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

Procedure & checklist

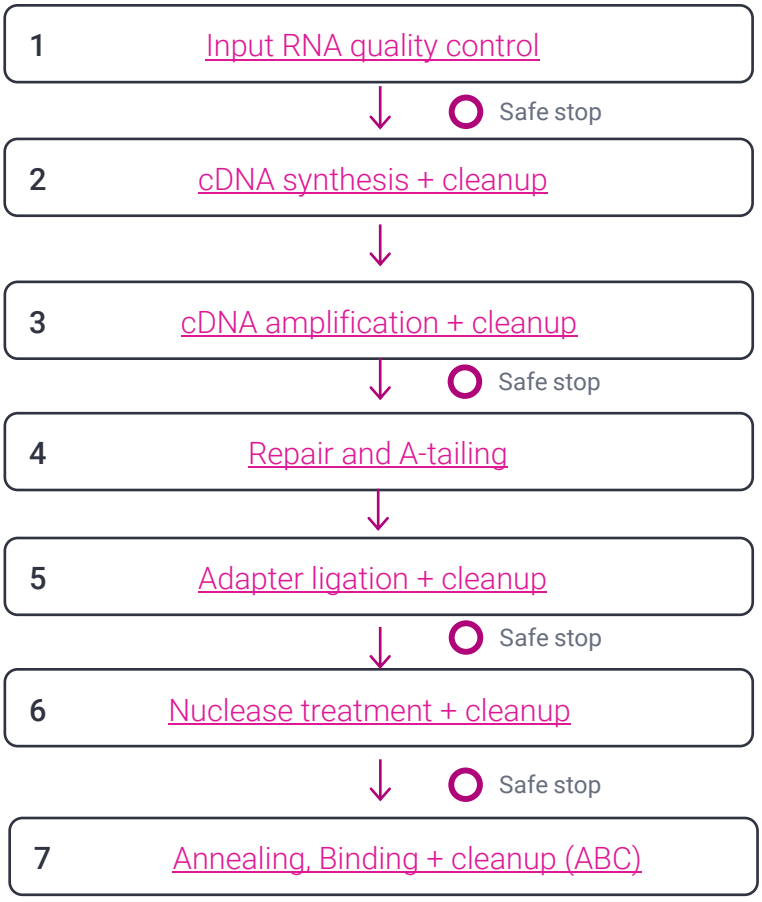
Before you begin

This procedure describes the workflow for constructing Iso-Seq libraries using SMRTbell prep kit 3.0 from RNA for sequencing on PacBio® Sequel® II/IIe, Vega™ 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
Number of SMRT® Cells per library prep	>8 acquisitions for Revio/Vega SPRQ™/SPRQ-Nx >2 SMRT Cells for Vega® (non-SPRQ) >10 SMRT Cells for Sequel® II/IIe
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)

102-396-000 REV09 AUG2026

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
SMRTbell® library preparation	
Iso-Seq® Express 2.0 Kit	PacBio® 103-071-500
Elution buffer (50 mL)	PacBio® 101-633-500
SMRTbell® prep kit 3.0	PacBio® 102-182-700
SMRTbell® barcoded adapter plate 3.0 (optional; for barcoding)	PacBio® 102-009-200
Revio SPRQ™ polymerase kit or Vega™ polymerase kit or Vega™ SPRQ polymerase kit or Sequel® II binding kit 3.2*	PacBio® 103-520-100 PacBio® 103-517-600 PacBio® 103-729-600 PacBio® 102-333-300
* Procedure for Sequel II binding kit 3.2 can be found in SMRT® Link Sample Setup	
Other supplies	
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.

Note: The Loading buffer is light sensitive and should be protected from light when not in use.

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.

Reagent and sample handling

Ensure that the DNA damage repair mix is stored at -20°C to avoid poor library performance.

Temperature-sensitive reagents Iso-Seq express 2.0 kit 103-071-500		
	Tube color	Reagent
	Purple	Iso-Seq RT buffer 103-103-900
cDNA synthesis	Orange	Iso-Seq RT primer mix 103-104-000
	Yellow	Iso-Seq RT enzyme mix 103-104-100
	Red	Iso-Seq cDNA PCR mix 103-104-200
	Blue	Iso-Seq Express TSO 2.0 103-104-300
	Green	Iso-Seq cDNA amplification primer 103-104-400

	Iso-Seq primer bc01 103-104-500
	Iso-Seq primer bc02 103-104-600
	Iso-Seq primer bc03 103-104-700
	Iso-Seq primer bc04 103-104-800
	Iso-Seq primer bc05 103-104-900
	Iso-Seq primer bc06 103-105-000
White	Iso-Seq primer bc07 103-105-100
	Iso-Seq primer bc08 103-105-200
	Iso-Seq primer bc09 103-105-300
	Iso-Seq primer bc10 103-105-400
	Iso-Seq primer bc11 103-105-500
	Iso-Seq primer bc12 103-105-600

SMRTbell prep kit 3.0

- Thaw the Repair buffer, Nuclease buffer, SMRTbell adapter and Elution buffer at room temperature.
- Mix reagent buffers with a brief vortex prior to use. Do not vortex enzymes.
- Quick-spin all reagents in microcentrifuge to collect liquid at bottom prior to use.
- Keep all temperature-sensitive reagents on ice.

Temperature-sensitive reagents		
Step used	**Tube**	**Reagent**
Repair and A-tailing	Blue	End repair mix
	Green	DNA repair mix
Adapter ligation	Orange	SMRTbell adapter
	Yellow	Ligation mix
	Red	Ligation enhancer
Nuclease treatment	Light green	Nuclease mix

Samples can be stored at 4°C at all safe stopping points listed in the protocol.

Anneal, bind, and cleanup

Thaw the following reagents at room temperature:

Component	Tube color
Annealing buffer	Light blue
Standard sequencing primer	Light green
Polymerase buffer	Yellow
Loading buffer	Green
Dilution buffer	Blue

Once thawed, place reaction buffers and sequencing primer on-ice prior to making master mix. The Loading buffer should be left at room temperature.

Note: The Loading buffer is light sensitive and should be protected from light when not in use.

Keep the following reagents on a cold block or ice:

1. Sequencing polymerase
2. Sequencing control

Bring the following reagents up to room temperature 30 minutes prior to use:

- Loading buffer
- SMRTbell cleanup beads

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

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

2.1 cDNA synthesis

✔	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* (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	Incubate 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 remove the cleared supernatant without disturbing the beads. Discard the supernatant.
	2.4.8	Slowly dispense 200 µL of 80% ethanol into the strip tube. Remove 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. • Remove 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 Incubate 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 aspirate 21 µL of the cleared eluate without disturbing the beads 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 55 µL of elution buffer to a new strip tube and transfer 45 µL of PCR amplified cDNA from Section 3.1 into the elution-buffer-added strip tube for a final volume of 100 µL. Add 90 µL (0.9x) of resuspended, room-temperature SMRTbell cleanup beads. **The correct ratio of beads to sample is critical at this step.** If targeting longer cDNA, 86 µL (0.86x) of SMRTbell cleanup beads can be used.
	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	Incubate 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 remove the cleared supernatant without disturbing the beads. Discard the supernatant.
	3.2.7	Slowly dispense 200 µL of 80% ethanol into the strip tube. Remove 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. • Remove 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	Incubate 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 aspirate the cleared eluate without disturbing the beads. **Transfer 47 µL of the eluate 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 short-term storage or **-20°C** for long-term storage.
	3.3.2	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	3.3.3	Proceed to the 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.
	5.2.2	Mix beads by pipetting 10 times or until evenly distributed.
	5.2.3	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	5.2.4	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads.
	5.2.5	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	5.2.6	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
	5.2.7	Slowly dispense **200 µL** of **80% ethanol** into each tube. Remove the 80% ethanol and discard.
	5.2.8	Repeat the previous step.
	5.2.9	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. • Remove residual 80% ethanol and discard.
	5.2.10	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.
	5.2.11	Quick-spin the tube strip in a microcentrifuge.
	5.2.12	Incubate at **room temperature** for **5 minutes** to elute DNA.
	5.2.13	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	5.2.14	Slowly aspirate the cleared eluate without disturbing the beads. Transfer eluate to a **new tube strip**. Discard old tube strip with beads.
	5.2.15	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	Incubate 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 remove the cleared supernatant without disturbing the beads. Discard the supernatant.
	6.2.7	Slowly dispense **200 µL** of **80% ethanol** into each tube. Remove 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. • Remove residual 80% ethanol and discard.
	6.2.10	Remove tube strip from the magnetic rack. **Immediately** add **26 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	Incubate 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 aspirate the cleared eluate without disturbing the beads. Transfer eluate 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	Proceed to Section 7 to prepare library for sequencing on Vega or Revio systems. **If necessary, dilute 25 µL of SMRTbell library to the concentrations indicated below. Failure to normalize libraries or pools of libraries to the appropriate concentration prior to ABC may result in low sequencing yield.**

SMRTbell library size	Concentration (ng/µL)
3–10 kb	<20 ng/µL
<3 kb	<10 ng/µL

or

Proceed to SMRT Link Sample Setup for preparing samples for sequencing with Sequel II/e.

6.2.17 Store SMRTbell libraries at 4°C if sequencing within two weeks. Store long-term at -20°C.

7. Annealing, binding, and cleanup (ABC)

This step is for preparing the libraries (25 µL) for sequencing on Revio or Vega systems. If samples are pooled prior to ABC or a custom volume is required, see Appendix section A1. The Polymerase kit used will depend on which sequencer or chemistry is being used.

✔	Step	Instructions
	7.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✔	Tube	Component	Volume
	Light blue	Annealing buffer	12.5 µL
	Light green	Standard sequencing primer	12.5 µL
		Total volume	**25 µL**

	7.2	Pipette-mix the **Annealing mix** and quick-spin to collect liquid.
	7.3	Add **25 µL** of **the Annealing mix** to each library. Total volume should equal **50 µL.**
	7.4	Pipette-mix each sample and quick-spin to collect liquid.
	7.5	Incubate at room temperature for **15 minutes**.
	7.6	During primer incubation, prepare the polymerase dilution (see below) and store on ice.
	7.7	To prepare the polymerase, add the following components to a new microcentrifuge tube on ice. Adjust component volumes for the number of samples being prepared, plus 10% overage.

Polymerase Dilution

✔	Tube	Component	Volume
	Yellow	Polymerase buffer	47 µL
	Purple	Sequencing polymerase	3 µL
		Total volume	**50 µL**

7.8	Pipette mix the **polymerase dilution** and quick-spin to collect liquid.
7.9	Add **50 µL of polymerase dilution** to primer annealed sample. Total volume should equal **100 µL**.
7.10	Pipette-mix each sample and quick-spin to collect liquid.
7.11	Incubate at **room temperature for 15 minutes.**
7.12	Proceed immediately to the next step of the protocol to remove excess polymerase.

Post-binding cleanup with 1.3X SMRTbell cleanup beads

7.13	Add **130 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample
7.14	Pipette-mix the beads until evenly distributed and quick-spin if necessary to collect all liquid from the sides of the tube.
7.15	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads
7.16	Place sample on an appropriate magnet and allow beads to separate fully from the solution
7.17	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant. **DO NOT USE EtOH.** Proceed immediately to the elution. It is important **not** to let the beads dry out.
7.18	Remove sample from the magnet and **immediately** add **Loading Buffer** to each tube and resuspend the beads by pipette mixing.

	Revio/Vega SPRQ polymerase kit	Vega polymerase kit (non-SPRQ)
Loading buffer	25 µL	50 µL

7.19	Quick-spin the samples to collect any liquid from the sides of the tube.
7.20	Incubate at **room temperature** for **15 minutes** to elute DNA
7.21	Place sample on magnet and allow beads to separate fully from the solution.
7.22	Slowly remove the cleared eluate without disturbing the beads. Transfer eluate to a **new tube**. Discard the old tube with beads
7.23	Use **1 µL** of sample to measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. **Important:** The **Qubit Flex** instrument is not compatible with measuring polymerase-bound library in Loading Buffer. Concentration readings will not be accurate.
7.24	Proceed to the **Loading Calculator** in SMRT Link v13.3 or higher to calculate the final dilution for adding the sample to the sequencing reagent plate. **The recommended loading concentration is 200 – 300 pM.**

PROTOCOL COMPLETE

Important: Polymerase-bound libraries can be stored at 4°C for up to 1 month, or at -20°C for up to 6 months prior to sequencing. Polymerase-bound libraries can withstand up to 4 freeze-thaw cycles. Note that the Loading buffer is light sensitive.

Appendix

A1. Annealing, binding, and cleanup (ABC) for custom volumes

This step is for preparing libraries for sequencing on PacBio sequencers. The sequencing polymerase is stable once bound to the HiFi library and can be stored at 4°C for 1 month or at -20°C for at least 6 months. Use the calculations below to determine custom reagent volumes based on input sample volume:

	SMRTbell library	Annealing buffer	Kinnex sequencing primer	Polymerase dilution
Volume (µL)	x	x/2	x/2	x*2
Example	100	50	50	200

See Section 7 for full protocol.

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
Updated for SPRQ chemistry and the Vega system	06	December 2024
Included Reagent and sample handling section, clarified polymerase kit part numbers	07	June 2025
Updated for Revio SPRQ-Nx	08	May 2026
Updated for Vega SPRQ-Nx	09	August 2026