Preparing Iso-Seq® v2 libraries using SMRTbell® prep kit 3.0

Procedure & checklist

Before you begin

This procedure describes the workflow for constructing Iso-Seq libraries using SMRTbell prep kit 3.0 from RNA for sequencing on PacBio® Sequel® II and Revio™ systems. For generating Kinnex™ libraries from Iso-Seq libraries, please refer to the Kinnex full-length RNA protocol.

Overview	
Samples	1–24
Workflow time	8 hours [for up to 24 samples]
RNA input	
Quality / size distribution	RIN (RNA integrity number) ≥7.0
Quantity	Total RNA 300 ng per library (minimum concentration 43 ng/µL per library)
cDNA Input	
Quantity	≥50ng per library for 1 SMRT® Cell 8M; ≥100ng per library for 1 Revio SMRT Cell

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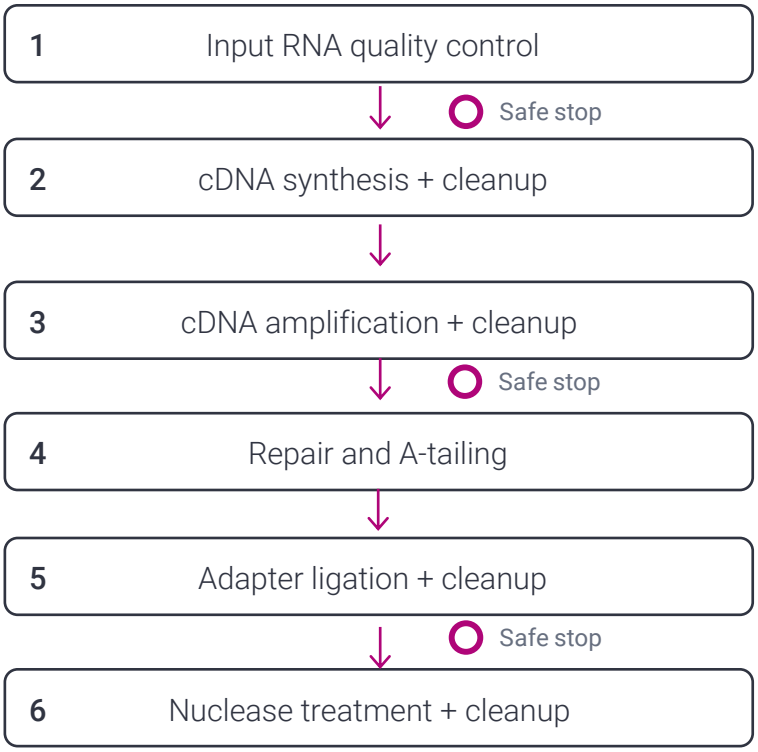
Workflow



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	
Iso-Seq® Express 2.0 Kit	PacBio® 103-071-500
SMRTbell® cleanup beads	PacBio® 102-158-300
Elution buffer (50 mL)	PacBio® 101-633-500
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 (MLS)
Nuclease-free water, molecular biology grade	Any MLS (e.g., Sigma-Aldrich W4502)
8-channel pipettes – P20 & P200)	Any MLS
Single-channel pipette – P2, P10, P20, P100 or P200	Any MLS
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Microcentrifuge	Any MLS
Magnetic separation rack compatible with 0.2 mL 8-tube strips	Any MLS (e.g., V&P Scientific, Inc. VP 772F4-1)
Thermal cycler compatible with 0.2 mL 8-tube strips	Any MLS

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 mins prior to use.

The workflow takes ~8hr to complete. If a stop is necessary, refer to workflow for safe stopping points.

Safety precautions

Refer to the Safety Data Sheet (SDS) for information on reagent hazards and protocols for safe handling, use, storage, and disposal.

Multiplexing best practices

Multiplexing can be achieved with one of the following methods.

1. Barcoded cDNA primers using Iso-Seq primers bc01–12 in step 3 of the protocol. To multiplex, use the Iso-Seq cDNA amplification primer in combination with Iso-Seq primers bc01–12 to amplify samples. After SMRTbell cleanup, Iso-Seq samples can be pooled and brought through a single SMRTbell prep kit 3.0 reaction. Each barcoded primer is sufficient for 2 reactions, with the Iso-Seq kit supporting a total of 24 reactions.
2. Barcoded adapters using SMRTbell Barcoded Adapter Plate 3.0. In this case, use barcoded adapters at step (5) “adapter ligation” in the workflow.
3. A combination of the above 2 approaches.

Workflow steps

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 the 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:

RIN	Quality recommendations
≥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 cDNA synthesis

In this step, total RNA samples are converted to first-strand cDNA products.

✔	Step	Instructions
	2.1.1	Quick-spin the Iso-Seq RT enzyme mix in the microcentrifuge 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.

Tube color	Reagent
Orange	Iso-Seq RT primer mix (103-104-000)
Purple	Iso-Seq RT buffer (103-103-900)
Red	Iso-Seq cDNA PCR mix (103-104-200)
Green	Iso-Seq cDNA amplification primer (103-104-400)
Blue	Iso-Seq Express TSO 2.0 (103-104-300)
White	Iso-Seq primer barcodes 01 – 12* (the number of primers thawed will depend on the number of samples processed) 103-104-500 through 103-105-600

*If processing only one sample, any of the 12 Iso-Seq barcoded primers can be used.

2.2 Primer annealing for first-strand synthesis

✔	Step	Instructions
	2.2.1	For each RNA sample to be processed, prepare reagent mix 1 on ice by adding the following components to each tube in the PCR strip tube.

✔	Tube color	Components	Volume
		Total RNA (300 ng)	<7 µL
	Orange	Iso-Seq RT primer mix	2 µL
		Nuclease-free water	Up to 9 µL
		Total volume	9 µL

	2.2.2	Thoroughly mix by pipetting up and down 10 times.
	2.2.3	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	2.2.4	Incubate in a thermal cycler with the following program. Set the lid temperature to 80°C.

Temperature	Time
70°C	5 min
20°C	hold
Proceed immediately to the next step	

2.3 Reverse transcription and template switching

✔	Step	Instructions
	2.3.1	For each RNA sample, prepare reagent mix 2 on ice 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.

✔	Tube color	Components	Volume
	Purple	Iso-Seq RT buffer (vortex briefly before use)	5 µL
		Nuclease-free Water	3 µL
	Yellow	Iso-Seq RT enzyme mix	2 µL
		Total volume added per reaction	10 µL

	2.3.2	Pipette-mix and quick-spin in a microcentrifuge 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 step 2.2	9 µL
		Reagent mix 2	10 µL
	Total volume added per reaction		19 µL

2.3.4 Thoroughly mix by pipetting up and down 10 times and then quick-spin to collect all liquid.

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

Temperature	Time
42°C	45 min
20°C	Hold

Proceed immediately to the next step.

2.3.6 Remove the sample tube from the thermal cycler and add 2 µL of Iso-Seq template switch oligo to the 19 µL reaction at room temperature for a total volume of 21 µL. Mix by pipetting up and down 10 times and then quick-spin to collect all liquid from the sides of the tube.

2.3.7 Return sample tube to thermal cycler and incubate with the following program. Set the lid temperature to 52°C.

Temperature	Time
42°C	15 min
4°C	hold

2.4 1.3X SMRTbell bead cleanup

✔	Step	Instructions
	2.4.1	For each sample, add 29 µL of elution buffer to the 21 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 the beads.
	2.4.6	Place the strip tubes in a magnetic separation rack until the 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 21 µL of elution buffer to the 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 the DNA.

2.4.14 Place the strip tube in a magnetic separation rack until the beads separate fully from the solution.

2.4.15 Slowly pipette off the cleared supernatant without disturbing the beads. Transfer 21 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

First-strand cDNA products are PCR-amplified and barcoded using barcoded Iso-Seq primers at this step.

3.1 cDNA amplification

✔	Step	Instructions
	3.1.1	For each sample, prepare reaction mix 3 on ice 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. Pipette mix master mix. Iso-Seq primer bc01–12 will be added to each sample individually and should not be added to the master mix.

✔	Tube color	Components	Volume
	Red	Iso-Seq cDNA PCR mix	25 µL
	Green	Iso-Seq cDNA amplification primer	2 µL
		Total volume	27 µL

3.1.2	On ice, add 27 µL of reaction mix 3 to the 21 µL of the eluted cDNA (from previous Section 2.4). Add 2 µL of Iso-Seq primer barcode 01–12 for a total volume of 50 µL.	

✔	Tube color	Component	Volume
	White	Iso-Seq primer barcode	2 µL

3.1.3	Thoroughly mix by pipetting up and down 10 times and then quick spin to collect all liquid.	
3.1.4.	Run the thermal cycler program below with the lid temperature set to 105°C. Keep sample on ice until thermal cycler lid has heated to 105°C.	

PCR program	
45 seconds at 98°C	1 cycle
10 seconds at 98°C 15 seconds at 60°C 3 minutes at 72°C	12 cycles
5 minutes at 72°C	
Hold at 4°C	

SAFE STOPPING POINT -- Store at 4°C or -20°C for long-term storage

3.2 Cleanup of amplified cDNA using 0.9X SMRTbell Cleanup beads

✔	Step	Instructions
	3.2.1	Add 45 µL (0.9x) of resuspended, room-temperature SMRTbell cleanup beads to the 50 µL of cDNA amplified reaction from Section 3.1. The correct ratio of beads to sample is critical at this step.
	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 elution buffer to the 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 the 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 1:4 dilution further to 1.5 ng/µL based on the Qubit reading if needed. • Run 1 µL on an Agilent Bioanalyzer using a High Sensitivity DNA kit.
	3.2.16	The expected recovery after cDNA amplification SMRTbell clean-up is >200 ng. A minimum of 100ng of total cDNA is recommended to proceed with the SMRTbell prep kit 3.0 (Step 4).

3.3. Pooling barcoded cDNA (skip if not multiplexing)

✔	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 100 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 color	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 if not multiplexing or if only using the cDNA amplification barcodes for multiplexing. OR Add **4 µL** of a SMRTbell barcoded adapter 3.0 to each sample to further multiplex samples.
	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 color	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 color	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	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

Revision history (description)	Version	Date
Initial release	01	April 2022
Adjusted lid temperature, in step 5.1.6, to 75°C.	02	April 2022
Modified cDNA amplification PCR cycles to 12 for monomer, changed cDNA amplification post-cleanup elution volume.	03	December 2023
Minor updates throughout	04	March 2024
Minor updates throughout	05	May 2024