Preparing Kinnex™-compatible cDNA using Iso-Seq® express 2.0 kit

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Procedure & checklist

Overview

This procedure describes the workflow for generating cDNA from polyadenylated (polyA) RNA using the Iso-Seq express 2.0 kit. The resulting cDNA can be used for either (1) generation of Kinnex concatenated libraries using Kinnex concatenation kit 2.0 or (2) construction of Iso-Seq monomer libraries using SMRTbell prep kit 3.0. The libraries are compatible with the PacBio® Vega™ and Revio® systems.

For generating Kinnex™ concatenated libraries, please refer to one of these protocols:

- Procedure & checklist - Preparing Kinnex libraries using Kinnex 8-fold prep kit
- Procedure & checklist - Preparing Kinnex libraries using Kinnex 12-fold prep kit

For generating monomer SMRTbell libraries, please refer to the protocol:

- Procedure & checklist - Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0

Overview	
Samples	1–24
Workflow time	3 hours for up to 24 samples
RNA input	
Quality / size distribution	RIN (RNA integrity number)/ RINe (RNA integrity number equivalent) ≥7.0
Quantity	Total RNA 300 ng (7 µL at 43 ng/µL) per cDNA synthesis

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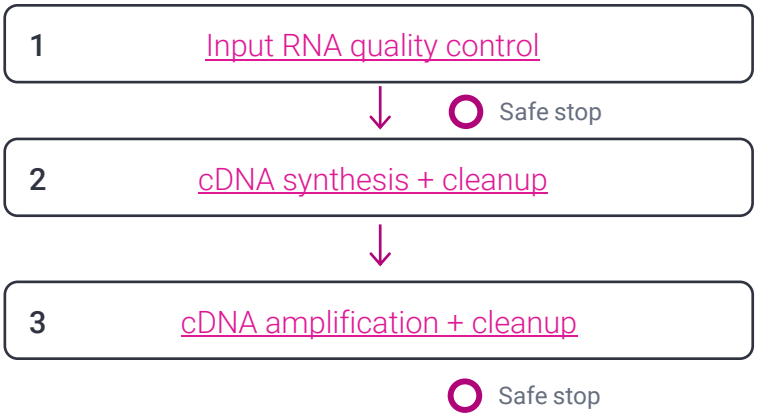
Workflow



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Materials and equipment

Recommended RNA and DNA sizing (use one of the following sizing systems)	
2100 Bioanalyzer instrument	Agilent Technologies G2939BA
RNA 6000 Nano kit	Agilent Technologies 5067-1511
DNA High Sensitivity DNA kit	Agilent Technologies 5067-4626
4150 or 4200 TapeStation System	Agilent Technologies G2992AA/ G2991BA
RNA ScreenTape Analysis	Agilent Technologies 5067-5576/5577/5578
High Sensitivity D5000 ScreenTape Assay	Agilent Technologies 5067-5592/5593/5594
D5000 ScreenTape Assay	Agilent Technologies 5067-5588/5589/5590
DNA quantitation	
Qubit Fluorometer	Thermo Fisher Scientific Q33238
Qubit 1X dsDNA HS Assay kit	Thermo Fisher Scientific Q33230
cDNA Generation	
Iso-Seq® express 2.0 kit	PacBio® 103-071-500
Elution buffer (50 mL)	PacBio® 101-633-500
SMRTbell® cleanup beads	PacBio® 102-158-300
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
Magnetic separation rack compatible with 0.2 mL 8-tube strips	Any MLS (e.g., V&P Scientific, Inc. VP772F4-1)
Thermal cycler compatible with 0.2 mL 8-tube strips	Any MLS
DNA LoBind tubes	Eppendorf 022431021
Microcentrifuge	Any MLS

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Before you begin

RNA quality

High-quality, intact total RNA is recommended to maximize recovery of full-length transcripts. Evaluate total RNA integrity using an Agilent TapeStation system with RNA ScreenTape Analysis or an equivalent method.

Recommended guidelines for evaluating RNA quality for this protocol:

- Use total RNA with a RINe ≥7.0; **RINe ≥8.0 is preferred**.
- Samples with **RINe <7.0** may be processed at discretion, with an increased risk of low cDNA yield and quality, downstream library failure, and reduced data quality.
- RNA should be free of contaminants that may inhibit reverse transcription.
- Minimize RNA degradation during handling and storage. Avoid RNase contamination, prolonged exposure to elevated temperature or extreme pH, and unnecessary freeze-thaw cycles.

Important: Iso-Seq library performance and the recovery of full-length transcripts depend on the integrity of the input RNA. Lower-quality RNA may be used with the expectation of reduced library and sequencing performance.

cDNA multiplexing with indexed primers

Twelve indexed cDNA primers are provided in the Iso-seq express 2.0 kit for sample barcoding: use the Iso-Seq cDNA amplification primer in combination with one of the Iso-Seq primers bc01–12 during amplification in Step 3 of this protocol. Each barcode primer is sufficient for 2 reactions, with the Iso-Seq kit supporting a total of 24 reactions.

Safety precautions

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

Reagent and sample handling best practices

- Room temperature is defined as any temperature in the range of 18–23°C for this protocol.
- Bring SMRTbell cleanup beads and Qubit 1X dsDNA HS reagents to room temperature for 30 minutes before use.
- Quick-spin all reagents before use to collect liquid at the bottom of the tube.
- Keep temperature-sensitive reagents on ice unless otherwise specified.
- Ensure the SMRTbell cleanup beads are thoroughly mixed to a homogenous suspension before accurately pipetting the required volume, as small changes in bead volume can significantly alter the size distribution of your sample.
- For cDNA amplification PCR, keep sample(s) on ice until the thermal cycler lid reaches 105°C to avoid digestion of primer by polymerase exonuclease activity.
- During bead binding and elution steps, pipette-mix until the beads are evenly distributed.
- Mix reactions by pipetting up and down 10 times, or until fully resuspended. Avoid introducing bubbles.

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

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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, or RNA Integrity Number equivalent (RINe) with Agilent TapeStation systems using RNA ScreenTape Analysis. See Figure 1 in the Appendix.
	1.2	Proceed to the next step of the protocol if sample quality is acceptable:

RIN/RINe	Quality recommendations
≥7.0	Recommended. Proceed to next step of the protocol.
<7.0	Decreased library success rate or reduced data quality

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

2. cDNA synthesis

In this step, first-strand cDNA was reverse transcribed from polyA RNA.

2.1 cDNA synthesis reagent preparation

✔	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.

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2.2 RT Primer annealing for first-strand synthesis

✔	Step	Instructions
	2.2.1	For each 300 ng RNA sample (≥43 ng/µL) to be processed, prepare Reaction Mix 1 on ice by adding the following components to each tube in the PCR strip tube. **Reaction Mix 1 (RM1):**

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

	2.2.2	Thoroughly mix RM1 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. Heated lid set at 80°C. Reaction volume set to 10 µL.

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 Reaction 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. Reaction Mix 2 (RM2):

✔	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 RM2 and quick-spin in a microcentrifuge to collect all liquid.

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2.3.3 **Add 10 µL of RM2** to the 9 µL from RM1 (Section 2.2) for a total volume of 19 µL.

✔	Tube	Reagent	Volume
		Previous RM 1 from step 2.2	9 µL
		RM 2	10 µL
		Total volume	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.
Heated lid set at 52°C. Reaction volume set to 25µL.

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.

✔	Tube color	Component	Volume
	Blue	Iso-Seq Express TSO 2.0	2 µL

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

2.3.8 Return sample tube to thermal cycler and incubate with the following program.
Heated lid set at 52°C. Reaction volume set to 25 µL.

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** (1.3X v/v) 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.

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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**, or enough to cover the beads, of **freshly prepared 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.

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3. cDNA amplification

First-strand cDNA products are PCR-amplified and barcoded using indexed Iso-Seq primers at this step. Select the **recommended number of PCR cycles** based on the downstream library prep option specified in **step 3.1.5.**

- 10 PCR cycles for Kinnex preparation (8 or 12-fold concatenation)
- 12 PCR cycles for SMRTbell prep kit 3.0 preparation (monomer)

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.** Reaction Mix 3 (RM3):

✔	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 RM3** to the 21 µL of the eluted cDNA (from step 2.4.15).	
3.1.3	Add **2 µL of the appropriate Iso-Seq primer barcode** to each sample for a total volume of 50 µL.	

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

3.1.4	Thoroughly mix by pipetting up and down 10 times and then quick spin to collect all liquid.	
3.1.5	Run the thermal cycler program below. Keep sample on ice until the thermal cycler lid has heated to 105°C. **Heated lid set at 105°C. Reaction volume set to 50 µL.**	

PCR program	Kinnex	SPK 3.0
45 seconds at 98°C	1 cycle	1 cycle
10 seconds at 98°C 15 seconds at 60°C 3 minutes at 72°C	10 cycles	12 cycles
5 minutes at 72°C	1 cycle	1 cycle
Hold at 4°C		

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

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3.2 Cleanup of amplified cDNA using SMRTbell Cleanup beads

Different SMRTbell cleanup ratios can be used to enrich for different lengths of cDNA. Select the cleanup ratio based on the intended downstream library preparation.

✔	Step	Instructions
	3.2.1	Transfer **45 µL of PCR-amplified cDNA** from Section 3.1 into a clean strip tube . **The correct bead-to-sample ratio is critical at this step.** Refer to the table below for the appropriate bead ratio selection. Add 49.5 µL (1.1x v/v) of resuspended, room-temperature SMRTbell cleanup beads. If targeting longer cDNA, **40.5** µL of SMRTbell cleanup beads (0.9X v/v) 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**, or enough to cover the beads, of **freshly prepared 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 the required volume of elution buffer and resuspend the beads by pipetting 10 times or until evenly distributed. Refer to the table below for the recommended elution volume for your selected downstream library prep kit:

SMRTbell cleanup bead Ratio	Bead volume (µL)	Recommended library prep kit
1.1X (v/v)	49.5 µL	Kinnex 8-fold Prep kit **or** Kinnex 12-fold Prep kit **or** SMRTbell Prep Kit 3.0
0.9X (v/v)	40.5 µL	Kinnex 8-fold Prep kit **or** SMRTbell Prep Kit 3.0

Selected library prep kit	Elution volume (µl)
Kinnex 8-/ 12-fold Prep Kit	24 µl
SMRTbell Prep Kit 3.0	47 µl

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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 the entire 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 1:5 dilution further to 1.5 ng/µL based on the Qubit reading if needed.
- Run on Agilent Bioanalyzer using High Sensitivity DNA kit; or on Agilent TapeStation using High Sensitivity D5000 ScreenTape Assay. See Figure 2 in the Appendix.

3.2.16 Refer to table below for the expected cDNA recovery after amplification and the minimum cDNA yield required to proceed with the selected downstream library prep kit.

Selected library prep kit	Expected yields per sample (ng)	Minimum yield for library prep (ng)
Kinnex 8-/ 12-fold Prep Kit	> 80ng	> 25ng
SMRTbell Prep Kit 3.0	> 200 ng	> 100ng

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

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. Refer to table below and ensure the total library input **is no less than minimum required yield and does not exceed the maximum total multiplex mass for corresponding elution volumn.** 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: For generating Kinnex concatenated libraries, please refer to one of the protocols: • Preparing Kinnex libraries using Kinnex 8-fold prep kit for cDNA with a peak size of 1.5 – 3 kb

Selected library prep kit	Maximum multiplex mass for library prep (ng)
Kinnex 8-/ 12-fold Prep Kit	35ng
SMRTbell Prep Kit 3.0	500ng

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- Preparing Kinnex libraries using Kinnex 12-fold prep kit for cDNA with a peak size of 1 – 2 kb

For generating monomer SMRTbell libraries, please refer to the protocol:

- Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0

PROTOCOL COMPLETE – Store at -20°C for long-term storage

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Appendix

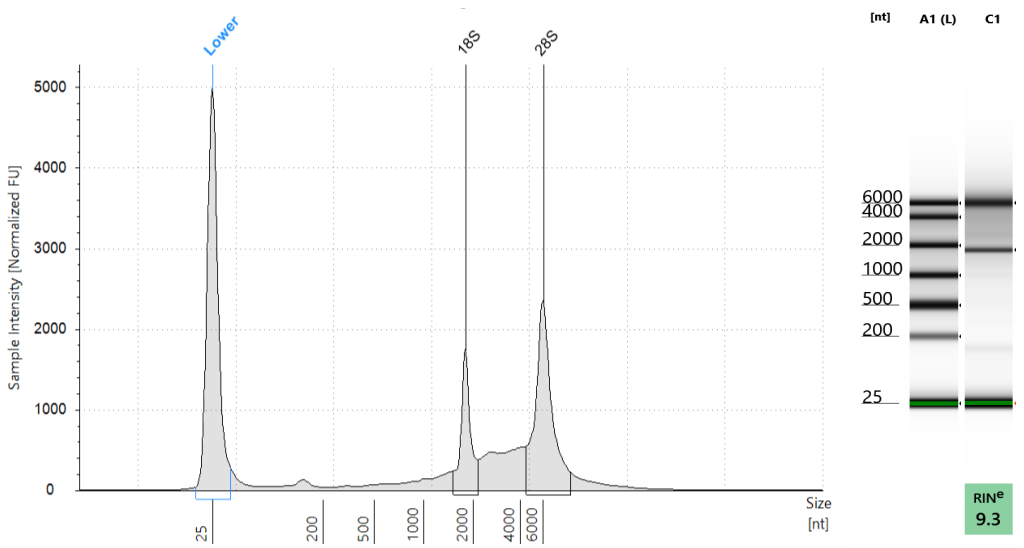

Figure 1. RNA quality control with TapeStation system

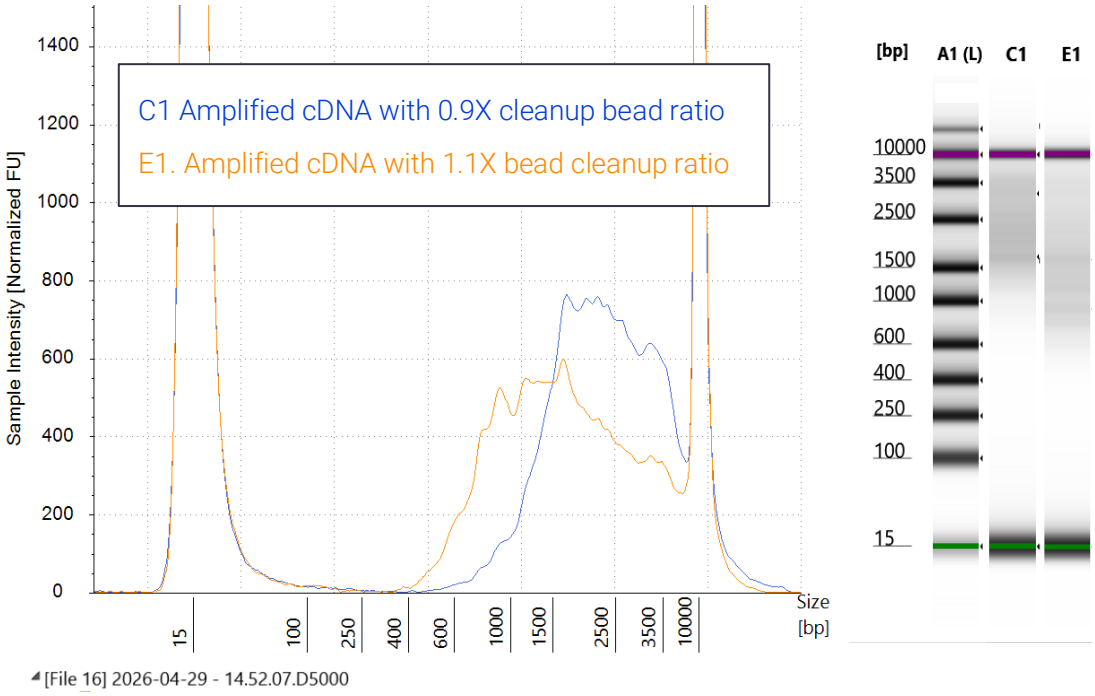

Figure 2. Size-selected amplified cDNA quality control with TapeStation system

APPENDIX COMPLETE

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FAQs

1. **Should I use 0.9x or 1.1x SMRTbell Cleanup bead ratio for post-cDNA amplification cleanup?**

   A 0.9X bead purification typically yields a cDNA peak of 2–3 kb, while a 1.1X bead purification typically yields a peak of 1–2 kb (see Figure 2 in the Appendix).

   Choose the bead ratio based on the desired cDNA size distribution and downstream Kinnex workflow:

   - 0.9X bead ratio plus the Kinnex 8-fold prep kit to enrich longer cDNA fragments.
   - 1.1X bead ratio plus the Kinnex 8-fold prep kit to retain shorter cDNA fragments and increase sequencing quality value.
   - 1.1X bead ratio plus the Kinnex 12-fold prep kit to retain shorter cDNA fragments and increase S-read throughput.

   For further enrichment of longer cDNA, use 0.86X (v/v) SMRTbell cleanup bead ratio (38.7µL of SMRTbell cleanup beads to 45uL of cDNA amplification reaction). Do not use a bead ratio below 0.86X, as this may result in low or no cDNA recovery.

2. **I want to multiplex more samples at cDNA level, do you have more cDNA barcodes?**

   Yes, If a higher multiplex level is desired, please contact Technical Support for an expanded set of 96 barcodes.

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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
Remove SPK3 library preparation	09	July 2026
Updated for new Kinnex kits	10	September 2026

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