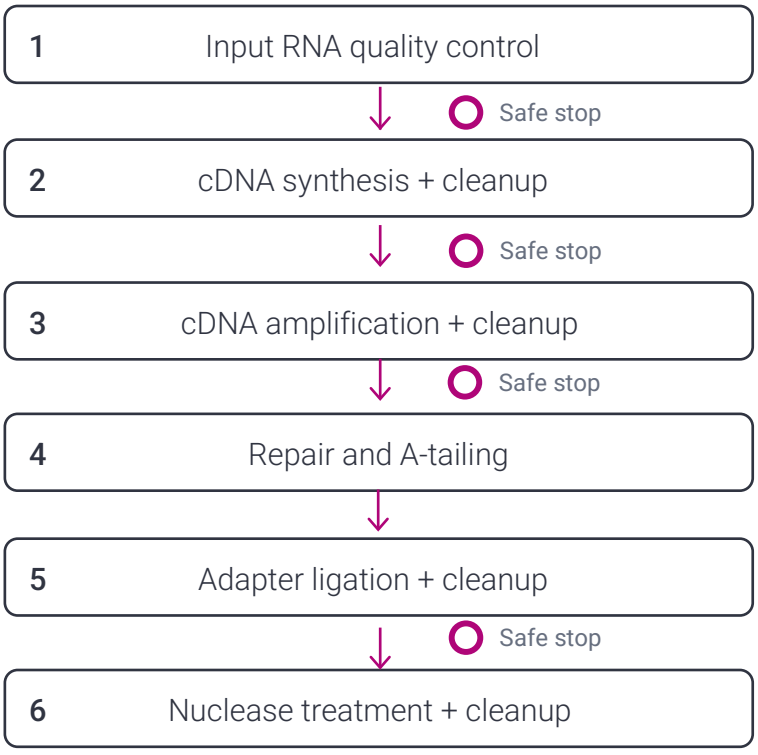
Procedure & checklist

Before you begin

This procedure describes the workflow for constructing Iso-Seq libraries from RNA for sequencing on PacBio Sequel® II and IIe systems.

Overview	
Samples	1–12
Workflow time	8 hours [for up to 12 samples]
RNA input	
Quality / size distribution	RIN (RNA integrity number) ≥7.0
Quantity	300ng per library
cDNA Input	
Quantity	≥ 160 ng per library for 1 SMRT Cell 8M

Workflow



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Required materials and equipment

RNA and DNA sizing	
2100 Bioanalyzer instrument	Agilent Technologies G2939BA
RNA 6000 Nano kit	Agilent Technologies 5067-1511
DNA High Sensitivity DNA kit	Agilent Technologies 5067-4626
DNA quantitation	
Qubit Fluorometer	Thermo Fisher Scientific Q33238
Qubit 1X dsDNA HS Assay kit	Thermo Fisher Scientific Q33230
cDNA synthesis and amplification	
NEBNext® Single Cell/Low Input cDNA Synthesis & Amplification Module*	NEB E6421S (24 reactions) or E6421L (96)
NEBNext® High-Fidelity 2X PCR Master Mix (for additional PCR reactions)	NEB M0541S
Elution buffer (50 mL)	PacBio 101-633-500
Iso-Seq Express oligo kit**	PacBio 101-737-500
SMRTbell® library preparation	
SMRTbell® prep kit 3.0	PacBio 102-182-700
SMRTbell® barcoded adapter plate 3.0 (optional; for barcoding**)	PacBio 102-009-200
200 Proof ethanol, molecular biology or ACS grade	Any major lab supplier
Nuclease-free water, molecular biology grade	Any major lab supplier (e.g., Sigma-Aldrich W4502)
8-channel pipettes – P20 & P200)	Any major lab supplier
Single-channel pipette – P2, P10, P20, P100 or P200	Any major lab supplier
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Microcentrifuge	Any major lab supplier
Magnetic separation rack compatible with 0.2 mL 8-tube strips	Any major lab supplier (e.g., V&P Scientific, Inc. VP 772F4-1)
Thermal cycler compatible with 0.2 mL 8-tube strips	Any major lab supplier

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

**Refer to multiplexing best practice for recommendation below.

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General best practices

Accurately pipette SMRTbell cleanup beads because small changes in volume can significantly alter the size distribution of your sample.

Equilibrate the SMRTbell cleanup beads at room temperature for 30 – 60 mins prior to use.

The workflow takes ~8hr to complete. Plan your experiments so that the entire workflow can be completed within an 8-hour day. If a stop is necessary, refer to workflow for safe stopping points.

Multiplexing best practice

Multiplexing could be achieved with one of the two following methods.

1. Barcoded adapters using SMRTbell Barcoded Adapter Plate 3.0. In this case, use barcoded adapters at step (5) “adapter ligation” in the workflow. Pooling of the barcoded libraries is described in Appendix 2.
2. Barcoded cDNA primers. See Appendix 3 for the 12 pairs of barcoded primer sequences that can be ordered from any oligo synthesis company. 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, which is described at the end of step (3) “cDNA amplification”.

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Procedure and checklist

1. Input RNA quality control

This protocol requires high-quality RNA. Prior to library preparation, evaluate the size distribution of the input RNA to determine whether it is suitable for the protocol.

✔	Step	Instructions
	1.1	Measure RNA Integrity Number (RIN) with an Agilent 2100 Bioanalyzer Instrument using the **RNA 6000 Nano kit.**
	1.2	Proceed to the next step of the protocol if sample quality is acceptable.

RNA Integrity Number (RIN)	
≥7.0	Recommended. Proceed to next step of the protocol.
<7.0	Increased library failure rates or reduced data quality.

SAFE STOPPING POINT – Store at -70°C or below

2. cDNA synthesis

2.1. Reagent preparation

✔	Step	Instructions
	2.1.1	Quick spin the NEBNext Single cell RT enzyme mix to collect liquid, then place on ice.
	2.1.2	Thaw the following components at room temperature, briefly vortex to mix, then quick spin to collect liquid and place on ice.

Reagent
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 (from Iso-Seq Express Oligo Kit)
Iso-Seq Express cDNA PCR Primer (from Iso-Seq Express Oligo Kit)) or custom barcoded cDNA primers if desired for multiplexing

	2.1.3	Thaw the NEBNext Cell Lysis Buffer at room temperature, briefly vortex to mix, quick spin to collect liquid and leave at room temperature. If the buffer appears cloudy after thawing, incubate briefly at 37°C to resuspend precipitant.

2.2. Primer annealing for first-strand synthesis

✔	Step	Instructions
	2.2.1	On ice, prepare **reagent mix 1** by adding the following components to each tube in the PCR strip tube in the order and volume listed below. See NEB's protocol for tube cap color.

✔	Components	Volume
	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**

	2.2.2	Thoroughly mix by vortexing the tube strip with two 2-second pulses.
	2.2.3	Quick spin the tube strip in a microcentrifuge to collect liquid.
	2.2.4	Incubate in a thermocycler with the following program. Set the **lid temperature to 80°C.**

Time	Temperature
5 min	70°C
Hold	4°C

2.3. Reverse transcription and template switching

✔	Step	Instructions

2.3.1 On ice, prepare **reagent mix 2** by adding the following components in the order and volume listed below. Adjust component volumes for the number of samples being prepared, plus 10% overage. See NEB's protocol for tube cap colors.

Components	Volume
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**

2.3.2 Thoroughly mix by vortexing with two 2-second pulses and then a quick spin to collect all liquid.

2.3.3 Add **10 µL** of **reaction mix 2** to the **9 µL** from **reaction mix 1** (section 2.2) for a total volume of **19 µL**.

✔	Tube	Reagent	Volume
	Previous	Reagent mix 1 from 2.2	9 µL
		Reagent mix 2	10 µL
	Total volume		19 µL

2.3.4 Thoroughly mix by vortexing with two 2-second pulses and then a quick spin to collect all liquid.

2.3.5 Incubate in a thermocycler with the following program. Set the **lid temperature to 52°C.**

Time	Temperature
75 min	42°C
Hold	4°C

2.3.6 On ice, add **1 µL** of **Iso-Seq express template switching oligo** to the **19 µL** reaction for a total volume of **20 µL**. Mix by vortexing with two 2-second pulses and then a quick spin to collect all liquid from the sides of the tube.

2.3.7 Incubate in a thermocycler with the following program. Set the **lid temperature to 52°C.**

Time	Temperature
15 min	42°C
Hold	4°C

2.4. 1.3X SMRTbell bead cleanup

✔	Step	Instructions
	2.4.1	Add **30 µL** of **elution buffer** to the **20 µL** reverse transcription and template switching reaction (section 2.3) for a total volume of **50 µL**.
	2.4.2	Add **65 µL** of resuspended, room-temperature SMRTbell cleanup beads.
	2.4.3	Mix beads by pipetting 10 times or until evenly distributed.
	2.4.4	Quick spin strip tubes in a microcentrifuge to collect liquid.
	2.4.5	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	2.4.6	Place the strip tubes in a magnetic separation rack until beads separate fully from the solution.
	2.4.7	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	2.4.8	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into the strip tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	2.4.9	Repeat the previous step.
	2.4.10	Remove residual 80% ethanol: • Remove the strip tube from the magnetic separation rack. • Quick spin the strip tube in a microcentrifuge. • Place the strip tube back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	2.4.11	Remove the strip tube from the magnetic rack. **Immediately** add **47 µL** of **elution buffer** to strip tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	2.4.12	Quick spin the strip tube in a microcentrifuge to collect liquid.
	2.4.13	Leave at **room temperature** for **5 minutes** to elute DNA.
	2.4.14	Place the strip tube in a magnetic separation rack until beads separate fully from the solution.
	2.4.15	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer **45.5 µL** of the supernatant to a new strip tube. Discard the old strip tube with beads.
	2.4.16	Proceed to the next step of the protocol.

3. cDNA amplification

3.1. cDNA amplification

✔	Step	Instructions

3.1.1 On ice, prepare **reaction mix 3** by adding the following components in the order and volume listed below. Adjust component volumes for the number of samples being prepared, plus 10% overage. See Appendix 3 for details on using barcoded cDNA primers. For tube cap colors, see NEB's protocol.

✔	Components	Volume
	NEBNext Single Cell cDNA PCR Master Mix	50 µL
	NEBNext Single Cell cDNA PCR Primer (or 12 µM Barcoded NEBNext Single Cell cDNA PCR Primer)	2 µL
	Iso-Seq Express cDNA PCR Primer (or 12 µM Barcoded Iso-Seq Express cDNA PCR Primer)	2 µL
	NEBNext Cell Lysis Buffer	0.5 µL
	Total volume	54.5 µL

3.1.2 Add **54.5 µL** of **reaction mix 3** to the **45.5 µL** of the eluted cDNA (from previous section 2.4) for a total volume of **100 µL**.

3.1.3 Thoroughly mix by vortexing with two 2-second pulses and then a quick spin to collect all liquid.

3.1.4 Place reaction in thermocycler block and run the program below. Set lid temperature to 105°C:

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

SAFE STOPPING POINT – Store at 4°C

3.2. Purification of amplified cDNA with SMRTbell cleanup beads

✔	Step	Instructions
	3.2.1	The concentration of SMRTbell cleanup beads will influence the size profile of the amplified cDNA at this step. Choose enrichment strategy and add the chosen volume of resuspended, room temperature SMRTbell cleanup beads to respective strip tube. **Transcript sizes and quality** — **SMRTbell cleanup bead volume** Typical transcripts, centered around 2 kb, for high-quality RNA — 86 µL Short transcripts <2kb or degraded samples with RIN < 7 — 95 µL Enrich for long transcripts >3 kb — 82 µL
	3.2.2	Mix beads by pipetting 10 times or until evenly distributed.
	3.2.3	Quick spin strip tubes in a microcentrifuge to collect liquid.
	3.2.4	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	3.2.5	Place the strip tubes in a magnetic separation rack until beads separate fully from the solution.
	3.2.6	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	3.2.7	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into the strip tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	3.2.8	Repeat the previous step.
	3.2.9	Remove residual 80% ethanol: • Remove the strip tube from the magnetic separation rack. • Quick spin the strip tube in a microcentrifuge. • Place the strip tube back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	3.2.10	Remove the strip tube from the magnetic rack. **Immediately** add **47 µL** of **low TE buffer** to strip tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	3.2.11	Quick spin the strip tube in a microcentrifuge to collect liquid.
	3.2.12	Leave at **room temperature** for **5 minutes** to elute DNA.
	3.2.13	Place the strip tube in a magnetic separation rack until beads separate fully from the solution.
	3.2.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer **47 µL** of the supernatant to a new strip tube. Discard the old strip tube with beads.
	3.2.15	**Recommended:** Measure concentration and size distribution of each cDNA sample. • Take a **1 µL** aliquot from each strip tube. Dilute each aliquot with **4 µL** of **elution buffer**. • Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. • Dilute sample further to **1.5 ng/µL** based on the Qubit reading. • Run **1 µL** on an Agilent Bioanalyzer using a High Sensitivity DNA kit.
	3.2.16	Proceed to the next step of the protocol if there is > **160 ng** of cDNA. If there is less than 160 ng of total cDNA, then go to Appendix 1 for additional cDNA amplification steps or pool samples if using barcoded cDNA primers.
		SAFE STOPPING POINT – Store at 4°C

3.3. Pooling barcoded cDNA (skip if not multiplexing with barcoded cDNA primers)

✔	Step	Instructions
	3.3.1	Using the concentration reading from the Qubit fluorometer, pool an equal mass of each barcoded cDNA sample. **Use the maximum total combined mass possible without exceeding 500 ng and not less than 160 ng in 46 µL**. Store any remaining purified amplified, barcoded cDNA at **4°C** for future use.
	3.3.2	Quick spin the tube strip in a microcentrifuge to collect liquid.
	3.3.3	Proceed to next step of the protocol.

4. Repair and A-tailing

✔	Step	Instructions
	4.1	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to the sample from the previous step at the specified volumes and skip RM1 steps.

Reaction Mix 1 (RM1)

✔	Tube	Component	Volume
	Purple	Repair buffer	8 µL
	Blue	End repair mix	4 µL
	Green	DNA repair mix	2 µL
		Total volume	14 µL

4.2	Thoroughly mix RM1 by pipetting 10 times.
4.3	Quick spin RM1 in a microcentrifuge to collect liquid.
4.4	Add **14 µL** of RM1 to each cDNA sample**.** Pipette mix 10 times and quick spin to collect liquid. Total reaction volume should be **60 µL.**
4.4	Incubate in a thermocycler with the following program. **Set the lid temperature to 75°C.**

Time	Temperature	Notes
30 min	37°C	Repair
5 min	65°C	A-tailing
Hold	4°C	

4.5	Proceed to the next step of the protocol.

5. Adapter ligation

5.1. Adapter ligation

✔	Step	Instructions
	5.1.1	Add **4 µL** of SMRTbell adapter (non-barcoded) to each sample from the previous step. OR Add **4 µL** of a SMRTbell barcoded adapter 3.0 to each sample to multiplex samples that were not barcoded with cDNA primers.
	5.1.2	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip RM2 steps.
	5.1.3	Thoroughly mix RM2 by pipetting 10 times.
	5.1.4	Quick spin RM2 in a microcentrifuge to collect liquid.
	5.1.5	Add **31 µL** of RM2 to each sample from previous step. Pipette mix 10 times and quick spin to collect liquid. Total volume should be **95 µL.**
	5.1.6	Incubate in a thermocycler with the following program. **Set the lid temperature to 75°C.**
	5.1.7	Proceed to the next step of the protocol.

Reaction Mix 2 (RM2)

✔	Tube	Component	Volume
	Yellow	Ligation mix	30 µL
	Red	Ligation enhancer	1 µL
		Total volume	**31 µL**

Time	Temperature	Notes
30 min	20°C	Ligation
Hold	4°C	

5.2. 1.3X SMRTbell bead cleanup

✔	Step	Instructions
	5.2.1	Add **124 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	5.1.2	Mix beads by pipetting 10 times or until evenly distributed.
	5.1.3	Quick spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	5.1.4	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	5.1.5	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	5.1.6	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	5.1.7	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	5.1.8	Repeat the previous step.
	5.1.9	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	5.1.10	Remove tube strip from the magnetic rack. **Immediately** add **40 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	5.1.11	Quick spin the tube strip in a microcentrifuge.
	5.1.12	Leave at **room temperature** for **5 minutes** to elute DNA.
	5.1.13	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	5.1.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
	5.1.15	Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at 4°C

6. Nuclease treatment

6.1. Nuclease treatment

✔	Step	Instructions
	6.1.1	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip RM3 steps.

Reaction Mix 3 (RM3)

✔	Tube	Component	Volume
	Light purple	Nuclease buffer	5 µL
	Light green	Nuclease mix	5 µL
		Total volume	**10 µL**

6.1.2	Thoroughly mix RM3 by pipetting 10 times.
6.1.3	Quick spin RM3 in a microcentrifuge to collect liquid.
6.1.4	Add **10 µL** of RM3 to each sample. Pipette mix 10 times and quick spin to collect liquid. Total volume should equal **50 µL.**
6.1.5	Incubate reaction in a thermocycler with the following program. **Set the lid temperature to 75°C.**

Time	Temperature	Notes
15 min	37°C	Nuclease treatment
Hold	4°C	

6.1.6	Proceed to the next step of the protocol.

6.2. 1.3X SMRTbell bead cleanup

✔	Step	Instructions
	6.2.1	Add **65 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	6.2.2	Mix beads by pipetting 10 times or until evenly distributed.
	6.2.3	Quick spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	6.2.4	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	6.2.5	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	6.2.6	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	6.2.7	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	6.2.8	Repeat the previous step.
	6.2.9	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	6.2.10	Remove tube strip from the magnetic rack. **Immediately** add **15 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	6.2.11	Quick spin the tube strip in a microcentrifuge.
	6.2.12	Leave at **room temperature** for **5 minutes** to elute DNA.
	6.2.13	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	6.2.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
	6.2.15	Measure concentration and size distribution of each cDNA sample. • Take a **1 µL** aliquot from each strip tube. Dilute each aliquot with **4 µL** of **elution buffer**. • Measure DNA concentration with a Qubit Fluorometer using the 1x dsDNA HS kit. • Dilute sample further to **1.5 ng/µL** based on the Qubit reading. • Run **1 µL** on an Agilent Bioanalyzer using a High Sensitivity DNA kit.
	6.2.16	If using SMRTbell barcoded adapters, pool samples as described in **Appendix 2**. Otherwise, proceed to sequencing using **Sample Setup in SMRT Link**.
	6.2.17	Store SMRTbell libraries at 4°C if sequencing within 2 weeks of prep. Otherwise, place libraries at -20°C for long-term storage. Minimize freeze-thaw cycles when working with libraries.

PROTOCOL COMPLETE

Appendix 1

Recommendations for additional cDNA amplification

The Sequel II and Sequel IIe Systems 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 may be required.

1. Set up the PCR by combining the following components together in the order and volumes listed below. Work on ice or cold block while setting up the reaction.

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 Required materials and equipment)

2. Thoroughly mix by vortexing with two 2-second pulses and then a quick spin to collect all liquid.

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

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

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

✔	Step	Instructions
	A1.1	Choose enrichment strategy and add the chosen volume of resuspended, room temperature SMRTbell cleanup beads to each respective strip tube. Use the same concentration of beads that were used for the original cDNA purification. **Transcript sizes and quality** / **SMRTbell cleanup bead volume** Typical transcripts, centered around 2 kb, for high-quality RNA — 86 µL Short transcripts <2kb or degraded samples with RIN < 7 — 95 µL Enrich for long transcripts >3 kb — 82 µL
	A1.2	Mix beads by pipetting 10 times or until evenly distributed.
	A1.3	Quick spin strip tubes in a microcentrifuge to collect liquid.
	A1.4	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	A1.5	Place the strip tubes in a magnetic separation rack until beads separate fully from the solution.
	A1.6	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	A1.7	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into the strip tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	A1.8	Repeat the previous step.
	A1.9	Remove residual 80% ethanol: • Remove the strip tube from the magnetic separation rack. • Quick spin the strip tube in a microcentrifuge. • Place the strip tube back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	A1.10	Remove the strip tube from the magnetic rack. **Immediately** add **47 µL** of **low TE buffer** to strip tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	A1.11	Quick spin the strip tube in a microcentrifuge to collect liquid.
	A1.12	Leave at **room temperature** for **5 minutes** to elute DNA.
	A1.13	Place the strip tube in a magnetic separation rack until beads separate fully from the solution.
	A1.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer **47 µL** of the supernatant to a new strip tube. Discard the old strip tube with beads.
	A1.15	Measure concentration and size distribution of each cDNA sample. • Take a **1 µL** aliquot from each strip tube. Dilute each aliquot with **4 µL** of **elution buffer**. • Measure DNA concentration with a Qubit Fluorometer using the 1x dsDNA HS kit. • Dilute sample further to **1.5 ng/µL** based on the Qubit reading. • Run **1 µL** on an Agilent Bioanalyzer using a High Sensitivity DNA kit.
	A1.16	Proceed to Repair and A-Tailing step if total amount of cDNA is greater than 160 ng.
		SAFE STOPPING POINT – Store at 4°C

Appendix 2

Pooling samples with SMRTbell barcoded adapters 3.0

✔	Step	Instructions
	A2.1	Using the final SMRTbell library concentration taken after nuclease treatment, **pool an equal mass of each adapter-barcoded sample.** Assuming transcript size distribution profiles are similar, this should provide a balanced representation. For more sensitive applications, or when the transcript profiles are significantly different, then equal molar pooling may be more appropriate.
	A2.2	Add **1.3X v/v** of resuspended, room-temperature SMRTbell cleanup beads to the pooled SMRTbell library.
	A2.3	Mix beads by pipetting 10 times or until evenly distributed.
	A2.4	Quick spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	A2.5	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	A2.6	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	A2.7	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	A2.8	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	A2.9	Repeat the previous step.
	A2.10	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	A2.11	Remove tube strip from the magnetic rack. **Immediately** add **15 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	A2.12	Quick spin the tube strip in a microcentrifuge.
	A2.13	Leave at **room temperature** for **5 minutes** to elute DNA.
	A2.14	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	A2.15	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
	A2.16	Recommended: Measure concentration and size distribution of the pooled sample. • Take a **1 µL** aliquot from each strip tube. Dilute each aliquot with **4 µL** of **elution buffer**. • Measure DNA concentration with a Qubit Fluorometer using the 1x dsDNA HS kit. • Dilute sample further to **1.5 ng/µL** based on the Qubit reading. • Run **1 µL** on an Agilent Bioanalyzer using a High Sensitivity DNA kit.

Appendix 3

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	April 2022
Adjusted lid temperature, in step 5.1.6, to 75°C.	02	April 2022

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