Technical overview – Kinnex library preparation using Kinnex full-length RNA kit

Sequel II and IIe systems ICS v11.0
Revio system ICS v13.0+
SMRT Link v13.0+

PN 103-344-700 Rev 01 | March 2024

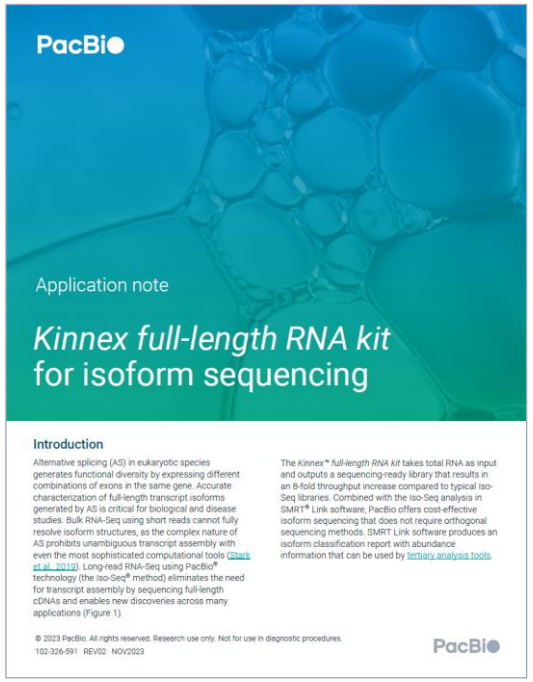
Kinnex library preparation using Kinnex full-length RNA kit

Technical Overview

1. Kinnex full-length RNA method overview
2. Kinnex full-length RNA library preparation workflow details
3. Kinnex full-length RNA sequencing preparation workflow details
4. Kinnex full-length RNA example sequencing performance data
5. Kinnex full-length RNA data analysis workflow overview
6. Technical documentation & applications support resources

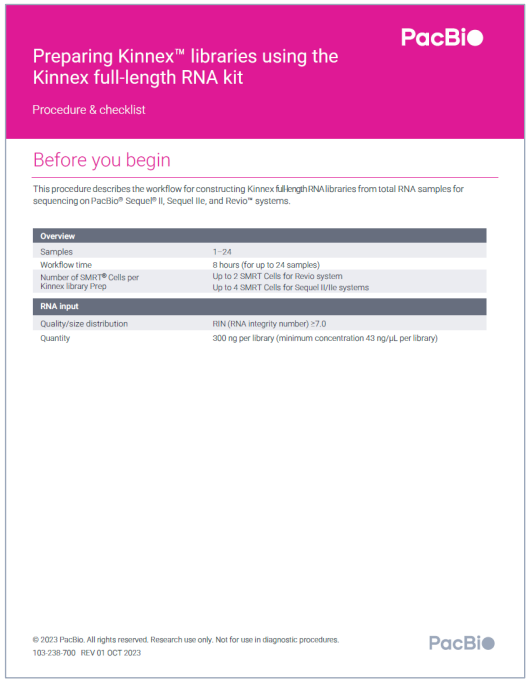
Kinnex library preparation using Kinnex full-length RNA kit: Getting started

Application-specific literature → **Application-specific protocol** → **Application-specific technical overview** → **Library preparation, sequencing & analysis**



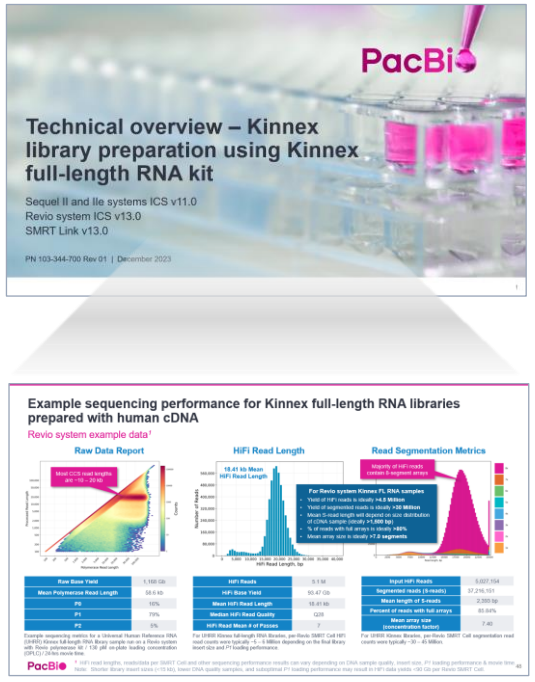
Application note – Kinnex full-length RNA kit for isoform sequencing (102-326-591)

Summary overview of application-specific library preparation and data analysis workflow recommendations.



Procedure & checklist – Preparing Kinnex libraries using the Kinnex full-length RNA kit (103-238-700)

Technical documentation containing application-specific library preparation protocol details.



Technical Overview – Kinnex library preparation using Kinnex full-length RNA kit (103-344-700)

Technical overview presentations describe sample preparation details for constructing Kinnex HiFi libraries for specific applications. Example sequencing performance data for a given application are also summarized.

cDNA synthesis & amplification
(Iso-Seq express 2.0 kit)

300 ng input total RNA per sample
RIN (RNA integrity number) ≥7.0
Sample multiplexing options available

Kinnex library preparation
(Kinnex PCR 8-fold kit + Kinnex concatenation kit)

Use amplified cDNA to generate Kinnex library containing 8-segment array
Sample multiplexing up to 48-plex supported

SMRT sequencing
(Sequel II/IIe & Revio systems)

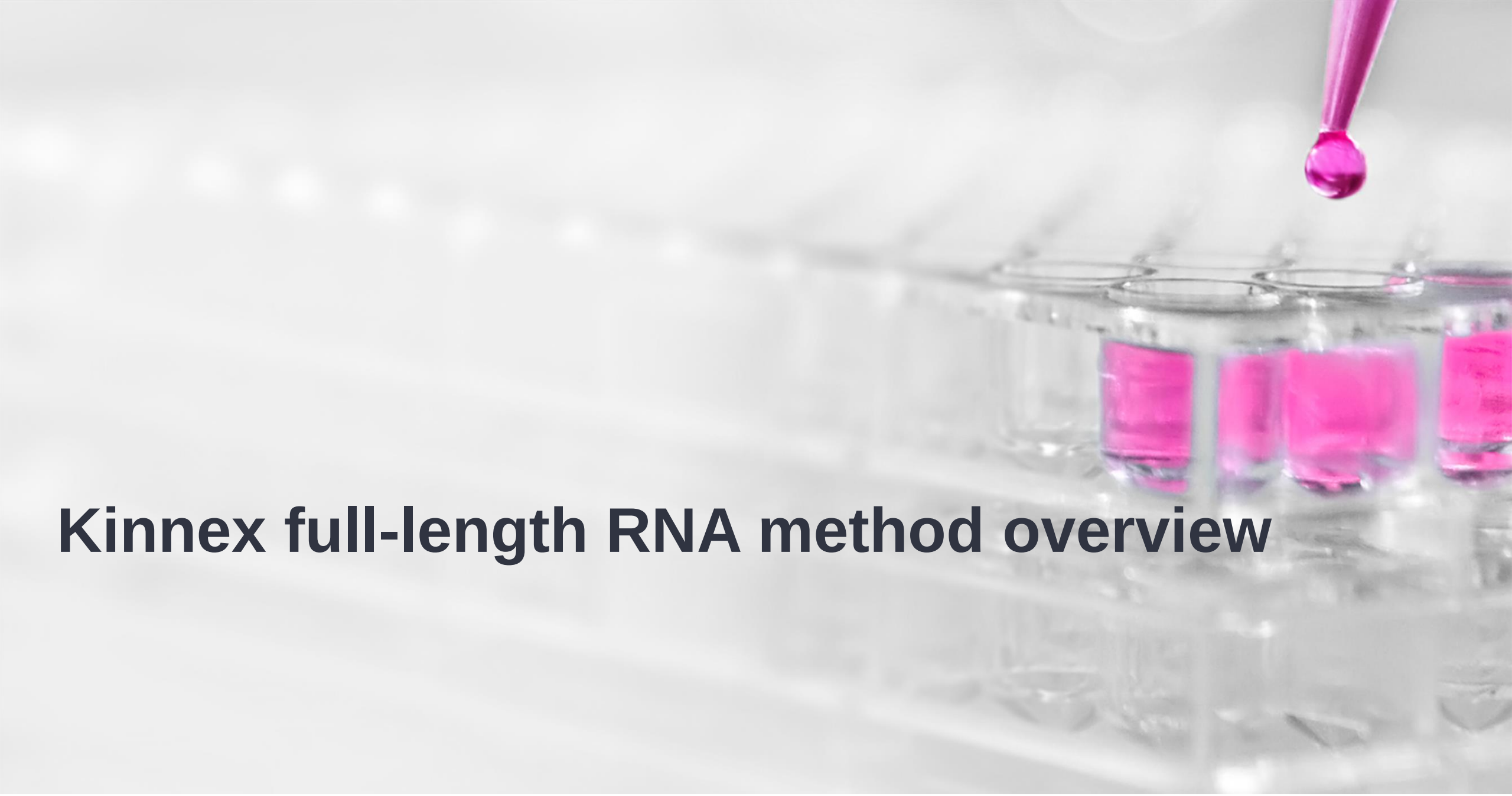
Perform ABC* and sequence Kinnex libraries on PacBio long-read systems

Data analysis
(SMRT Link)

Use SMRT Link Read Segmentation data utility to split arrayed transcript HiFi reads
Use SMRT Link Iso-Seq analysis application to identify novel genes and isoforms with abundance information

PacBio

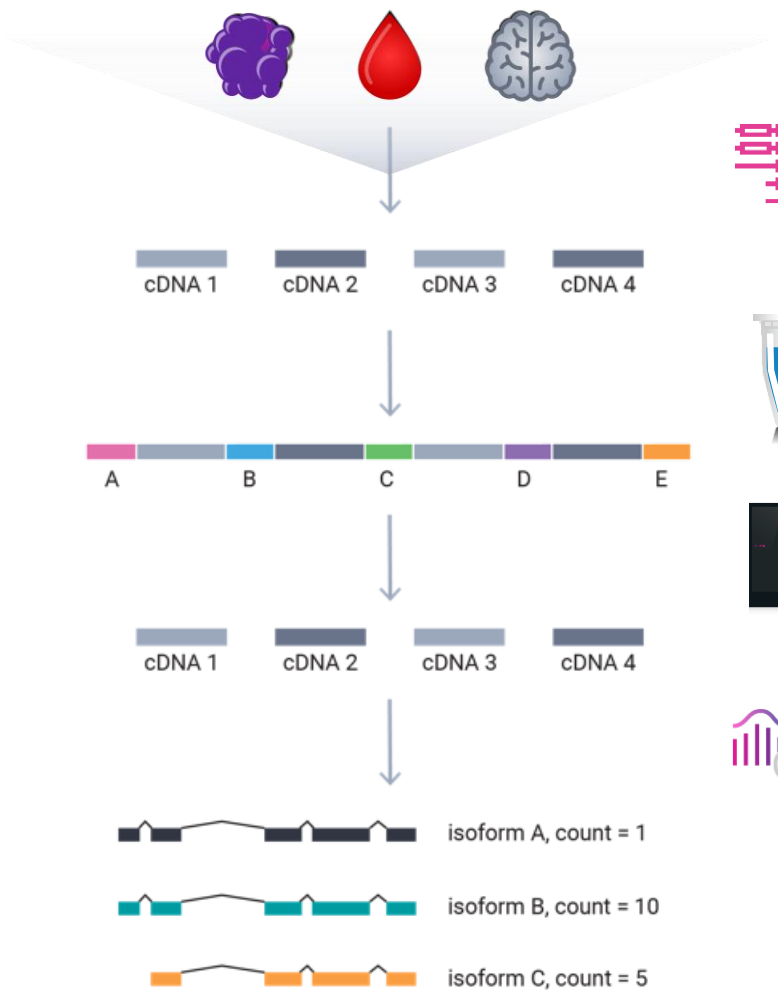
* ABC = Anneal primer / Bind polymerase / Clean up bound complex



Kinnex full-length RNA method overview

Kinnex full-length RNA method overview

Use Kinnex full-length RNA kit to perform high-accuracy, full-length isoform sequencing with PacBio long-read systems



cDNA generation

Kinnex library preparation

PacBio sequencing

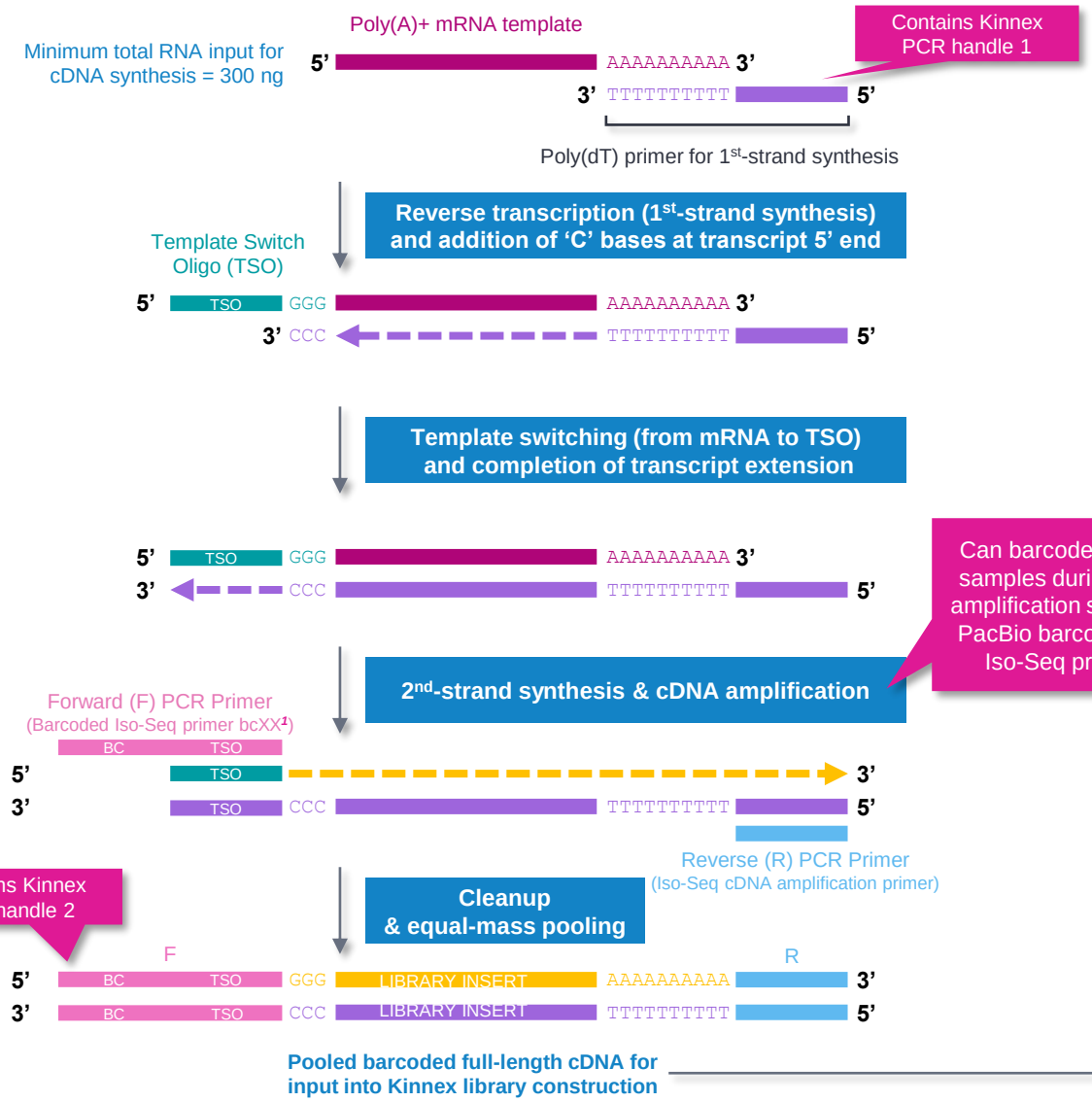
Read segmentation + Isoform analysis

- Input 300 ng total RNA, RIN ≥7
- Generate up to 12-plex barcoded cDNA using Iso-Seq express 2.0 kit (103-071-500)
- 2-day Kinnex library preparation using Kinnex full-length RNA kit (103-072-000)
- SMRT Link Run Design support for 'Kinnex full-length RNA' application type with auto-analysis (read segmentation + isoform analysis)[1]
- SMRT Link Iso-Seq isoform-classification software to identify novel genes and isoforms with abundance information

[1] Kinnex full-length RNA kit requires SMRT Link v13.0 or higher.

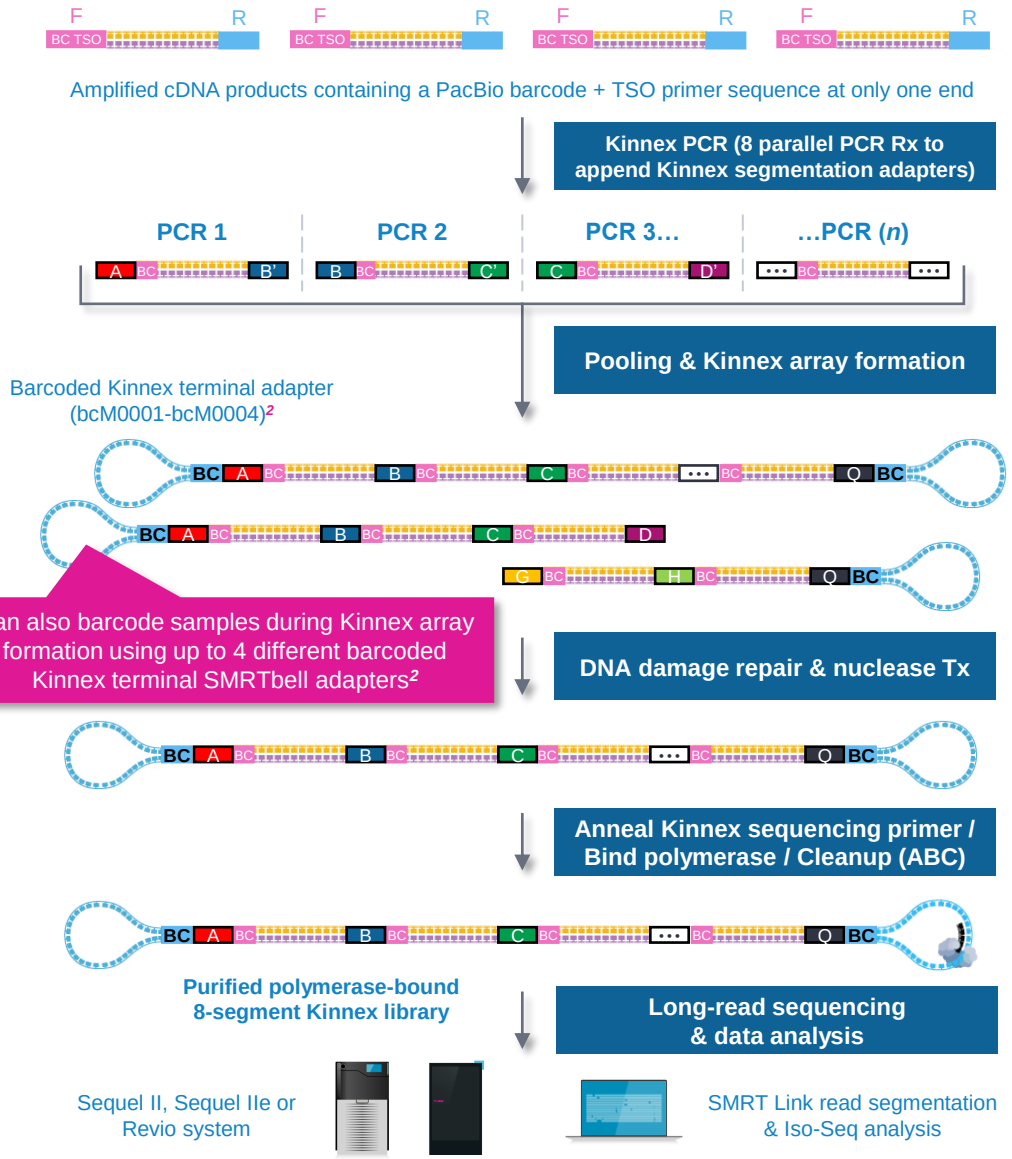
Kinnex full-length RNA method overview (cont.)

Full-length cDNA synthesis & amplification



Can barcode up to 12 samples during cDNA amplification step using PacBio barcoded Fwd Iso-Seq primers[1]

Kinnex library prep, sequencing & analysis



[1] Twelve barcoded Iso-Seq primers (Iso-Seq primer bc01–12) are available for cDNA amplification step.

[2] Kinnex adapter barcode sequences can be downloaded from SMRT Link Data Management module.

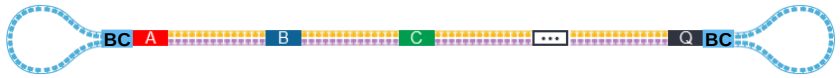
Kinnex full-length RNA library preparation procedure description

Procedure & checklist – Preparing Kinnex libraries using the Kinnex full-length RNA kit (103-238-700) describes the workflow for constructing Kinnex libraries from total RNA samples using the **Iso-Seq express 2.0 kit** and **Kinnex full-length RNA kit** for sequencing on PacBio Sequel II, Sequel IIe, and Revio systems

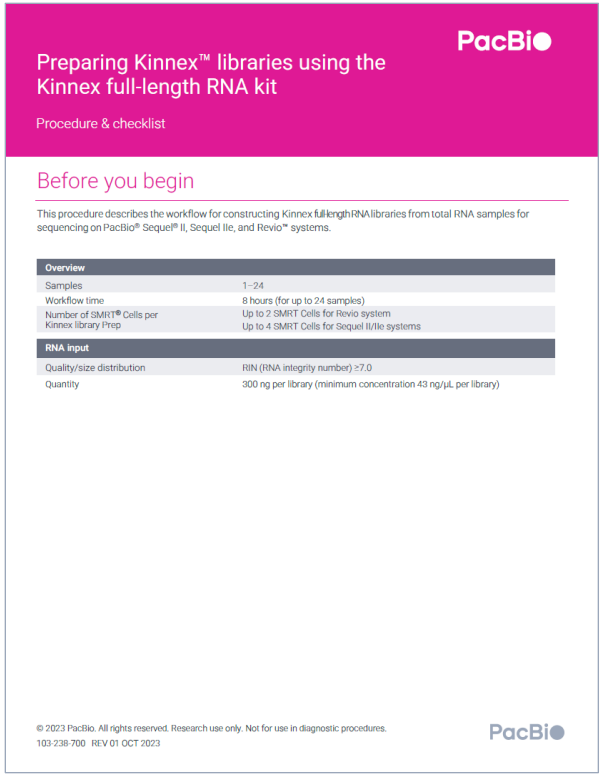
Overview	
Samples	1–24
Workflow time	8 hours (for up to 24 samples)
Number of SMRT® Cells per Kinnex library Prep	Up to 2 SMRT Cells for Revio system Up to 4 SMRT Cells for Sequel II/IIe systems
RNA input	
Quality/size distribution	RIN (RNA integrity number) ≥7.0
Quantity	300 ng per library (minimum concentration 43 ng/µL per library)

Kinnex full-length RNA kit
103-072-000 (12 rxn)

Iso-Seq express 2.0 kit[1]
103-071-500 (24 rxn)



Kinnex full-length RNA library template (~12–16 kb)
Contains 8 concatenated full-length cDNA segments


PacBio

Preparing Kinnex™ libraries using the Kinnex full-length RNA kit

Procedure & checklist

Before you begin

This procedure describes the workflow for constructing Kinnex full-length RNA libraries from total RNA samples for sequencing on PacBio® Sequel® II, Sequel IIe, and Revio™ systems.

Overview	
Samples	1–24
Workflow time	8 hours (for up to 24 samples)
Number of SMRT® Cells per Kinnex library Prep	Up to 2 SMRT Cells for Revio system Up to 4 SMRT Cells for Sequel II/IIe systems
RNA input	
Quality/size distribution	RIN (RNA integrity number) ≥7.0
Quantity	300 ng per library (minimum concentration 43 ng/µL per library)

© 2023 PacBio. All rights reserved. Research use only. Not for use in diagnostic procedures.
103-238-700 REV 01 OCT 2023

PacBio

PacBio Documentation (103-238-700)

- Kinnex full-length RNA library prep protocol uses **Kinnex full-length RNA kit and Iso-Seq express 2.0 kit**
  - → **Do not use** SMRTbell prep kit 3.0 with this protocol

[1] **Note:** Iso-Seq express 2.0 kit (103-071-500) is **not included** in Kinnex full-length RNA bundle kit (103-072-000) and must be purchased separately from PacBio.

Kinnex full-length RNA kit bundle and Iso-Seq express 2.0 kit components

Kinnex full-length RNA kit bundle and Iso-Seq express 2.0 kit provide full support for Kinnex library prep workflow

Iso-Seq express 2.0 kit (103-071-500)

Includes Iso-Seq Express template switching oligo, barcoded cDNA PCR Primers, and other reagents needed for performing 1st-strand cDNA synthesis and PCR amplification of cDNA products generated from input total RNA.

Kinnex full-length RNA kit bundle (103-072-000)

Includes Kinnex PCR kit, Kinnex concatenation and ancillary DNA cleanup reagents needed for incorporation of Kinnex segmentation adapters and Kinnex array formation for generating Kinnex full-length RNA libraries.

Iso-Seq express 2.0 kit components

Component		Description
1		**Iso-Seq RT buffer** • For 1st-strand cDNA synthesis
2		**Iso-Seq RT primer mix** • For 1st-strand cDNA synthesis
3		**Iso-Seq RT enzyme mix** • For 1st-strand cDNA synthesis
4		**Iso-Seq template switch oligo** • For 1st-strand cDNA synthesis
5		**Iso-Seq cDNA PCR mix** • Enzyme nucleotide mix for cDNA amplification
6		**Iso-Seq cDNA amplification primer** • Reverse primer for cDNA amplification
7		**Iso-Seq primers (bc01 – bc12)** • Barcoded forward primers (bc01 – bc04) for cDNA amplification

Kinnex full-length RNA kit components

Component		Description
1		**Kinnex PCR 8-fold kit (12 rxn)** • Contains reagents for Kinnex PCR to incorporate segmentation adapters
2		**Kinnex concatenation kit (12 rxn)** • Contains reagents for Kinnex array formation and SMRTbell template construction • Includes barcoded Kinnex adapter mixes (bcM0001 – bcM0004)
3		**SMRTbell cleanup beads** • For DNA cleanup
4		**Elution buffer** • For DNA cleanup

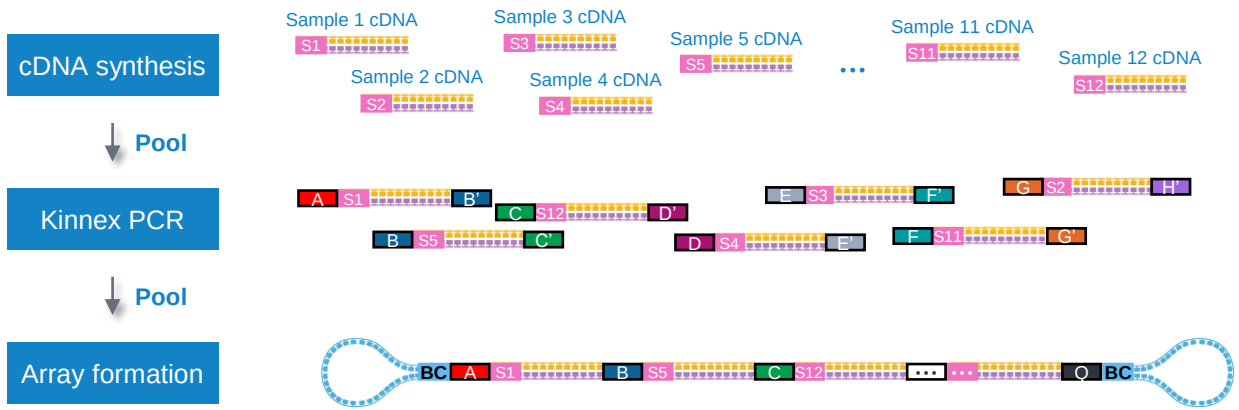
Kinnex full-length RNA library barcoding options for sample multiplexing

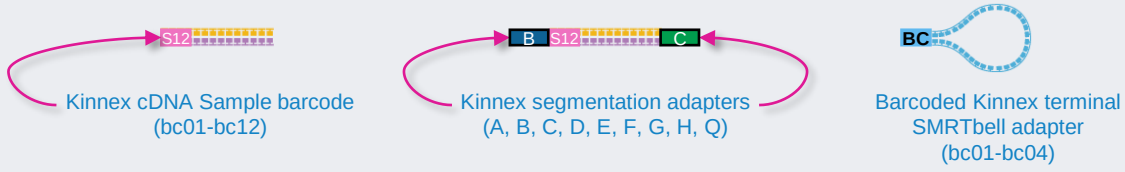
Kinnex full-length RNA library preparation procedure supports up to 48-plex sample multiplexing

Kinnex full-length RNA library preparation procedure supports **up to 48-plex** sample multiplexing through combined use of:

→ 12 different barcoded cDNA amplification PCR primers (bc01 – bc12)

→ 4 different barcoded Kinnex terminal SMRTbell adapters (bc01 – bc04)





Kinnex full-length RNA experimental design considerations

Kinnex full-length RNA application use case recommendations for PacBio systems

Example application	Human genetics disease studies	Biopharma for identifying highly expressed targets	Plant & animal whole genome annotation
Experimental goal	Isoform discovery and quantification of moderate-to-rare transcripts	Isoform discovery of high expressed transcripts	Comprehensive transcript annotation in a species
Example study design	Disease vs. normal tissues with multiple replicates	Disease cohort with >20+ samples	Plant or animal with multiple tissue types
Target depth of coverage per sample	10 M reads per sample	5 M reads per sample	≤5 M reads per tissue (of same species)
Sample multiplexing[1]	**Sequel II/IIe system:** Up to 2 samples per SMRT Cell 8M (2-plex)	**Sequel II/IIe system:** Up to 3 samples per SMRT Cell 8M (3-plex)	**Sequel II/IIe system:** Up to 3 tissue types per SMRT Cell 8M (3-plex)
	Revio system: Up to 4 samples per Revio SMRT Cell (4-plex)	**Revio system:** Up to 8 samples per Revio SMRT Cell (8-plex)	**Revio system:** Up to 8 tissue types per Revio SMRT Cell (8-plex)[2]
Expected data throughput (per SMRT Cell)	**Sequel II/IIe system:** 15 M reads per SMRT Cell 8M divided by *N* samples		
	Revio system: 40 M reads divided by *N* samples		
Kinnex library prep protocol	Procedure & checklist – Preparing Kinnex libraries using the Kinnex full-length RNA kit (103-238-700)		
Total RNA input into Kinnex library prep workflow	300 ng total RNA (RIN ≥7) for 1st-strand cDNA synthesis		
SMRT Link data analysis workflows	Read Segmentation and Iso-Seq analysis application with option to "pool reads and cluster together" to get a master isoform classification file with per-sample full-length read counts		
Community data analysis tools	Annotation & quantification: PIGEON, SQANTI3, Differential analysis: TappAS, Fusion calling: pbfusion, Visualization: SWAN		

[1] Kinnex concatenation kit (103-071-800) can support up to 48-plex sample multiplexing through the combined use of 12 different barcoded cDNA amplification primers and 4 different barcoded Kinnex terminal SMRTbell adapters during Kinnex full-length RNA library construction.

[2] If targeting <5 M transcripts reads per sample → can multiplex up to 12 tissues types per Revio SMRT Cell.

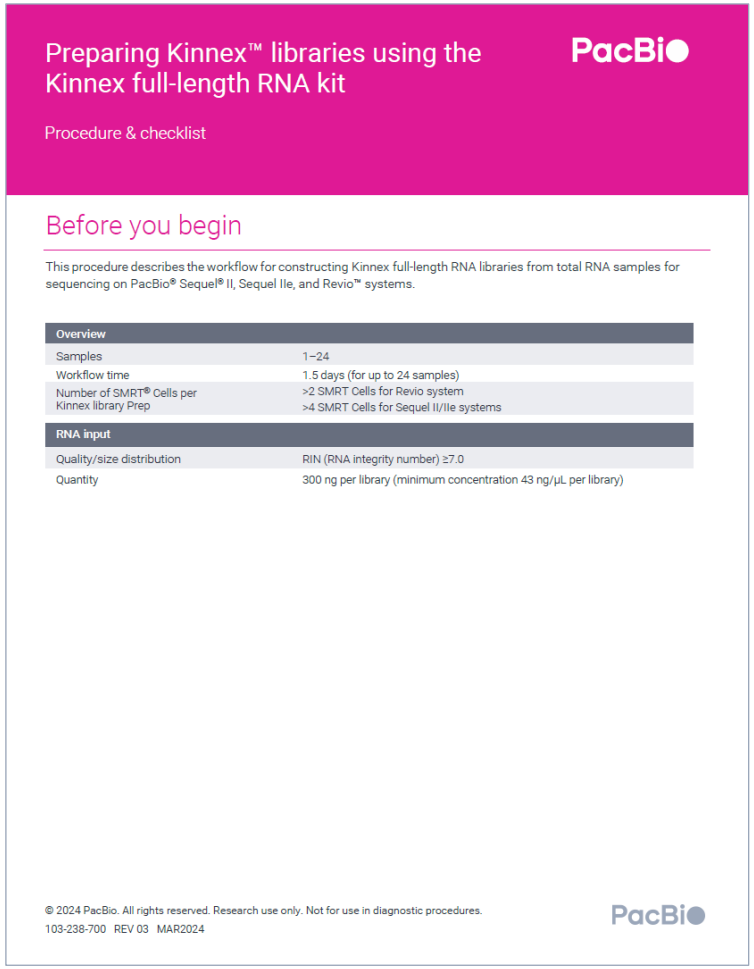
Kinnex full-length RNA library preparation workflow details

Procedure & checklist – Preparing Kinnex libraries using the Kinnex full-length RNA kit (103-238-700)

Procedure & checklist 103-238-700 describes the workflow for constructing Kinnex libraries from total RNA samples using the **Iso-Seq express 2.0 kit** and **Kinnex full-length RNA kit** for sequencing on PacBio Sequel II, Sequel IIe, and Revio systems[1]

Procedure & checklist contents

1. Total RNA input QC recommendations and general best practices for reagent & sample handling.
2. Enzymatic workflow steps for cDNA synthesis and amplification.
3. Enzymatic workflow steps for construction of 8-segment Kinnex arrays from amplified cDNA.
4. Enzymatic workflow steps for DNA damage repair & nuclease treatment of Kinnex libraries.
5. Workflow steps for final cleanup of Kinnex SMRTbell libraries using SMRTbell cleanup beads.



Preparing Kinnex™ libraries using the Kinnex full-length RNA kit

PacBio

Procedure & checklist

Before you begin

This procedure describes the workflow for constructing Kinnex full-length RNA libraries from total RNA samples for sequencing on PacBio® Sequel® II, Sequel IIe, and Revio™ systems.

Overview	
Samples	1–24
Workflow time	1.5 days (for up to 24 samples)
Number of SMRT® Cells per Kinnex library Prep	>2 SMRT Cells for Revio system >4 SMRT Cells for Sequel II/IIe systems
RNA input	
Quality/size distribution	RIN (RNA integrity number) ≥7.0
Quantity	300 ng per library (minimum concentration 43 ng/µL per library)

© 2024 PacBio. All rights reserved. Research use only. Not for use in diagnostic procedures.

103-238-700 REV 03 MAR2024

PacBio

PacBio Documentation (103-238-700)

[1] Refer to **SMRT Link Sample Setup** for recommended sample setup conditions for sequencing Kinnex full-length RNA library samples on PacBio Sequel II/IIe & Revio systems.

Kinnex full-length RNA library construction workflow overview

Procedure & checklist – Preparing Kinnex libraries using the Kinnex full-length RNA kit (103-238-700)

	Cleanup	DNA QC	Walk-away time*
1 Input RNA quality control	N/A	Qubit RNA HS assay Bioanalyzer RNA 6000 Nano kit	N/A
Safe stop			
2 cDNA synthesis + cleanup	1.3X SMRTbell cleanup beads	N/A	1.5 hrs
3 cDNA amplification + cleanup	0.9X SMRTbell cleanup beads	Qubit dsDNA HS assay Bioanalyzer HS DNA assay	1.5 hrs
Safe stop			
4 Kinnex PCR + cleanup	1.05X SMRTbell cleanup beads	Qubit dsDNA HS assay	1.5 – 2.0 hrs
5 Kinnex array formation + cleanup	1.0X SMRTbell cleanup beads	N/A	2.0 hrs
Safe stop			
6 Nuclease treatment + cleanup	1.0X SMRTbell cleanup beads	Qubit dsDNA HS assay Femto Pulse gDNA 165 kb kit	0.6 hrs

Day 1 (~5 hrs*)

Day 2 (~3 hrs*)

* **Excludes** hands-on time for setting up enzymatic reaction steps and additional time required to perform DNA sizing QC and DNA concentration QC.

General best practices recommendations for preparing Kinnex full-length RNA libraries

Reagent and sample handling

- Take care to 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.
- In cDNA amplification and Kinnex PCR, keep sample(s) on ice until thermal cycler lid has reached 105°C to avoid digestion of primers by polymerase exonuclease activity.
- This workflow takes ~8 hrs to complete.
    - If a stop is necessary, refer to the workflow for safe stopping points.

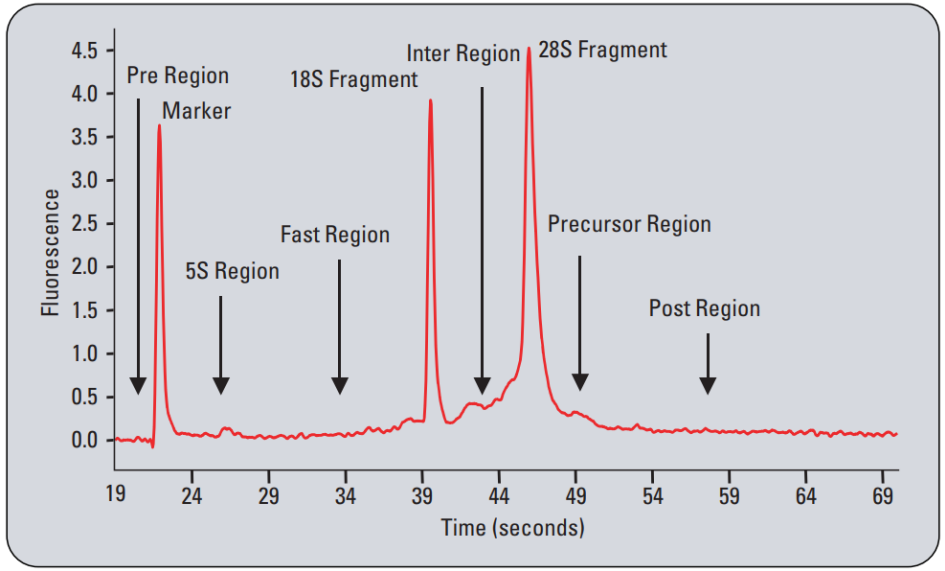
* **Room temperature** (RT) is defined as any temperature in the range of 18 – 23°C for this protocol.

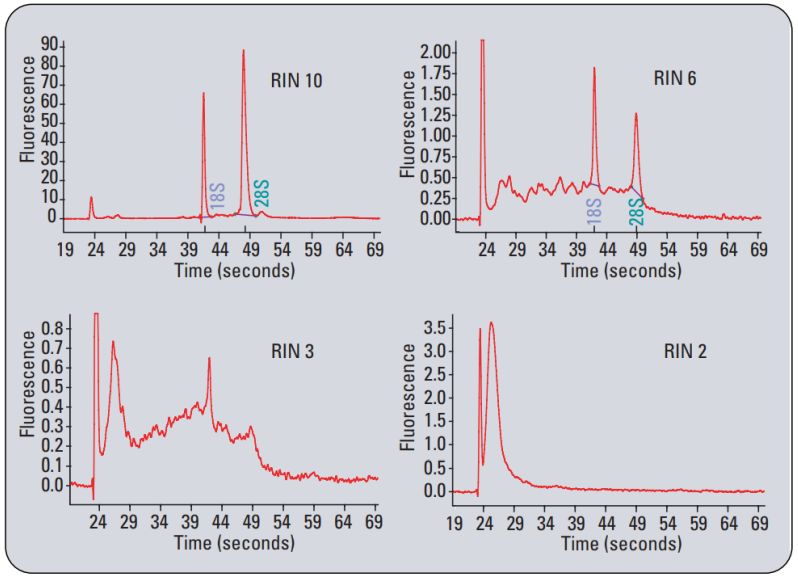
Input RNA quality control

Input RNA quality control is highly recommended before proceeding to the MAS-Seq library prep workflow

1. Input RNA quality control
   - Safe stop
2. cDNA synthesis + cleanup
3. cDNA amplification + cleanup
   - Safe stop
4. Kinnex PCR + cleanup
5. Kinnex array formation + cleanup
   - Safe stop
6. Nuclease treatment + cleanup

- 300 ng of total RNA per library (minimum concentration 43 ng/µL per library) is required for this procedure
- Sample QC of input total RNA samples should be assessed by measuring RNA Integrity Number (RIN) using a Bioanalyzer 2100 instrument (Agilent Technology) with RNA 6000 Nano kit[1]
  - RIN ≥7.0 (ideally ≥8.0) is sufficient for Kinnex full-length RNA protocol
  - Samples with RIN <7.0 can be processed, but risk of significant underperformance or even failure is greatly increased
- RIN score (1 to 10) is related to ratio of the area under 28s and 18s fragment peaks and also takes into account signal intensity above baseline in the Inter-Region and Fast Region since this is where degradation products appear
- Higher RIN numbers are correlated with better overall sample quality and lower degradation





Left: Bioanalyzer electropherogram detailing the regions that are indicative of RNA quality. **Right:** Sample electro-pherograms corresponding to different RNA Integrity Number (RIN) scores. Samples range from intact (RIN 10), to degraded (RIN 2). Images from Agilent Application Note: RNA Integrity Number (RIN) – Standardization of RNA Quality Control (5989-1165EN)

[1] Refer to *Agilent Application Note: RNA Integrity Number (RIN) – Standardization of RNA Quality Control* (5989-1165EN)

cDNA synthesis + cleanup

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

1 Input RNA quality control

Safe stop

2 cDNA synthesis + cleanup

3 cDNA amplification + cleanup

Safe stop

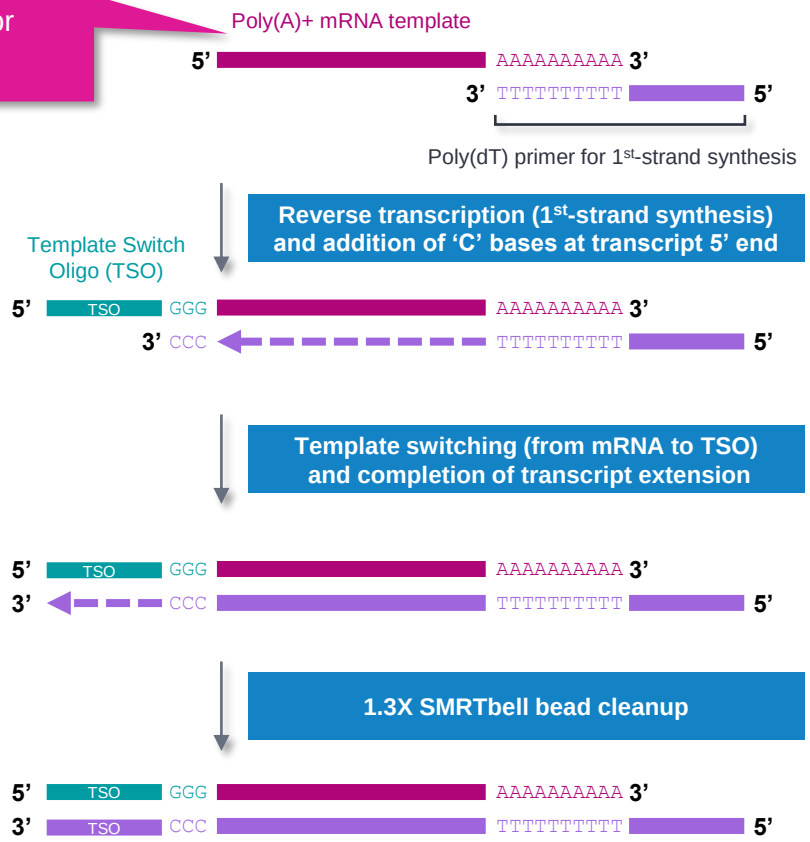
4 Kinnex PCR + cleanup

5 Kinnex array formation + cleanup

Safe stop

6 Nuclease treatment + cleanup

- Minimum total RNA input for cDNA synthesis = **300 ng**



cDNA synthesis + cleanup (cont.)

Procedural notes

1 Input RNA quality control

Safe stop

2 cDNA synthesis + cleanup

3 cDNA amplification + cleanup

Safe stop

4 Kinnex PCR + cleanup

5 Kinnex array formation + cleanup

Safe stop

6 Nuclease treatment + cleanup

2.1 Thawing reagents for first-strand synthesis

✓	Step	Instructions
	2.1.1	Quick-spin the Iso-Seq RT enzyme mix in the microcentrifuge to collect liquid, then place on ice.
		Thaw the following components at room temperature, briefly vortex to mix, then quick-spin to collect liquid and place on ice.

Reagent
Iso-Seq RT primer mix (103-104-000)

- After thawing specified reagents **on ice**, perform a quick spin to collect liquid, then **place on ice**

2.2 Primer annealing for first-strand synthesis

✓	Step	Instructions
		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.

✓	Components	Volume
2.2.1	Total RNA (300 ng)	<7 µL

- Set up primer annealing reaction mix **on ice** and then transfer to thermal cycler for incubation

2.3 Reverse transcription and template switching

✓	Step	Instructions
		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.

✓	Components	Volume
2.3.1	Iso-Seq RT buffer (vortex briefly before use)	5 µL

- Set up reverse transcription reaction mix **on ice** and then transfer to thermal cycler for incubation

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.

- Perform **1.3X** SMRTbell bead cleanup

PacBio

cDNA amplification + cleanup

In this step, first-strand cDNA products are PCR-amplified and barcoded using barcoded Iso-Seq primers

1 Input RNA quality control

Safe stop

2 cDNA synthesis + cleanup

3 cDNA amplification + cleanup

Safe stop

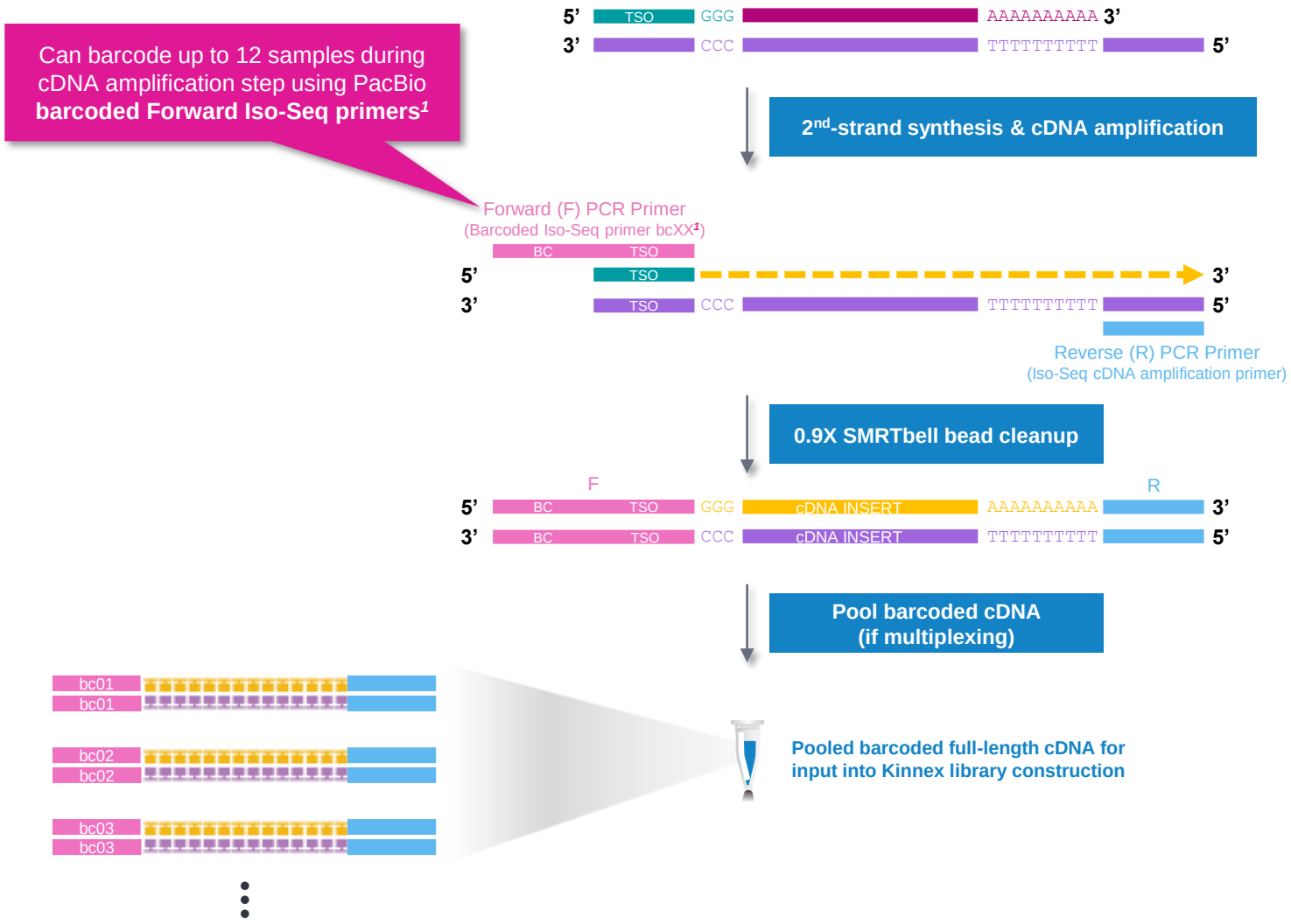
4 Kinnex PCR + cleanup

5 Kinnex array formation + cleanup

Safe stop

6 Nuclease treatment + cleanup

Can barcode up to 12 samples during cDNA amplification step using PacBio **barcoded Forward Iso-Seq primers**[1]



[1] Twelve barcoded Iso-Seq primers (Iso-Seq primer bc01–12) are available for cDNA amplification step.

cDNA amplification + cleanup

Procedural notes

1 Input RNA quality control

↓ Safe stop

2 cDNA synthesis + cleanup

↓

3 cDNA amplification + cleanup

↓ Safe stop

4 Kinnex PCR + cleanup

↓

5 Kinnex array formation + cleanup

↓ Safe stop

6 Nuclease treatment + cleanup

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.

✔	Components	Volume
	Iso-Seq cDNA PCR mix	25 µL
	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 bc01–12 for a total volume of 50 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	10 cycles	
15 seconds at 60°C		
3 minutes at 72°C		
5 minutes at 72°C		
Hold at 4°C		

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

- Set up cDNA amplification reaction mix **on ice**
- **Do not add Barcoded Forward Iso-Seq primers to master mix reaction**

- Add desired Barcoded Forward Iso-Seq primer (**select one of bc01-bc12**) to each individual cDNA amplification reaction

- After setting up reactions on ice, add PCR reactions to thermal cycler **after the lid has preheated to 105°C** to avoid digestion of primers by polymerase exonuclease activity

cDNA amplification + cleanup (cont.)

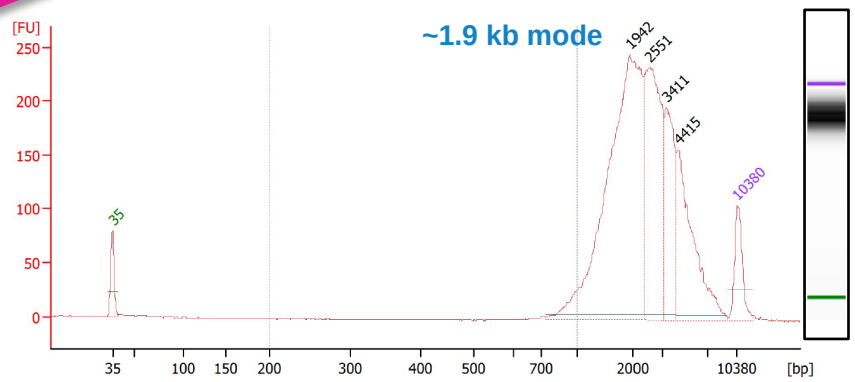
Procedural notes

1 Input RNA quality control
↓ Safe stop
2 cDNA synthesis + cleanup
↓
3 cDNA amplification + cleanup
↓ Safe stop
4 Kinnex PCR + cleanup
↓
5 Kinnex array formation + cleanup
↓ Safe stop
6 Nuclease treatment + cleanup

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.
	3.2.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer 24 µL of the supernatant to a new strip tube. Discard the old strip tube with beads.
		Recommended: Measure concentration and size distribution of each cDNA sample.
	3.2.15	• 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 >100 ng. A minimum of 55 ng of total cDNA is recommended to proceed with Kinnex PCR (Step 4). If less than 55 ng but more than 25 ng is recovered, proceed with Kinnex PCR but expect lower yields. Do not proceed with less than 25 ng.

- Perform **0.9X** SMRTbell bead cleanup[1]
- Perform DNA concentration QC using Qubit ds DNA HS assay and DNA sizing QC using Bioanalyzer



Example Bioanalyzer DNA sizing QC analysis results for amplified full-length cDNA generated from a universal human RNA reference (UHRR) total RNA sample.

- Expected yield of purified cDNA product is **>100 ng**
- Minimum cDNA amount needed to proceed with Kinnex PCR = **55 ng** (**do not proceed with <25 ng**)

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 for a total mass of 55 ng. 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.

- If multiplexing, perform **equal-mass pooling** of each barcoded cDNA sample after 0.9X SMRTbell bead cleanup

PacBio

* **Note:** Non-Kinnex full-length RNA (bulk monomer) Iso-Seq protocol (***Procedure & checklist – Preparing Iso-Seq libraries using SMRTbell prep kit 3.0*** [102-396-000] offers 3 options for post-cDNA amplification SMRTbell bead cleanup: 0.82X, 0.86X, and 0.95X → For simplification, Kinnex full-length RNA protocol only specifies to use 0.9X.

Kinnex PCR

In this step, incorporate programmable Kinnex segmentation adapter sequences into amplified cDNA products

1 Input RNA quality control

Safe stop

2 cDNA synthesis + cleanup

3 cDNA amplification + cleanup

Safe stop

4 Kinnex PCR + cleanup

5 Kinnex array formation + cleanup

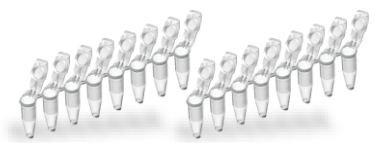
Safe stop

6 Nuclease treatment + cleanup

Set up 8 parallel Kinnex PCR reactions per sample with premixed Kinnex primers to generate amplified cDNA products containing programmable sequences at both ends

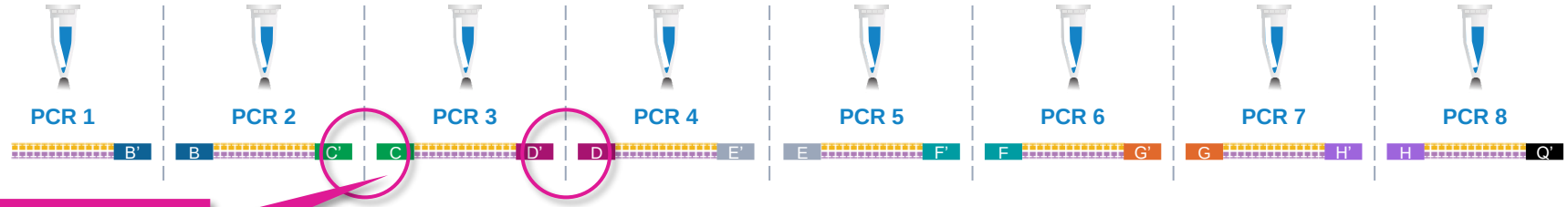
Barcoded cDNA sample after 0.9X SMRTbell bead cleanup

Distribute barcoded cDNA sample + Kinnex PCR reaction mix into 8 PCR tubes or 8 wells in a 96-well plate



OR

Perform Kinnex PCR by adding premixed Kinnex primer pair solutions



Example: Amplified cDNA products from Kinnex PCR 3 contain flanking segmentation adapter sequences that are **complementary** to the ends of cDNA products from Kinnex PCR 2 & Kinnex PCR 4

Pool 8 Kinnex PCR products together and perform 1.05X SMRTbell bead cleanup

Kinnex PCR-amplified cDNA library insert sample ready for Kinnex array formation step

PacBio

Kinnex PCR (cont.)

Procedural notes

1. Input RNA quality control
2. cDNA synthesis + cleanup
3. cDNA amplification + cleanup
4. Kinnex PCR + cleanup
5. Kinnex array formation + cleanup
6. Nuclease treatment + cleanup

Safe stop

4.1 Kinnex PCR

✔	Step	Instructions
	4.1.1	Thaw primers. The entire volume of primers can be transferred to an 8-tube strip for ease of use with a multi-channel pipette.

	8X concatenation	Tube color	P/N
1	Kinnex primer mix A	Orange	103-107-800
2	Kinnex primer mix B	Orange	103-107-900
3	Kinnex primer mix C	Orange	103-108-000
4	Kinnex primer mix D	Orange	103-108-100
5	Kinnex primer mix E	Orange	103-108-200
6	Kinnex primer mix F	Orange	103-108-300
7	Kinnex primer mix G	Orange	103-108-400
8	Kinnex primer mix HQ	Orange	103-108-500

	4.1.2	Briefly vortex to mix, then quick-spin to collect liquid and place the primer mixes on ice.
	4.1.3	Thaw the following components, briefly vortex to mix, then quick-spin to collect liquid and place on ice. Add the components on ice in a LoBind tube.

Master mix components	Volume for 8X concatenation*
PCR-grade water	88-X µL
Kinnex PCR mix (103-107-700)	110 µL
55 ng of amplified cDNA from Step 3.2.16	X µL
Total volume	198 µL

X = 55 (ng)/purified pooled DNA concentration from step 3.2.16 (single-plex) or step 3.2.3 (multiplex)
*10% overage included

	4.1.4.	Aliquot 22.5 µL of Master Mix 1 into each of the 8 PCR tubes (for 8X concatenation).
	4.1.5	Add 2.5 µL of Kinnex primer mix into each of 8 PCR tubes from Step 4.4.
		Set up the thermal cycler program listed below with the lid set to 105˚C. Keep sample(s) on ice until the lid is heated to 105°C.
		The duration of PCR is approximately 1 hour.

	Step	Temperature	Duration	Cycles
4.1.6	Initial denaturation	98°C	3 min	1
	Denaturation	98°C	20 s	9
	Annealing	68°C	30 s	
	Extension	72°C	4 min	

- Can transfer entire volume of primers to PCR tubes for ease of use with multi-channel pipettes (8 primer mix tubes)

- Set up Kinnex PCR reactions **ON ICE**
- PCR polymerase 3'→5' exonuclease activity negatively impacts amplification yield if prepared at room temp.

- **Critical step! Correct setup of all 8 Kinnex PCR reactions is required** – any missing/incorrect MAS primer pairs will result in no/low SMRTbell yield

- Set up on ice and add PCR reaction to thermal cycler after lid has preheated to 105°C to avoid digestion of primers by polymerase exonuclease activity

Kinnex PCR (cont.)

Procedural notes

1 Input RNA quality control

Safe stop

2 cDNA synthesis + cleanup

3 cDNA amplification + cleanup

Safe stop

4 Kinnex PCR + cleanup

5 Kinnex array formation + cleanup

Safe stop

6 Nuclease treatment + cleanup

4.2 Pooling of 8 Kinnex PCR products and 1.05X SMRTbell cleanup

✔	Step	Instructions
	4.2.1	Add 23 µL from each of the 8 PCR reactions into a 1.5 mL tube for a total volume of 184 µL. An equal volume of each PCR product is necessary for efficient array assembly.
	4.2.2	Add 193 µL (**1.05X** v/v) of resuspended, room-temperature SMRTbell cleanup beads to a tube of pooled Kinnex PCR amplicon. **The correct ratio of beads to pooled sample is critical at this step.**
	4.2.3	Pipette-mix the beads until evenly distributed.
	4.2.4	Quick-spin the tube in a microcentrifuge to collect liquid.
	4.2.5	Leave at **room temperature** for **10 minutes** to allow the DNA to bind beads
	4.2.6	Place the tube in a magnetic separation rack until the beads separate fully from the solution.
	4.2.7	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	4.2.8	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into the tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	4.2.9	Repeat the previous step.
	4.2.10	Remove residual 80% ethanol: • Remove the tube from the magnetic separation rack. • Quick-spin the tube in a microcentrifuge. • Place the tube back in the magnetic separation rack until the beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	4.2.11	Remove the tube from the magnetic rack. **Immediately** add **40 µL** of **elution buffer** to the tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	4.2.12	Quick-spin the tube in a microcentrifuge to collect liquid.
	4.2.13	Leave at **room temperature** for **5 minutes** to elute DNA.
	4.2.14	Place tube in a magnetic separation rack until beads separate fully from the solution.
	4.2.15	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new LoBind tube**. Discard old tube with beads.
	4.2.16	Make a 1:10 dilution of the sample in elution buffer and measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Typical yield is 6–12 µg.

- Pool exactly 23 µL from each Kinnex PCR reaction in a clean 1.5 mL DNA LoBind tube[1] for a total combined volume of 184 µL

- Add exactly 193 µL of SMRTbell cleanup beads (1.05X)
- Kinnex PCR mix significantly increases stringency of SMRTbell clean up beads, so accurate pipetting is critical

- Perform DNA concentration QC to verify there is sufficient yield of Kinnex PCR products (min. 4 µg) to proceed to Kinnex array formation step

[1] DNA concentration QC measurements for individual Kinnex PCR reactions prior to pooling are not required.

Kinnex array formation

In this step, assemble cDNA transcripts (“segments”) containing programmable ends into a linear array

1 Input RNA quality control

↓ Safe stop

2 cDNA synthesis + cleanup

↓

3 cDNA amplification + cleanup

↓ Safe stop

4 Kinnex PCR + cleanup

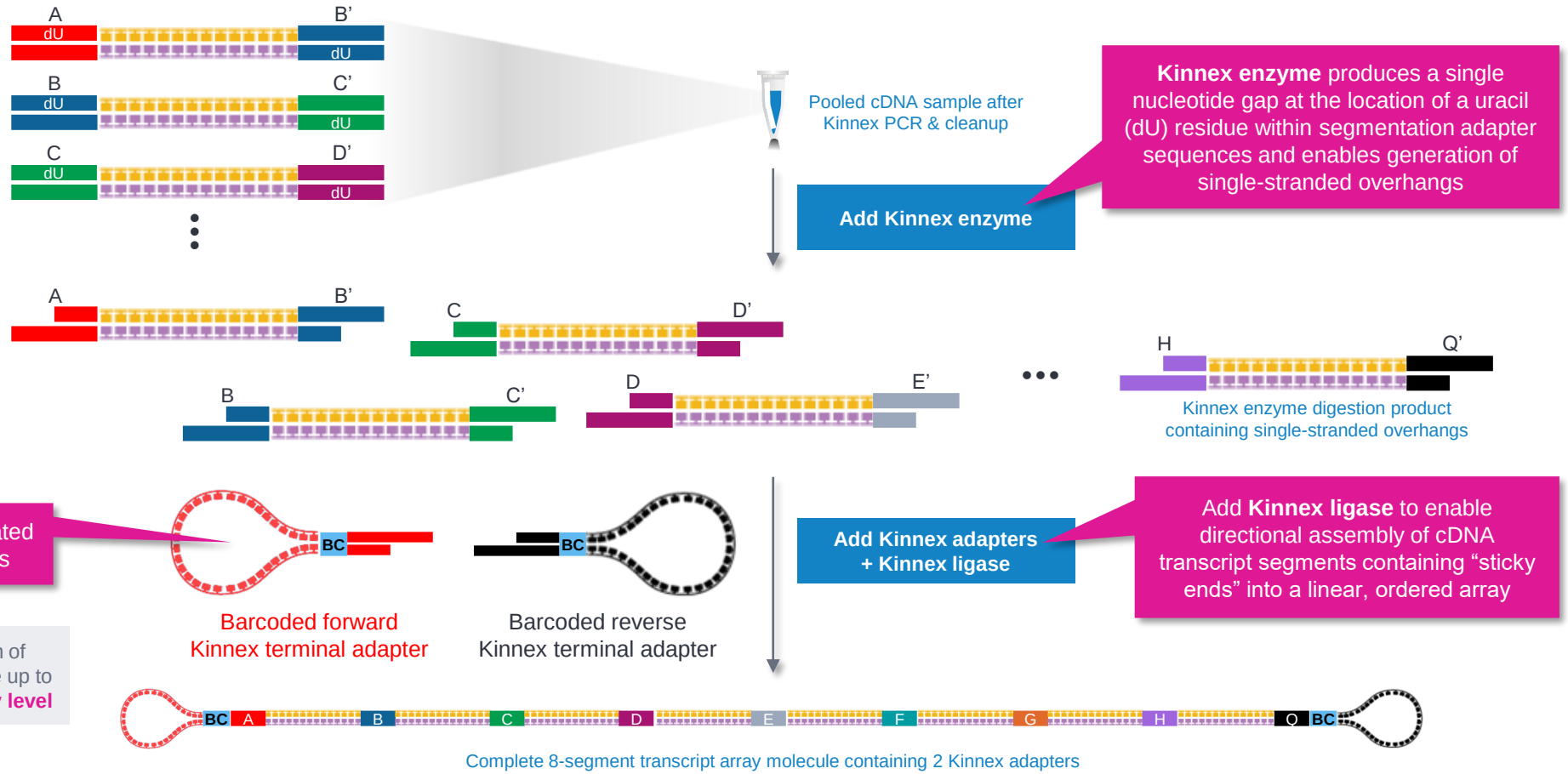
↓

5 Kinnex array formation + cleanup

↓ Safe stop

6 Nuclease treatment + cleanup

Treat pooled Kinnex PCR products with Kinnex enzyme to create single-stranded overhangs to enable subsequent directional assembly of cDNA transcripts into a linear, ordered array



Barcoded Kinnex terminal adapters[1] are ligated to specific overhang sequences at array ends

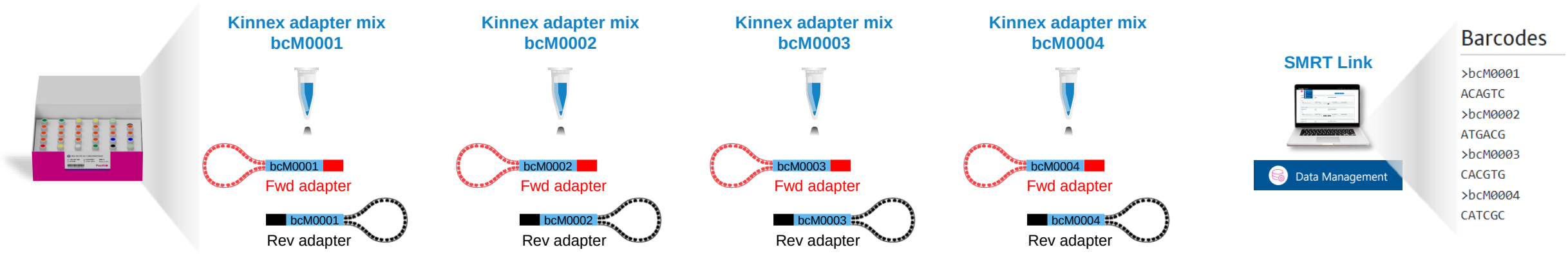
Kinnex library prep workflow supports incorporation of PacBio barcodes at the array formation step to enable up to **4-plex sample multiplexing at the SMRTbell library level**

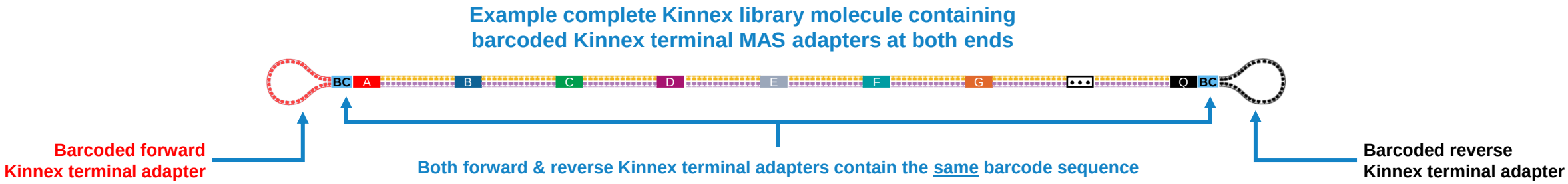
[1] **Note:** Four barcoded terminal Kinnex adapters (Kinnex adapter bcM0001-M0004) are available for Kinnex array formation step. **Note:** Kinnex concatenation workflow is not compatible with standard SMRTbell adapters from SMRTbell prep kit 3.0 and is also not compatible with SMRTbell barcoded adapter plate 3.0.

Kinnex array formation (cont.)

Kinnex terminal adapters incorporate barcode sequences to enable up to 4-plex sample multiplexing at the library level

- Kinnex adapters contain barcode sequences[1] to enable (optional) sample multiplexing at the SMRTbell library level (up to 4-plex)
  - Forward and reverse Kinnex adapter pairs are pre-mixed in Kinnex concatenation kits
  - Kinnex concatenation kits contain a total of 4 barcoded Kinnex adapter mixes (bcM0001-bcM0004) to enable multiplexing of up to 4 samples per SMRT Cell





[1] Kinnex adapter barcode sequences can be downloaded from SMRT Link Data Management module.

Kinnex array formation (cont.)

Kinnex terminal adapters use a new design that enables improved SMRT sequencing performance

- Kinnex adapters enable:
  - Longer polymerase read length → Improved HiFi conversion rate (HiFi reads/Total *P1* reads)
  - Improved *P1* loading efficiency

Forward and reverse Kinnex terminal adapters have **different structures**

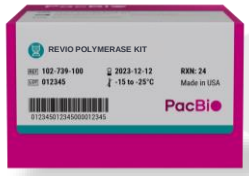
Annealed & bound Kinnex library molecule

- New Kinnex adapter design requires a different sequencing primer (Kinnex sequencing primer 103-179-000)

Sequel II binding kit 3.2 and cleanup beads (102-333-300)

Sequencing primer 3.2

Kinnex sequencing primer (103-179-000)



Revio polymerase kit[2] (102-817-600)

Standard sequencing primer

Kinnex sequencing primer (103-179-000)

[1] If needed, Kinnex sequencing primer may be provided free-of-charge to any customers that are still using older Sequel II binding kits or older Revio polymerase kits that do not already contain the primer.

[2] Revio polymerase kit includes SMRTbell cleanup beads.

Kinnex array formation (cont.)

Procedural notes

1. Input RNA quality control
2. cDNA synthesis + cleanup
3. cDNA amplification + cleanup
4. Kinnex PCR + cleanup
5. Kinnex array formation + cleanup
6. Nuclease treatment + cleanup

Safe stop

5.1 Kinnex array formation

✔	Step	Instructions
	5.1.1	In a 0.2 mL PCR tube, add 5 µg of sample from Step 4.2.15, in 39 µL of volume (128 ng/µL). Dilute with elution buffer going into this step if the sample is too concentrated.
		Add 2 µL of Kinnex adapter bc01–04 (select one barcode per library preparation). Note: if combining multiple barcoded Kinnex libraries for sequencing, make sure each library uses one of the 4 different Kinnex barcoded adapters.

Tube color	Components	Volume
Blue	Kinnex adapter bc	2 µL

	5.1.2	Add the following components in the listed order. If processing multiple samples, make a master mix with 10% overage. Pipette mix master mix.

Tube color	Components	Volume

	5.1.4	After running the Kinnex primer digestion/ligation program, add 2 µL of DNA repair mix directly to the Kinnex primer digestion/ligation sample.
	5.1.5	Thoroughly mix by pipetting up and down 10 times and then quick spin to collect all liquid.
	5.1.6	Run the DNA Damage Repair Program with the lid set to >55°C.

Step	Temperature	Duration
1	45°C	30 min
2	4°C	Hold

5.2 1X SMRTbell bead cleanup

✔	Step	Instructions
	5.2.1	Add 1X v/v (60 µL) of resuspended, room temperature SMRTbell cleanup beads to each sample.
	5.2.2	Pipette-mix the beads until evenly distributed and quick-spin in a microcentrifuge to collect liquid.

- Recommended input amount to proceed with Kinnex array formation is **5 µg** of Kinnex PCR amplicons (from Step 4)
  - Proceeding with <3 µg is **not recommended** since lower input amounts may lead to insufficient final library yields to enable optimal sequencing results
- **IMPORTANT:** If combining multiple barcoded Kinnex libraries for sequencing, make sure each library uses one of the 4 different Kinnex barcoded adapters
- Perform **DNA Damage Repair** step to repair nicked / damaged DNA sites within newly formed Kinnex array products
- Perform **1X** SMRTbell bead cleanup at room temp.

Nuclease treatment & cleanup

Perform nuclease treatment and final SMRTbell bead cleanup to remove incomplete SMRTbell template arrays

1 Input RNA quality control

↓ Safe stop

2 cDNA synthesis + cleanup

↓

3 cDNA amplification + cleanup

↓ Safe stop

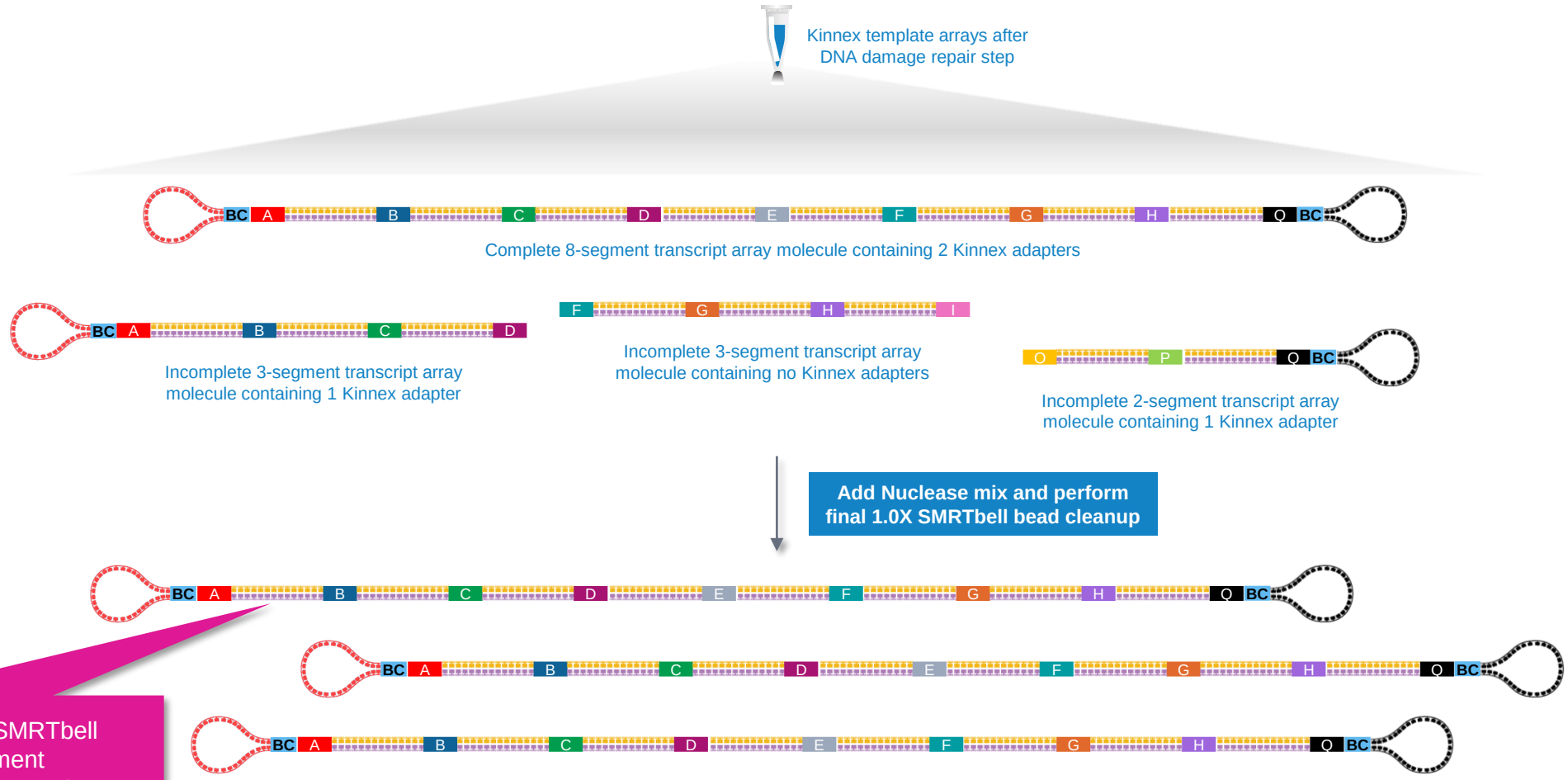
4 Kinnex PCR + cleanup

↓

5 Kinnex array formation + cleanup

↓ Safe stop

6 Nuclease treatment + cleanup



- After nuclease treatment, most remaining SMRTbell templates are complete (full-length) 8-segment transcript array molecules capped with Kinnex adapters

Nuclease treatment & cleanup (cont.)

Procedural notes

1 Input RNA quality control

↓ Safe stop

2 cDNA synthesis + cleanup

↓

3 cDNA amplification + cleanup

↓ Safe stop

4 Kinnex PCR + cleanup

↓

5 Kinnex array formation + cleanup

↓ Safe stop

6 Nuclease treatment + cleanup

6.1 Nuclease treatment

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

Nuclease master mix

Tube	Component	Volume
Light purple	Nuclease buffer (103-110-200)	5 µL
Light green	Nuclease mix (103-110-100)	5 µL
	Total volume	10 µL

	6.1.2	Add 10 µL of Nuclease Master mix to each sample. The total volume should be 50 µL.
	6.1.3	Thoroughly mix by pipetting up and down 10 times and then quick spin to collect all liquid.
	6.1.4	Run the nuclease treatment program with the lid set to >47°C.

Step	Temperature	Duration
1	37°C	15 min
2	4°C	Hold

- Perform nuclease treatment for **15 min**

Nuclease treatment & cleanup (cont.)

Procedural notes

1 Input RNA quality control

↓ Safe stop

2 cDNA synthesis + cleanup

↓

3 cDNA amplification + cleanup

↓ Safe stop

4 Kinnex PCR + cleanup

↓

5 Kinnex array formation + cleanup

↓ Safe stop

6 Nuclease treatment + cleanup

Final Kinnex library yield is typically sufficient to load **≥2 SMRT Cells**

6.2 Final cleanup with 1X SMRTbell cleanup beads

Step	Instructions
6.2.1	Add 50 µL SMRTbell cleanup beads to each sample from the previous step. Pipette-mix the beads until evenly distributed.
6.2.2	Quick-spin the tube strip in a microcentrifuge to collect all liquid.
6.2.3	Leave at room temperature for 10 minutes to allow DNA to bind the beads.
6.2.4	Place the tube strip in a magnetic separation rack until the beads separate fully from the solution.
6.2.5	Slowly pipette off the cleared supernatant without disturbing the beads and discard the supernatant.
6.2.6	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.
6.2.7	Repeat the previous step.
6.2.8	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from
6.2.13	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new PCR tube strip. Discard old tube strip with beads.
6.2.14	Take a 1 µL aliquot from each tube. Make a 1:5 dilution of the sample in elution buffer and measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Calculate the total mass. Expect 10-25% recovery of the starting Kinnex-PCR product.
	Recommended: Further dilute each aliquot to 250 pg/µL with the Femto Pulse dilution buffer. Measure the final SMRTbell library size distribution with a Femto Pulse system.
6.2.15	Proceed to SMRT Link Sample Setup to prepare the SMRTbell library for sequencing. **DNA concentration must be less than 60 ng/µl to go into ABC.** Using a concentration above 60 ng/µl will result in lower loading during sequencing.
6.2.16	Store SMRTbell libraries at 4°C if sequencing within the week. Long-term storage should be at -20°C. Minimize freeze-thaw cycles when handling SMRTbell libraries.
	PROTOCOL COMPLETE

- Perform **DNA concentration QC** on final purified Kinnex RNA library using a Qubit dsDNA HS assay
  - Typical final SMRTbell library yield from 5 µg of input DNA into Kinnex array formation is **~10 – 25%** – a much higher observed yield might suggest incomplete digestion of partial SMRTbell templates
  - **Troubleshooting tip:** If SMRTbell library yield is higher than expected and *P1* loading is lower than expected, consider repeating the nuclease treatment step

- Perform **DNA sizing QC** on final purified Kinnex full-length RNA library using a Femto Pulse system (expected final library insert size is **~12 – 16 kb**)

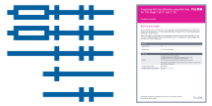
- Kinnex full-length RNA final SMRTbell library concentration must be **≤60 ng/ µL** to proceed with SMRT Link sample setup (ABC[1])
  - → Using a concentration above 60 ng/µL will result in lower loading during sequencing

Kinnex full-length RNA library prep inputs & expected step yields

Final Kinnex library yield is typically sufficient to load ≥2 SMRT Cells

Kinnex FL RNA

Preparing Kinnex libraries using the Kinnex full-length RNA kit (103-238-700)



Library insert generation

cDNA synthesis → **SMRTbell bead cleanup** → **cDNA amplification** → **SMRTbell bead cleanup**

IN: 300 ng of total RNA per sample

OUT: ≥100 ng of amplified cDNA per sample

Kinnex PCR

Kinnex PCR → **SMRTbell bead cleanup**

IN: 55 ng of amplified cDNA (single or multiplex)

OUT: 6-12 µg of amplified products after pooling

Kinnex array formation

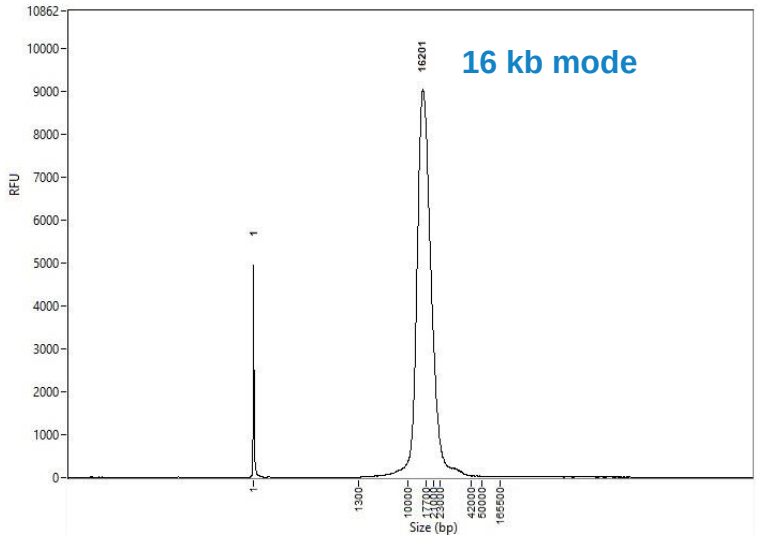
Kinnex digestion / Ligation → **DNA damage repair** → **SMRTbell cleanup** → **Nuclease treatment** → **SMRTbell bead cleanup**

IN: 5 µg of pooled Kinnex PCR products

OUT: 80% recovery from Kinnex digestion Rx input

IN: 40 µL of DDR reaction products

OUT: 10-25% recovery from Kinnex digestion Rx input



Example Femto pulse DNA sizing QC analysis results for final Kinnex full-length RNA library prepared with human universal human reference RNA (UHRR) total RNA sample.

Example Kinnex full-length RNA library prep yields

Total RNA input for cDNA synthesis	300 ng
cDNA input for Kinnex array formation	5900 ng
Post-nuclease treatment & final library cleanup yield (%)[1]	1460 ng (24.7%)

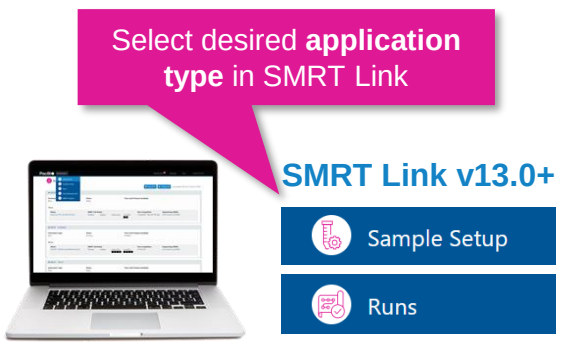
[1] **Post-nuclease treatment & final cleanup yields typically ranged from ~10% to ~25% when using UHRR total RNA samples for Kinnex full-length RNA library construction.**

Final Kinnex library yield is typically sufficient to load **≥2 SMRT Cells**

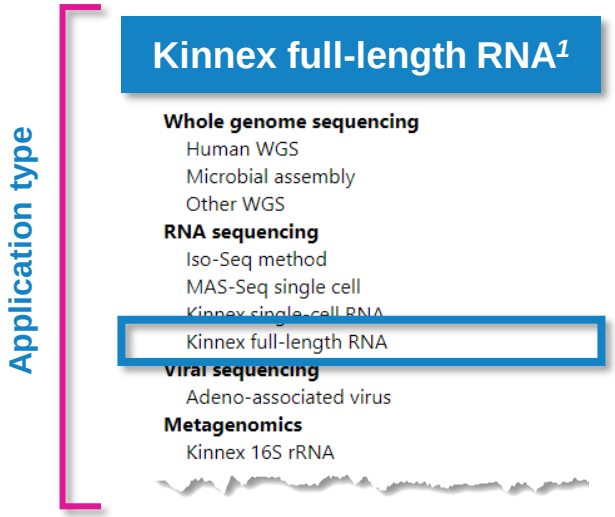
Kinnex full-length RNA sequencing preparation workflow details

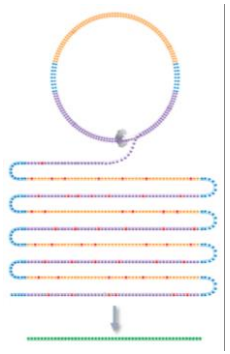
Sample Setup & Run Design recommendations for Kinnex full-length RNA libraries

SMRT Link supports Kinnex full-length RNA sequencing preparation & analysis workflow for PacBio systems[1]



Recommended target *P1* loading range for Revio system is **~50 – 70%**



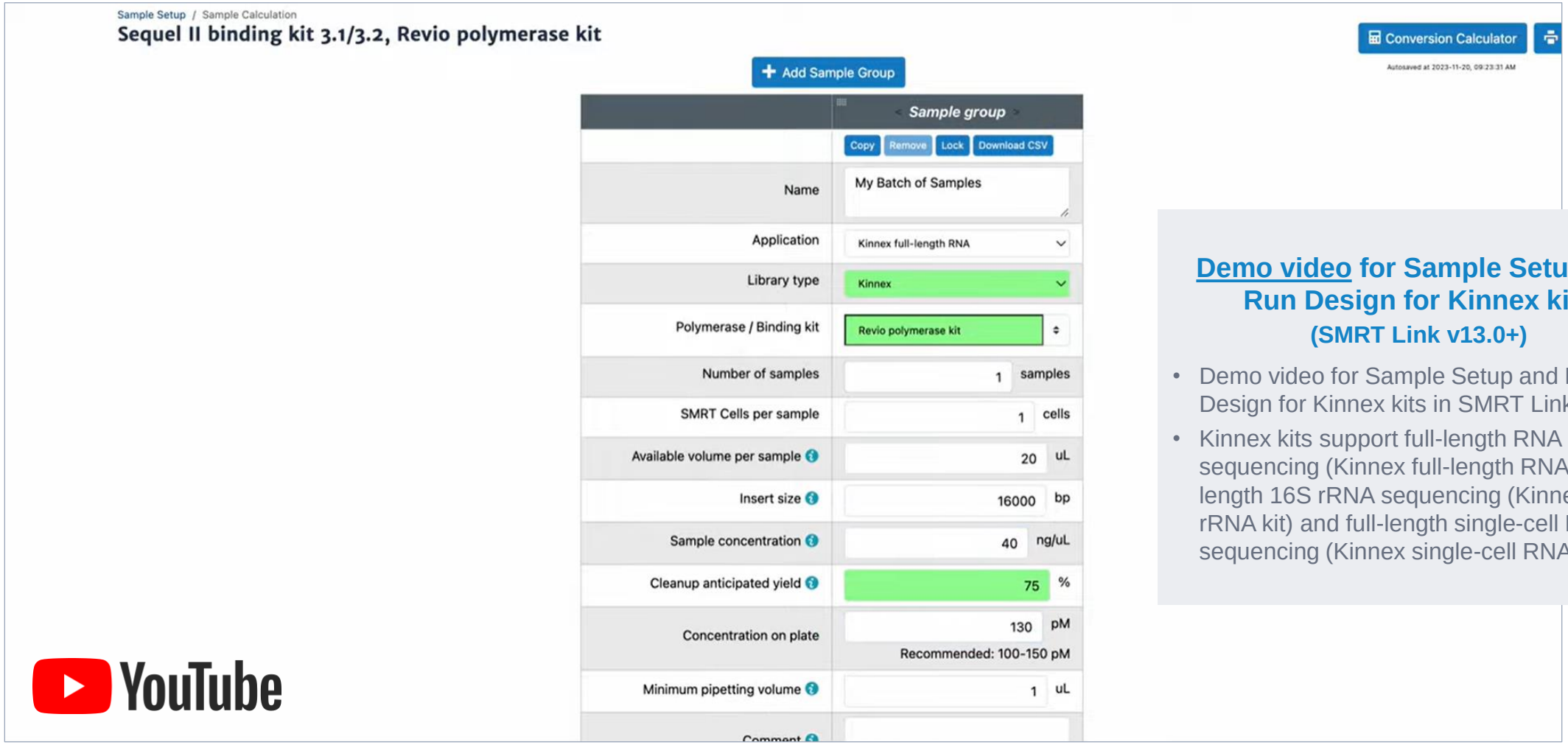


SMRT Link module	Key setup parameters For Kinnex libraries	Sequel II/IIe system recommended settings for Kinnex libraries	Revio system recommended settings for Kinnex libraries
Sample setup	**Library type**	Kinnex	
	Primer	Kinnex sequencing primer	
	Binding/Polymerase kit[1]	Sequel II binding kit 3.2 (includes Kinnex sequencing primer)	Revio polymerase kit (includes Kinnex sequencing primer)
	Concentration on plate	40 – 60 pM	100 – 150 pM
Runs → Run design	**Adapter / Library type**	SMRTbell Adapter Design = SMRTbell Kinnex Prep Kit	Library type = Kinnex
	Movie collection time	30 hrs	24 hrs
	Use adaptive loading	YES	
	On-instrument CCS	CCS Analysis Output - Include Low Quality Reads = NO CCS Analysis Output - Include Kinetics Information = NO	Consensus Mode = MOLECULE

[1] Kinnex full-length RNA kit requires SMRT Link v13.0 or higher.

SMRT Link Sample Setup and Run Design for Kinnex kits video demonstration

Video demonstration of SMRT Link Sample Setup and Run Design setup procedure for Kinnex kits supporting full-length RNA sequencing, single-cell RNA sequencing and full-length 16S rRNA sequencing



Demo video for Sample Setup and Run Design for Kinnex kits
(SMRT Link v13.0+)

- Demo video for Sample Setup and Run Design for Kinnex kits in SMRT Link v13.0+
- Kinnex kits support full-length RNA sequencing (Kinnex full-length RNA kit), full-length 16S rRNA sequencing (Kinnex 16S rRNA kit) and full-length single-cell RNA sequencing (Kinnex single-cell RNA kit)

SMRT Link Sample Setup procedure for Kinnex full-length RNA libraries

Revio system

	Sample group
	Copy · Remove · Lock · Download CSV
Name	Kinnex full-length RNA library demo
Application	Kinnex full-length RNA
Library type	Kinnex
Polymerase / Binding kit	Revio polymerase kit
Number of samples	1 samples
SMRT Cells per sample	1 cells
Available volume per sample	20 uL
Insert size	16000 bp
Sample concentration	40 ng/uL
Cleanup anticipated yield	75 %
Concentration on plate	130 pM Recommended: 100-150 pM
Minimum pipetting volume	1 uL
Comment	Kinnex library containing array of 8 FL cDNA segments

Sequel II and IIe systems

	Sample group
	Copy · Remove · Lock · Download CSV
Name	Kinnex full-length RNA library demo
Application	Kinnex full-length RNA
Library type	Kinnex
Polymerase / Binding kit	Sequel® II Binding Kit 3.2
Number of samples	1 samples
SMRT Cells per sample	1 cells
Available volume per sample	20 uL
Insert size	16000 bp
Sample concentration	40 ng/uL
Cleanup anticipated yield	75 %
Concentration on plate	50 pM Recommended: 40-60 pM
Minimum pipetting volume	1 uL
Comment	Kinnex library containing array of 8 FL cDNA segments

- Select **application type** to autofill fields in green

IMPORTANT: Specify **Library type = Kinnex**

- Library type field determines sequencing primer type to use for annealing step
  - → Kinnex libraries require use of **Kinnex sequencing primer**[1]

- Select **Revio polymerase kit** for Revio system and **Sequel II Binding Kit 3.2** for Sequel II/IIe systems

- Recommended Kinnex full-length RNA library input concentration for sample setup is **20 – 60 ng/µL**

- Recommended OPLC range is **100 – 150 pM** for Revio system and **40 – 60 pM** for Sequel II/IIe systems

- **Recommended target *P1* loading range**
- Revio system: **~50 – 70%**
- Sequel II and IIe systems: **~60 – 80%**

[1] Sample Setup **Library type** field specifies structure of SMRTbell library and determines sequencing primer type to use for annealing step. For Kinnex libraries, the forward and reverse Kinnex terminal SMRTbell adapters have different structures and require use of Kinnex sequencing primer for primer annealing step. (View Video tutorial – ***SMRT Link Sample Setup and Run Design setup procedure for Kinnex kits*** at: YouTube Link)

SMRT Link Run Design procedure for Revio system

Sample and run information

Select desired **application type** to autofill Library Type, Polymerase Kit & Movie Acquisition Time recommended settings

Specify **Kinnex** library type (instead of Standard or AAV)[1]

Specify **Revio polymerase kit**

Kinnex full-length RNA

Plate 1, Well A01: Kinnex full-length RNA library demo

Application (Required)	Kinnex full-length RNA
Plate Well (Required)	Plate 1, Well A01
Well Name (Required)	Kinnex full-length RNA library demo
Well Comment	
Library Type (Required)	Kinnex
Insert Size (bp) (Required)	16000
Polymerase Kit (Required)	Revio polymerase kit
Movie Acquisition Time (hours)	24

Specify **Use Adaptive Loading = YES**

Use Adaptive Loading: YES / NO

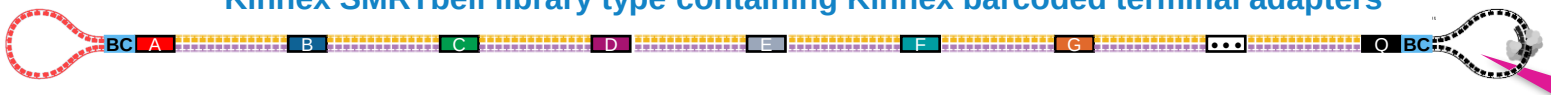
Specify **Insert Size**

Recommend **24 hrs** movie collection for Revio Kinnex samples

Standard SMRTbell library type containing standard SPK3 barcoded terminal adapters

Forward and reverse standard terminal adapters have the **same structure**

Kinnex SMRTbell library type containing Kinnex barcoded terminal adapters



Forward and reverse Kinnex terminal adapters have **different structures**

Library Type field determines which adapter finding algorithm is used during post-primary analysis[1]

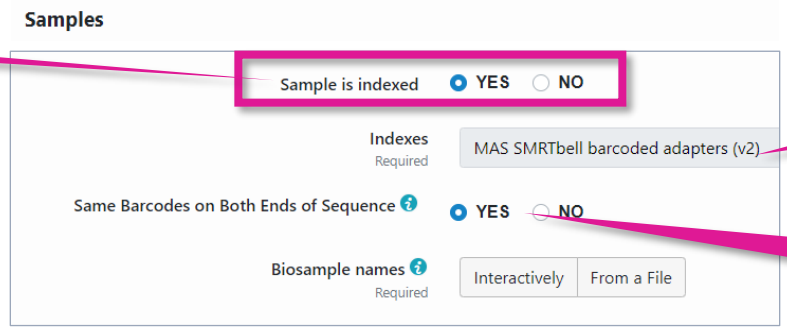
[1] **Note:** When sequencing a Kinnex library sample, if 'Standard' library type is mistakenly selected instead of 'Kinnex' then a higher missing adapter rate (> 95%) and a slight degradation in barcode demultiplexing performance (~93-96% barcoded HiFi read yield) will be observed.

SMRT Link Run Design procedure for Revio system (cont.)

Sample indexing (barcoding) information

Kinnex full-length RNA

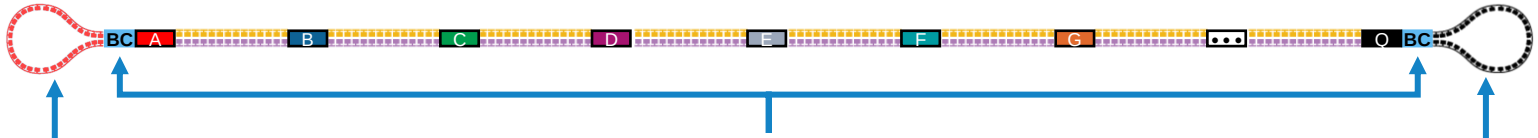
Default = **YES** for **Sample is indexed**



Specify **Indexes** FASTA
= **MAS SMRTbell barcoded adapters (v2)**

Specify **YES** for **Same barcodes on both ends of sequences**

Example complete Kinnex library molecule containing barcoded Kinnex terminal MAS adapters[1] at both ends

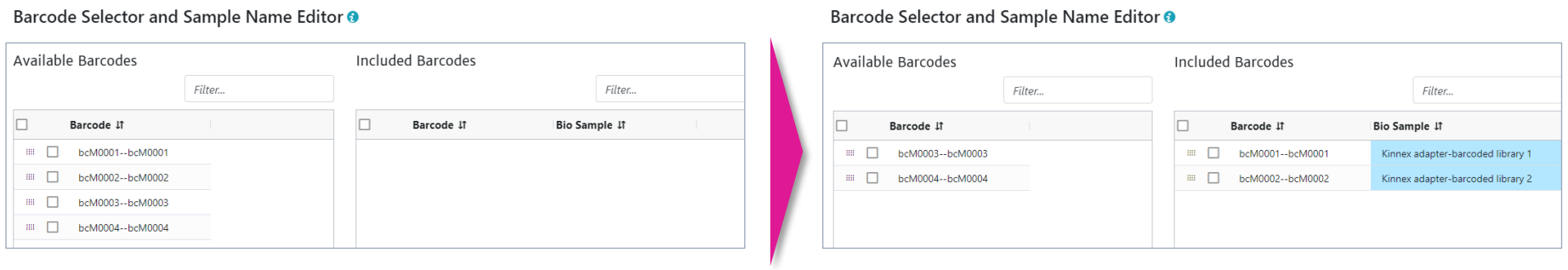


Barcoded forward Kinnex terminal adapter

Both forward & reverse Kinnex terminal adapters contain the same barcode sequence

Barcoded reverse Kinnex terminal adapter

Example interactive biosample name specification for a multiplexed Kinnex library sample



SMRT Link

Data Management

MAS SMRTbell barcoded adapter indexes

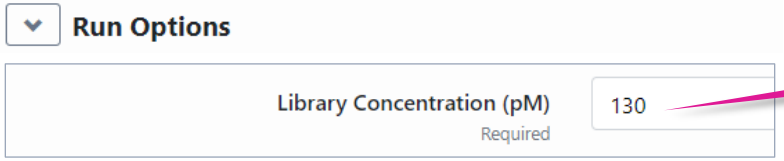
```
>bcM0001
ACAGTC
>bcM0002
ATGACG
>bcM0003
CACGTG
>bcM0004
CATCGC
```

[1] Four barcoded terminal Kinnex adapters (Kinnex adapter bcM0001-bcM0004) are available for Kinnex array formation step. Kinnex adapter barcode sequences can be downloaded from SMRT Link Data Management module.

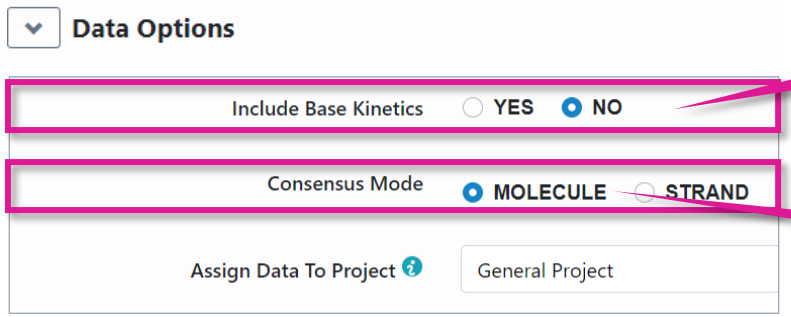
SMRT Link Run Design procedure for Revio system (cont.)

Run options and data options

Kinnex full-length RNA



On-plate loading concentration is **required** for Revio samples



Default = **NO** for **Include Base Kinetics**

Default **Consensus Mode = MOLECULE**[1]

Can leave Include Base Kinetics and Consensus Mode fields at their default settings for Kinnex library samples

[1] **Molecule Consensus Mode** → generates a single circular consensus sequencing read for a given library insert molecule. **Strand Consensus Mode** → generates a consensus sequence for each strand (forward & reverse) of a given library insert molecule.

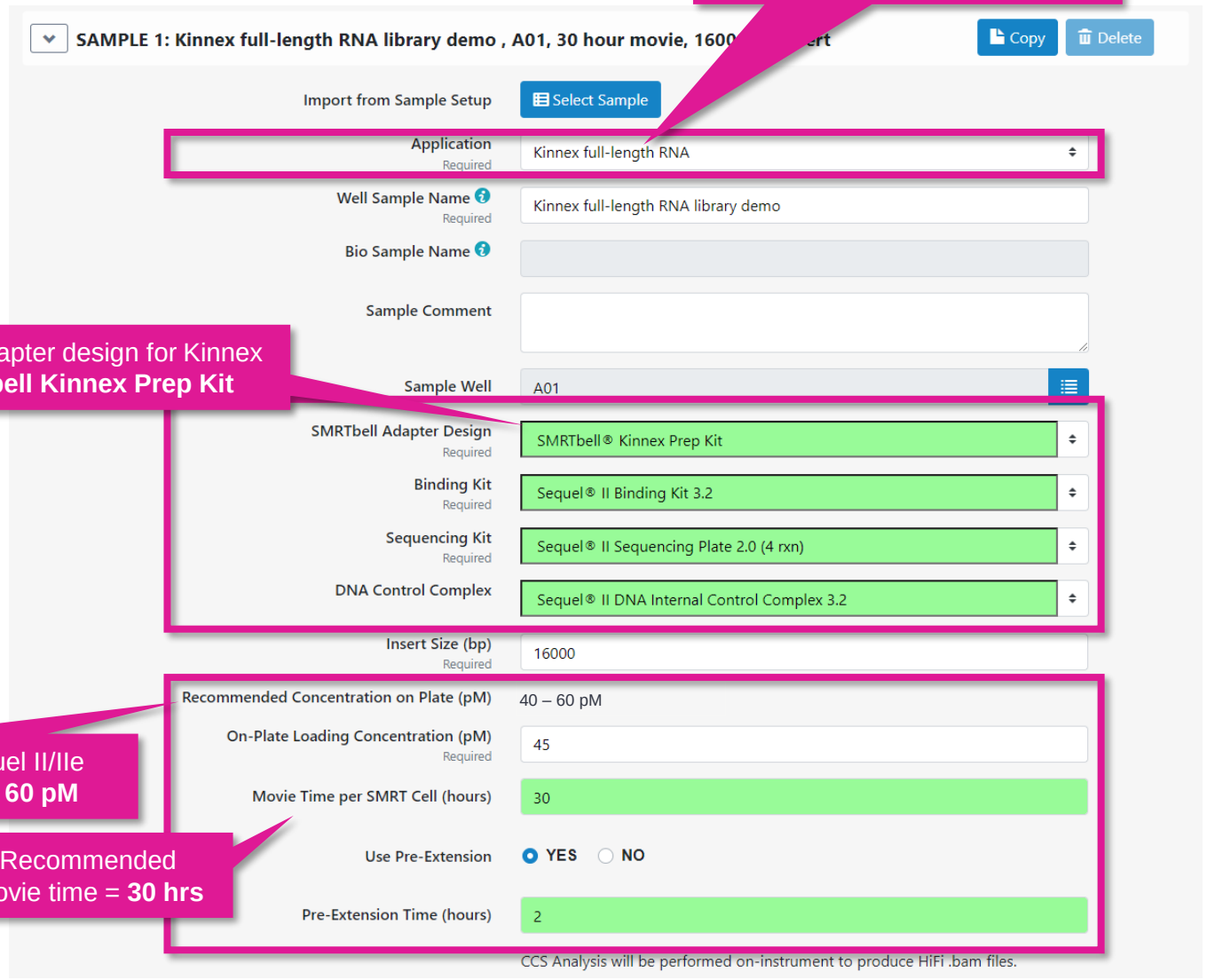
SMRT Link Run Design procedure for Sequel II/IIe systems

Sample information and run information

Kinnex full-length RNA

- Select desired Kinnex application from the **Application** field drop-down menu
- The following fields are auto-populated with default recommended values and high-lighted in green:
  - ❑ **SMRTbell Adapter Design**
    - → SMRTbell Kinnex Prep Kit
  - ❑ **Binding Kit**
    - → Sequel II Binding Kit 3.2
  - ❑ **Sequencing Kit**
    - → Sequel II Sequencing Plate 2.0 (4 rxn or 1 rxn)
  - ❑ **DNA Control Complex**
    - → Sequel II DNA Internal Control Complex 3.2
  - ❑ **Movie Time per SMRT Cell**
    - → 30 hrs
  - ❑ **Pre-Extension Time**
    - → 2 hrs

SMRTbell Adapter Design field determines which adapter finding algorithm is used during post-primary analysis[1]



Example sample information entered into a Sequel IIe system run design worksheet for a Kinnex full-length RNA library sample.

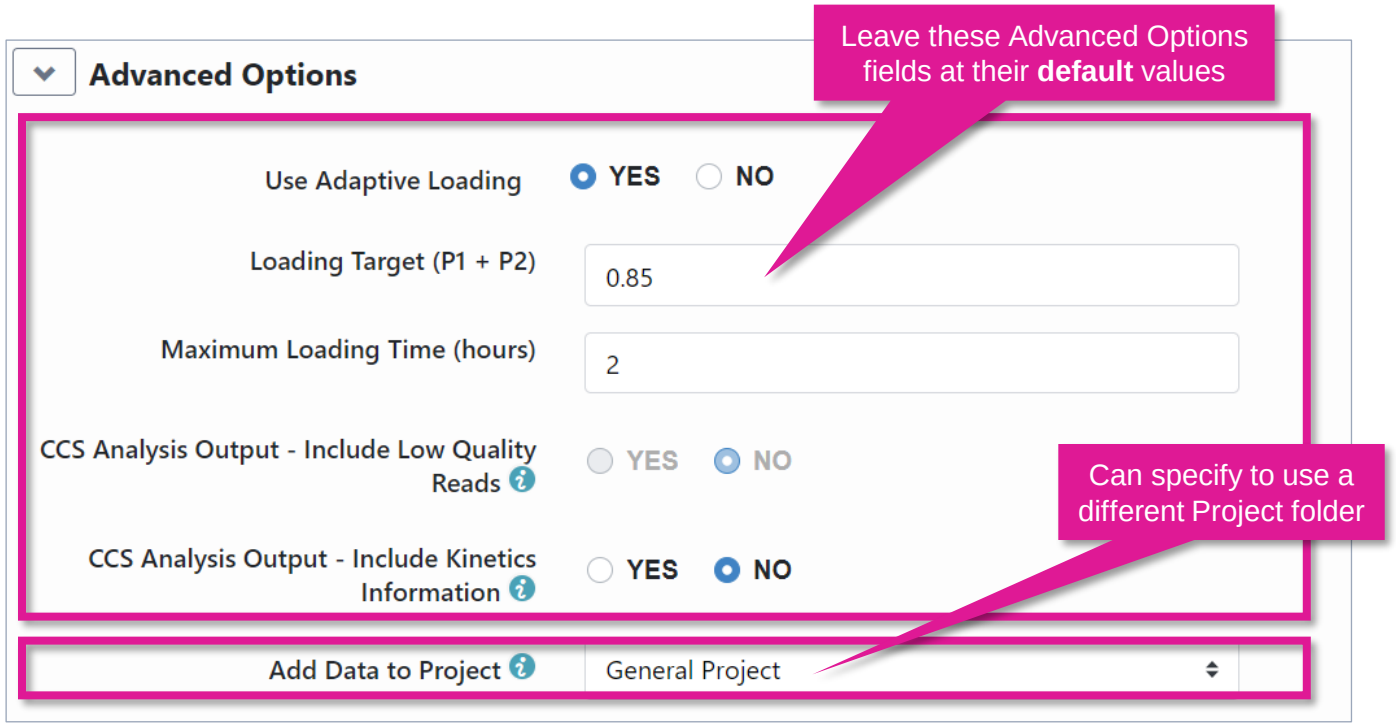
[1] **Note:** When sequencing a Kinnex library sample, if 'Overhand-SMRTbell Prep Kit 3.0' is mistakenly selected instead of 'SMRTbell Kinnex Prep kit' in the SMRTbell Adapter Design field, then a higher missing adapter rate (> 95%) and a slight degradation in barcode demultiplexing performance (~93-96% barcoded HiFi read yield) will be observed.

SMRT Link Run Design procedure for Sequel II/IIe systems (cont.)

Advanced options

Kinnex full-length RNA

- For all Kinnex library samples, leave the following **Advanced Options** fields at their **default settings**
  - ❑ **Use Adaptive Loading**
    - → YES
  - ❑ **Loading Target (P1 + P2)**
    - → 0.85
  - ❑ **Maximum Loading Time**
    - → 2 hours
  - ❑ **CCS Analysis Output - Include Low Quality Reads**
    - → NO
  - ❑ **CCS Analysis Output - Include Kinetics Information**
    - → NO
  - ❑ **Pre-Extension Time**
    - → 2 hrs
- If desired, specify to use an alternative project folder for the **Add Data to Project** field



Example default Advanced Options settings entered into a Sequel IIe system run design worksheet for a Kinnex full-length RNA library sample.

SMRT Link Run Design procedure for Sequel II/IIe systems (cont.)

Barcoded sample options

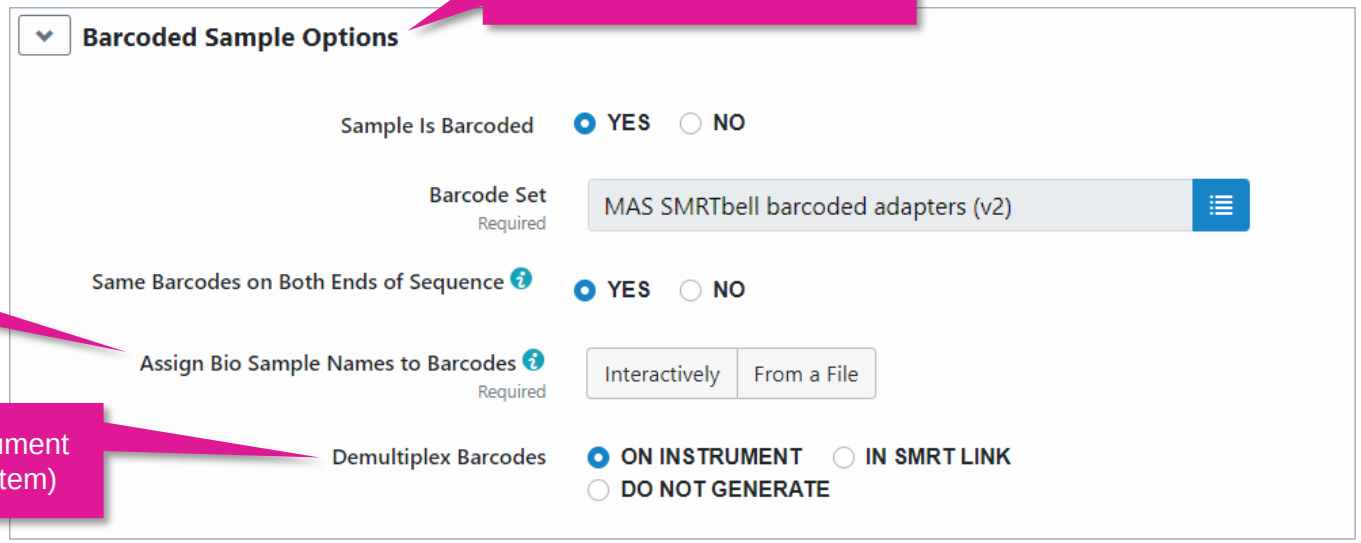
Kinnex full-length RNA

- For Kinnex library samples, can leave most **Barcoded Sample Options** fields at their **default settings**

Specify Bio Sample Names, either interactively or by downloading a CSV file (**Interactively** or **From a file**)

If desired, specify to perform barcode demultiplexing on-instrument or in SMRT Link (default = **On-instrument** for Sequel IIe system)

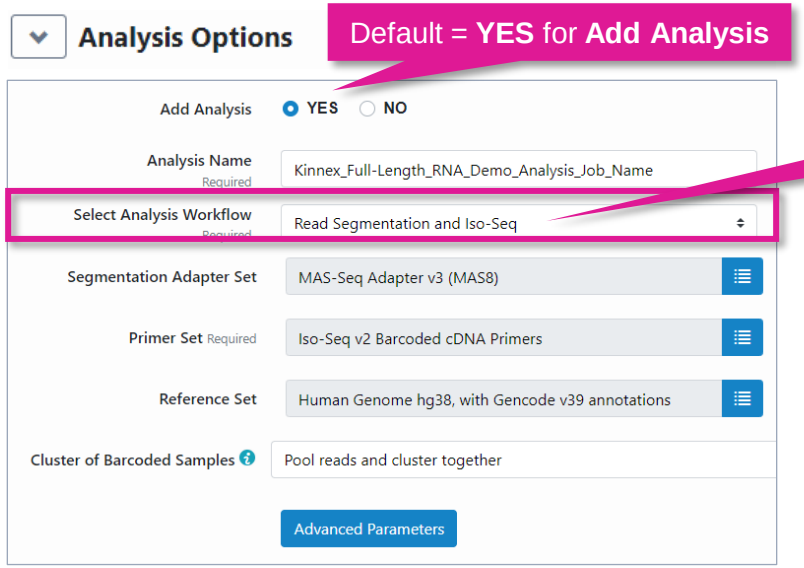
Can leave most of these fields at their default **values**



Example default Barcoded Sample Options settings entered into a Sequel IIe system run design worksheet for a Kinnex full-length RNA library sample.

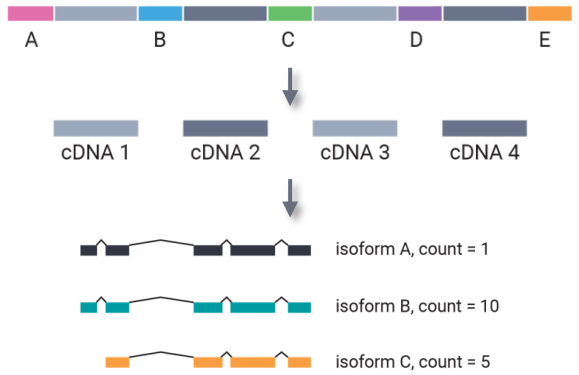
SMRT Link Run Design analysis options for Revio system and Sequel II/IIe systems

Kinnex full-length RNA



Analysis Workflow is automatically filled in (Default = Read Segmentation and Iso-Seq)

Read Segmentation and Iso-Seq

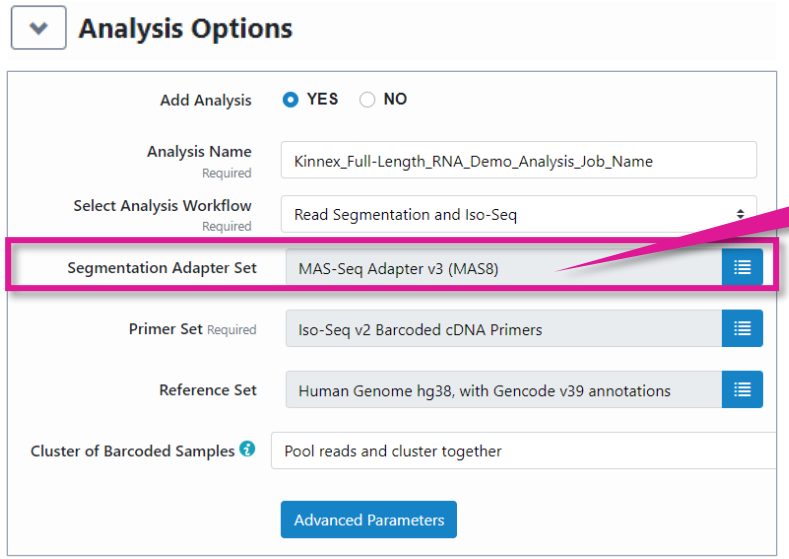


Perform isoform-classification analysis to identify novel genes & isoforms with abundance information (raw counts & normalized counts per million)

See ***SMRT Link User Guide*** documentation for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application.

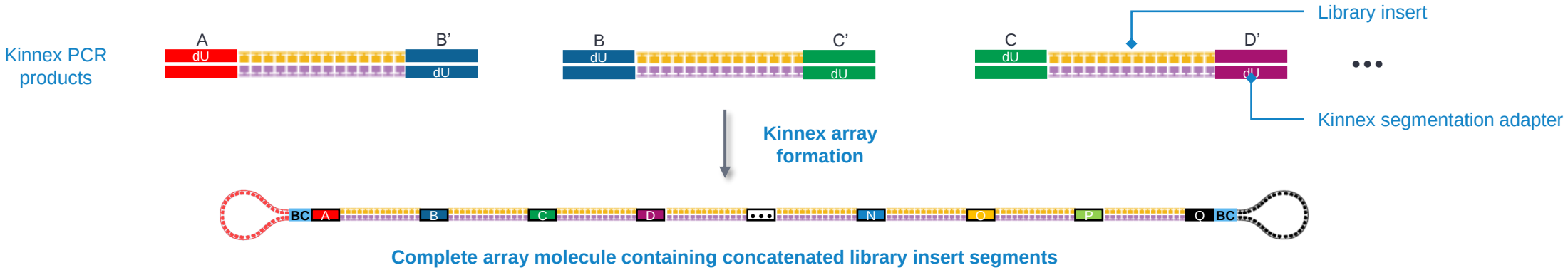
SMRT Link Run Design analysis options for Revio system and Sequel II/IIe systems (cont.)

Kinnex full-length RNA



Specify **Segmentation Adapter Set** that corresponds to the Kinnex library concatenation method used

→ For Kinnex full-length RNA samples, specify **MAS-Seq Adapter v3 (MAS8)**



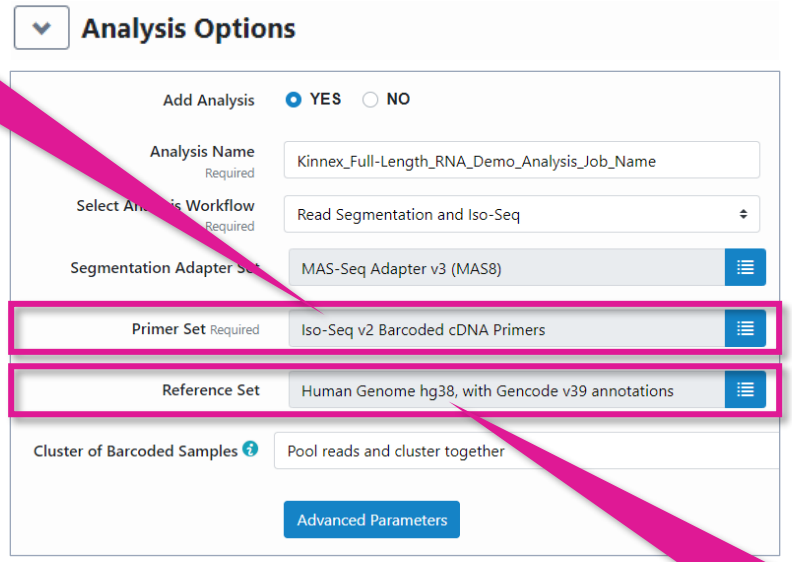
Complete array molecule containing concatenated library insert segments

See ***SMRT Link User Guide*** documentation for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application.

SMRT Link Run Design analysis options for Revio system and Sequel II/IIe systems (cont.)

Specify **Primer Set** used for cDNA amplification

Kinnex full-length RNA



Specify primer sequence file in FASTA format to **identify cDNA primers for removal** (include the 5' and 3' cDNA primers)

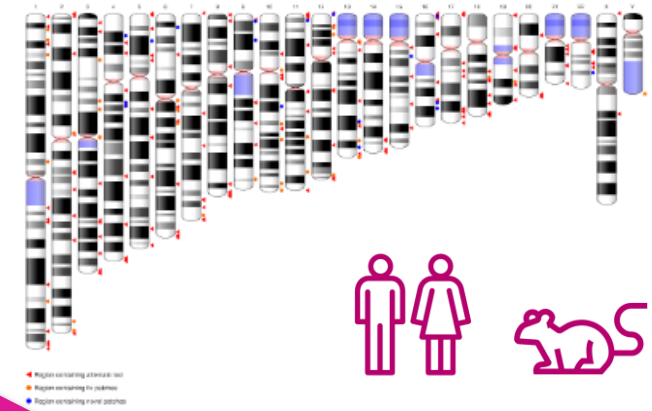
Forward (F) PCR primer
(Barcoded Iso-Seq primer bcXX[2])

Reverse (R) PCR primer
(Iso-Seq cDNA amplification primer)

For Kinnex full-length RNA analysis, select 'Iso-Seq v2 Barcoded cDNA primers'

```
>IsoSeqX_bc01_5p
CTACACGACGCTCTTCCGATCTACTACACGCAATGAAGTCGCAGGGTTGGG
>IsoSeqX_bc02_5p
CTACACGACGCTCTTCCGATCTACTAGTAGCAATGAAGTCGCAGGGTTGGG
>IsoSeqX_bc03_5p
CTACACGACGCTCTTCCGATCTAGTGTACGCAATGAAGTCGCAGGGTTGGG
                    ⋮
>IsoSeqX_bc11_5p
CTACACGACGCTCTTCCGATCTGTAGATAGCAATGAAGTCGCAGGGTTGGG
>IsoSeqX_bc12_5p
CTACACGACGCTCTTCCGATCTGTATGACGCAATGAAGTCGCAGGGTTGGG
>IsoSeqX_3p
AAGCAGTGGTATCAACGCAGAGTAC
```

Specify reference genome & annotation sets to align high quality isoforms to, and to collapse isoforms mapped to the same genomic loci.



Specify **Reference** Set. Default sets are:

- Human Genome hg38, with Gencode v39 annotations
- Mouse Genome mm39, with Gencode vM28 annotations

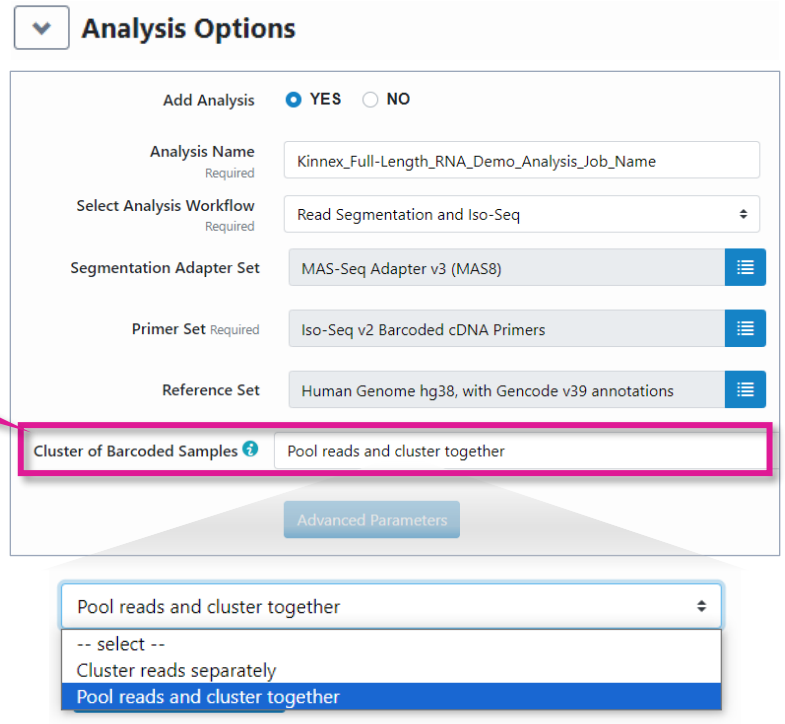
PacBio

See ***SMRT Link User Guide*** documentation for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application. To view Iso-Seq v2 Barcoded cDNA primer sequences, go to SMRT Link Data Mangement module and select to view 'Barcode Sets' > 'Iso-Seq v2 Barcoded cDNA Primers'.

SMRT Link Run Design analysis options for Revio system and Sequel II/IIe systems (cont.)

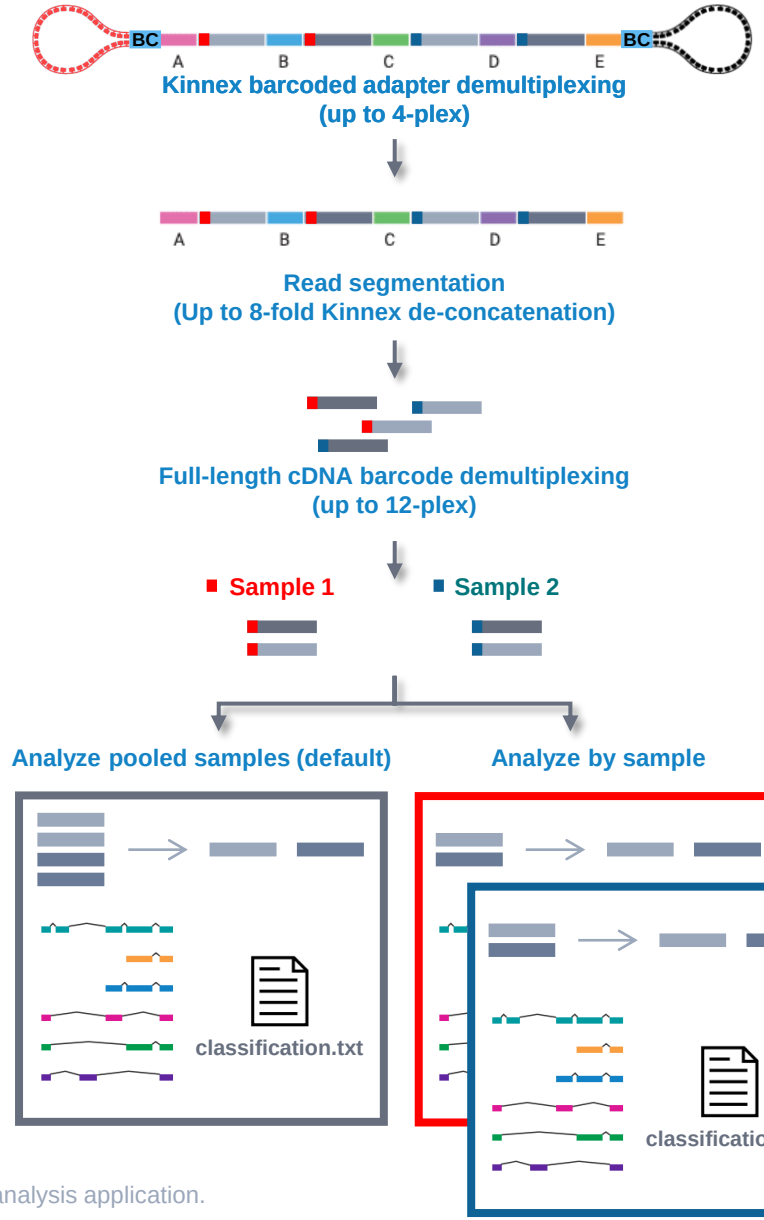
Specify **how to perform read clustering for barcoded samples**

Kinnex full-length RNA



Specification of **Cluster of Barcoded Samples** setting **determines whether all FLNC reads will be pooled for clustering** (Does not apply to non-barcoded samples.)

Clustering options for Kinnex full-length RNA samples



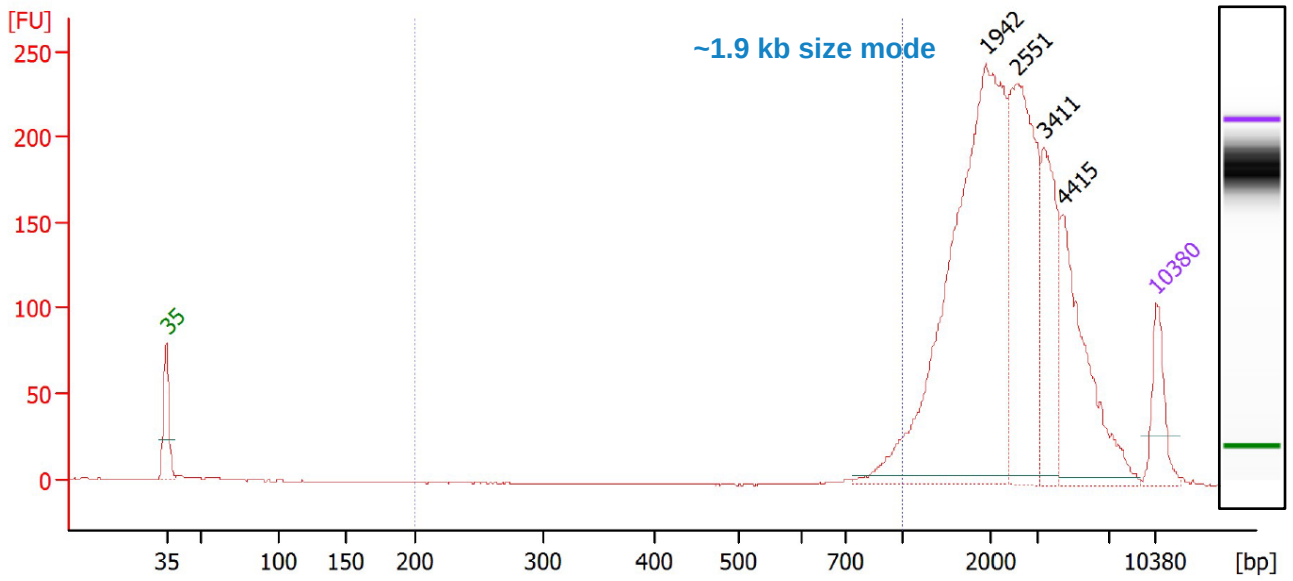
See ***SMRT Link User Guide*** documentation for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application.

Kinnex full-length RNA example sequencing performance data

Example Kinnex full-length RNA library preparation QC results

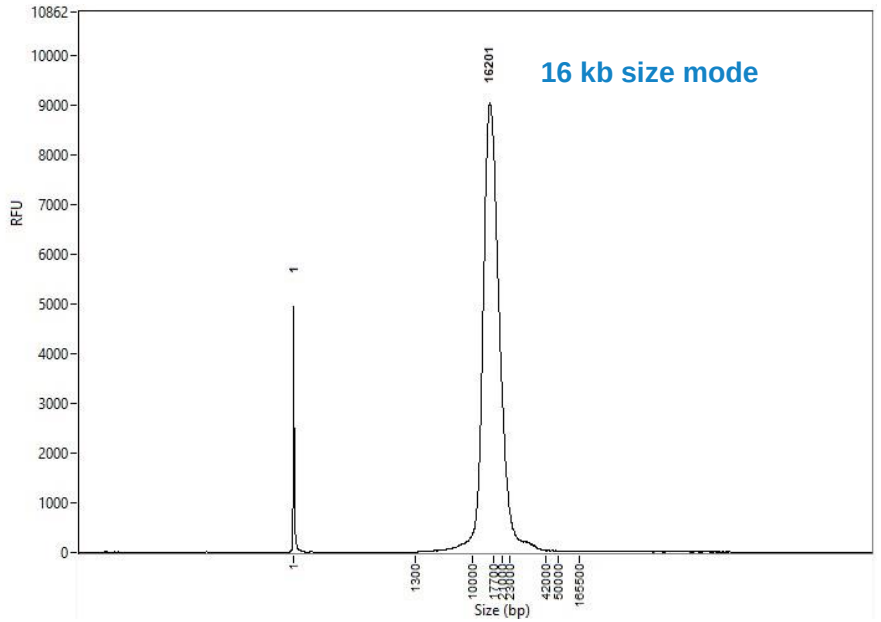
Kinnex full-length RNA library prepared with human UHRR total RNA

Amplified full-length cDNA QC



Example Bioanalyzer DNA sizing QC analysis results for amplified full-length cDNA generated from a universal human RNA reference (UHRR) total RNA sample.

Final Kinnex full-length RNA library QC



Example Femto Pulse DNA sizing QC analysis results for final Kinnex full-length RNA library.

Final Kinnex library yield is typically sufficient to load **≥2 SMRT Cells**

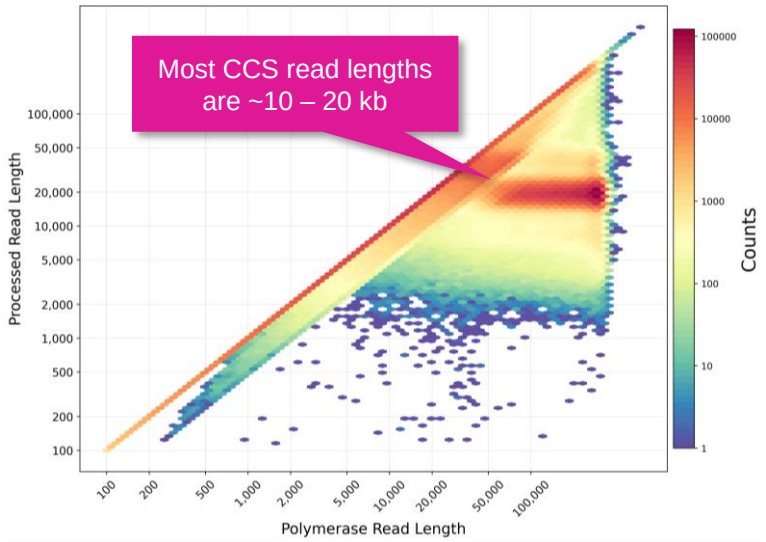
Total RNA input for cDNA synthesis	300 ng
cDNA input for Kinnex array formation	5900 ng
Post-nuclease treatment & final library cleanup yield (%)[1]	1460 ng (24.7%)

[1] Post-nuclease treatment & final cleanup yields typically ranged from ~10% to ~25% when using UHRR total RNA samples for Kinnex full-length RNA library construction.

Example sequencing performance for Kinnex full-length RNA libraries prepared with human cDNA

Sequel IIe system example data[1]

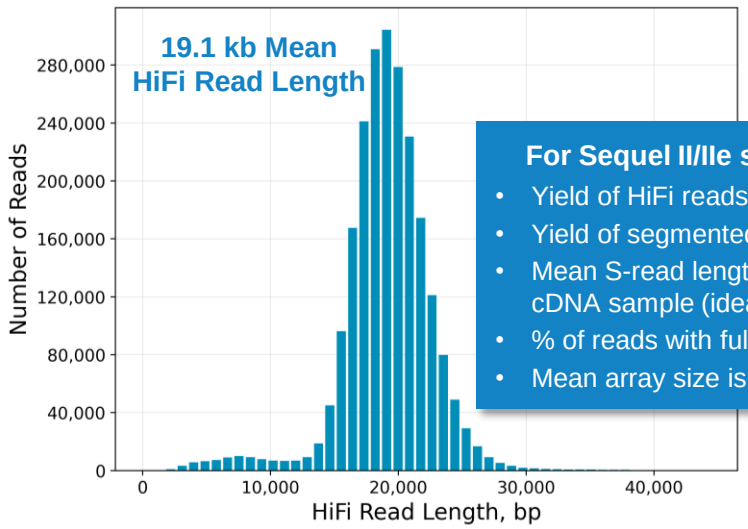
Raw Data Report



Raw Base Yield	652 Gb
Mean Polymerase Read Length	115.36 kb
P0	28%
P1	71%
P2	1%

Example sequencing metrics for a Universal Human Reference RNA (UHRR) Kinnex full-length RNA library sample run on a Sequel IIe system with Binding Kit 3.2 (Polymerase 2.2) / 80 pM on-plate loading concentration (OPLC) / 30-hrs movie time / 2-hrs pre-extension time.

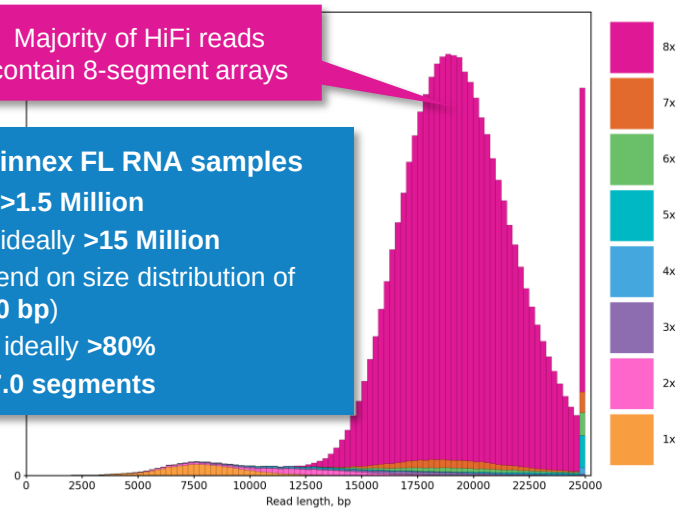
HiFi Read Length



HiFi Reads	2.3 M
HiFi Base Yield	43.2 Gb
Mean HiFi Read Length	19.1 kb
Median HiFi Read Quality	Q32
HiFi Read Mean # of Passes	10

For UHRR Kinnex full-length RNA libraries, per-SMRT Cell 8M HiFi read counts typically ranged from ~2 – 3 Million depending on the final library insert size.

Read Segmentation Metrics



For Sequel II/IIe system Kinnex FL RNA samples

- Yield of HiFi reads is ideally **>1.5 Million**
- Yield of segmented reads is ideally **>15 Million**
- Mean S-read length will depend on size distribution of cDNA sample (ideally **>1,500 bp**)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>7.0 segments**

Reads	2,260,039
Segmented reads (S-reads)	17,213,165
Mean length of S-reads	2,420 bp
Percent of reads with full arrays	91.07%
Mean array size (concentration factor)	7.62

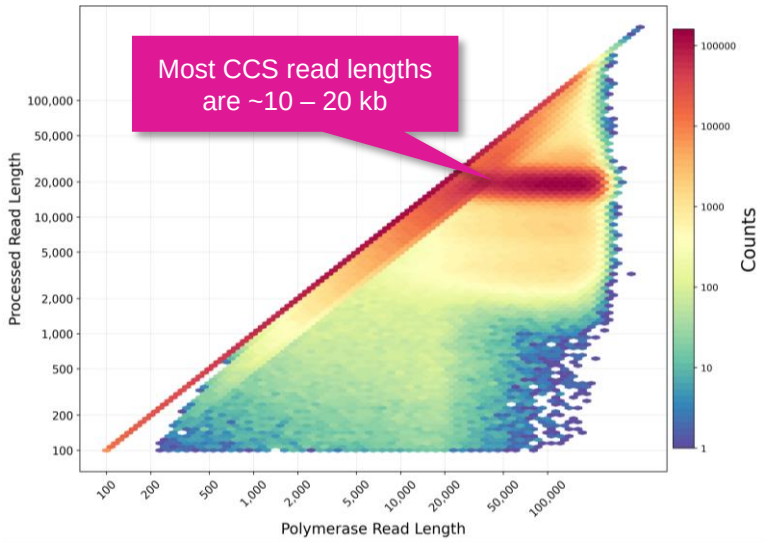
For UHRR Kinnex libraries, per-SMRT Cell 8M segmentation read counts were typically ~15 – 20 Million.

[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time.
Note: Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in HiFi data yields <30 Gb per Sequel II SMRT Cell 8M.

Example sequencing performance for Kinnex full-length RNA libraries prepared with human cDNA

Revio system example data[1]

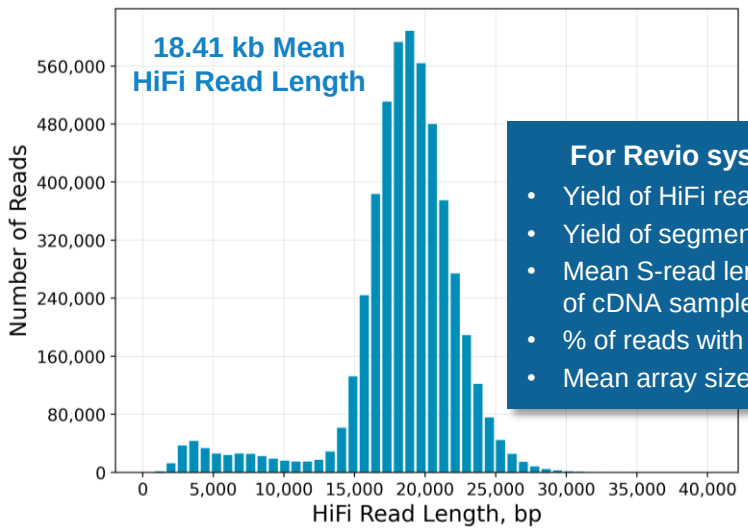
Raw Data Report



Raw Base Yield	1,168 Gb
Mean Polymerase Read Length	58.6 kb
P0	16%
P1	79%
P2	5%

Example sequencing metrics for a Universal Human Reference RNA (UHRR) Kinnex full-length RNA library sample run on a Revio system with Revio polymerase kit / 130 pM on-plate loading concentration (OPLC) / 24-hrs movie time.

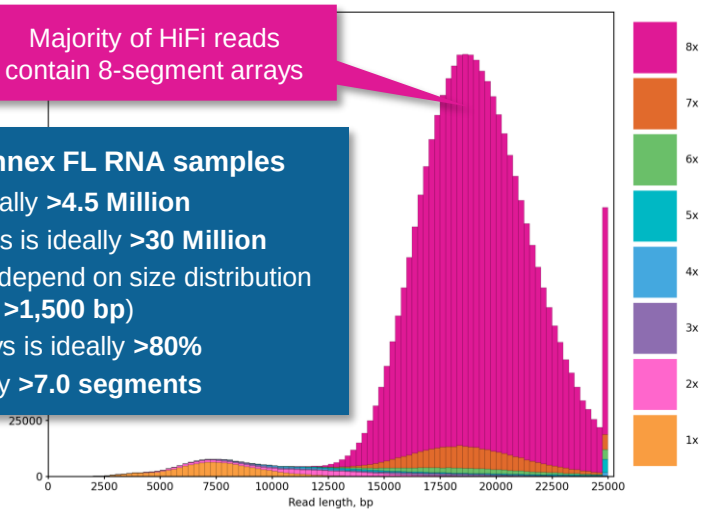
HiFi Read Length



HiFi Reads	5.1 M
HiFi Base Yield	93.47 Gb
Mean HiFi Read Length	18.41 kb
Median HiFi Read Quality	Q28
HiFi Read Mean # of Passes	7

For UHRR Kinnex full-length RNA libraries, per-Revio SMRT Cell HiFi read counts were typically ~5 – 6 Million depending on the final library insert size and *P1* loading performance.

Read Segmentation Metrics



For Revio system Kinnex FL RNA samples

- Yield of HiFi reads is ideally **>4.5 Million**
- Yield of segmented reads is ideally **>30 Million**
- Mean S-read length will depend on size distribution of cDNA sample (ideally **>1,500 bp**)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>7.0 segments**

Input HiFi Reads	5,027,154
Segmented reads (S-reads)	37,216,151
Mean length of S-reads	2,393 bp
Percent of reads with full arrays	85.84%
Mean array size (concentration factor)	7.40

For UHRR Kinnex libraries, per-Revio SMRT Cell segmentation read counts were typically ~30 – 45 Million.

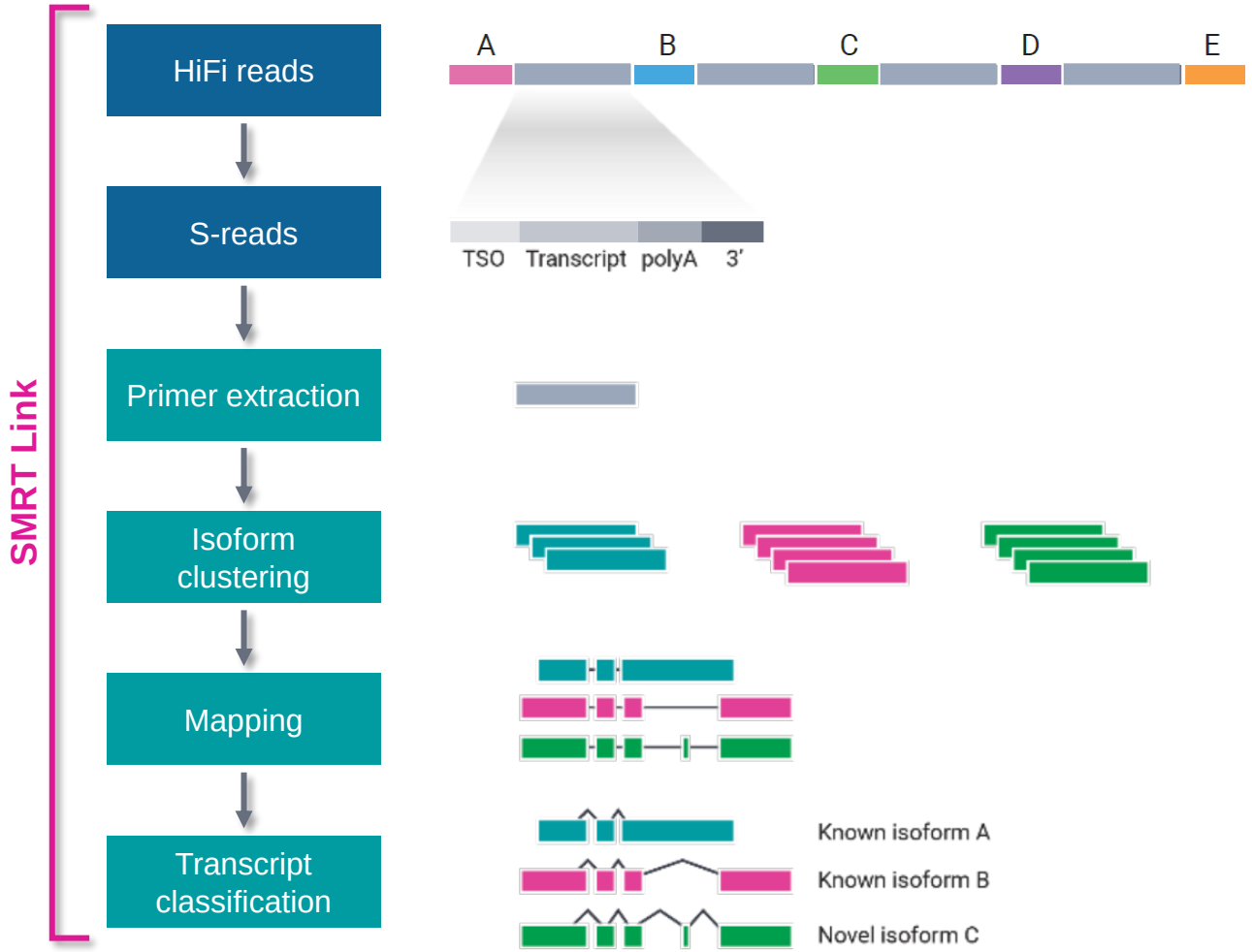
[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Note: Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in HiFi data yields <90 Gb per Revio SMRT Cell.

Kinnex full-length RNA data analysis workflow overview

Kinnex full-length RNA bioinformatics workflow overview

SMRT Link Read segmentation and Iso-Seq workflow processes HiFi reads generated from Kinnex full-length RNA libraries to produce classified isoforms with read counts that are compatible with tertiary analysis tools

SMRT Link read segmentation and Iso-Seq workflow[1]



Read segmentation

- HiFi reads are segmented into individual segmented reads (S-reads) that represent the original cDNA sequences

Primer extraction

- Primers and polyA tails are removed, but also used to orient the read into 5' → 3' orientation

Isoform clustering

- FLNC reads are clustered by their sequencing similarity to produce isoform consensus sequences
- This step is the last step of Iso-Seq analysis if no genome is provided

Mapping

- If a genome is provided, isoform consensus sequences from the previous step are mapped and further collapsed by their exonic structures to produce isoforms as GFF files for visualization

Transcript classification[2]

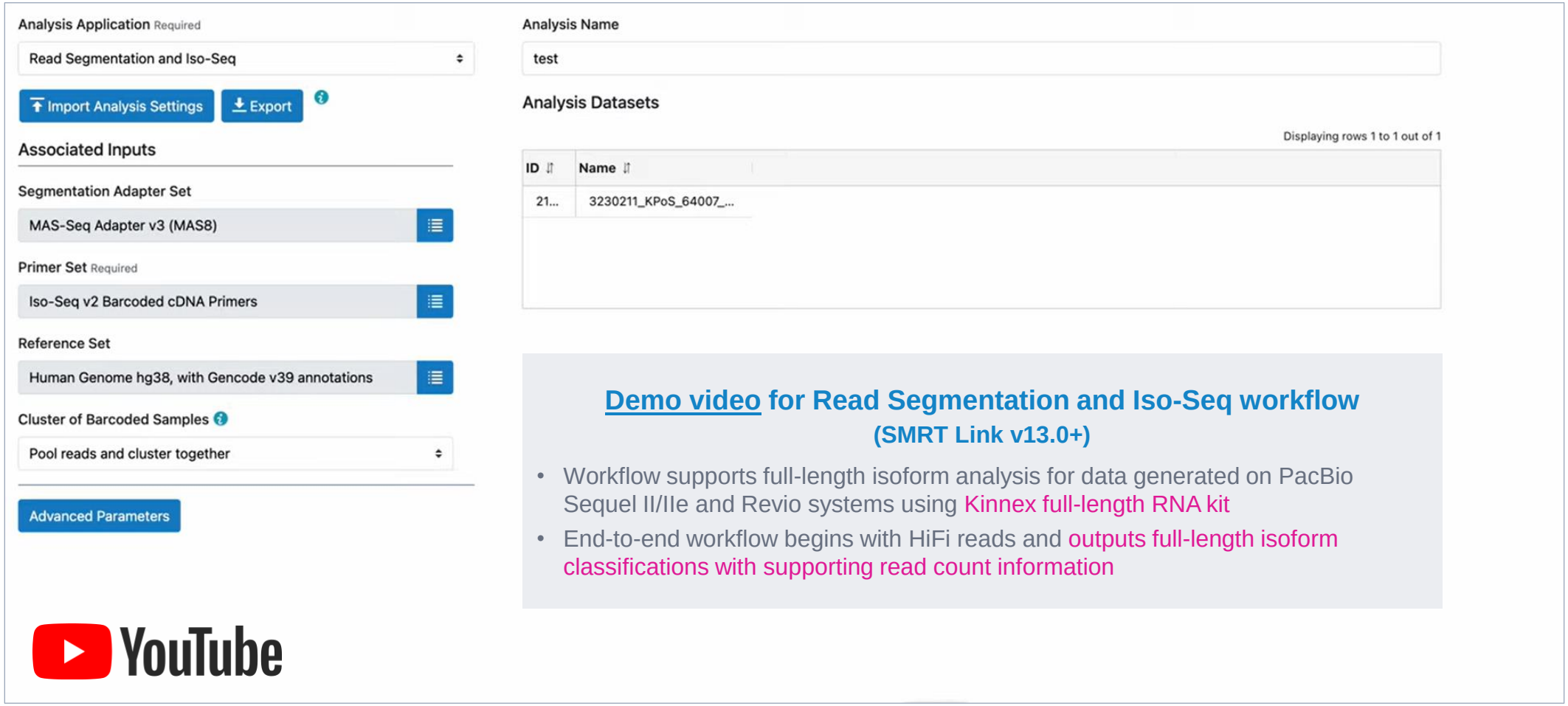
- If an annotation (e.g., Gencode) is provided, isoforms are classified against it using pigeon (the PacBio implementation of SQANTI3) to identify known and novel genes/isoforms
- The Iso-Seq workflow can jointly analyze pooled sample reads to produce a unified isoform annotation with per-sample read counts, both raw and normalized as counts per million (CPM)

[1] See ***SMRT Link User Guide*** (Documentation) for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application. (A video tutorial is also available for viewing.)

[2] Note: SMRT Link only supports transcript classification for human and mouse samples. Non-human/mouse samples will require customized annotation GTF files to be run *via* the command line.

SMRT Link Read Segmentation and Iso-Seq analysis video demonstration

Video demonstration of SMRT Link Read Segmentation and Iso-Seq application workflow for analysis of Kinnex full-length RNA samples



Kinnex full-length RNA bioinformatics workflow recommendations

SMRT Link Read Segmentation and Iso-Seq workflow common considerations and recommendations for analysis of Kinnex full-length RNA data[1]

Analysis recommendations for Iso-Seq data based on reference genome and annotation availability[1]

- With SMRT Link v13, the **Read segmentation and Iso-Seq workflow** analysis application supports human and mouse reference genomes and annotations to produce classified isoforms with read counts.
- If working with other organisms, see table below for analysis recommendations

Available reference or annotation	Analysis workflow recommendation
Human or mouse	• Use the Iso-Seq workflow with preloaded human / mouse annotation to get mapped, unique isoforms with classifications and read count information (FASTA, GFF, TXT).
Model organism with good annotation	• Run Iso-Seq workflow with uploaded reference genome to get mapped, unique isoforms (FASTA, GFF) • Generate pigeon-compliant annotation and use the command line for isoform classification with read count information (TXT)
Non-model organism with genome	• Run Iso-Seq workflow with uploaded reference genome to get mapped, unique isoforms (FASTA, GFF)
No genome	• Run Iso-Seq workflow without reference genome to get unique isoforms (FASTA)

PacBio

[1] See ***Application note – Kinnex full-length RNA kit for isoform sequencing*** (102-326-591).

Kinnex full-length RNA bioinformatics workflow recommendations (cont.)

SMRT Link Read Segmentation and Iso-Seq workflow common considerations and recommendations for analysis of Kinnex full-length RNA data[1]

Sequencing depth recommendations for Iso-Seq data based on experimental goals and study design

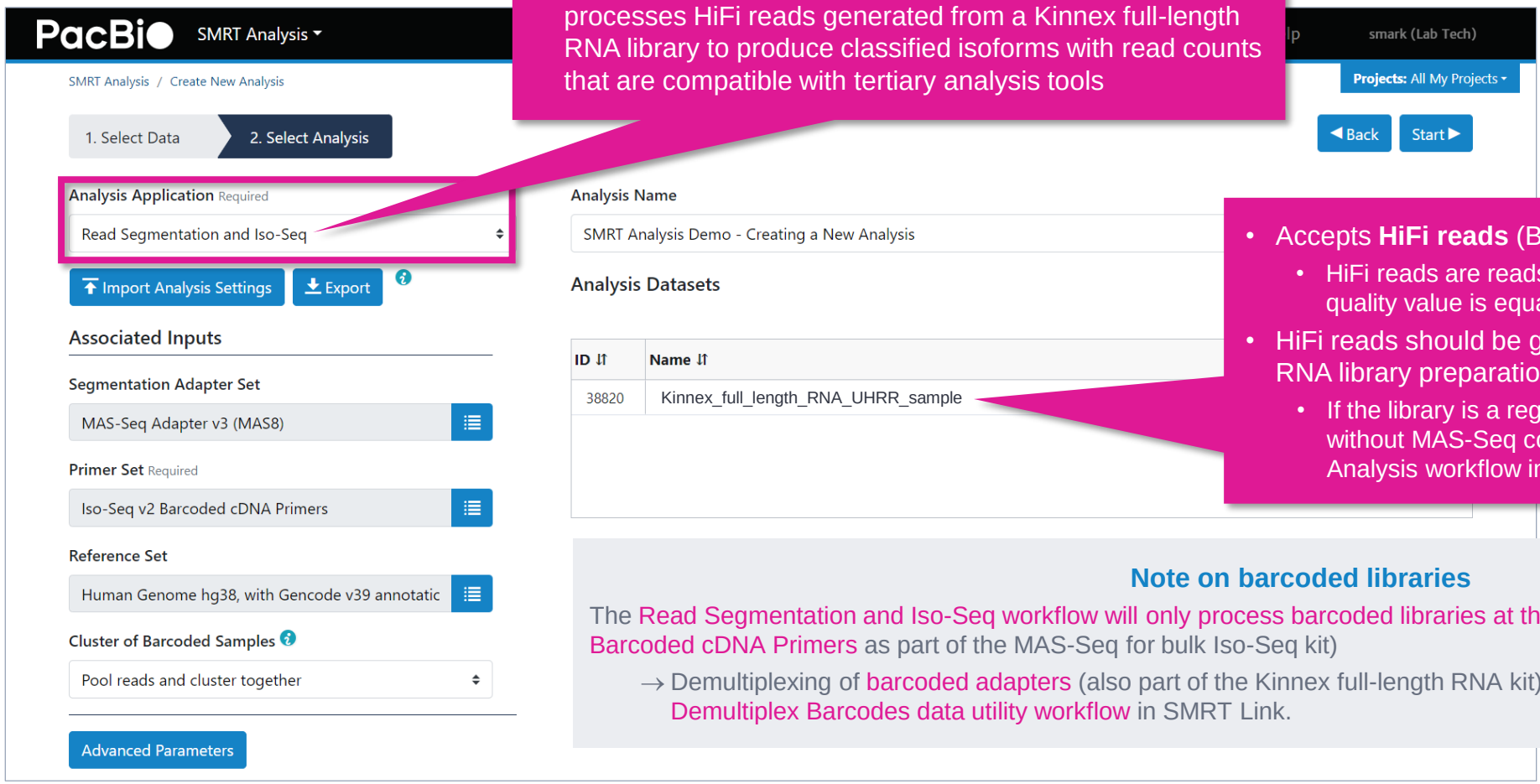
Example application	Human genetics disease studies	Biopharma for identifying highly expressed targets	Plant & animal whole genome annotation
Experimental goal	Isoform discovery and quantification of moderate-to-rare transcripts	Isoform discovery of high expressed transcripts	Comprehensive transcript annotation in a species
Example study design	Disease vs. normal tissues with multiple replicates	Disease cohort with >20+ samples	Plant or animal with multiple tissue types
Target depth of coverage per sample	10 M reads per sample	5 M reads per sample	≤5 M reads per tissue (of same species)
Sample multiplexing[1]	**Sequel II/IIe system:** Up to 2 samples per SMRT Cell 8M (2-plex)	**Sequel II/IIe system:** Up to 3 samples per SMRT Cell 8M (3-plex)	**Sequel II/IIe system:** Up to 3 tissue types per SMRT Cell 8M (3-plex)
	Revio system: Up to 4 samples per Revio SMRT Cell (4-plex)	**Revio system:** Up to 8 samples per Revio SMRT Cell (8-plex)	**Revio system:** Up to 8 tissue types per Revio SMRT Cell (8-plex)[2]
SMRT Link data analysis workflows	Read Segmentation and Iso-Seq analysis application with option to “pool reads and cluster together” to get a master isoform classification file with per-sample full-length read counts		

[1] See ***Application note – Kinnex full-length RNA kit for isoform sequencing*** (102-326-591).

SMRT Link Read Segmentation and Iso-Seq analysis application setup

Specify **Read Segmentation and Iso-Seq** analysis application in SMRT Link[1]

- **Read Segmentation and Iso-Seq** analysis application processes HiFi reads generated from a Kinnex full-length RNA library to produce classified isoforms with read counts that are compatible with tertiary analysis tools



- Accepts **HiFi reads** (BAM format) as input
  - HiFi reads are reads generated with CCS analysis whose quality value is equal to or greater than 20
- HiFi reads should be generated using the Kinnex full-length RNA library preparation protocol (103-238-700)
  - If the library is a regular (non-Kinnex) Iso-Seq monomer library without MAS-Seq concatenation, use the SMRT Link Iso-Seq Analysis workflow instead

Note on barcoded libraries

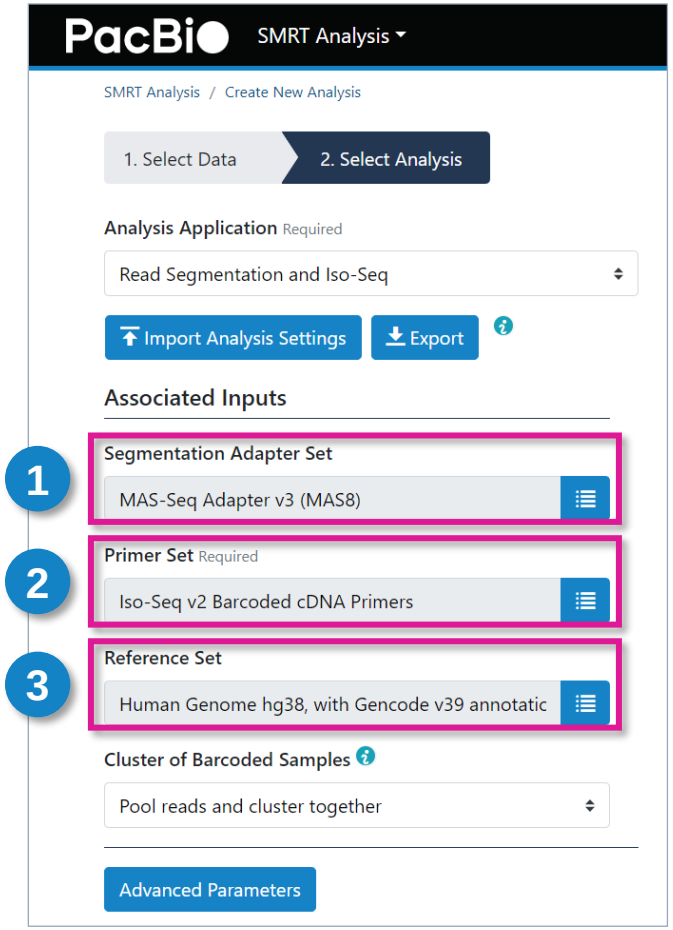
The Read Segmentation and Iso-Seq workflow will only process barcoded libraries at the cDNA level (such as using Iso-Seq v2 Barcoded cDNA Primers as part of the MAS-Seq for bulk Iso-Seq kit)

→ Demultiplexing of barcoded adapters (also part of the Kinnex full-length RNA kit) should first be performed by running the Demultiplex Barcodes data utility workflow in SMRT Link.

[1] See ***SMRT Link User Guide*** (Documentation) for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application.

SMRT Link Read Segmentation and Iso-Seq analysis application setup (cont.)

Specify **Read Segmentation and Single-Cell Iso-Seq** analysis application required associated inputs[1]



1. Segmentation Adapter Set (Default = MAS-Seq Adapter v3 (MAS8))

- Specify a FASTA file, provided by PacBio, containing segmentation adapters. If you need a custom segmentation adapter set, click Advanced Parameters and use a custom FASTA file formatted as described in the SMRT Link User Guide documentation.

2. Primer Set (Required) (Default = Iso-Seq v2 Barcoded cDNA Primers)

- Specify a primer sequence file in FASTA format to identify cDNA primers for removal. The primer sequence includes the 5' and 3' cDNA primers
- Primer IDs must be specified using the suffix `_5p` to indicate 5' cDNA primers and the suffix `_3p` to indicate 3' cDNA primers. The 3' cDNA primer should not include the `T`s and is written in reverse complement. (See the SMRT Link User Guide for example Iso-Seq v2 Barcoded cDNA Primer IDs and sequences)
- Each primer sequence must be unique

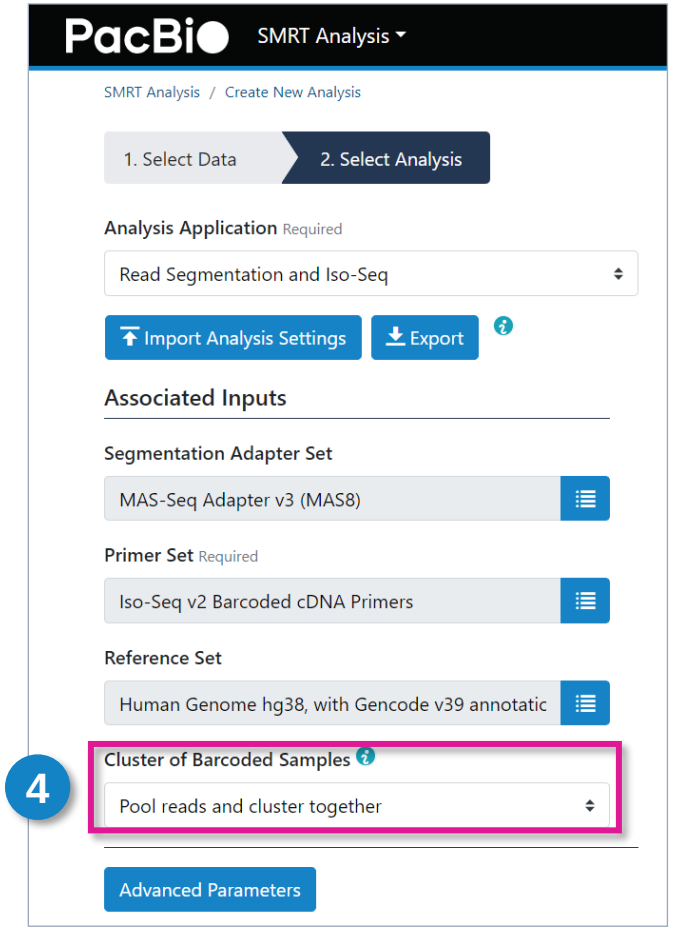
3. Reference Set (Required)

- Specify one of two default reference genome and annotation sets to align high quality isoforms to, and to collapse isoforms mapped to the same genomic loci. The default sets are `Human_hg38_Gencode_v39` and `Mouse_mm39_Gencode_vM28` annotations
- Alternatively, choose other reference genomes (but not with annotations) that were custom-uploaded to SMRT Link
- The Reference Set can be left blank. If blank, the workflow will stop after the isoform clustering step (`isoseq cluster`)

[1] See ***SMRT Link User Guide*** (Documentation) for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application.

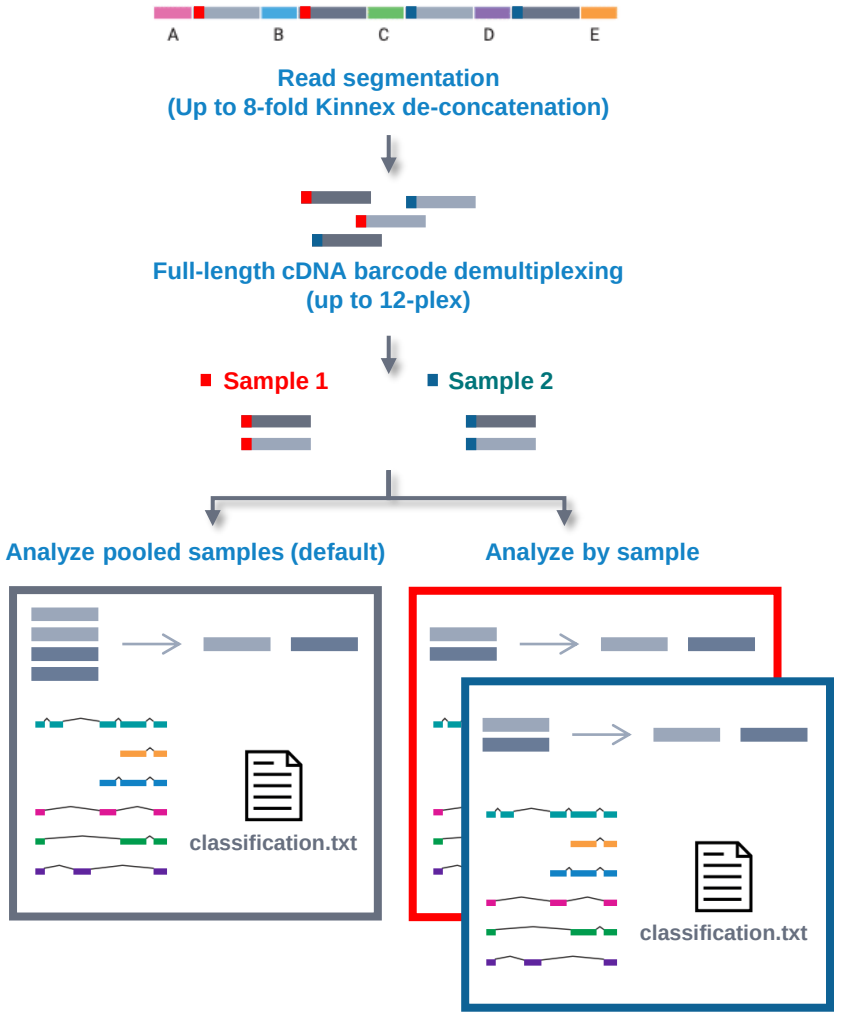
SMRT Link Read Segmentation and Iso-Seq analysis application setup (cont.)

Specify **Read Segmentation and Single-Cell Iso-Seq** analysis application required associated inputs[1]



4. Cluster of Barcoded Samples

- This option specifies barcoded samples that were barcoded at the cDNA level, where the (barcoded) cDNA primers are specified in the Primer Set option. This option does **not** address libraries that were barcoded using barcoded adapters
- Specify whether all FLNC reads will be pooled for clustering, then demultiplexed based on pooled result. **Note:** This setting does **not** apply to non-barcoded samples
- Specify **Pool reads and cluster together** if barcoded samples are from the **same** species, but different tissues, or samples of the same genes but different individuals. The samples are clustered with **all** barcodes pooled
- Specify **Cluster reads separately** if barcoded samples are from different species. The samples are clustered **separately** by barcode
- In either case, the samples on the results page are automatically named `BioSample_1` through `BioSample_N`



Example SMRT Link Read Segmentation data utility processing results[1] for Kinnex full-length RNA library prepared with human UHRR sample

SMRT Link Read Segmentation data utility job report – Summary Metrics and Segmentation Statistics

Summary Metrics

Value	Analysis Metric
6,400,421	Reads
48,911,387	Segmented reads (S-reads)
2,111	Mean length of S-reads
91.89 %	Percent of reads with full arrays
7.64	Mean array size (concatenation factor)

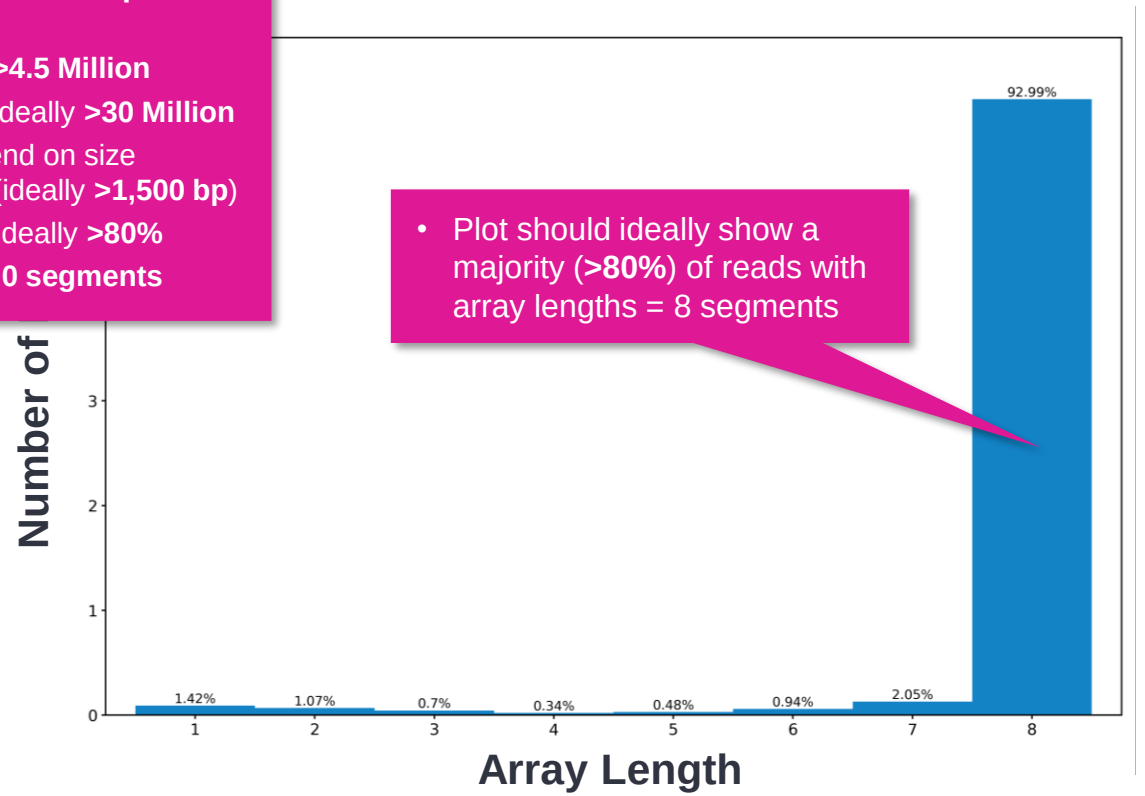
Example Revio system data shown.

For Revio system with optimal sample *P1* loading:

- Yield of HiFi reads is ideally **>4.5 Million**
- Yield of segmented reads is ideally **>30 Million**
- Mean S-read length will depend on size distribution of cDNA sample (ideally **>1,500 bp**)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>7.0 segments**

- **Reads:** Number of input arrayed HiFi reads
- **Segmented reads (S-reads):** Number of generated S-reads
- **Mean length of S-reads:** Mean read length of generated S-reads
- **Percent of reads with full arrays:** Percentage of input HiFi reads containing all adapter sequences in the order listed in the segmentation adapter FASTA file
- **Mean array size:** Mean number of fragments (or S-reads) found in input reads

Segmentation Statistics



- Plot should ideally show a majority (**>80%**) of reads with array lengths = 8 segments

Histogram distribution of the number of S-reads per HiFi read. (Example Revio system data shown.)

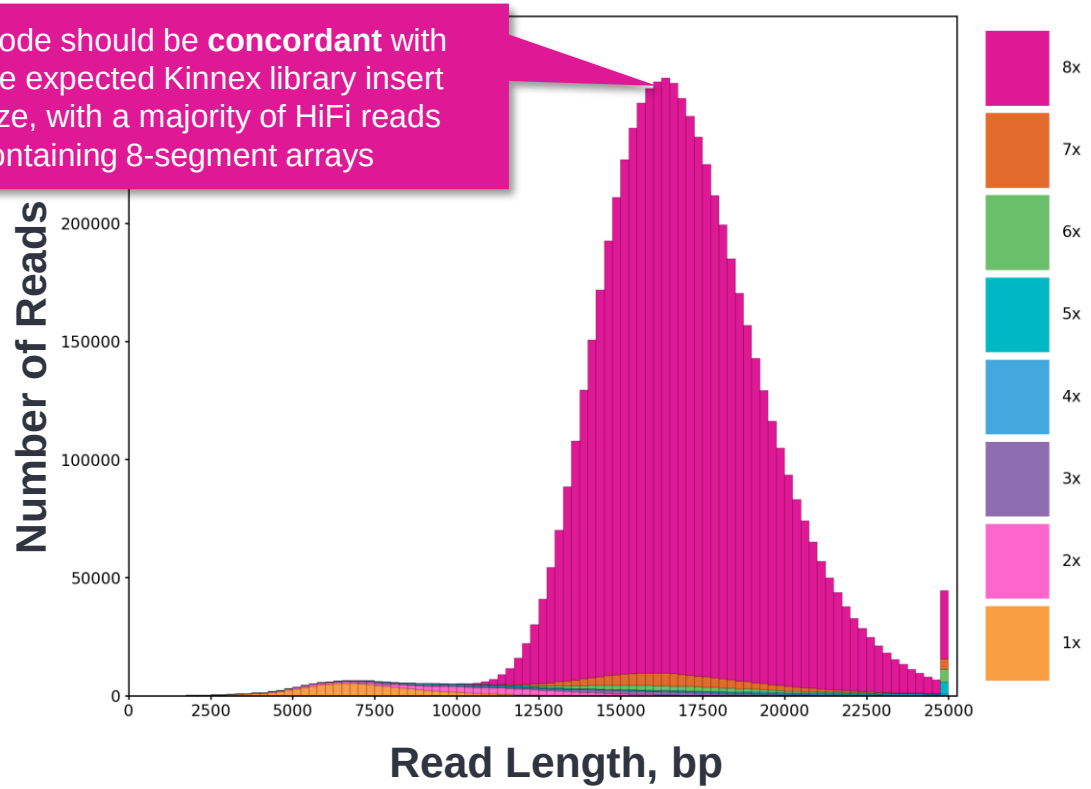
[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Note: Refer to **SMRT Link MAS-Seq troubleshooting guide** (102-994-400) for example performance metrics typically achievable with Kinnex libraries under optimal *P1* loading conditions. For Sequel IIe systems, we recommend aiming for ~60 – 80% *P1* loading. For Revio system, we recommend aiming for ~50 – 70% *P1* loading.

Example SMRT Link Read Segmentation data utility processing results[1] for Kinnex full-length RNA library prepared with human UHRR sample (cont.)

SMRT Link Read Segmentation data utility job report – Length of Reads and S-read Length Distribution

Length of Reads

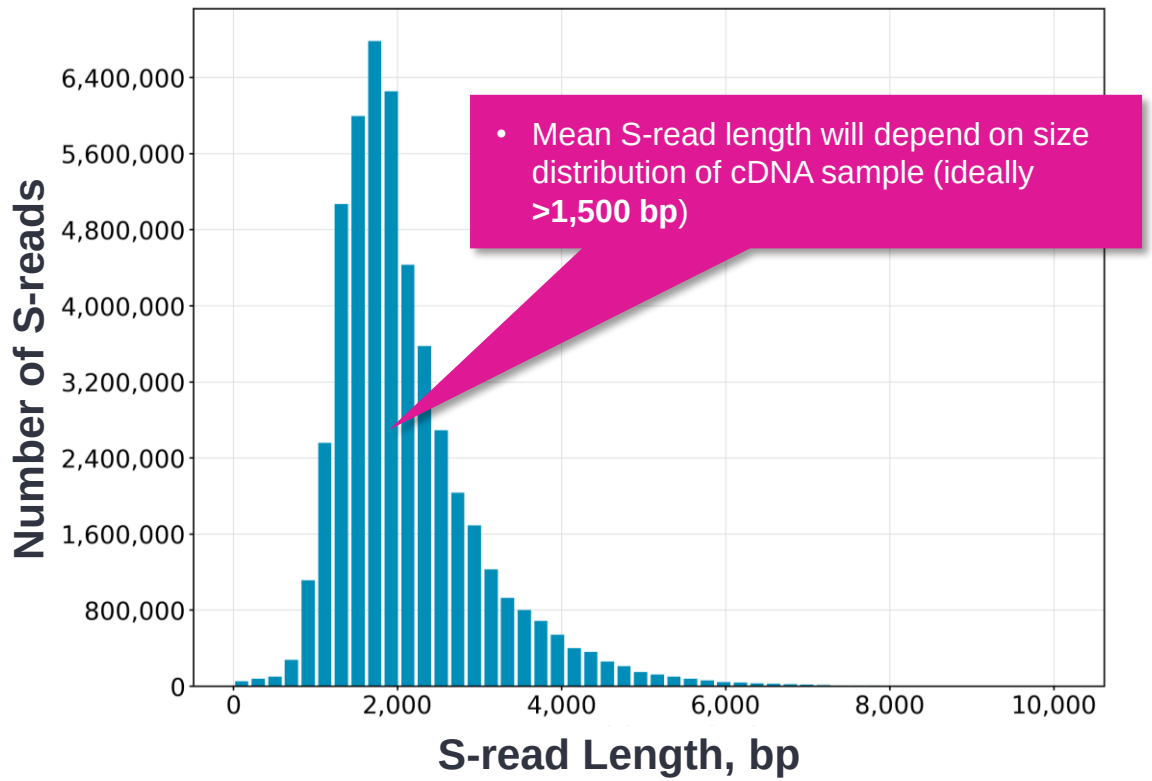
- Mode should be **concordant** with the expected Kinnex library insert size, with a majority of HiFi reads containing 8-segment arrays



Histogram distribution of the number of HiFi reads by read length, in base pairs. (Example Revio system data shown.)

S-read Length Distribution

- Mean S-read length will depend on size distribution of cDNA sample (ideally **>1,500 bp**)



Histogram distribution of the number of S-reads by HiFi read length, in base pairs. (Example Revio system data shown.)

[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Note: Refer to **SMRT Link MAS-Seq troubleshooting guide** (102-994-400) for example performance metrics typically achievable with Kinnex libraries under optimal *P1* loading conditions. For Sequel IIe systems, we recommend aiming for ~60 – 80% *P1* loading. For Revio system, we recommend aiming for ~50 – 70% *P1* loading.

Example SMRT Link Iso-Seq analysis results for Kinnex full-length RNA library prepared with human UHRR sample

SMRT Link Iso-Seq analysis job report – Read Classification statistics

Summary Metrics

Value	Analysis Metric
46,396,697	Reads
44,875,144	Reads with 5' and 3' Primers
44,784,675	Non-Concatamer Reads with 5' and 3' Primers
44,739,994	Non-Concatamer Reads with 5' and 3' Primers and Poly-A Tail (FLNC Reads)
1,981	Mean Length of FLNC Reads
12	Unique Primers
3,739,595	Mean Reads per Primer
4,379,484	Max. Reads per Primer
3,143,785	Min. Reads per Primer
1,521,553	Reads without Primers
96.22%	Percent Bases in Reads with Primers
96.72%	Percent Reads with Primers

Example Revio system data shown.

- **Reads:** Total number of CCS reads
- **Reads with 5’ and 3’ Primers:** Number of CCS reads with 5’ and 3’ cDNA primers detected
- **Non-Concatemer Reads with 5’ and 3’ Primers:** Number of nonconcatemer CCS reads with 5’ and 3’ primers detected
- **Non-Concatemer Reads with 5’ and 3’ Primers and Poly-A Tail (FLNC Reads):** Number of non-concatemer CCS reads with 5’ and 3’ primers and polyA tails detected. This is usually the number for full-length, nonconcatemer (FLNC) reads, unless polyA tails are not present in the sample
- **Mean Length of FLNC Reads:** Mean length of the non-concatemer CCS reads with 5' and 3' primers and polyA tails detected
- **Unique Primers:** Number of unique primers in the sequence
- **Mean Reads per Primer:** Mean number of CCS reads per primer
- **Max. Reads per Primer:** Maximum number of CCS reads per primer
- **Min. Reads per Primer:** Minimum number of CCS reads per primer
- **Reads without Primers:** Number of CCS reads without a primer
- **Percent Bases in Reads with Primers:** Percentage of bases in CCS reads in the sequence data that contain primers
- **Percent Reads with Primers:** Percentage of CCS reads in the sequence data that contain primers

Example SMRT Link Iso-Seq analysis results for Kinnex full-length RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Read Classification statistics

Primer Data

Bio Sample Name	Primer Name	CCS Reads	Mean Primer Quality	Reads with 5' and 3' Primers	Non-Concatamer Reads with 5' and 3' Primers	Non-Concatamer Reads wit
BioSample_1	IsoSeqX_bc01_5p--IsoSeqX_3p	3,648,886	99.6	3,648,886	3,641,475	3,638,289
BioSample_2	IsoSeqX_bc02_5p--IsoSeqX_3p	3,826,584	99.7	3,826,584	3,818,994	3,815,554
BioSample_3	IsoSeqX_bc03_5p--IsoSeqX_3p	4,296,994	99.7	4,296,994	4,288,437	4,284,413
BioSample_4	IsoSeqX_bc04_5p--IsoSeqX_3p	3,588,147	99.7	3,588,147	3,580,832	3,577,622
BioSample_5	IsoSeqX_bc05_5p--IsoSeqX_3p	4,543,731	99.7	4,543,731	4,534,661	4,530,441
BioSample_6	IsoSeqX_bc06_5p--IsoSeqX_3p	4,606,161	99.7	4,606,161	4,596,642	4,592,382
BioSample_7	IsoSeqX_bc07_5p--IsoSeqX_3p	4,009,556	99.7	4,009,556	4,001,451	3,997,812
BioSample_8	IsoSeqX_bc08_5p--IsoSeqX_3p	3,651,440	99.7	3,651,440	3,644,176	3,641,097
BioSample_9	IsoSeqX_bc09_5p--IsoSeqX_3p	4,390,535	99.7	4,390,535	4,381,510	4,377,425
BioSample_10	IsoSeqX_bc10_5p--IsoSeqX_3p	4,049,234	99.7	4,049,234	4,041,043	4,037,382
BioSample_11	IsoSeqX_bc11_5p--IsoSeqX_3p	3,323,541	99.7	3,323,541	3,316,702	3,313,724
BioSample_12	IsoSeqX_bc12_5p--IsoSeqX_3p	3,372,840	99.7	3,372,840	3,365,834	3,362,745
Bio Sample 4	No Primer	1,603,738	0.0	0	0	0

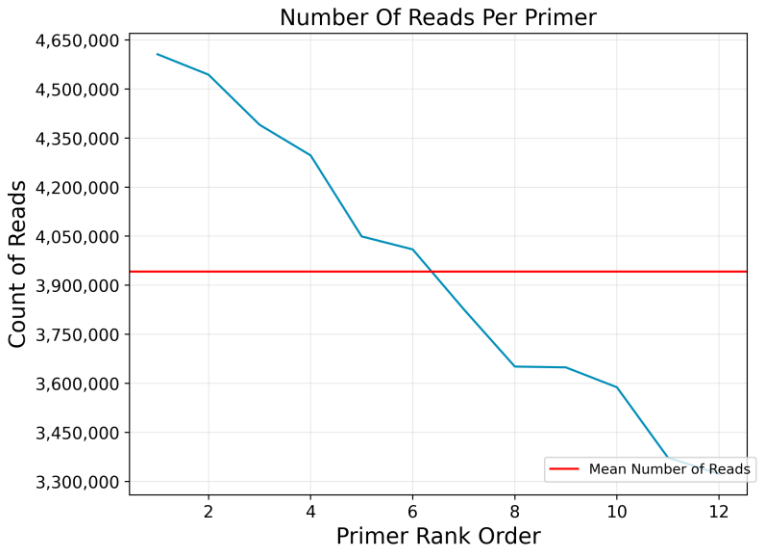
Example Revio system data shown.

- **Bio Sample Name:** Name of the biological sample associated with the primer
- **Primer Name:** A string containing the pair of primer indices associated with this biological sample
- **CCS Reads:** Number of CCS reads associated with the primer
- **Mean Primer Quality:** Mean primer quality associated with the primer
- **Reads with 5’ and 3’ Primers:** Number of CCS reads with 5’ and 3’ cDNA primers detected
- **Non-Concatemer Reads with 5’ and 3’ Primers:** Number of non-concatemer CCS reads with 5’ and 3’ primers detected
- **Non-Concatemer Reads with 5’ and 3’ Primers and Poly-A Tail:** Number of non-concatemer CCS reads with 5’ and 3’ primers and polyA tails detected. This is usually the number for full-length, non-concatemer (FLNC) reads, unless polyA tails are not present in the sample.

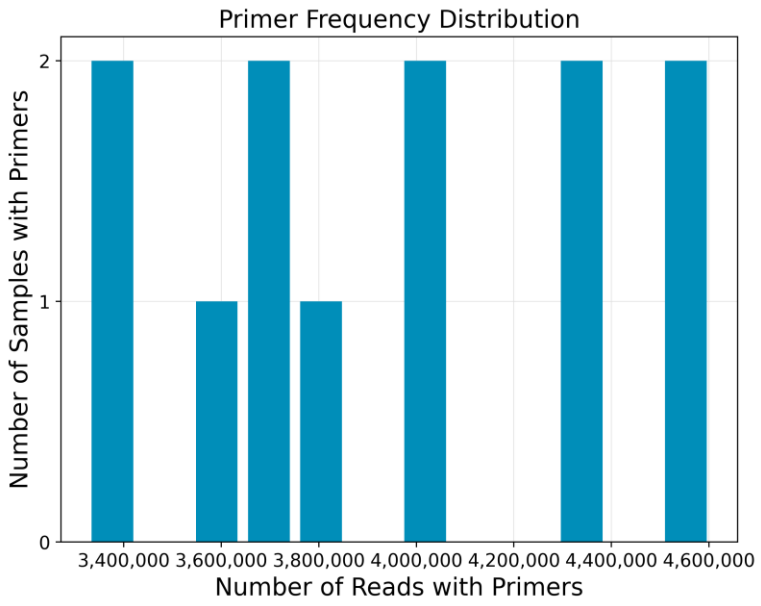
Example SMRT Link Iso-Seq analysis results for Kinnex full-length RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Read Classification statistics

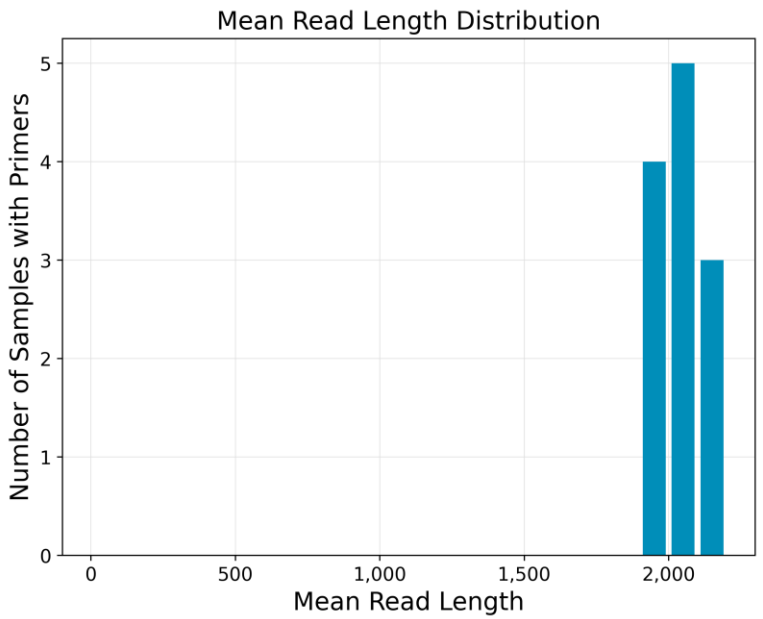
Primer Read Statistics



Number Of Reads Per Primer: Maps the number of reads per primer, sorted by primer ranking



Primer Frequency Distribution: Maps the number of samples with primers by the number of reads with primers

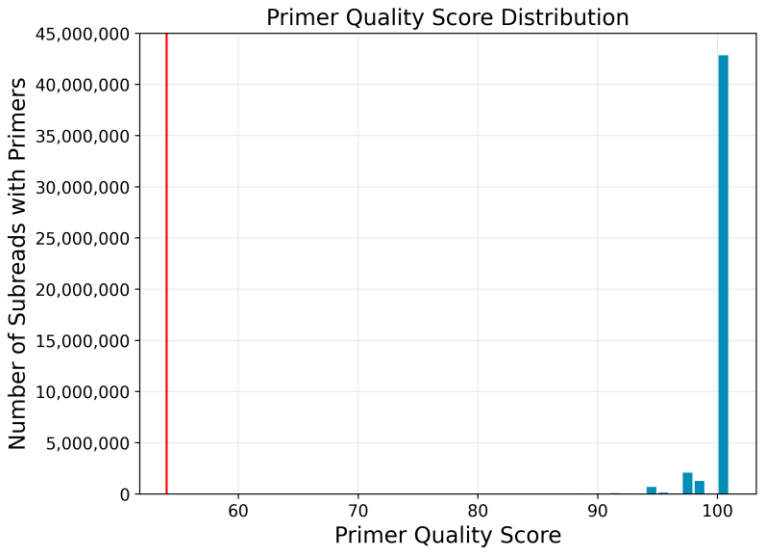


Mean Read Length Distribution: Maps the read mean length against the number of samples with primers

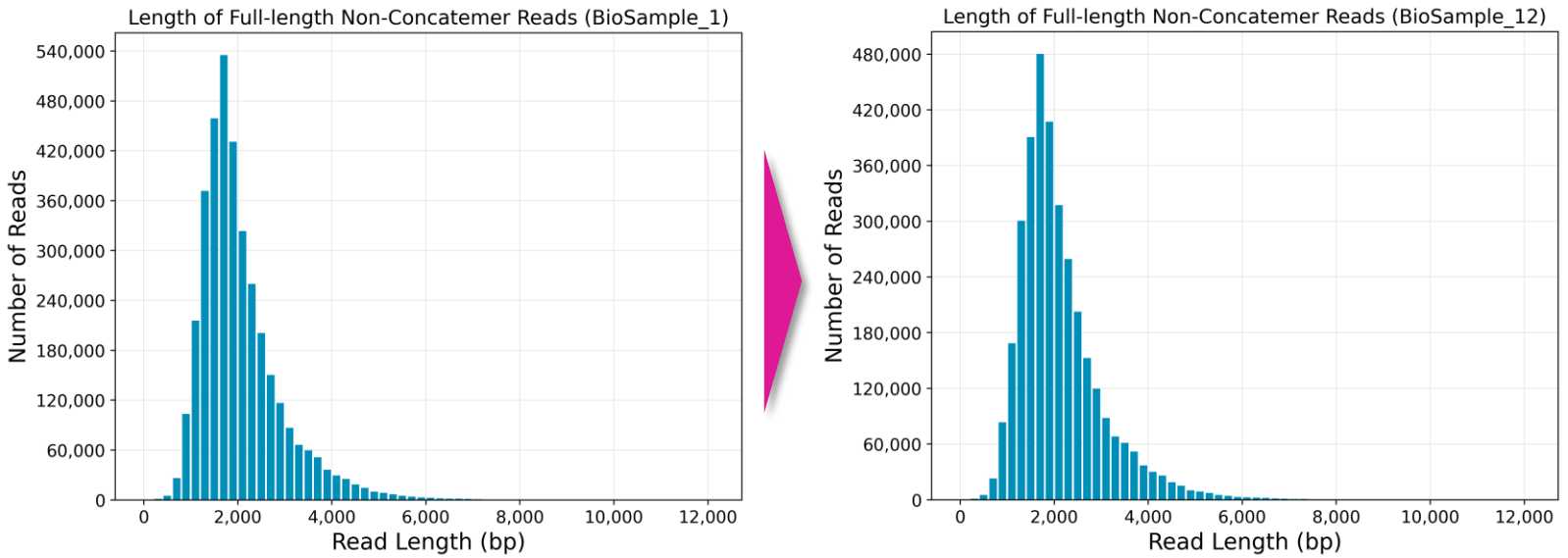
Example SMRT Link Iso-Seq analysis results for Kinnex full-length RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Read Classification statistics

Primer Quality Scores



Length of Full-length Non-Concatemer Reads



Primer Quality Score Distribution: Histogram of primer scores

Length of Full-Length Non-Concatemer Reads: Per-sample histograms of the read length distribution of non-concatemer CCS reads with 5' and 3' primers and polyA tails detected

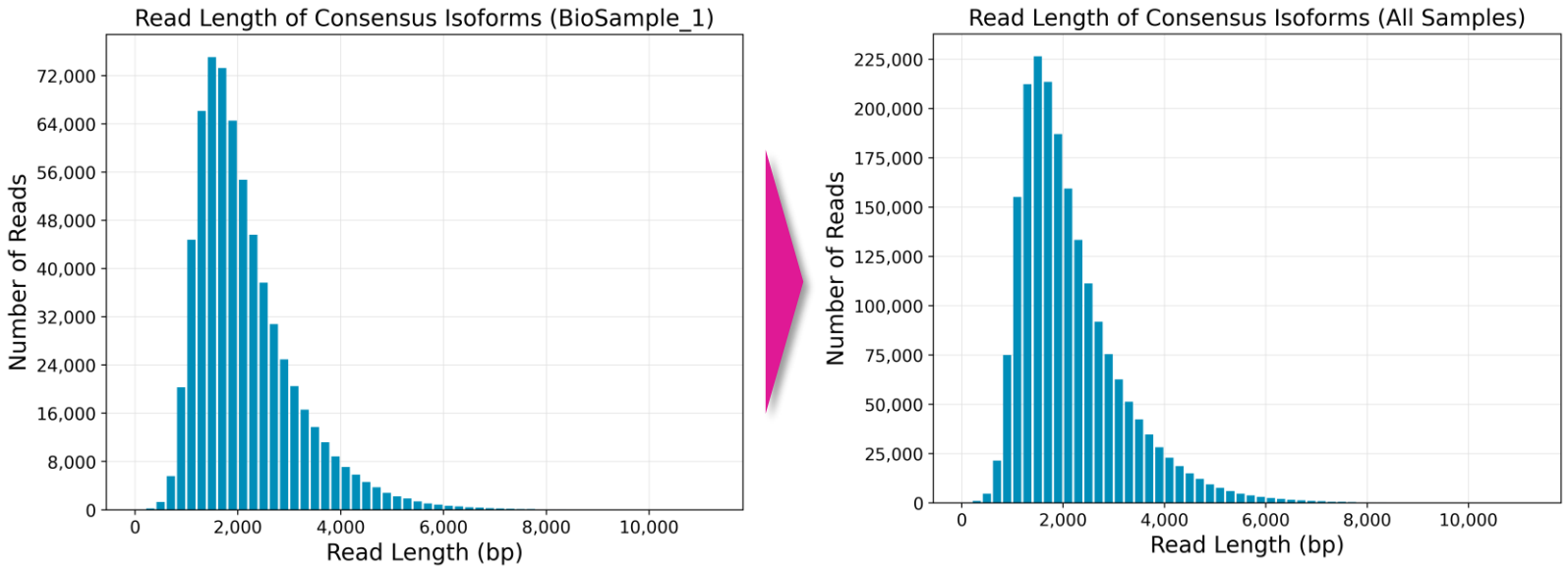
Example SMRT Link Iso-Seq analysis results for Kinnex full-length RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Transcript Clustering statistics

Summary Metrics

Sample Name	Number of High-Quality Isoforms
BioSample_1	650,495
BioSample_2	666,648
BioSample_3	712,671
BioSample_11	617,944
BioSample_12	621,931
All Samples	2,001,226

Length of Consensus Isoforms



- **Sample Name:** Sample name for which the following metrics apply
- **Number of High-Quality Isoforms:** Number of consensus isoforms that have an estimated accuracy above the specified threshold

- **Length of Consensus Isoforms:** Per-sample histograms of the consensus isoform lengths and the distribution of isoforms exceeding a read length cutoff. Also includes a single histogram plot for all samples.

Example SMRT Link Iso-Seq analysis results for Kinnex full-length RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Transcript Mapping and Classification statistics

Summary Metrics (All samples)

Sample Name	Total Unique Genes	Total Unique Genes, filtered	Total Unique Isoforms	Total Unique Isoforms, filtered
BioSample_1	127,560	18,576	317,862	127,517
BioSample_2	131,678	18,833	325,744	129,550
BioSample_3	143,056	19,089	348,790	135,483
BioSample_4	125,866	18,529	315,326	126,923
BioSample_5	148,422	19,451	360,453	138,918
BioSample_6	149,269	19,495	361,022	138,742
BioSample_7	136,195	18,867	336,140	132,612
BioSample_8	128,393	18,608	317,667	126,641
BioSample_9	145,921	19,291	353,923	136,903
BioSample_10	137,496	18,999	338,295	133,063
BioSample_11	120,079	18,308	301,435	122,466
BioSample_12	120,440	18,355	303,142	123,454

Example Revio system data shown.

- **Sample Name:** Sample name for which the following metrics apply
- **Total unique genes:** The total number of unique genes across all cells.
- **Total unique genes, filtered:** The total number of unique genes, after filtering out reads based on the SQANTI transcript filtering criteria.
- **Total unique isoforms:** The total number of unique isoforms across all cells
- **Total unique isoforms, filtered:** The total number of unique isoforms across all cells, after filtering out reads based on the SQANTI transcript filtering criteria.

Example SMRT Link Iso-Seq analysis results for Kinnex full-length RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Transcript Mapping and Classification statistics

Transcript Classification, filtered (All samples)

Category	Count	CAGE Detected	CAGE Detected, (%)	polyA Detected	polyA Detected, (%)
FSM	171662	83485	48.63%	92562	53.92%
ISM	257444	32719	12.70%	158888	61.71%
NIC	149131	97650	65.47%	78854	52.87%
NNC	106396	67432	63.37%	57440	53.98%
Antisense	1501	324	21.58%	923	61.49%
Fusion	3446	2021	58.64%	1901	55.16%
More junctions	83	49	59.03%	55	66.26%
Genic intron	0	0	0.00%	0	0.00%
Genic genomic	1166	524	44.93%	704	60.37%
Intergenic	3073	285	9.27%	2441	79.43%

Example Revio system data shown.[1]

- **Category:** Transcript classification[2] assigned by the classification and filtering tool `pigeon`, based on the SQANTI3 software
- **Count:** The number of transcripts, after filtering out reads based on the SQANTI filtering criteria, in a specific classification
- **CAGE Detected:** The number of transcripts where the transcription start site falls within 50 bp of an annotated CAGE (Cap Analysis of Gene Expression) peak site
- **CAGE Detected, (%):** The percentage of transcripts where the transcription start site falls within 50 bp of an annotated CAGE peak site
- **polyA Motif Detected:** The number of transcripts where a known polyA motif is detected upstream of the transcription end site
- **polyA Motif Detected, (%):** The percentage of transcripts where a known polyA motif is detected upstream of the transcription end site

PacBio

[1] **Note:** Unfiltered transcript classification data are also displayed in the Iso-Seq analysis job report.

[2] Refer to the ***SMRT Link User Guide*** (Documentation) for descriptions of transcript classification categories (e.g., FSM – Full splice match, ISM – Incomplete splice match, etc.).

Example SMRT Link Iso-Seq analysis results for Kinnex full-length RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Transcript Mapping and Classification statistics

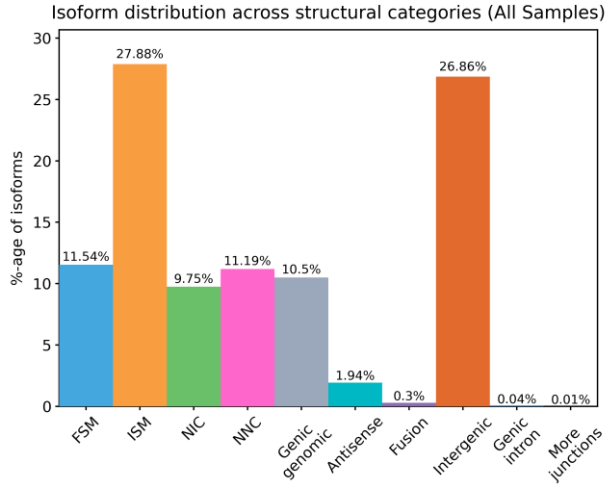
Transcript Classification Plots

Transcript Classification Plots, Filtered

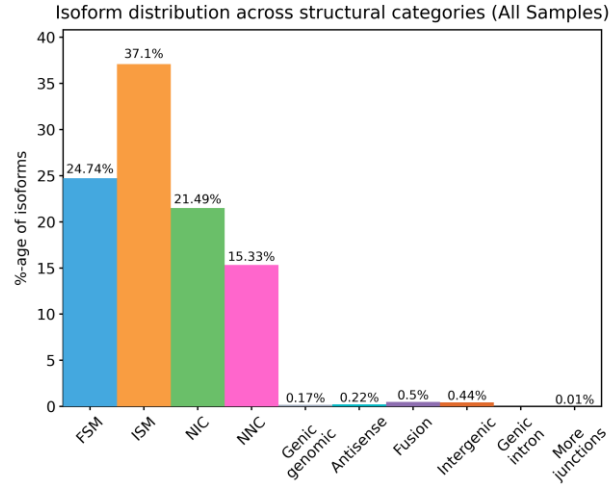
Isoform distributions across structural categories:

- Distribution of the % of isoforms by structural categories

Example Revio system data shown.



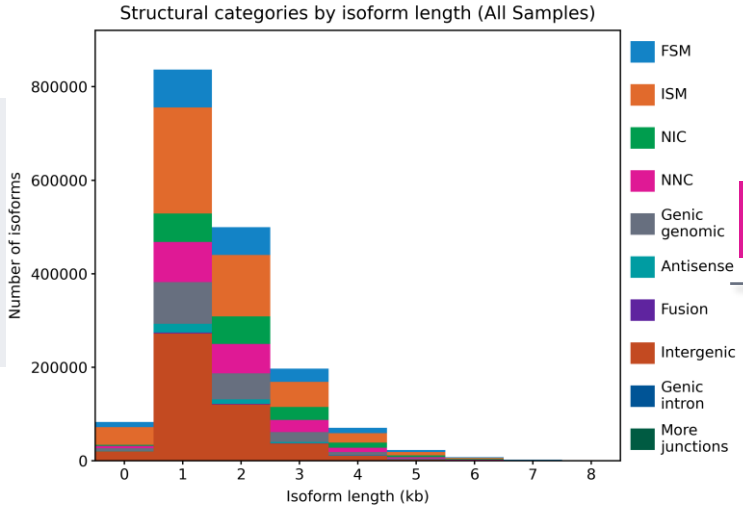
Filter out reads based on the SQANTI3 transcript filtering criteria



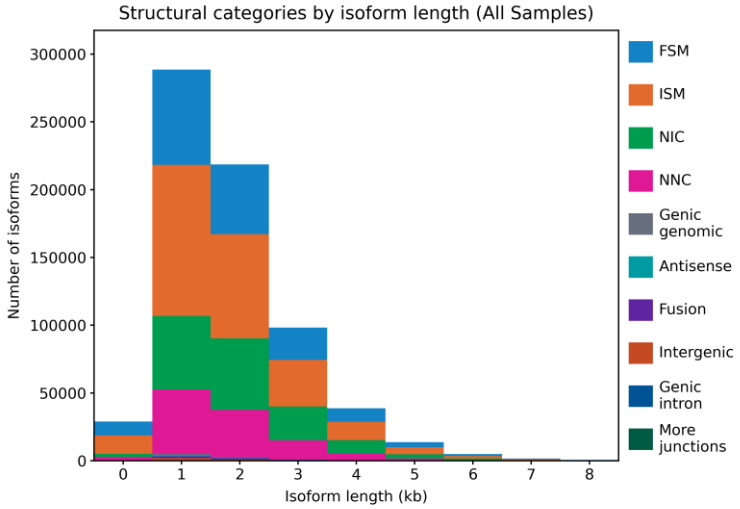
Structural categories by isoform lengths:

- Histogram display of the number of isoforms by their length in kb and their structural category

Example Revio system data shown.



Filter out reads based on the SQANTI3 transcript filtering criteria

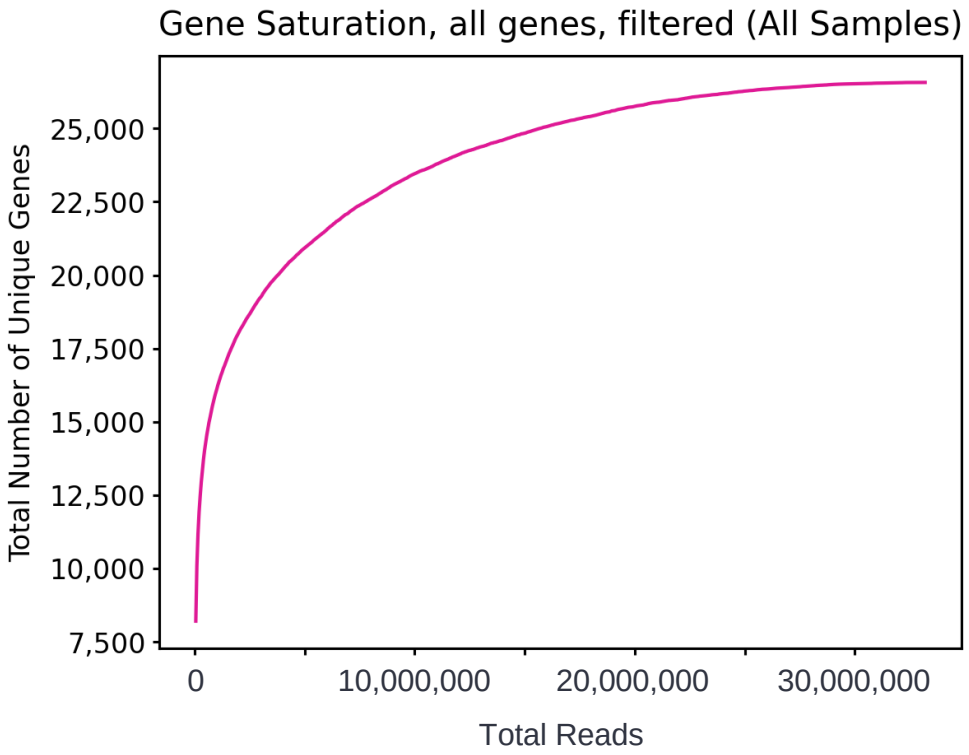


PacBio

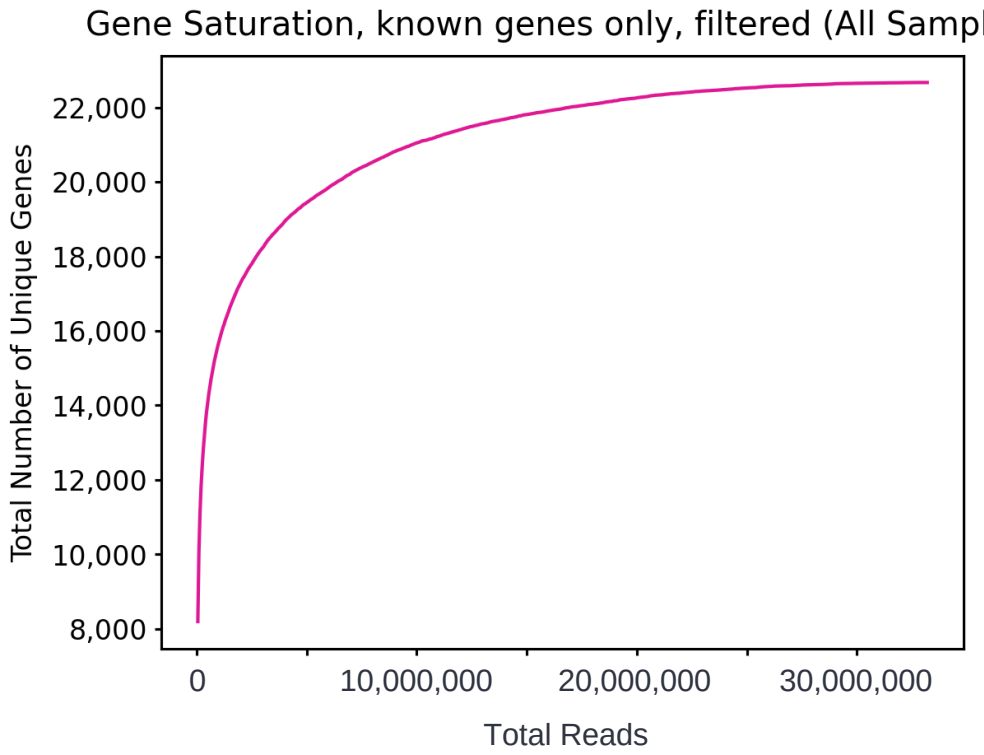
Example SMRT Link Iso-Seq analysis results for Kinnex full-length RNA library prepared with human UHRR sample (cont.)

SMRT Link Iso-Seq analysis job report – Transcript Mapping and Classification statistics

Gene Saturation



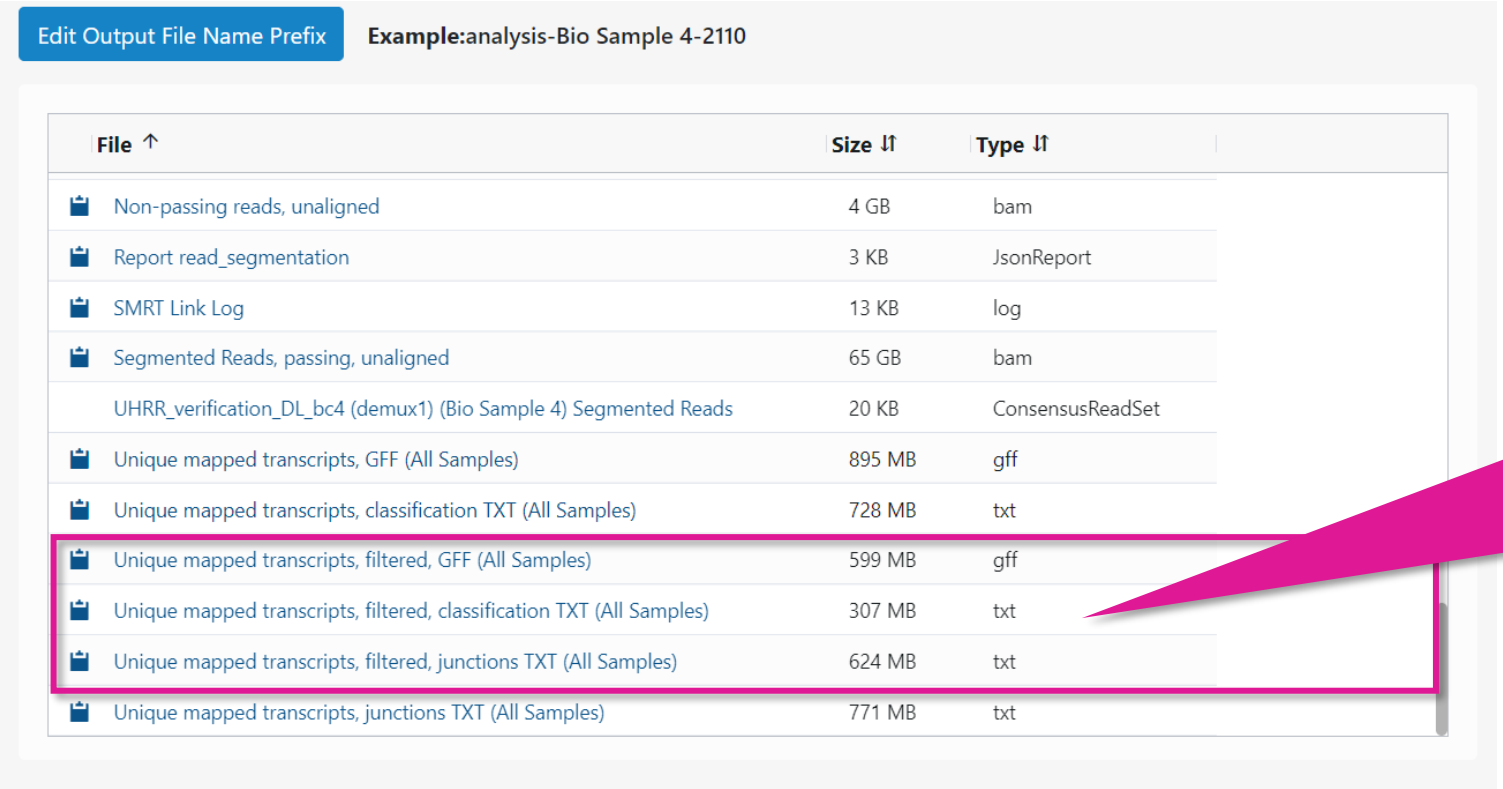
Gene Saturation, all genes, filtered: Saturation plot showing the level of gene saturation for all genes, after filtering out reads based on the SQANTI transcript filtering criteria



Gene Saturation, known genes only, filtered: Saturation plot showing the level of gene saturation, for unique known genes only (genes annotated in the reference annotation) per cell, after filtering out reads based on the SQANTI transcript filtering criteria

Example SMRT Link Iso-Seq analysis results for Kinnex full-length RNA library prepared with human UHRR sample (cont.)

File Downloads tab



Refer to SMRT Link user guide for descriptions of downloadable output files

- These files are useful for **visualizing** isoform structures in Integrative Genomics Viewer (IGV) / UCSC genome browser and enable understanding of why an isoform is novel/known, etc.
  - GFF file containing unique mapped transcripts after filtering
  - Text file containing unique mapped transcript classifications against annotations, after filtering
  - Text file containing information about unique mapped transcript junctions, after filtering

Files shown in the File Downloads tab are available on the analysis results page. Additional files are also available on the SMRT Link server in the analysis output directory.

Technical documentation & applications support resources

Technical resources for Kinnex full-length RNA library preparation, sequencing & data analysis

RNA sample preparation resources

- Note: The products below have not been tested or validated by PacBio but are listed here as examples of third-party kits used by other PacBio customers for isolating total RNA for PacBio RNA sequencing (Iso-Seq) applications

Kit type	Product name
mRNA isolation	Ambion Poly(A) Purist MAG Kit [Link]
Total RNA isolation	Qiagen RNeasy Plus Kits [Link]
	Sigma Spectrum Plant Total RNA Kit [Link]
	iNtRON Easy Spin Total RNA [Link]
	TRIzol Reagent can be used to isolate total RNA from tissues or cells, including lipid-rich and difficult samples [Link]
RNA stabilization & storage	RNALater is an aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA [Link]

Technical resources for Kinnex full-length RNA library preparation, sequencing & data analysis (cont.)

Kinnex full-length RNA library preparation literature & other resources

- Application note – Kinnex full-length RNA kit for isoform sequencing (102-326-591)
- Procedure & checklist – Preparing Kinnex libraries using Kinnex full-length RNA kit (103-238-700)
- Technical overview – Kinnex kits for single-cell RNA, full-length RNA and 16S rRNA sequencing (103-343-700)
- Technical overview – Kinnex library preparation using Kinnex full-length RNA kit (103-344-700)
- Video tutorial – SMRT Link Sample Setup and Run Design setup procedure for Kinnex kits [Link]
- Whitepaper – Bulk and single-cell isoform sequencing for human disease research (102-326-576)

Data analysis resources

- Application note – Bioinformatics tools for full length isoform sequencing (102-326-593)
- SMRT Link MAS-Seq troubleshooting guide (102-994-400)
- SMRT Link software installation guide [Link]
- SMRT Link user guide [Link]
- SMRT Tools reference guide [Link]
- Video tutorial – Read Segmentation and Iso-Seq workflow in SMRT Link [Link]

Technical resources for Kinnex full-length RNA library preparation, sequencing & data analysis (cont.)

Publications

- Schertzer, M.D. et al. (2023) Cas13d-mediated isoform-specific RNA knockdown with a unified computational and experimental toolbox. BioRxiv preprint [Link]
- Al'Khafaji, A.M. et al. (2023) High-throughput RNA isoform sequencing using programmable cDNA concatenation. Nature biotechnology. [Link]

Webinars

- PacBio Iso-Seq social club webinar (2022) – Introduction to Iso-Seq method [Link]
- PacBio Iso-Seq social club webinar (2022) – SQANTI3 for isoform classification and annotation [Link]
- PacBio Iso-Seq social club webinar (2022) – TappAS for isoform differential expression analysis [Link]

Example PacBio data sets

Application	Dataset	Data type	PacBio system
Kinnex full-length RNA sequencing	Homo sapiens – universal human reference RNA (UHRR) [Link]	HiFi long read	Sequel II & Revio systems
	Homo sapiens – HG002 [Link]	HiFi long read	Revio system
	Homo sapiens – Heart [Link]	HiFi long read	Revio system
	Homo sapiens – Cerebellum [Link]	HiFi long read	Revio system

www.pacb.com

Research use only. Not for use in diagnostic procedures. © 2024 Pacific Biosciences of California, Inc. (“PacBio”). All rights reserved. Information in this document is subject to change without notice. PacBio assumes no responsibility for any errors or omissions in this document. Certain notices, terms, conditions and/or use restrictions may pertain to your use of PacBio products and/or third-party products. Refer to the applicable PacBio terms and conditions of sale and to the applicable license terms at pacb.com/license. Pacific Biosciences, the PacBio logo, PacBio, Circulomics, Omniome, SMRT, SMRTbell, Iso-Seq, Sequel, Nanobind, SBB, Revio, Onso, Apton, Kinnex, and PureTarget are trademarks of PacBio.