Technical overview – Kinnex library preparation using Kinnex single-cell RNA kit

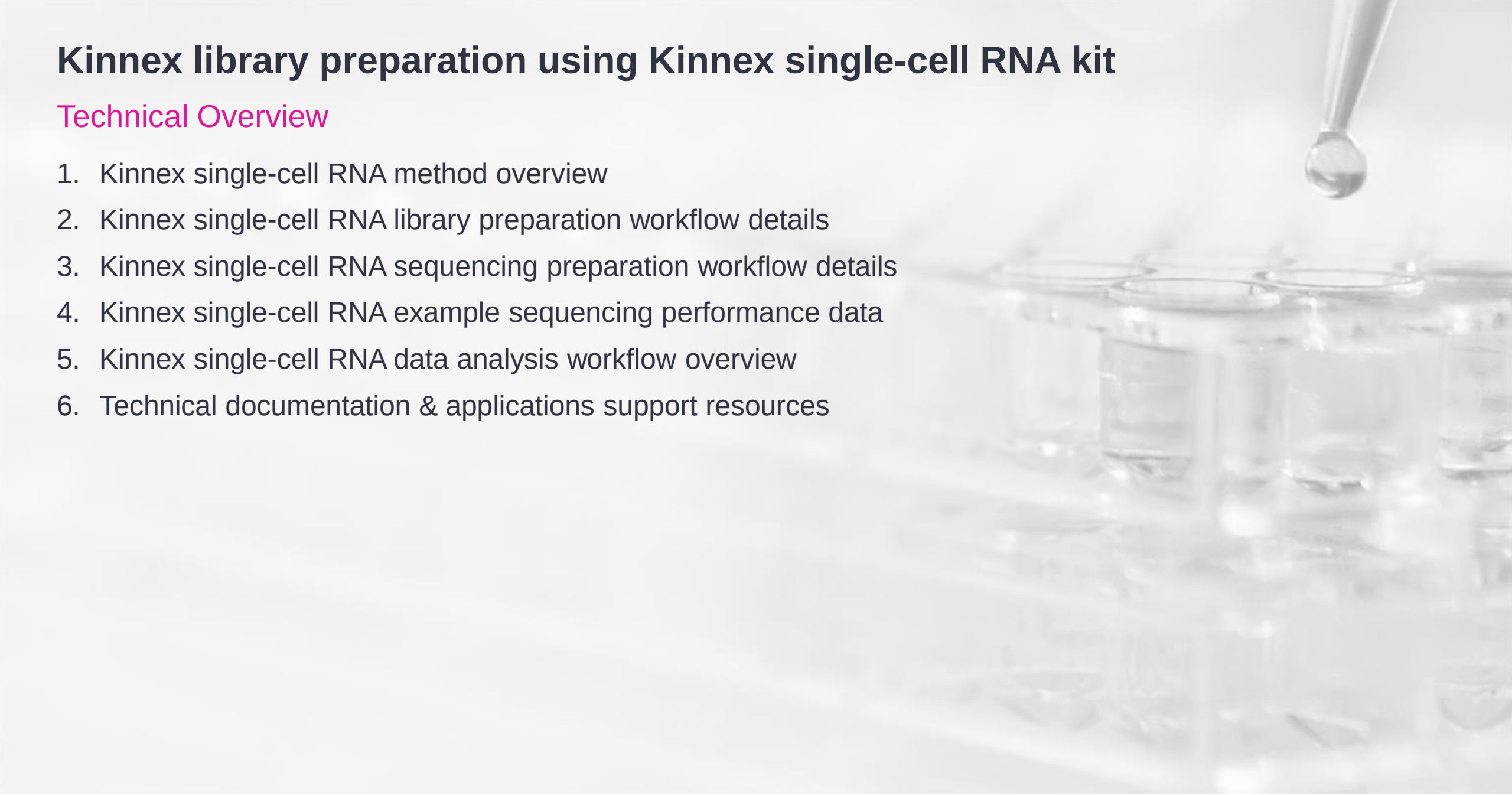
Sequel II and IIe systems ICS v11.0
Revio system ICS v13.1
SMRT Link v13.1

PN 103-344-600 Rev 01 | April 2024

Kinnex library preparation using Kinnex single-cell RNA kit

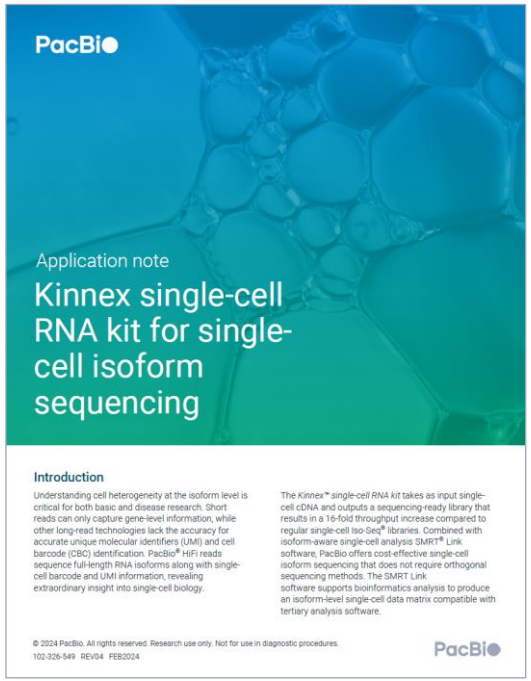
Technical Overview

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



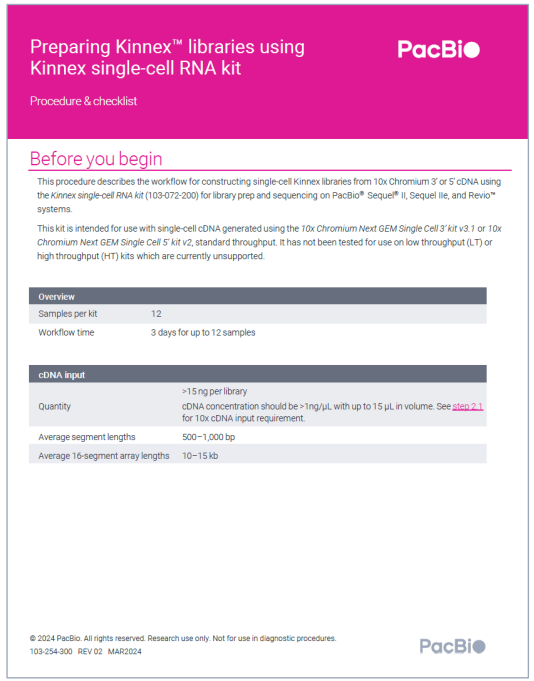
Kinnex library preparation using Kinnex single-cell RNA kit: Getting started

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



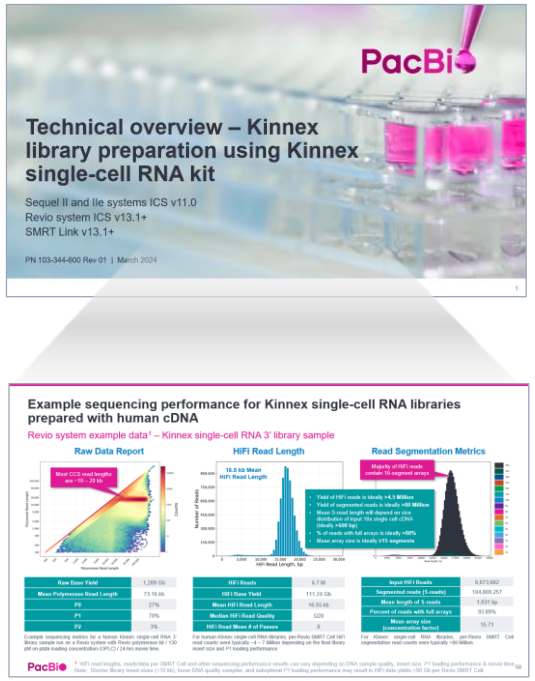
Application Note - Kinnex single-cell RNA for single-cell isoform sequencing (102-326-549)

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



Procedure & checklist – Preparing Kinnex libraries using Kinnex single-cell RNA kit (102-254-300)

Technical documentation containing application-specific library preparation protocol details.



Technical Overview: Kinnex library preparation using Kinnex single-cell RNA kit (103-344-600)

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.

Single-cell cDNA synthesis

Use 10x Genomics Chromium system to perform 3’ or 5’ single-cell cDNA synthesis (3,000 – 10,000 cells input)

Kinnex library preparation (Kinnex single-cell RNA kit)

Use ≥15 ng of 10x Chromium 3’ or 5’ single-cell cDNA input to generate Kinnex library containing 16-segment array

SMRT sequencing (Sequel II/IIe & Revio systems)

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

Data analysis (SMRT Link)

Use Read Segmentation data utility to split arrayed transcript HiFi reads

Use Single-cell Iso-Seq analysis application to identify novel genes and isoforms

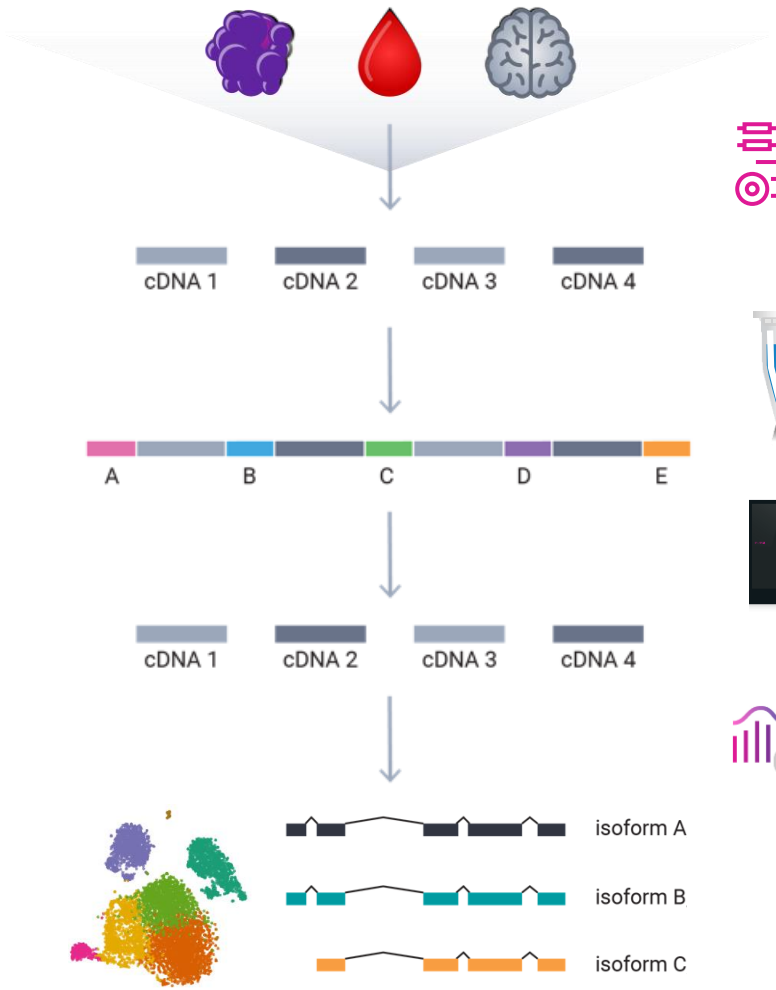
PacBio

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

Kinnex single-cell RNA method overview

Kinnex single-cell RNA method overview

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



Single-cell cDNA generation

Kinnex library preparation

PacBio sequencing

Read segmentation + single-cell Isoform analysis

- 10x Chromium Single Cell 3’ kit (v3.1) and 5’ kit (v2)
- 15–75 ng cDNA input
- 3,000 to 10,000 target cell recovery
- 2-day Kinnex library preparation using Kinnex single-cell RNA kit
- Barcoded Kinnex adapters support up to 4-plex multiplexing
- SMRT Link Run Design support for ‘Kinnex single-cell RNA’ application type option with auto-analysis (read segmentation + single-cell isoform analysis)[1]
- SMRT Link single-cell Iso-Seq isoform-classification software to identify novel genes and isoforms
- Output compatible with tertiary single-cell analysis tools (e.g., *Seurat*, *Scanpy*, *Kana*)

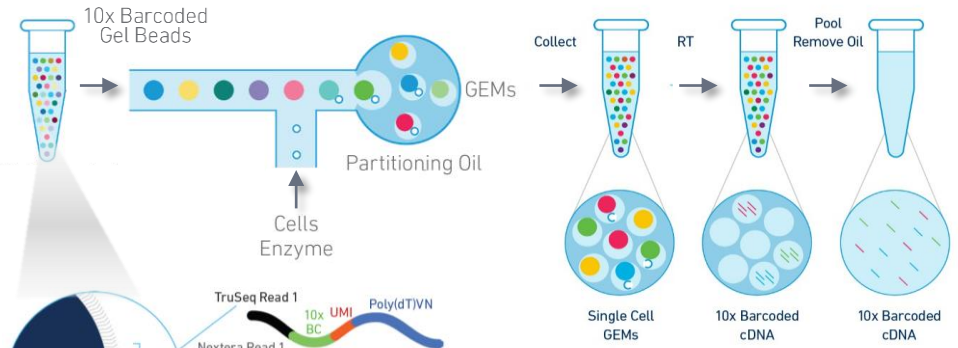
[1] Kinnex full-length RNA kit requires SMRT Link v13.0 or higher.

Kinnex single-cell RNA method overview

Single-cell cDNA sample preparation[1]

Chromium Controller/X/iX

Chromium Next GEM Single Cell 5' or 3' Kit



cDNA amplification & cleanup

Full-length 10x barcoded single-cell cDNA for input into PacBio Kinnex single-cell RNA library construction

PacBio

Procedure & checklist (103-254-300)

Kinnex library prep, sequencing & analysis

PacBio

Kinnex single-cell RNA kit (103-072-200)

10x single-cell cDNA input

Template switch oligo (TSO) artifact removal

cDNA with expected Fwd & Rev primer sequences

Kinnex segmentation adapter incorporation & array formation

16-segment Kinnex array construct containing barcoded SMRTbell adapters at both ends

DNA damage repair & nuclease treatment / ABC[2]

PacBio long-read systems

Sequel II, Sequel IIe or Revio system

SMRT Link

Read segmentation & single-cell Iso-Seq analysis

[1] Refer to 10x Genomics Support website to download 10x Chromium user guides and other documentation.

[2] ABC = Anneal sequencing primer / Bind polymerase / Complex cleanup

Single-cell cDNA sample preparation[1]

10x GENOMICS

Poly(dT) primer for 1st-strand synthesis

CBC = 10x cell barcode
UMI = Unique molecular identifier

5' [CBC][UMI]TTTTTTTTTT 3'
3' AAAAAAAAAA 5'
Poly(A)+ mRNA template

Example 10x single cell 3' cDNA workflow is shown here

Samples incorporate a 10x cell barcode during single-cell cDNA synthesis

Reverse transcription (1st-strand synthesis) and addition of 'C' bases at transcript 5' end

5' [CBC][UMI]TTTTTTTTTT CCC 3'
3' AAAAAAAAAA GGG TSO 5'
Template Switch Oligo (TSO)

Template switching (from mRNA to TSO) and completion of transcript extension

5' [CBC][UMI]TTTTTTTTTT CCC 3'
3' AAAAAAAAAA GGG TSO 5'

2nd-strand synthesis & cDNA amplification

10x Forward PCR Primer

5' [CBC][UMI]TTTTTTTTTT CCC TSO 3'
3' AAAAAAAAAA GGG TSO 5'
TSO
10x Reverse PCR Primer

cDNA cleanup

5' [CBC][UMI]TTTTTTTTTT CCC TSO 3'
3' [CBC][UMI]AAAAAAAAAA GGG TSO 5'

10x-barcoded single-cell cDNA for input into PacBio Kinnex single-cell RNA library construction

10x & PacBio key protocol steps

PacBio

Procedure & checklist (103-254-300)

Kinnex library prep, sequencing & analysis

PacBio

F R — cDNA with expected structure (TSO primer sequence at only one end)

TSO artifact removal

F R F R R R — cDNA with template switch oligo (TSO) artifact (TSO primer sequences at both ends)

F R

Kinnex PCR (16 parallel PCR Rx to append Kinnex segmentation adapters)

PCR 1 (A B') | PCR 2 (B C') | PCR 3… (C D') | …PCR (*n*)

Barcoded Kinnex terminal adapter (bcM0001-bcM0004)[2]

Pooling & Kinnex array formation

BC A B C … Q BC
BC A B C D
O P Q BC

Samples can be barcoded during Kinnex array formation using up to 4 different barcoded Kinnex SMRTbell adapters[2]

DNA damage repair & nuclease Tx

BC A B C … Q BC

Anneal Kinnex sequencing primer / Bind polymerase / Cleanup (ABC)

BC A B C … Q BC

Purified polymerase-bound 16-segment Kinnex library

Long-read sequencing & data analysis

Sequel II, Sequel IIe or Revio system

SMRT Link read segmentation & single-cell Iso-Seq analysis

[1] Refer to 10x Genomics Support website to download 10x Chromium user guides and other documentation.

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

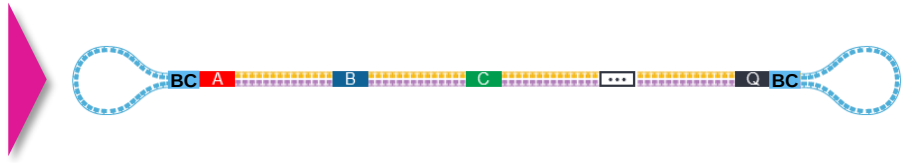
Kinnex single-cell RNA library preparation procedure description

Procedure & checklist – Preparing Kinnex libraries using the Kinnex single-cell RNA kit (103-254-300) describes the workflow for constructing single-cell Kinnex libraries from 10x Chromium 3’ or 5’ cDNA using the Kinnex single-cell RNA kit (103-072-200) for library prep and sequencing on PacBio Sequel II, Sequel IIe, and Revio systems

Overview	
Samples per kit	12
Workflow time	3 days for up to 12 samples

cDNA input	
Quantity	>15 ng per library cDNA concentration should be >1ng/µL with up to 15 µL in volume. See step 2.1 for 10x cDNA input requirement.
Average segment lengths	500–1,000 bp
Average 16-segment array lengths	10–15 kb

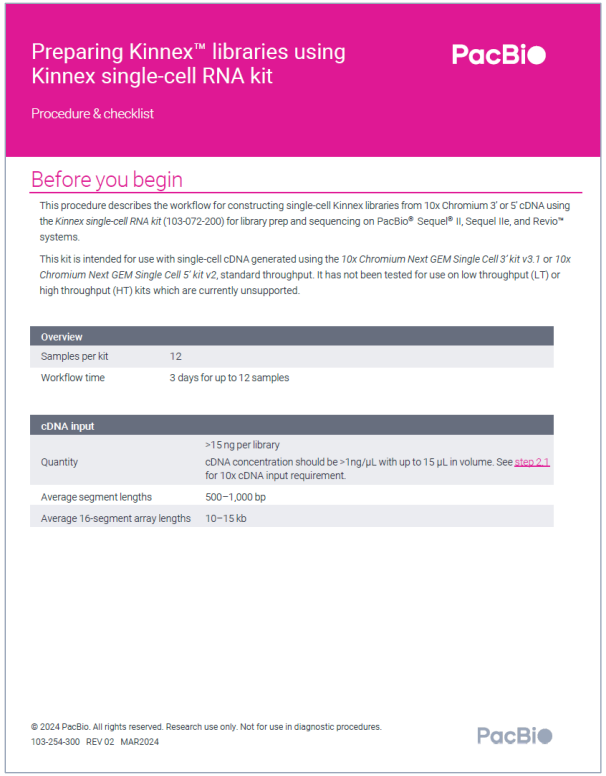
Kinnex single-cell RNA kit
103-072-200 (12 rxn)



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

For use with single-cell cDNA generated with 10x Chromium Next GEM Single Cell 3’ kit v3.1 or 10x Chromium Next GEM Single Cell 5’ kit v2, standard throughput[1]

Kinnex single-cell RNA library preparation procedure supports up to 4-plex sample multiplexing through use of 4 different barcoded Kinnex SMRTbell adapters[2]



Preparing Kinnex™ libraries using Kinnex single-cell RNA kit

PacBio

Procedure & checklist

Before you begin

This procedure describes the workflow for constructing single-cell Kinnex libraries from 10x Chromium 3’ or 5’ cDNA using the *Kinnex single-cell RNA kit* (103-072-200) for library prep and sequencing on PacBio® Sequel® II, Sequel IIe, and Revio™ systems.

This kit is intended for use with single-cell cDNA generated using the *10x Chromium Next GEM Single Cell 3’ kit v3.1* or *10x Chromium Next GEM Single Cell 5’ kit v2*, standard throughput. It has not been tested for use on low throughput (LT) or high throughput (HT) kits which are currently unsupported.

Overview	
Samples per kit	12
Workflow time	3 days for up to 12 samples

cDNA input	
Quantity	>15 ng per library cDNA concentration should be >1ng/µL with up to 15 µL in volume. See step 2.1 for 10x cDNA input requirement.
Average segment lengths	500–1,000 bp
Average 16-segment array lengths	10–15 kb

103-254-300 REV 02 MAR2024

PacBio

PacBio Documentation (103-254-300)

- Kinnex full-length RNA library prep protocol uses **Kinnex single-cell RNA kit**
  → **Do not use** SMRTbell prep kit 3.0 with this protocol

[1] Kit has not been tested for use on low throughput (LT) or high throughput (HT) kits which are currently unsupported. Please contact PacBio Technical Support for questions about compatibility of the Kinnex single-cell RNA library preparation workflow with other 10x Chromium kits.

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

Kinnex single-cell RNA kit bundle components

Kinnex single-cell RNA kit bundle provides full support for Kinnex library prep workflow

Kinnex single-cell RNA kit (103-072-200)

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 single-cell RNA libraries from input 10x Chromium Single Cell 5' and 3' cDNA.

Kinnex single-cell RNA kit components

Component		Description
1		**Kinnex capture beads kit (12 rxn)** • Contains reagents for removing template-switch oligo (TSO) artifacts from single-cell cDNA
2		**Kinnex single cell concatenation kit (12 rxn)** • Contains reagents for Kinnex array formation and SMRTbell template construction • Includes barcoded Kinnex adapter mixes (bcM0001 – bcM0004) • Also contains Kinnex capture primer oligos for TSO artifact removal
3		**SMRTbell cleanup beads** • For DNA cleanup
4		**Elution buffer** • For DNA cleanup

Kinnex single-cell RNA experimental design considerations

Kinnex single-cell RNA application use case recommendations for PacBio systems

	Sequel II and IIe systems	Revio system
Experimental goal	Characterize alternative splicing in single cells / cell types	
Sample multiplexing[1]	Not recommended	Up to 2 samples per Revio SMRT Cell (2-plex)
Cell input into 10x Chromium single cell 3' or 5' cDNA generation workflow	3,000 – 10,000 cells for running a single (non-multiplexed) sample on one Sequel II SMRT Cell 8M)	3,000 – 6,000 cells per sample if multiplexing 2 samples per Revio SMRT Cell (2-plex) 8,000 – 10,000 cells per sample if running a single (non-multiplexed) sample on one Revio SMRT Cell
Expected coverage	Obtain ≥3,000 – 10,000 unique reads/single cell	Obtain up to ~10,000 unique reads/single cell
Kinnex library prep protocol	Procedure & checklist – Preparing Kinnex libraries using Kinnex single-cell RNA kit (103-254-300)	
Single-cell cDNA input into Kinnex library prep workflow	15-75 ng of 10x Chromium 3' or 5' single cell cDNA	
SMRT Link data analysis workflows	Read Segmentation and Single-cell Iso-Seq Analysis	
Community data analysis tools	Annotation & quantification: SQANTI3 Differential analysis: TappAS Fusion calling: pbfusion Visualization: SWAN	

[1] Kinnex single-cell concatenation kit (103-242-000) can support up to 4-plex sample multiplexing per SMRT Cell through the use of four different barcoded Kinnex terminal SMRTbell adapters during Kinnex single-cell RNA library construction.

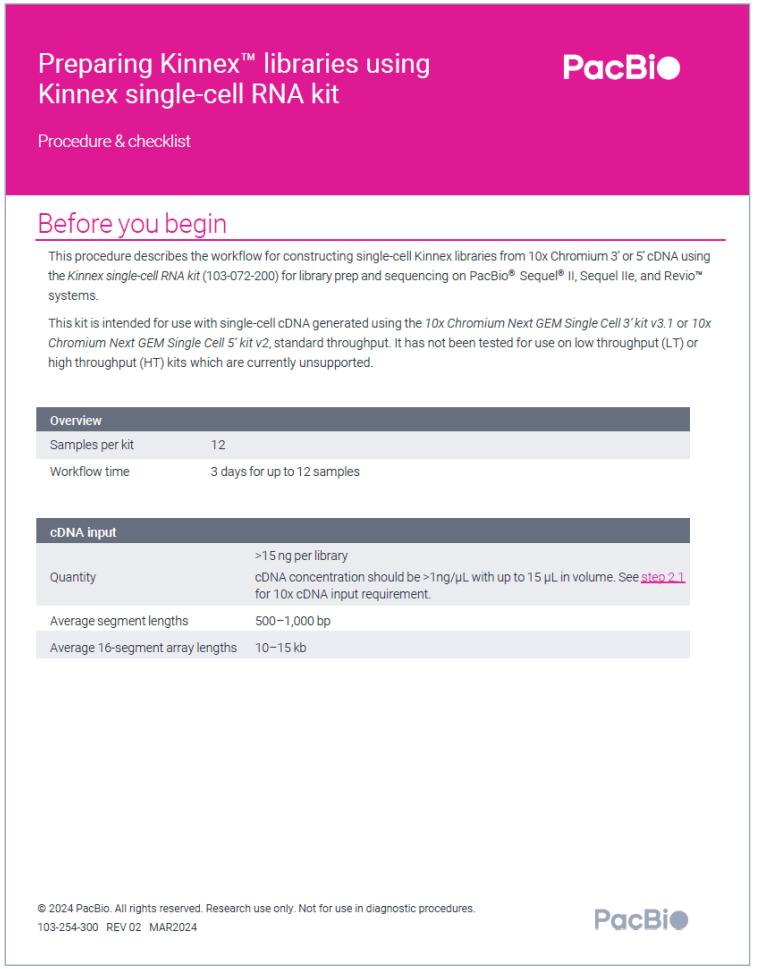
Kinnex single-cell RNA library preparation workflow details

Procedure & checklist – Preparing Kinnex libraries using the Kinnex single-cell RNA kit (103-254-300)

Procedure & checklist 103-254-300 describes the workflow for constructing single-cell Kinnex libraries from 10x Chromium 3' or 5' cDNA using the Kinnex single-cell RNA kit (103-072-200) for library prep and sequencing on PacBio Sequel II, Sequel IIe, and Revio systems[1]

Procedure & checklist contents

1. General best practices for reagent & sample handling and 10x single cell cDNA input recommendations.
2. Enzymatic workflow steps for removal of template-switch oligo (TSO) artifacts from input 10x single cell cDNA samples.
3. Enzymatic workflow steps for construction of 16-segment Kinnex arrays from 10x single cell cDNA.
4. Enzymatic workflow steps for DNA damage repair & nuclease treatment of Kinnex single-cell RNA SMRTbell libraries.
5. Workflow steps for final cleanup of Kinnex single-cell RNA SMRTbell libraries using SMRTbell cleanup beads.



Preparing Kinnex™ libraries using Kinnex single-cell RNA kit

PacBio

Procedure & checklist

Before you begin

This procedure describes the workflow for constructing single-cell Kinnex libraries from 10x Chromium 3' or 5' cDNA using the *Kinnex single-cell RNA kit* (103-072-200) for library prep and sequencing on PacBio® Sequel® II, Sequel IIe, and Revio™ systems.

This kit is intended for use with single-cell cDNA generated using the *10x Chromium Next GEM Single Cell 3' kit v3.1* or *10x Chromium Next GEM Single Cell 5' kit v2*, standard throughput. It has not been tested for use on low throughput (LT) or high throughput (HT) kits which are currently unsupported.

Overview	
Samples per kit	12
Workflow time	3 days for up to 12 samples

cDNA input	
Quantity	>15 ng per library cDNA concentration should be >1ng/µL with up to 15 µL in volume. See step 2.1 for 10x cDNA input requirement.
Average segment lengths	500–1,000 bp
Average 16-segment array lengths	10–15 kb

103-254-300 REV 02 MAR2024

PacBio

PacBio Documentation (103-254-300)

[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 single-cell RNA library construction workflow overview

Procedure & checklist – Preparing Kinnex libraries using Kinnex single-cell RNA kit (103-254-300)

	Cleanup	DNA QC	Walk-away time*
1 Input cDNA quality control	N/A	Qubit HS assay Bioanalyzer HS DNA kit	N/A
2 TSO PCR	1.5X SMRTbell cleanup beads	Qubit HS assay	1.5 hrs
Safe stop			
3 TSO artifact removal	1.5X SMRTbell cleanup beads	Qubit HS assay	1.5 hrs
Safe stop			
4 Kinnex PCR	1.5X SMRTbell cleanup beads	Qubit HS assay	1.5 – 2.0 hrs
Safe stop			
5 Kinnex array formation	1.2X SMRTbell cleanup beads	Qubit HS assay	1.5 hrs
Safe stop			
6 DNA damage repair	1.2X SMRTbell cleanup beads	N/A	30 min
Safe stop			
7 Nuclease Tx + final cleanup	1.2X SMRTbell cleanup beads	Qubit HS assay Femto Pulse gDNA 165 kb kit	1.5 hrs

Day 1 (~6 – 6.5 hrs*)

Day 2 (~2 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 single-cell RNA libraries

cDNA Input

- Use an optimal input range of 3,000 – 10,000 cells* for the 10x Chromium single cell 3’ cDNA generation workflow
- Follow the best practices in the **10x Chromium user guide**.
- Input cDNA quality control is highly recommended before proceeding to the Kinnex single-cell RNA workflow

Refer to 10x Genomics Support website to download 10x Chromium user guides and other documentation.

DNA sizing and quantitation QC

- Perform DNA concentration measurements with a **Qubit fluorometer** using the Qubit 1X dsDNA High Sensitivity (HS) Assay Kit.
- Perform DNA sizing measurements with a **Bioanalyzer system** using the High Sensitivity DNA Kit (for input cDNA QC) or with a **Femto Pulse system** using the Genomic DNA 165 kb Kit (for final SMRTbell library QC)

Qubit 4 fluorometer and 1X ds DNA High Sensitivity Assay Kit (Thermo Fisher Scientific)

Bioanalyzer 2100 System and High Sensitivity DNA Kit (Agilent Technologies)

Femto Pulse System and Genomic DNA 165 Kit (Agilent Technologies)

* **Note:** Cell capture efficiency of the 10x Chromium single cell workflow is **~60%**. For example, to achieve a **target cell recovery** of ~5,000 cells, approximately 10,000 cells can be used for input into the 10x Chromium single cell workflow.

General best practices recommendations for preparing Kinnex single-cell RNA libraries (cont.)

Reagent and sample handling

- Thaw repair buffer, nuclease buffer, and elution buffer at room temperature.
- Briefly vortex reagent buffers & Kinnex adapters prior to use. Enzyme mixes **do not** require vortexing.
- Quick spin all reagents to collect liquid at tube bottom prior to use.
- Keep all temperature-sensitive reagents on ice.
- Bring SMRTbell cleanup beads and Qubit 1X dsDNA HS reagents to room temperature for 30-60 minutes prior to use.
- Thoroughly pipette-mix all bead binding and elution steps until beads are distributed evenly in solution.
- Pipette mix all library prep reactions by pipetting up and down 10 times.
- Wide-bore pipette tips are recommended help to minimize foaming specifically when resuspending Kinnex capture beads
- Samples can be stored at 4°C at all safe stopping points listed in the protocol.
- 1.5X SMRTbell cleanup is recommended before Kinnex array formation.

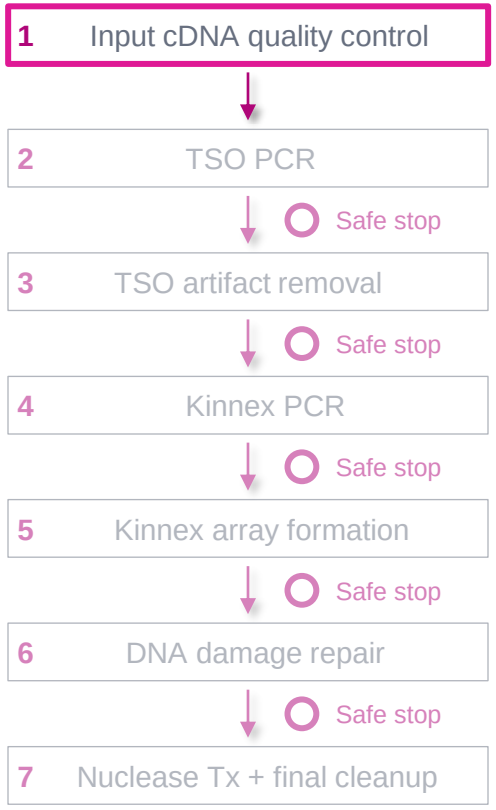
Temperature-sensitive reagents

Step used	Tube color	Reagent
TSO PCR and Kinnex PCR	Green	Kinnex single-cell PCR mix 103-244-500
	Yellow	Kinnex 3' capture primer mix 103-182-400
	Red	Kinnex 5' capture primer mix 103-182-200
	Orange	Kinnex primers premix (A-PQ)
		103-107-800 A
		103-107-900 B
		103-108-000 C
		103-108-100 D
		103-108-200 E
		103-108-300 F
		103-108-400 G
		103-153-000 H
		103-153-100 I
		103-153-200 J
		103-153-300 K
		103-153-400 L
		103-153-500 M
		103-153-600 N
		103-153-700 O
		103-153-800 PQ
Kinnex array formation	Light green	Kinnex single-cell enzyme 103-243-800
	Yellow	Kinnex single-cell ligase 103-244-000
	White	Kinnex single-cell ligase buffer 103-244-100
	Red	Kinnex single-cell ligation additive 103-244-400
	Blue	Kinnex adapter mix
		bc01 103-109-600
		bc02 103-109-700
		bc03 103-109-800
		bc04 103-109-900
DNA damage repair Nuclease treatment	Green	DNA repair mix 103-110-000
	Purple	Repair buffer 102-244-300
	Light green	Nuclease mix 103-110-100
	Light purple	Nuclease buffer 103-110-200

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

Input cDNA quality control

Input cDNA quality control is highly recommended before proceeding to the Kinnex single-cell RNA library prep workflow



- We recommend using an optimal range of 3,000 – 10,000 cells input into 10x Chromium 3' or 5' single cell workflow[1]
- Protocol requires a **minimum of 15 ng** of 10x Chromium single cell cDNA (**maximum of 75 ng** per library)
  - If your cDNA sample amounts are between **16 – 59 ng**, then normalize all samples to 15 ng
  - If your cDNA sample amounts are >**75 ng**, then normalize all samples to 75 ng
  - If your cDNA sample amounts are between **60 – 75 ng**, normalization is not required.
- Evaluate the size distribution of each input cDNA sample to determine whether it is suitable for the protocol (average cDNA fragment size should be between 500 – 1,500 bp)
  - 10x single cell cDNA samples measured with a Bioanalyzer system typically show a peak at ~1 – 1.8 kb

✔	Step	Instructions
	1.1	Bring the Qubit 1X dsDNA HS working solution and standards to room temperature.
	1.2	Pulse vortex or pipette mix each sample to homogenize the DNA in solution.
	1.3	Quick spin each sample to collect liquid.
	1.4	Take a 1 µL aliquot from each sample.
	1.5	Measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit.
	1.6	Dilute each sample to 1.0-1.5 ng/µL in elution buffer or water, based on the Qubit reading.
	1.7	Measure DNA size distribution with a Bioanalyzer system using the High Sensitivity DNA Kit.
	1.8	Proceed to the next step of the protocol if sample quality is acceptable.

[1] Refer to 10x Genomics Chromium Single Cell 3' Reagent Kits User Guide – v3.1 (CG000204)

Input cDNA quality control (cont.)

Example Bioanalyzer DNA sizing QC results for single cell 3’ cDNA prepared with the 10x Chromium system

1 Input cDNA quality control

2 TSO PCR

Safe stop

3 TSO artifact removal

Safe stop

4 Kinnex PCR

Safe stop

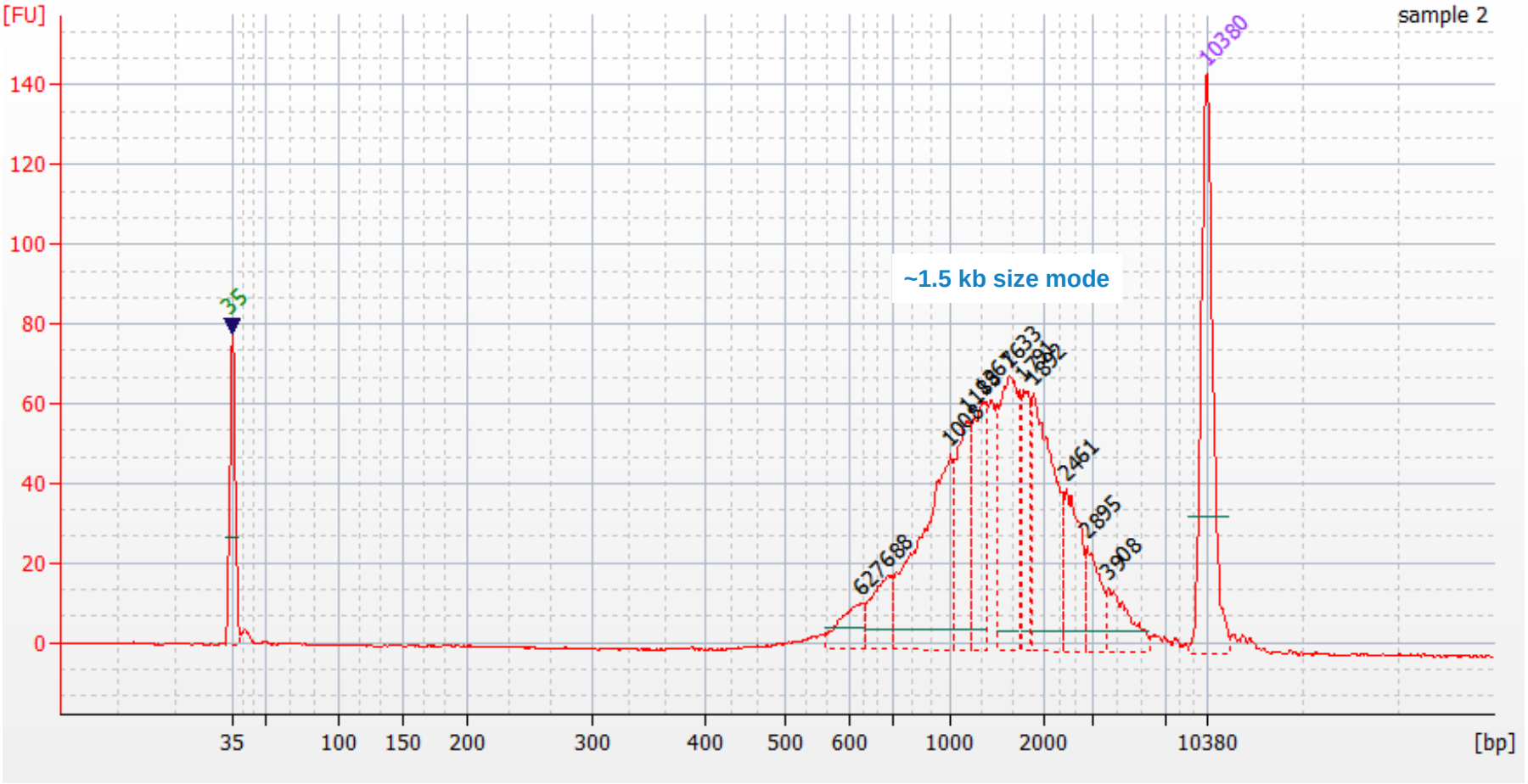
5 Kinnex array formation

Safe stop

6 DNA damage repair

Safe stop

7 Nuclease Tx + final cleanup



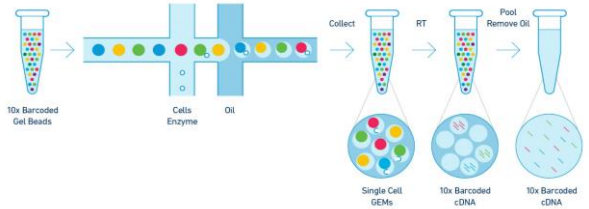
Bioanalyzer DNA sizing QC analysis results for a 10x Chromium single cell 3’ cDNA sample prepared from a human GM12878 cell line.

TSO PCR & TSO artifact removal

Perform Steps 2 & 3 to remove template switch oligo (TSO) priming artifacts generated during 10x cDNA synthesis

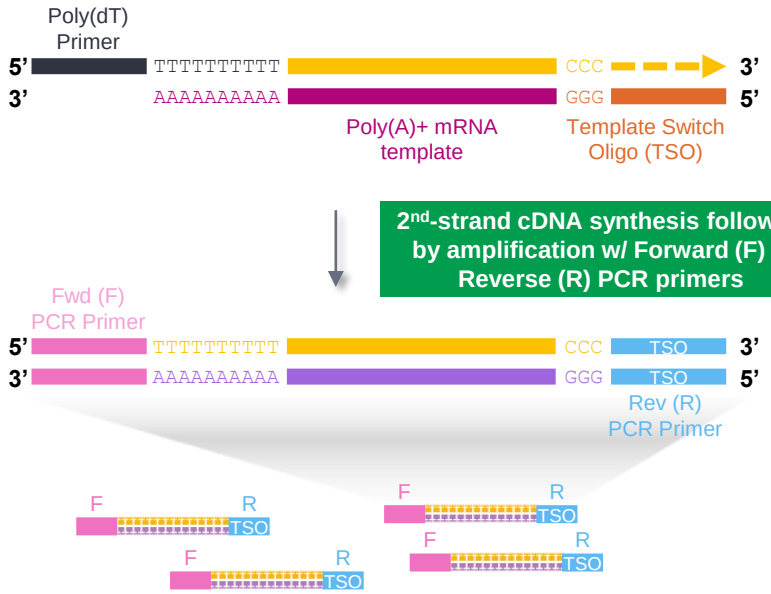
1 Input cDNA quality control
2 TSO PCR
Safe stop
3 TSO artifact removal
Safe stop
4 Kinnex PCR
Safe stop
5 Kinnex array formation
Safe stop
6 DNA damage repair
Safe stop
7 Nuclease Tx + final cleanup

TSO priming artifacts can occur if the TSO acts as a nonspecific primer on poly(A)+ mRNA



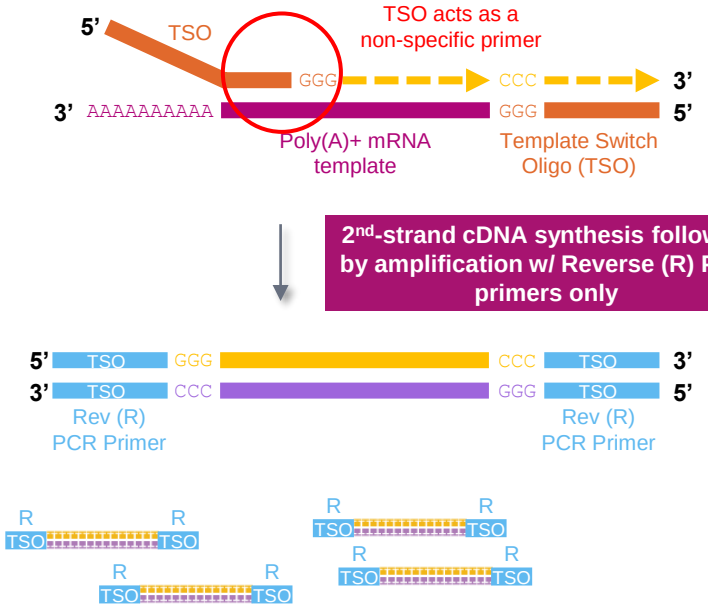
- Up to ~50% of cDNA products from the 10x Chromium single cell cDNA preparation workflow may contain a TSO priming artifact instead of the correct structure

Example synthesis of 10x 3' cDNA products containing correct structure



☑ Amplified (full-length) 10x cDNA products with correct structure (TSO sequence at one end)

Example synthesis of 10x 3' cDNA products containing TSO priming artifact



☒ Amplified (non-full length) 10x cDNA products with TSO priming artifacts (TSO sequences at both ends)

TSO PCR & TSO artifact removal (cont.)

Role of Kinnex capture primers and Kinnex capture beads

1 Input cDNA quality control

2 TSO PCR

Safe stop

3 TSO artifact removal

Safe stop

4 Kinnex PCR

Safe stop

5 Kinnex array formation

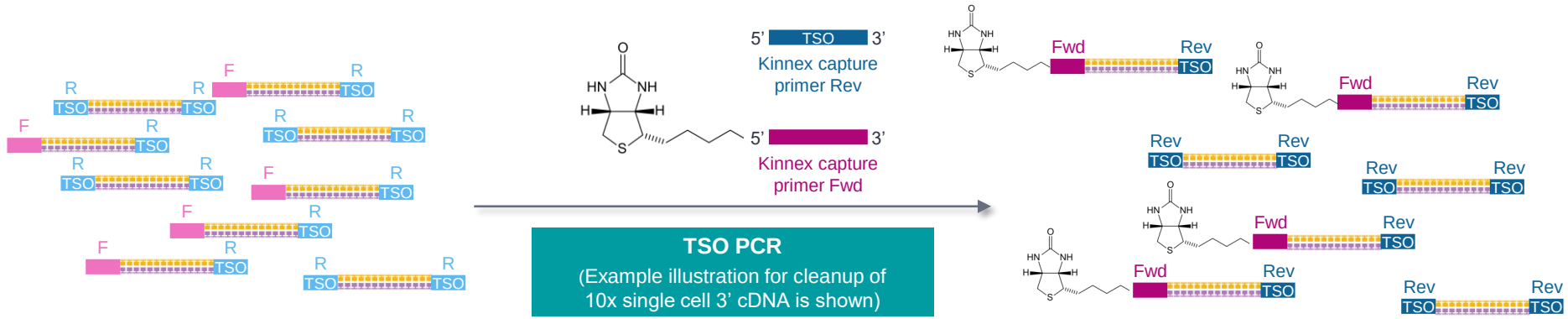
Safe stop

6 DNA damage repair

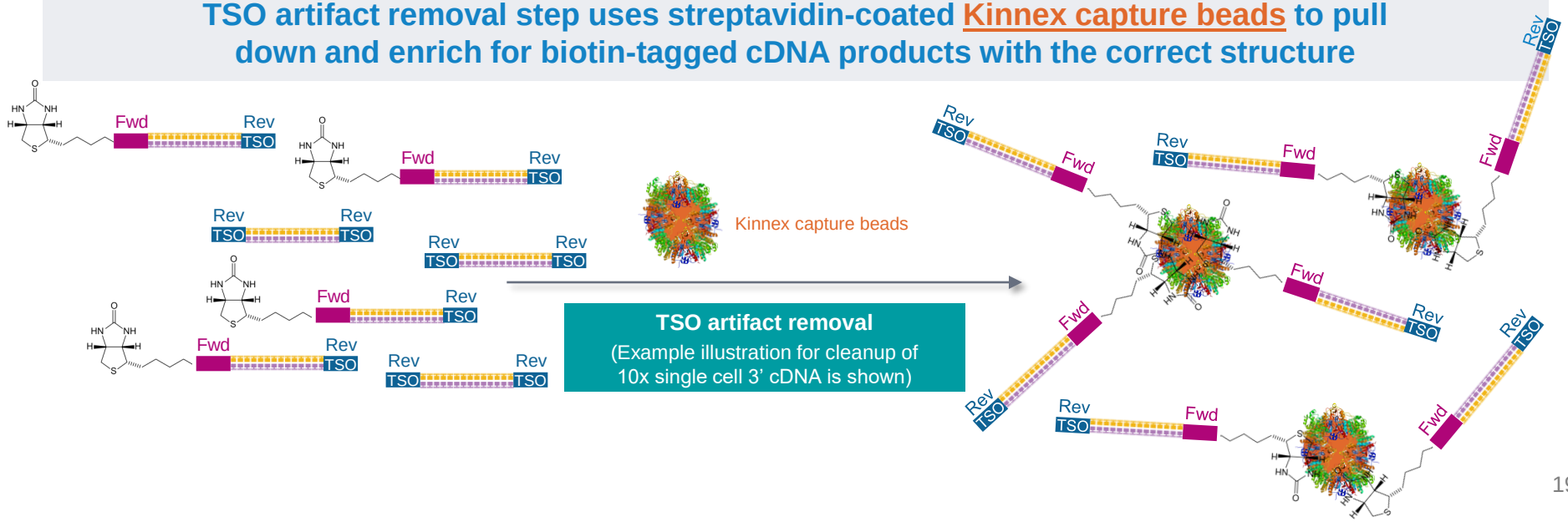
Safe stop

7 Nuclease Tx + final cleanup

TSO PCR step in Kinnex single-cell RNA procedure uses a modified PCR primer (Kinnex capture primer Fwd) to incorporate a biotin tag into desired cDNA products with the correct structure



TSO artifact removal step uses streptavidin-coated Kinnex capture beads to pull down and enrich for biotin-tagged cDNA products with the correct structure



TSO PCR & TSO artifact removal (cont.)

Procedural notes

1 Input cDNA quality control

↓

2 TSO PCR

↓ Safe stop

3 TSO artifact removal

↓ Safe stop

4 Kinnex PCR

↓ Safe stop

5 Kinnex array formation

↓ Safe stop

6 DNA damage repair

↓ Safe stop

7 Nuclease Tx + final cleanup

2. TSO PCR

Step	Instructions
	Normalize cDNA sample input to 15 ng if it is between 15 ng and 59 ng using elution buffer. Normalize cDNA sample input to 75 ng if it is higher than 75 ng using elution buffer. For cDNA amounts between 60–75ng, proceed without normalizing. Select either the Kinnex 3' or 5' capture primer mix depending on the 10x Genomics kit used. Set up the following PCR reaction on ice (RM1).
2.1	**Reaction Mix 1 (RM1):**

Tube color	Component	Volume
	Nuclease-free water	Up to 50 µl
Green	Kinnex single-cell PCR mix (103-244-500)	25 µL
Red	Kinnex 5' capture primer mix (103-182-200)	10 µL
	or	
Yellow	Kinnex 3' capture primer mix (103-182-400)	
	10x 5' or 3' cDNA library (1–5 ng/µL)	Up to 15 µL
	Total volume	**50 µL**

2.2	Pipette-mix **RM1**.
2.3	Quick spin **RM1** in a microcentrifuge to collect liquid.
2.4	Select the TSO PCR program based on cDNA input. Keep sample on ice until thermal cycler lid has heated to 105°C.

- If needed, **normalize** cDNA sample input amounts to 15 ng or 75 ng
- ***IMPORTANT!*** Select either the Kinnex 3' or 5' capture primer mix depending on the 10x Genomics kit used
- Set up TSO PCR reactions **ON ICE**
- PCR polymerase 3'→5' exonuclease activity negatively impacts amplification yield if prepared at room temp.
- For **lower** cDNA sample inputs (15 ng), use a **higher** number of PCR cycles (5)

TSO PCR program (15 ng input)

Heated lid set at 105°C

Step	Time	Temperature	Cycles
1	3 min	98°C	1
2	20 sec	98°C	
3	30 sec	65°C	5
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

Or TSO PCR program (60-75 ng input)

Heated lid set at 105°C

Step	Time	Temperature	Cycles
1	3 min	98°C	1
2	20 sec	98°C	
3	30 sec	65°C	3
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

PacBio

TSO PCR & TSO artifact removal (cont.)

Procedural notes

1. Input cDNA quality control
2. TSO PCR
   - Safe stop
3. TSO artifact removal
   - Safe stop
4. Kinnex PCR
   - Safe stop
5. Kinnex array formation
   - Safe stop
6. DNA damage repair
   - Safe stop
7. Nuclease Tx + final cleanup

3. TSO artifact removal

✔	Step	Instructions
	3.1	Bring Kinnex capture beads kit to room temperature. Resuspend the beads by vortexing.
	3.2	Transfer 10 µL resuspended Kinnex capture beads per sample to a PCR tube. Scale up the amount of beads if processing more than 4 samples (with 10% overage). If preparing more than 40 µL of beads, use a 1.5 mL LoBind tube instead of PCR tube.
	3.3	Place the tube on the magnet until the beads separate fully from the solution.
	3.4	Carefully remove and discard the supernatant while the tube remains on the magnet. Avoid touching the bead pellet with the pipette tip.
	3.5	• Remove the tube from the magnet. • Add 40 µL Kinnex bead binding buffer along the inside wall of the tube where the beads are collected and gently resuspend by pipetting using wide bore tips. DO NOT VORTEX. Note: the solution may be viscous. Highly recommend using wide bore tips to avoid foaming. When excess bubbles are present, lower cDNA recovery is expected. • Quick-spin the tube in a microcentrifuge if needed. Note: Scale up the volume of Kinnex capture binding buffer accordingly if preparing more than 40 µL of beads.
	3.6	Place the tube on the magnet until the beads separate fully from the solution and remove the supernatant.
	3.18	Add 2 µL Kinnex enzyme to the sample with capture beads to cleave the captured DNA products from Kinnex capture beads.
	3.19	Pipette-mix each sample and a very quick spin in a microcentrifuge to collect liquid.
	3.20	Run the TSO artifact removal program. **TSO artifact removal program** Heated lid set at 47°C

Step	Time	Temperature
1	30 min	37°C
2	Hold	4°C

	3.21	Place the tube on the magnet for 1 minute and move the supernatant containing the library to a fresh tube.

- Bring Kinnex capture beads to **room temperature** and resuspend by vortexing
- **Critical step! For all Kinnex capture bead handling steps: P**ipette mix with care and avoid generating bubbles by using **wide bore tips** for mixing (**do not vortex**)
  - When excess bubbles are present, lower cDNA recovery is expected
- Add Kinnex enzyme to **cleave** captured cDNA products from Kinnex capture beads
- Keep the **supernatant** after treatment with Kinnex enzyme and placement on the magnet
- After completing TSO artifact removal step, perform cleanup with 1.5X SMRTbell cleanup beads and proceed to Kinnex PCR (Step 4) if sample quantity is acceptable (minimum 25 ng)

PacBio

TSO PCR & TSO artifact removal (cont.)

TSO artifact video demonstration

1. Input cDNA quality control
2. TSO PCR
   - Safe stop
3. TSO artifact removal
   - Safe stop
4. Kinnex PCR
   - Safe stop
5. Kinnex array formation
   - Safe stop
6. DNA damage repair
   - Safe stop
7. Nuclease Tx + final cleanup

View Video tutorial – PacBio Kinnex single-cell RNA TSO artifact removal demo for Kinnex single-cell RNA kit at: YouTube Link

Kinnex PCR

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

1 Input cDNA quality control

2 TSO PCR

Safe stop

3 TSO artifact removal

Safe stop

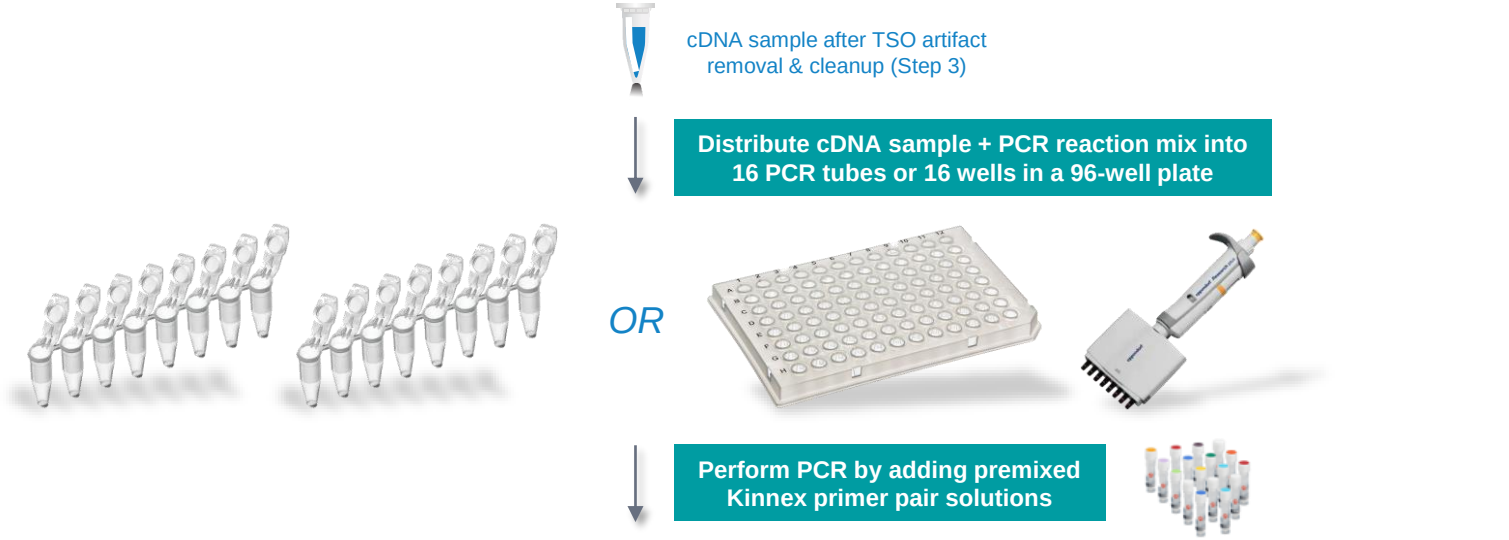
4 Kinnex PCR

Safe stop

7 Nuclease Tx + final cleanup

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

Set up 16 parallel PCR reactions/sample with premixed Kinnex primers to generate amplified cDNA products containing programmable sequences at both ends.



Kinnex PCR (cont.)

Procedural notes

1 Input cDNA quality control
↓
2 TSO PCR
↓ Safe stop
3 TSO artifact removal
↓ Safe stop
4 Kinnex PCR
↓ Safe stop
5 Kinnex array formation
↓ Safe stop
6 DNA damage repair
↓ Safe stop
7 Nuclease Tx + final cleanup

- 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

4. Kinnex PCR

✓	Step	Instructions
		Set up the following PCR reaction mix per sample on ice. Reaction Mix 2 (**RM2**):

✓	Master mix components	Volume for 16X concatenation*
	PCR-grade water	176−X µL
4.1	Kinnex single-cell PCR mix (103-244-500)	220 µL
	25 ng of amplified cDNA from Step 3.38	X µL
	Total volume	396 µL

X = 25 (ng) / purified pooled cDNA concentrations from Step 3.38
*10% overage included

4.2	Quick-spin **RM2** in a microcentrifuge to collect liquid.	
4.3	Add 22.5 µL of **RM2** to a new PCR tube on ice. Repeat this step to prepare a total of 16 tubes per sample (each containing 22.5 µL of **RM2**).	
	Add 2.5 µL of Kinnex primers premix into each of 16 PCR tubes on ice according to the table below.	

	PCR tube	Kinnex primers premix	PN
4.4	1	Kinnex primers premix A	103-107-800
	2	Kinnex primers premix B	103-107-900
	15	Kinnex primers premix O	103-153-700
	16	Kinnex primers premix PQ	103-153-800

4.5	Pipette-mix each sample. The total volume of each tube should be 25.0 µL.	
4.6	Quick-spin the strip tubes in a microcentrifuge to collect liquid.	
	Run the Kinnex PCR program with heated lid set to 105°C. Keep sample on ice until thermal cycler lid has heated to 105°C.	

- 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 16 Kinnex PCR reactions is required** – any missing/incorrect Kinnex primer pairs will result in no/low SMRTbell yield

Kinnex PCR program

Heated lid set at 105°C

Step	Time	Temperature	Cycles
1	3 min	98°C	1
2	20 sec	98°C	
3	30 sec	68°C	9
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

- After Kinnex PCR step, pool entire volume of all 16 reactions into a clean 1.5 mL LoBind tube[1] and perform cleanup with 1.5X SMRTbell cleanup beads

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

PacBio

Kinnex array formation

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

1 Input cDNA quality control

2 TSO PCR

Safe stop

3 TSO artifact removal

Safe stop

4 Kinnex PCR

Safe stop

5 Kinnex array formation

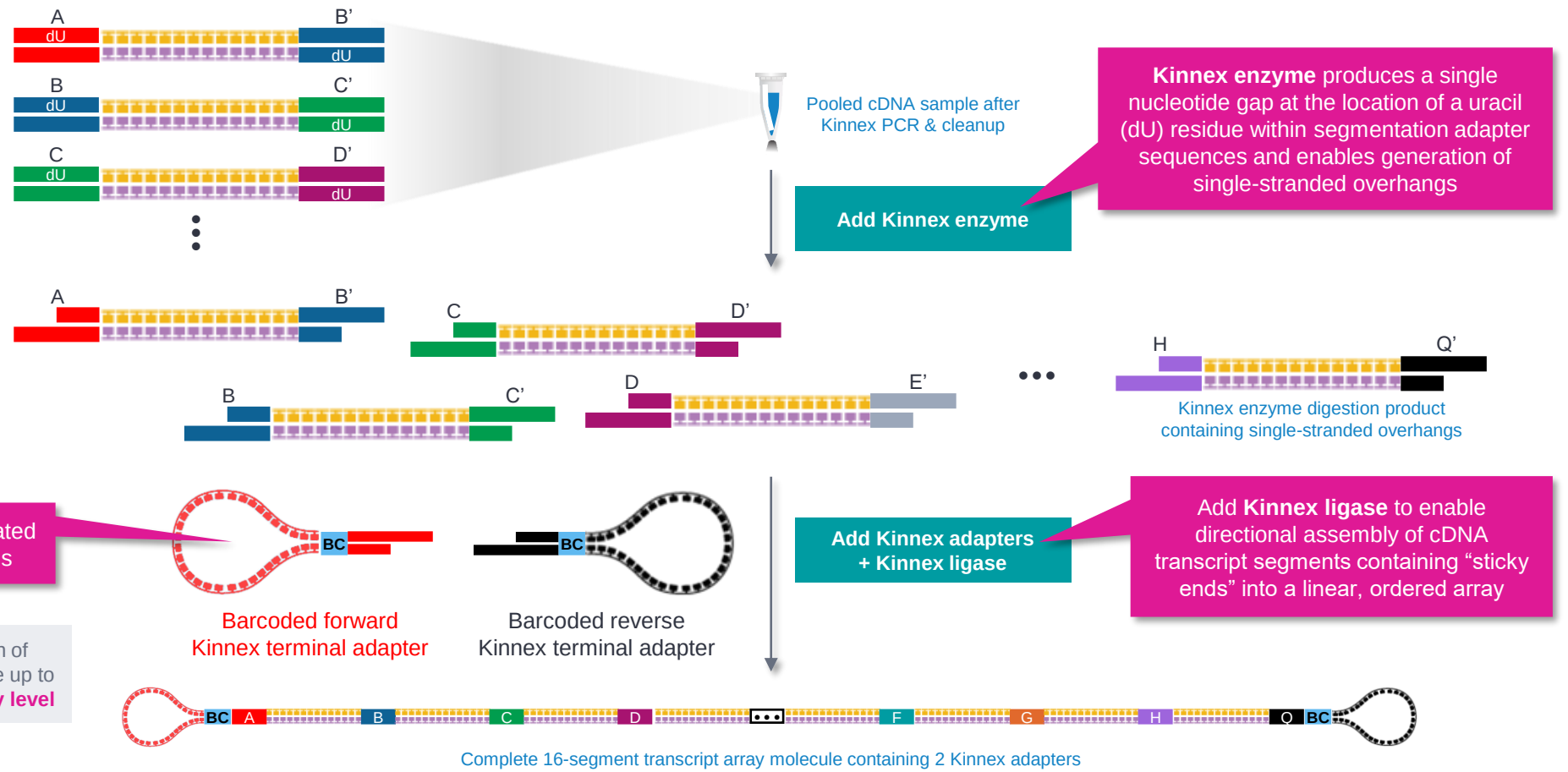
Safe stop

6 DNA damage repair

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**

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

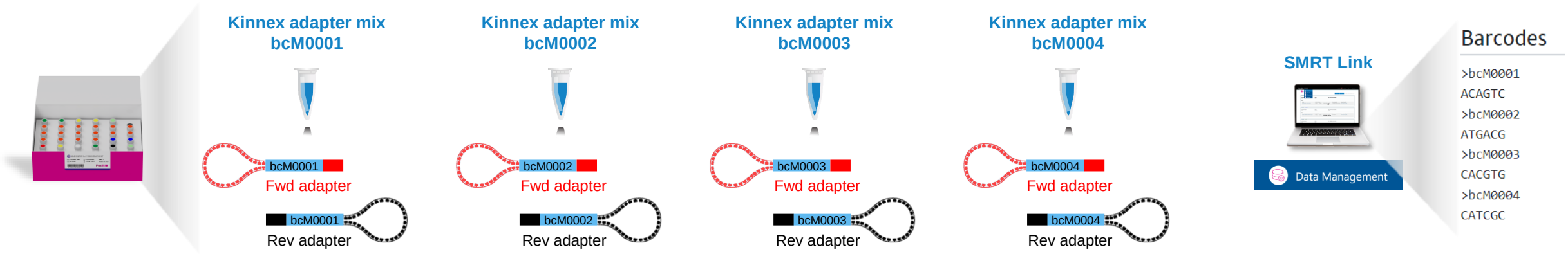


[1] **Note:** Single-cell Kinnex single-cell RNA library prep workflow described in this **Procedure & checklist** (103-254-300) is not compatible with standard SMRTbell adapters from SMRTbell prep kit 3.0 and is also not compatible with SMRTbell barcoded adapter plate 3.0.

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



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

Barcoded forward Kinnex terminal adapter

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

Barcoded reverse Kinnex terminal adapter

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

Kinnex array formation (cont.)

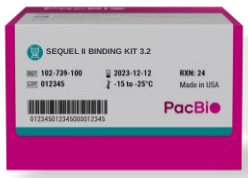
Kinnex terminal adapters use a new design that enables improved SMRT sequencing performance

- 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

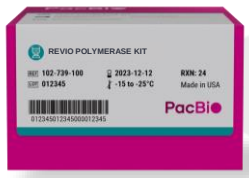
- New Kinnex adapter design requires a different sequencing primer (Kinnex sequencing primer 103-179-000)



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 cDNA quality control
2. TSO PCR
   - Safe stop
3. TSO artifact removal
   - Safe stop
4. Kinnex PCR
   - Safe stop
5. **Kinnex array formation**
   - Safe stop
6. DNA damage repair
   - Safe stop
7. Nuclease Tx + final cleanup

5. Kinnex array formation

Step	Instructions
5.1	In a 0.2 mL PCR tube, add **5 µg** of sample from Step 4.22, in 47 µL of volume (106 ng/µl). Dilute with elution buffer going into this step if sample is too concentrated.
5.2	Add 10 µL of Kinnex enzyme to create single-stranded extensions on PCR-amplified cDNA fragments to enable subsequent directional assembly of 16 PCR products.
5.3	Pipette-mix each sample.
5.4	Run the Kinnex primer digestion program.
5.5	Add 3 µL of Kinnex adapter barcode 01–04 (use a single barcode per sample) and 20 µL of Kinnex ligation additive to each sample for a total volume of 80 µL.
5.6	Pipette-mix each sample.
5.7	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample in the order and volume listed below. **Reaction Mix 3 (RM3):**

Tube color	Component	Volume
Blue	Kinnex barcode adapter	3 µL
Red	Kinnex ligation additive	20 µL
	Total volume	23 µL

Tube color	Component	Volume
White	Kinnex single-cell ligase buffer 103-244-100	10 µL
Yellow	Kinnex single-cell ligase 103-244-000	10 µL
	Total volume	20 µL

Step	Instructions
5.12	Run the Kinnex array ligation program.

- 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

Kinnex primer digestion program

Heated lid set at 47°C

Step	Time	Temperature
1	30 min	37°C
2	Hold	4°C

- ***IMPORTANT:*** Always add MAS adapters and MAS ligation additive to the sample BEFORE adding MAS ligase
- If combining multiple barcoded Kinnex libraries for sequencing, make sure each library uses one of the 4 different Kinnex barcoded adapters

Kinnex array ligation program

Heated lid set at 52°C

Step	Time	Temperature
1	60 min	42°C
2	Hold	4°C

- After Kinnex array formation step, perform cleanup with 1.2X SMRTbell cleanup beads and proceed to DNA damage repair (Step 6)

DNA damage repair & nuclease treatment

Perform DNA damage repair to repair nicked/damaged DNA sites and perform nuclease treatment to remove incomplete SMRTbell template arrays

1 Input cDNA quality control

2 TSO PCR

Safe stop

3 TSO artifact removal

Safe stop

4 Kinnex PCR

Safe stop

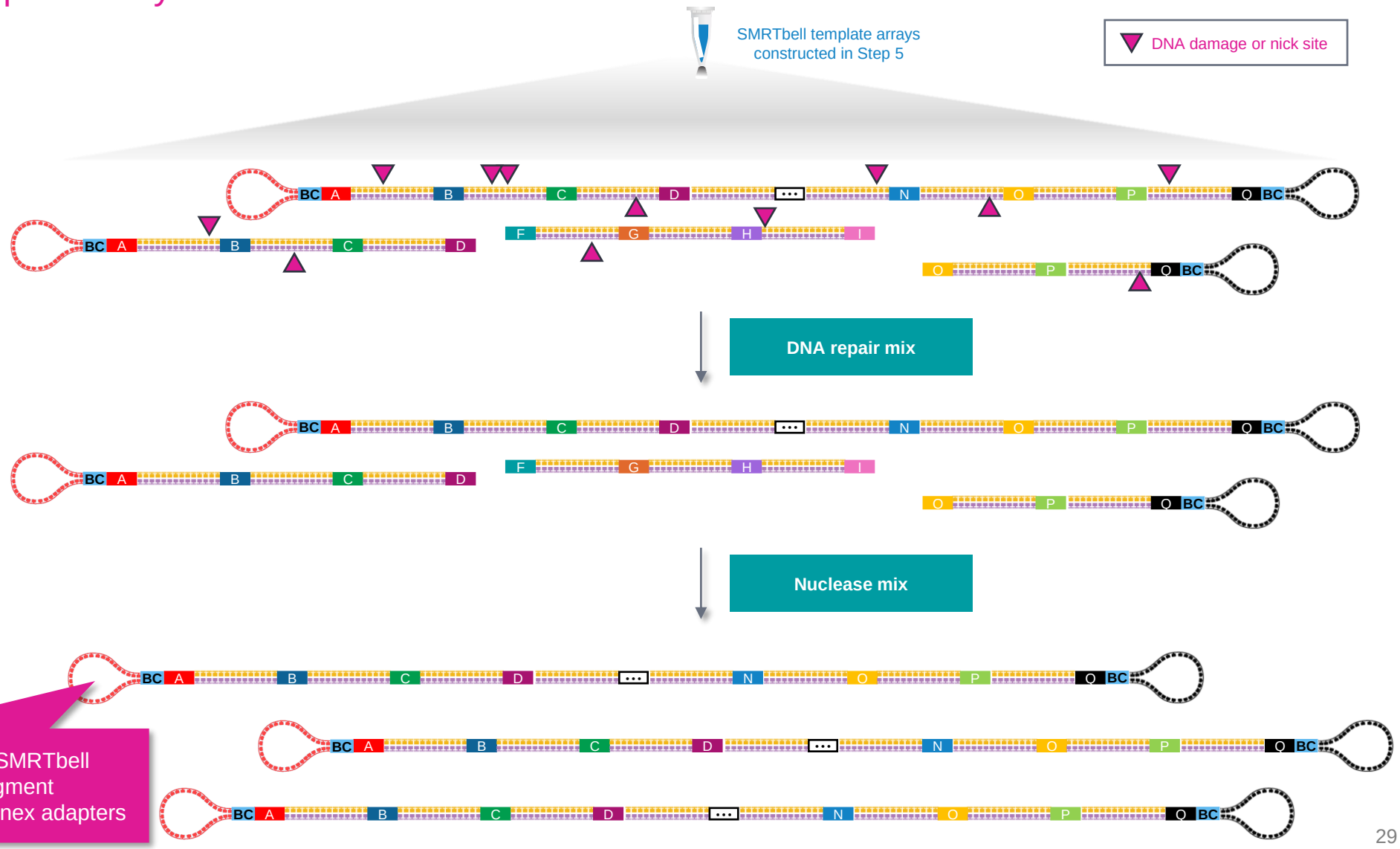
5 Kinnex array formation

Safe stop

6 DNA damage repair

Safe stop

7 Nuclease Tx + final cleanup



- After nuclease treatment, most remaining SMRTbell templates are complete (full-length) 16-segment transcript array molecules capped with Kinnex adapters

DNA damage repair

Procedural notes

1 Input cDNA quality control
↓
2 TSO PCR
↓ Safe stop
3 TSO artifact removal
↓ Safe stop
4 MAS PCR
↓ Safe stop
5 MAS array formation
↓ Safe stop
6 DNA damage repair
↓ Safe stop
7 Nuclease Tx + final cleanup

6. DNA damage repair

✓	Step	Instructions
	6.1	In a 0.2 mL PCR tube, add 42 µL of sample from Step 5.26
		Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample in the order and volume listed below.
		Reaction Mix 4 (RM4):
	6.2	

✓	Tube color	Component	Volume
	Purple	Repair buffer 102-696-100	6 µL
	Green	DNA repair mix 102-696-000	2 µL
		Total volume	8 µL

	6.3	Pipette-mix **RM4**.
	6.4	Quick-spin **RM4** in a microcentrifuge to collect liquid.
	6.5	Add 8 µL of **RM4** to each sample. Total volume should equal 50 µL.
	6.6	Pipette-mix each sample.
	6.7	Quick-spin the strip tube in a microcentrifuge to collect liquid.
	6.8	Run the DNA damage repair program.

- **DO NOT** proceed with more than 5 µg of Kinnex array input per individual DDR reaction (in a 50 µL reaction volume)
  - Adding more than 5 µg per reaction can result in incomplete digestion of partial (non-full length) SMRTbell template arrays, resulting in low *P1* loading performance during sequencing

DNA damage repair program

Heated lid set at 47°C

Step	Time	Temperature
1	30 min	37°C
2	Hold	4°C

- After DNA damage repair step, perform cleanup with 1.2X SMRTbell cleanup beads and proceed to Nuclease treatment (Step 7)

Nuclease treatment

Procedural notes

1 Input cDNA quality control

2 TSO PCR

Safe stop

3 TSO artifact removal

Safe stop

4 MAS PCR

Safe stop

5 MAS array formation

Safe stop

6 DNA damage repair

Safe stop

7 Nuclease Tx + final cleanup

7. Nuclease treatment

Step	Instructions
7.1	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below. **Reaction Mix 5 (RM5):** Tube color: Light purple — Component: Nuclease buffer 103-110-200 — Volume: 5 µL Tube color: Light green — Component: Nuclease mix 103-110-100 — Volume: 5 µL Total volume: 10 µL
7.2	Pipette-mix **RM5**.
7.3	Quick-spin **RM5** in a microcentrifuge to collect liquid.
7.4	Add 10 µL of **RM5** to each sample. Total volume should equal 50 µL.
7.5	Pipette-mix each sample.
7.6	Quick-spin the strip tube in a microcentrifuge to collect liquid.
	Run the nuclease treatment program.

- **1-hr** nuclease treatment

Nuclease treatment program

Heated lid set at 47°C

Step	Time	Temperature
1	60 min	37°C
2	Hold	4°C

- After Nuclease treatment step, perform final cleanup with 1.2X SMRTbell cleanup beads (Step 8)

Final cleanup with SMRTbell cleanup beads

Procedural notes

1. Input cDNA quality control
2. TSO PCR
   - Safe stop
3. TSO artifact removal
   - Safe stop
4. MAS PCR
   - Safe stop
5. MAS array formation
   - Safe stop
6. DNA damage repair
   - Safe stop
7. Nuclease Tx + final cleanup

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

7.8 Final cleanup with 1.2X SMRTbell cleanup beads

Step	Instructions
7.8	Add 60 µL SMRTbell cleanup beads to each sample from the previous step. Pipette-mix the beads until evenly distributed.
7.9	Quick-spin the tube strip in a microcentrifuge to collect all liquid.
7.10	Leave at room temperature for 10 minutes to allow DNA to bind beads.
7.11	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
7.12	Slowly pipette off the cleared supernatant without disturbing the beads. It is recommended to save the supernatant in another tube strip in case of poor DNA recovery.
7.13	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.
7.14	Repeat the previous step.
7.15	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. Pipette off residual 80% ethanol and discard.
7.16	Remove the tube strip from the magnetic rack. Immediately add 26 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
7.17	Quick-spin the tube strip in a microcentrifuge to collect liquid.
7.18	Leave at room temperature for 5 minutes to elute DNA.
7.19	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
7.20	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new 0.5 mL LoBind tube. Discard old tube strip with beads.
7.21	Take a 1 µL aliquot from each tube. Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Calculate the total mass. Recommended: Further dilute each aliquot to 250 pg/µL with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system.
7.22	Proceed to SMRT Link Sample Setup to prepare the SMRTbell library for sequencing. **DNA concentration must be less than 20 ng/µl to go into ABC. Using a concentration above 20 ng/µl will result in lower loading during sequencing.**
7.23	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 single-cell RNA library using a Femto Pulse system (expected final library insert size is **~10 – 15 kb**)

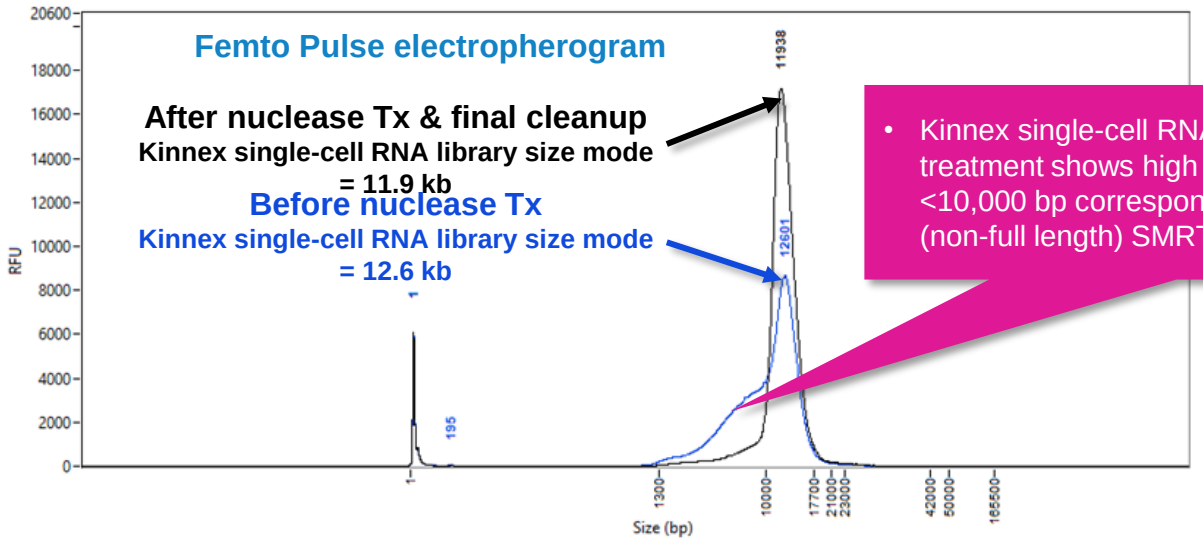
- Kinnex single-cell RNA final SMRTbell library concentration must be **≤20 ng/ µL** to proceed with SMRT Link sample setup (ABC[1])
  - → Using a concentration above 20 ng/µL will result in lower loading during sequencing

[1] ABC = Anneal sequencing primer / Bind polymerase / Complex cleanup.

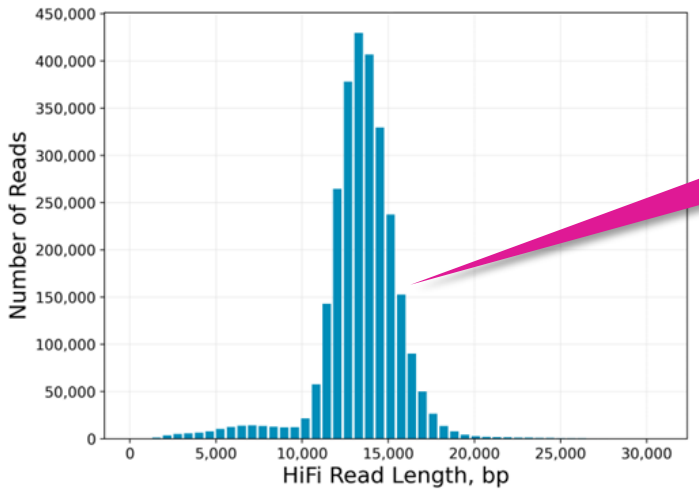
Final cleanup with SMRTbell cleanup beads (cont.)

Example Femto Pulse DNA sizing QC results for Kinnex single-cell RNA library before nuclease treatment and after nuclease treatment & final cleanup

1. Input cDNA quality control
2. TSO PCR
   - Safe stop
3. TSO artifact removal
   - Safe stop
4. MAS PCR
   - Safe stop
5. MAS array formation
   - Safe stop
6. DNA damage repair
   - Safe stop
7. **Nuclease Tx + final cleanup**



- Kinnex single-cell RNA library before nuclease treatment shows high amounts of smaller fragments <10,000 bp corresponding mostly to **incomplete** (non-full length) SMRTbell template arrays



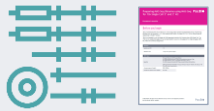
- HiFi read length mode is consistent with expected final library insert size

Kinnex single-cell RNA library prep inputs & expected step yields

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

Kinnex single-cell

Preparing Kinnex libraries using Kinnex single-cell RNA kit (103-254-300)



Library insert generation

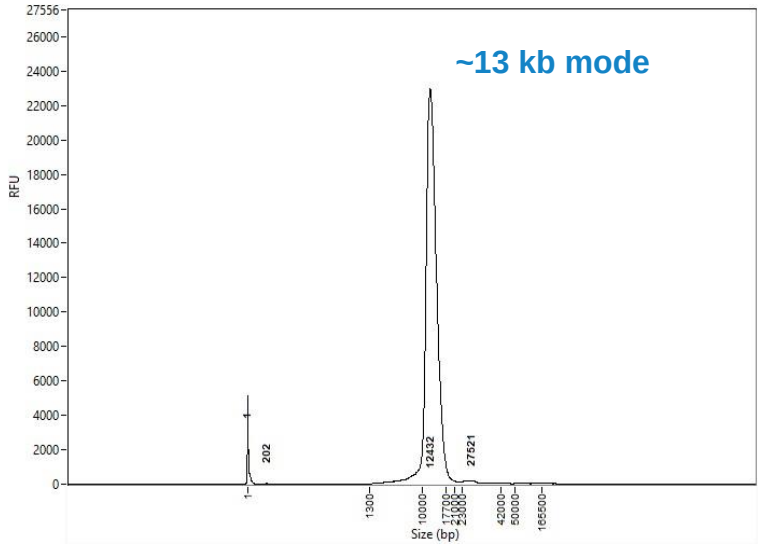
TSO PCR	SMRTbell bead cleanup	TSO removal	SMRTbell bead cleanup
IN: 15 ng, 60-75 ng or 75 ng of 10x 3' or 5' cDNA	**OUT: ~200 ng – 1 µg of amplified products**	**IN:** 150 ng – 1 µg of TSO PCR products	**OUT: ≥50 ng of TSO artifact-depleted cDNA**

Kinnex PCR

Kinnex PCR	SMRTbell bead cleanup
Min. IN: 50 ng* of TSO artifact-depleted cDNA	**OUT: ≥10 µg of amplified products after pooling**

Kinnex array formation

Kinnex digestion	Ligation	SMRTbell cleanup	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 single-cell library generated for a 10x Chromium single-cell 3' cDNA sample prepared from a human cell line (HG002).

Example Kinnex single-cell RNA library prep yields

10x single cell 3' cDNA input	15 ng
cDNA input for Kinnex array formation	5000 ng
Post-nuclease treatment & final library cleanup yield (%)[1]	1100 ng (22.0%)

[1] **Post-nuclease treatment & final cleanup yields typically ranged from ~10% to ~25% when using single-cell 3' cDNA samples for Kinnex single-cell RNA library construction.**

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

Kinnex single-cell RNA sequencing preparation workflow details

Sample Setup & Run Design recommendations for Kinnex single-cell RNA libraries

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

Select desired **application type** in SMRT Link

SMRT Link v13.1+

- Sample Setup
- Runs

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

Application type

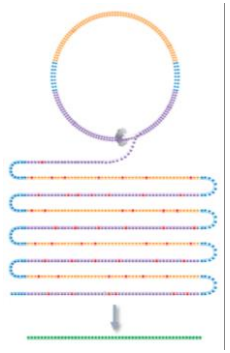
Kinnex single-cell RNA[1]

Whole genome sequencing
- Human WGS
- Microbial assembly
- Other WGS

RNA sequencing
- Iso-Seq method
- MAS-Seq single cell
- Kinnex single-cell RNA
- Kinnex full-length RNA

Viral sequencing
- Adeno-associated virus

Metagenomics
- Kinnex 16S rRNA



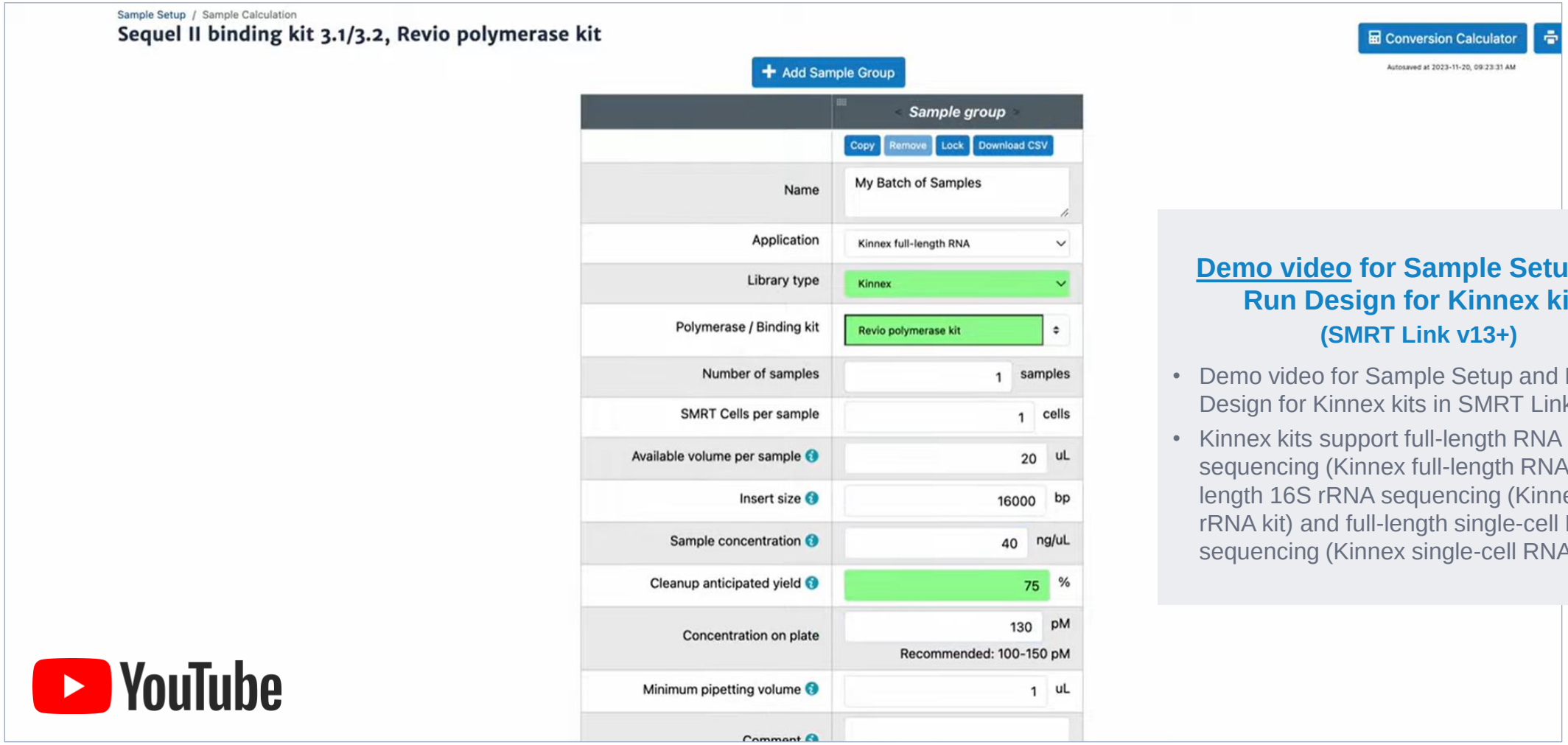
HiFi read

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 single-cell RNA kit requires SMRT Link v13.1 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+)

- Demo video for Sample Setup and Run Design for Kinnex kits in SMRT Link v13+
- 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 single-cell RNA libraries

Revio system

Sequel II and IIe systems

Sample group	
	Copy · Remove · Lock · Download CSV
Name	Kinnex single-cell 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	15000 bp
Sample concentration	20 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 16 sc-cDNA segments

Sample group	
	Copy · Remove · Lock · Download CSV
Name	Kinnex single-cell 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	15000 bp
Sample concentration	20 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 16 sc-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

- Kinnex single-cell RNA library input concentration should be normalized to **20 ng/μL** for sample setup

- 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**

Single-cell RNA

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

Application (Required)	Kinnex single-cell RNA
Plate Well (Required)	Plate 1, Well A01
Well Name (Required)	Kinnex single-cell RNA library demo
Well Comment	
Library Type (Required)	Kinnex
Insert Size (bp) (Required)	15000
Polymerase Kit (Required)	Revio polymerase kit
Movie Acquisition Time (hours)	24

Specify **Use Adaptive Loading = YES**

Use Adaptive Loading: YES / NO

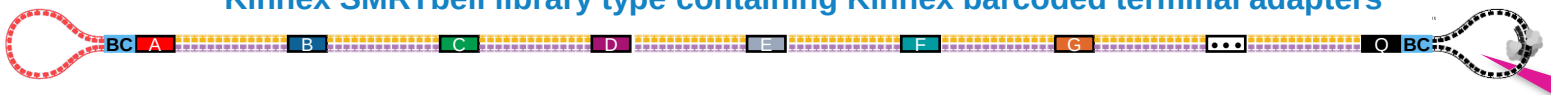
Specify **Insert Size**

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]

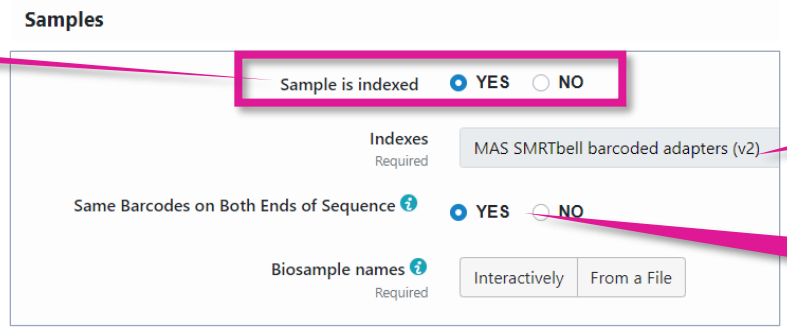
[1] **Note:** When sequencing a Kinnex library sample, if 'Standard' library type is mistakenly selected instead of 'Kinnex' then a higher missing adapter rate (> 95%) and a slight degradation in barcode demultiplexing performance (~93-96% barcoded HiFi read yield) will be observed.

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

Sample indexing (barcoding) information

Single-cell RNA

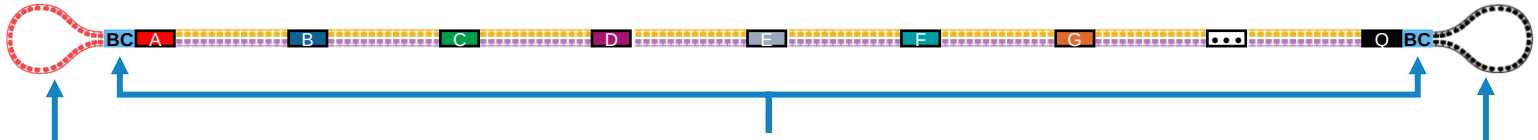
Default = **YES** for **Sample is indexed**



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

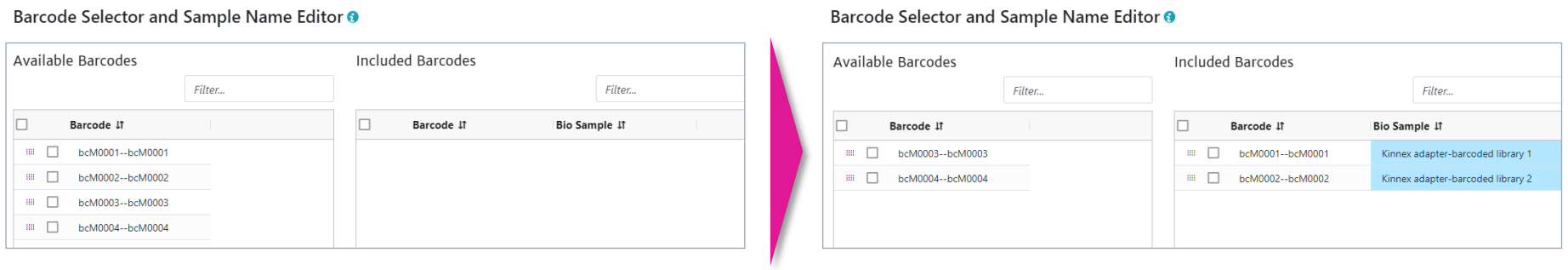


Barcoded forward Kinnex terminal adapter

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

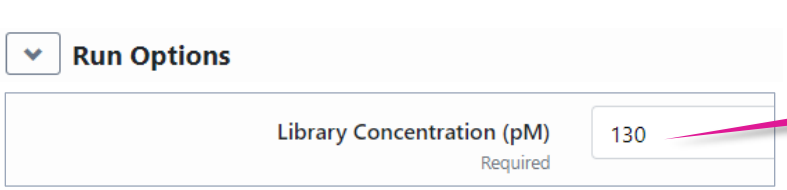
```
>bcM0001
ACAGTC
>bcM0002
ATGACG
>bcM0003
CACGTG
>bcM0004
CATCGC
```

[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.

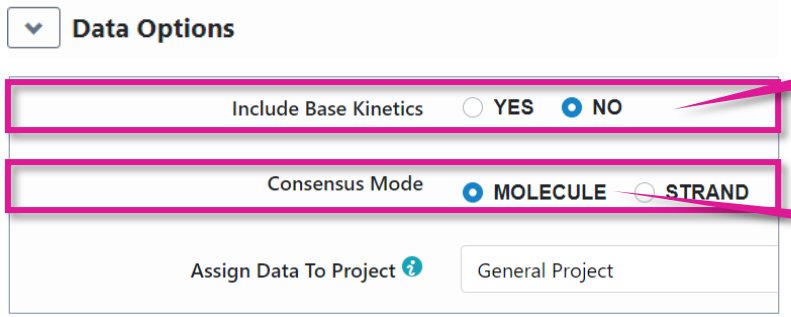
SMRT Link Run Design procedure for Revio system (cont.)

Run options and data options

Kinnex single-cell 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.

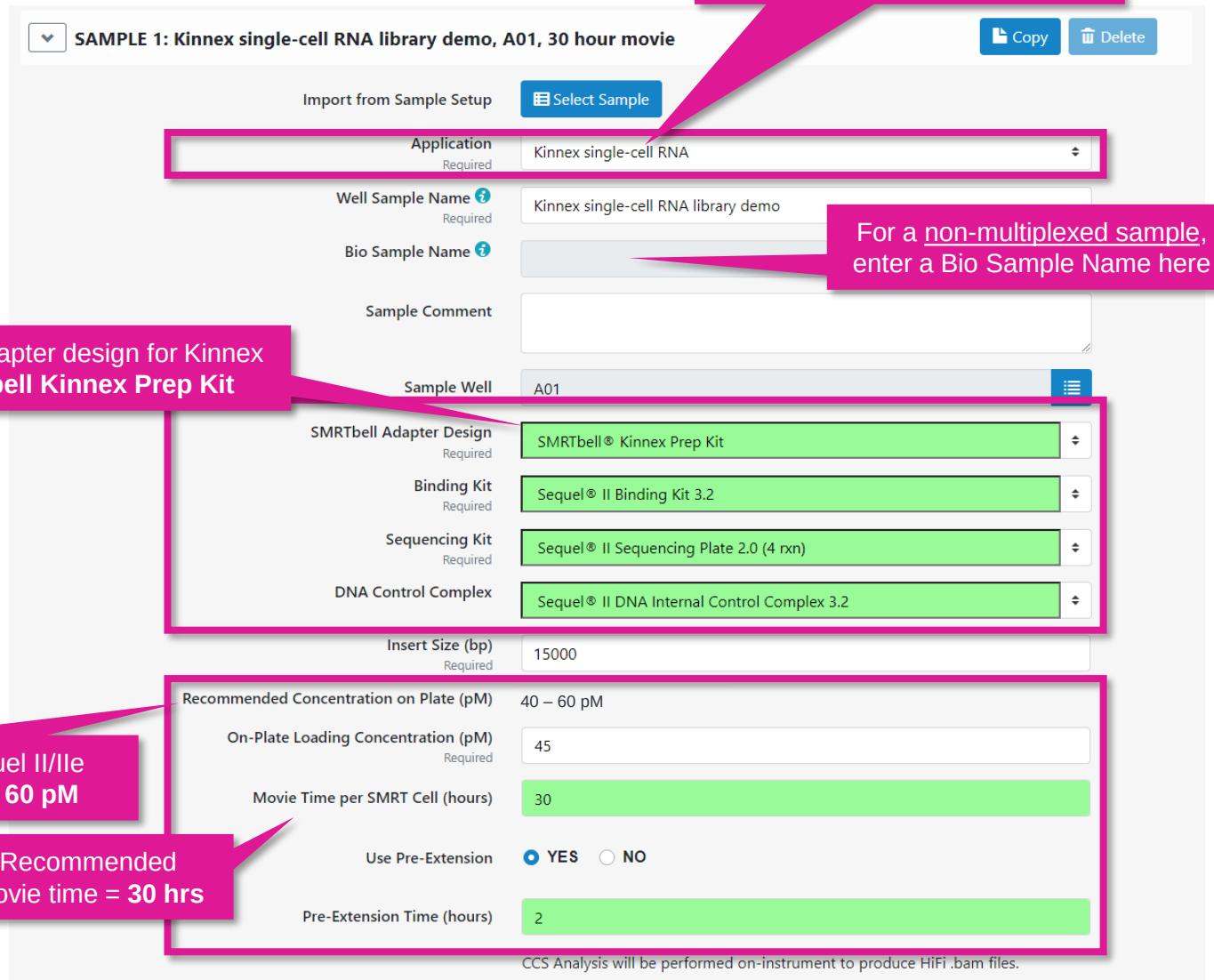
SMRT Link Run Design procedure for Sequel II/IIe systems

Sample information and run information

Kinnex single-cell 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 single-cell RNA library sample.

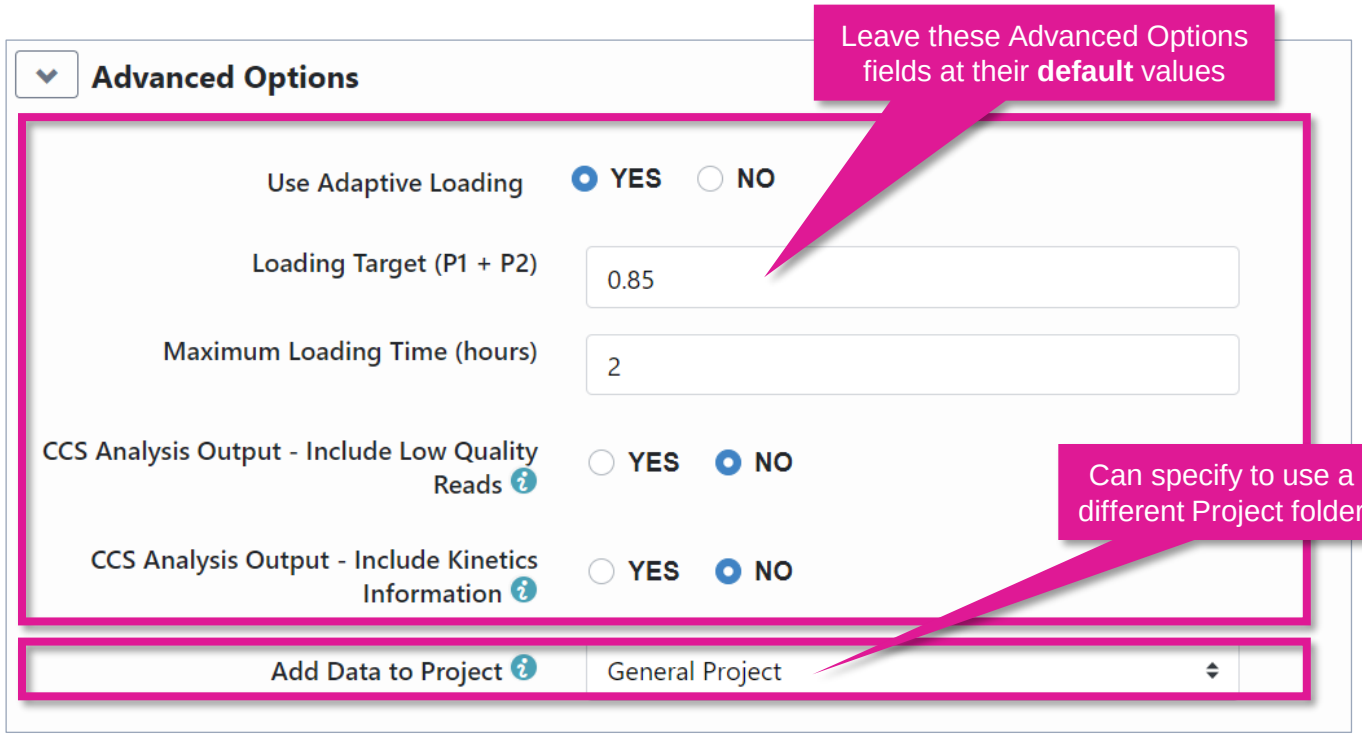
[1] **Note:** When sequencing a Kinnex library sample, if 'Overhand-SMRTbell Prep Kit 3.0' is mistakenly selected instead of 'SMRTbell Kinnex Prep kit' in the SMRTbell Adapter Design field, then a higher missing adapter rate (> 95%) and a slight degradation in barcode demultiplexing performance (~93-96%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

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

Advanced options

Kinnex single-cell 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 single-cell RNA library sample.

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

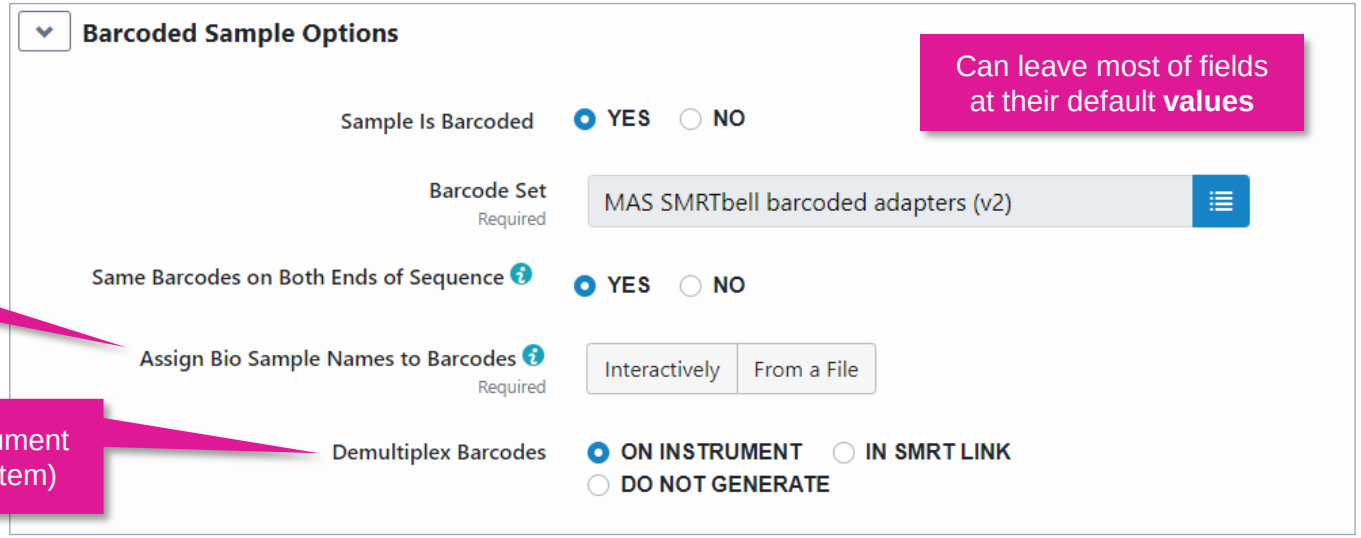
Barcoded sample options

Single-cell RNA

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

Non-multiplexed Kinnex single-cell RNA library

Multiplexed Kinnex single-cell RNA library



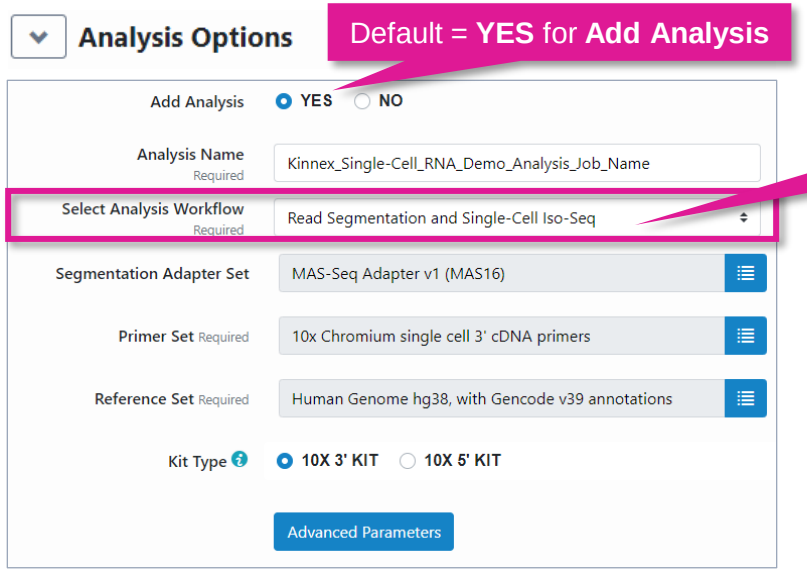
Specify Bio Sample Names, either interactively or by downloading a CSV file (**Interactively** or **From a file**)

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

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

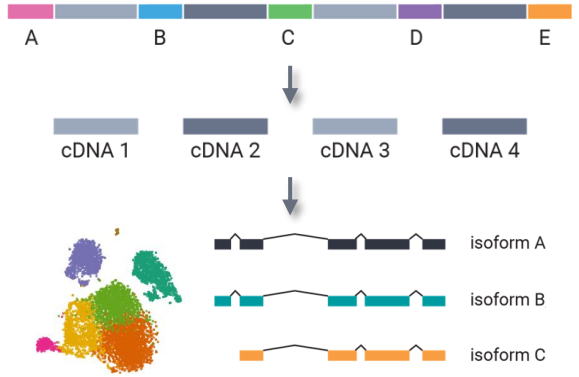
SMRT Link Run Design analysis options for Revio system and Sequel II/IIe systems

Kinnex single-cell RNA



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

Read Segmentation and Single-Cell Iso-Seq



Perform isoform-classification analysis
to identify novel genes & isoforms

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 single-cell RNA

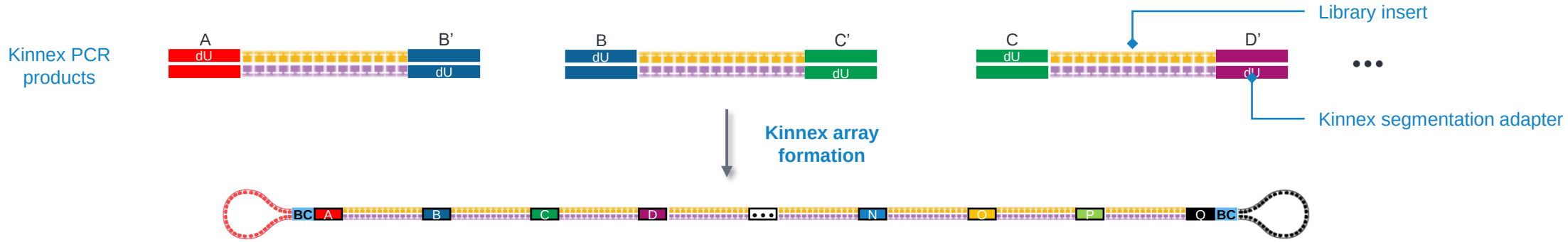
Analysis Options

Add Analysis	YES / NO
Analysis Name (Required)	Kinnex_Single-Cell_RNA_Demo_Analysis_Job_Name
Select Analysis Workflow (Required)	Read Segmentation and Single-Cell Iso-Seq
Segmentation Adapter Set	MAS-Seq Adapter v1 (MAS16)
Primer Set (Required)	10x Chromium single cell 3' cDNA primers
Reference Set (Required)	Human Genome hg38, with Gencode v39 annotations
Kit Type	10X 3' KIT / 10X 5' KIT

Advanced Parameters

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

→ For Kinnex single-cell RNA samples, specify **MAS-Seq Adapter v1 (MAS16)**



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 single-cell cDNA amplification

Kinnex single-cell RNA

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

10x Forward (F) PCR primer

10x Reverse (R) PCR primer

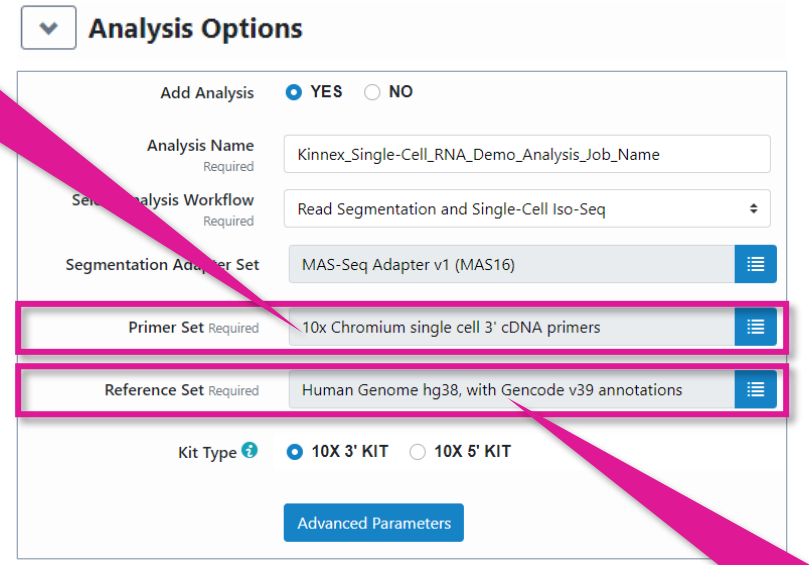
For Kinnex single-cell 3’ RNA analysis, select ‘10x Chromium single cell 3’ cDNA primers’

```
>5p
AAGCAGTGGTATCAACGCAGAGTACATGGG
>3p
AGATCGGAAGAGCGTCGTGTAG
```

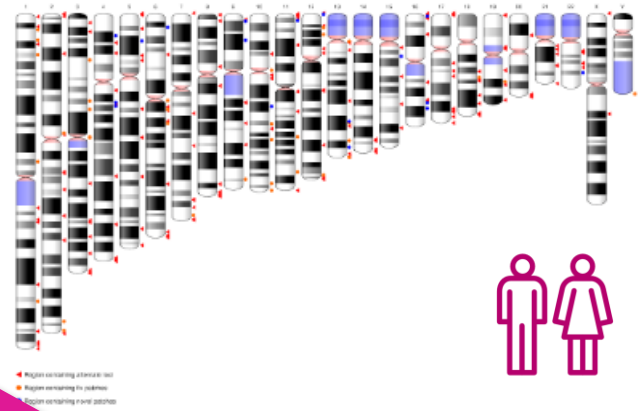
OR

For Kinnex single-cell 5’ RNA analysis, select ‘10x Chromium single cell 5’ cDNA primers’

```
>5p
CTACACGACGCTCTTCCGATCT
>3p
GTACTCTGCGTTGATACCACTGCTT
```



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 set is:

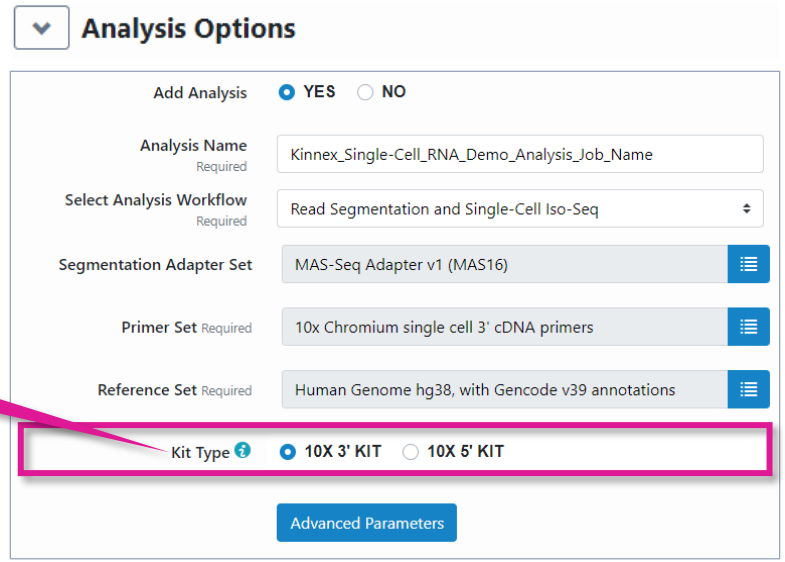
- Human Genome hg38, with Gencode v39 annotations

See ***SMRT Link User Guide*** documentation for detailed descriptions of parameter settings for Read Segmentation and Iso-Seq analysis application. To view 10x Chromium single cell (5’ and 3’) cDNA primer sequences, go to SMRT Link Data Mangement module and select to view ‘Barcode Sets’ > ‘10x Chromium single cell 5’ cDNA primers’ or ‘10x Chromium single cell 3’ cDNA primers’.

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

Kinnex single-cell RNA

Specify **Kit Type** used for single-cell cDNA generation



Specification of Kit Type (10x 3’ Kit or 10x 5’ Kit) **determines which set of 10x barcode sequences to use**, and also affects **UMI and single-cell barcode design settings**[1]

For Kinnex single-cell 3’ RNA analysis, select ‘10x 3’ Kit’

OR

For Kinnex single-cell 5’ RNA analysis, select ‘10x 5’ Kit’

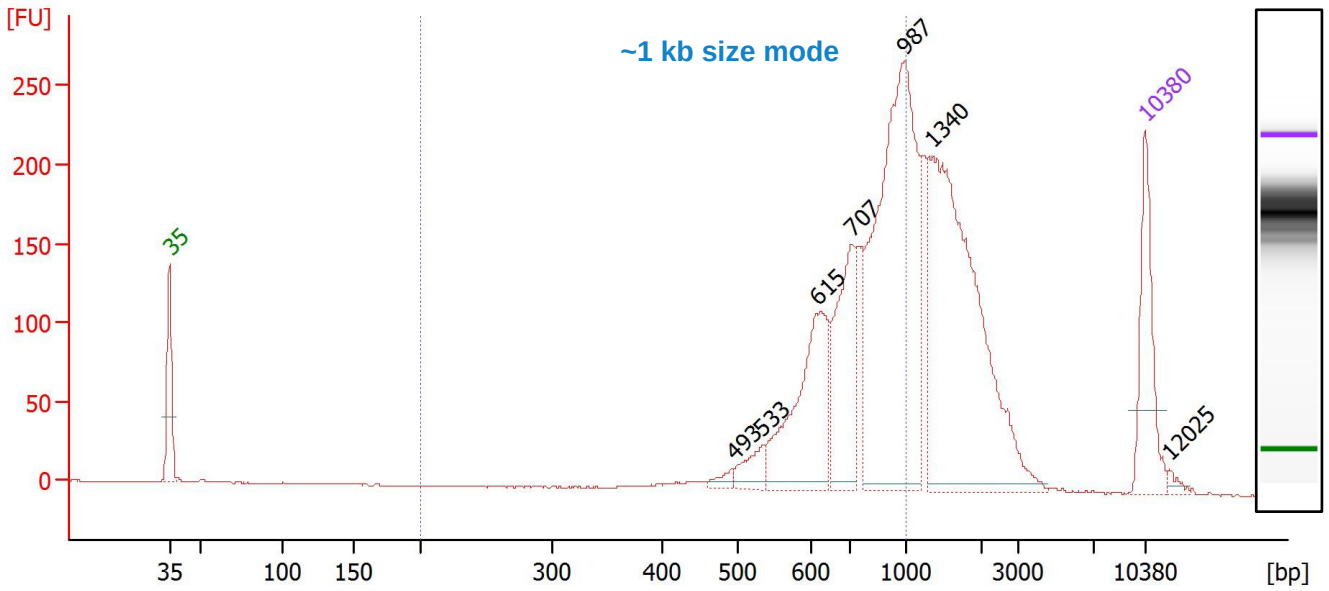
See ***SMRT Link User Guide*** documentation for detailed descriptions of parameter settings for Read Segmentation and Single-Cell Iso-Seq analysis application.

Kinnex single-cell RNA example sequencing performance data

Example Kinnex single-cell RNA library preparation QC results

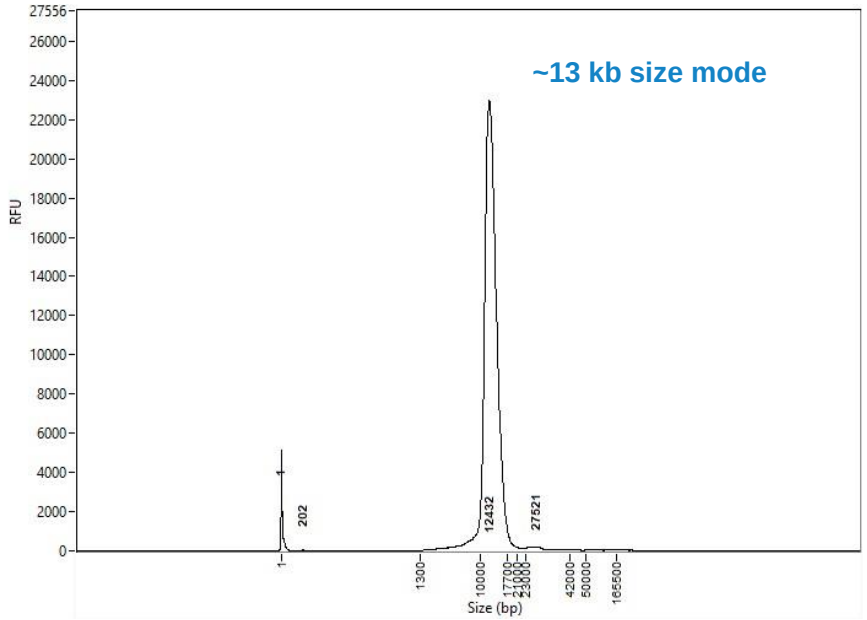
Kinnex single-cell 3’ RNA library prepared with human cDNA

Kinnex PCR DNA sizing QC (Single-cell 3’ cDNA)



Example Bioanalyzer DNA sizing QC analysis results for Kinnex PCR products generated for a 10x Chromium single-cell 3’ cDNA samples prepared from a human cell line (HG002).

Final Kinnex single-cell 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**

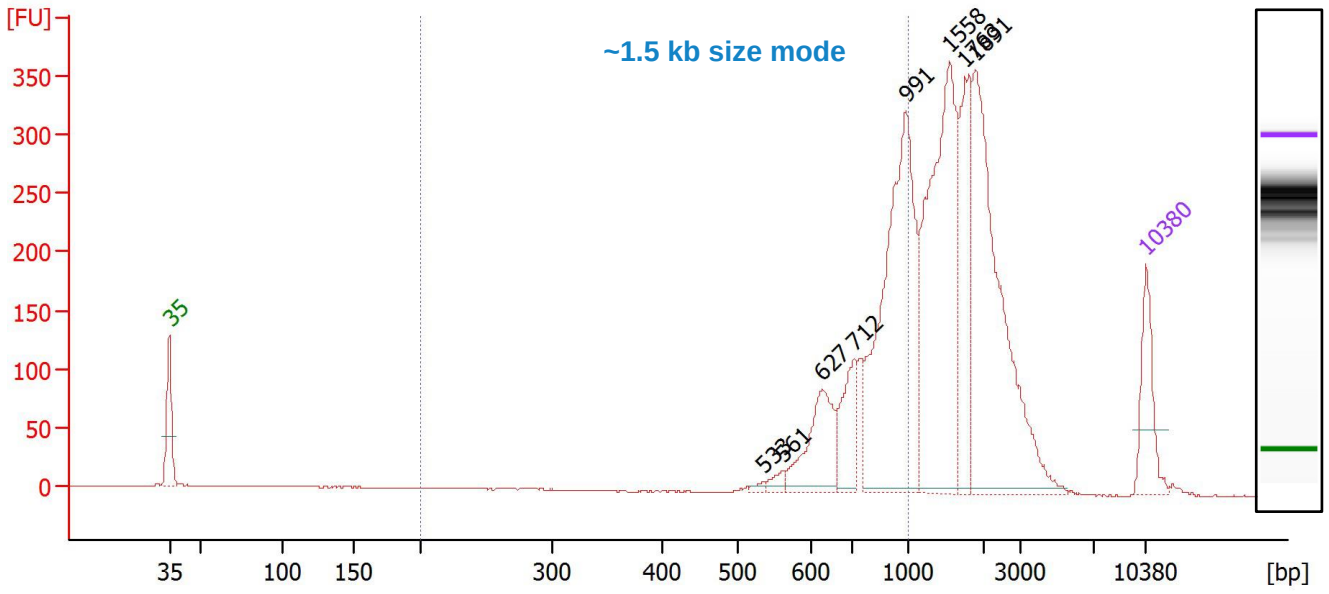
10x single cell 5’ cDNA input	15 ng
Kinnex array input for DDR	5000 ng
Post-nuclease treatment & final library cleanup yield (%)[1]	1100 ng (22.0%)

[1] Post-nuclease treatment & final cleanup yields typically ranged from ~10% to ~25% when using single-cell 3’ cDNA samples for Kinnex single-cell RNA library construction.

Example Kinnex single-cell RNA library preparation QC results (cont.)

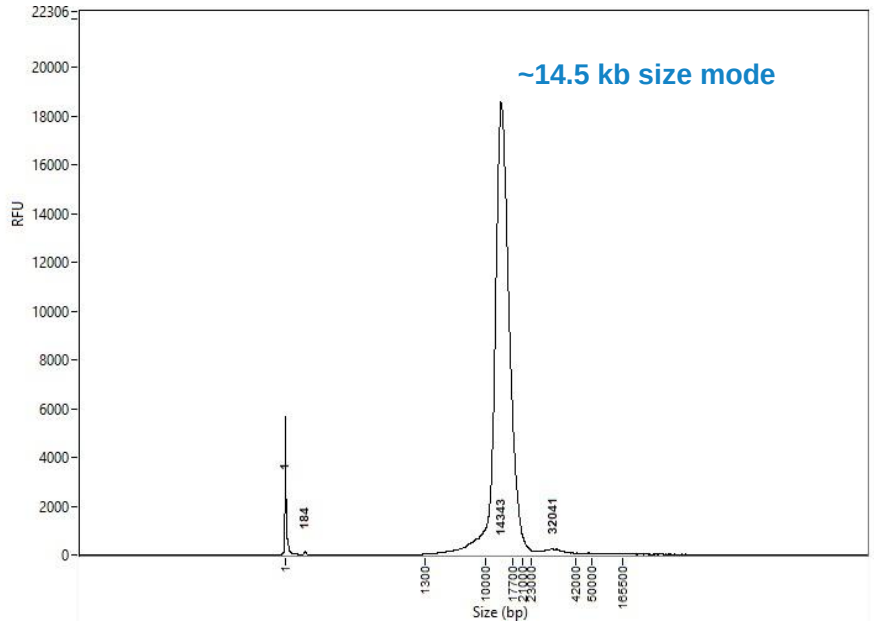
Kinnex single-cell 5’ RNA library prepared with human cDNA

Kinnex PCR DNA sizing QC (Single-cell 5’ cDNA)



Example Bioanalyzer DNA sizing QC analysis results for Kinnex PCR products generated for a 10x Chromium single-cell 5’ cDNA samples prepared from a human cell line (HG002).

Final Kinnex single-cell 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**

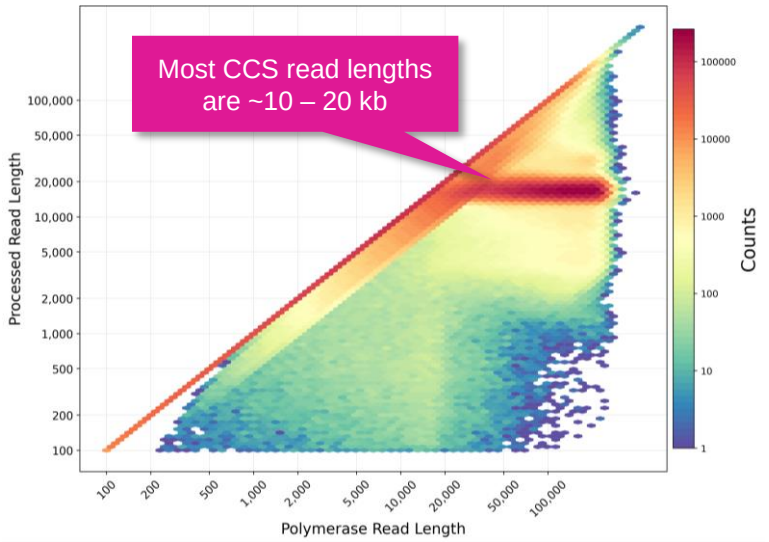
10x single cell 5’ cDNA input	15 ng
Kinnex array input for DDR	5000 ng
Post-nuclease treatment & final library cleanup yield (%)[1]	1008 ng (20.2%)

[1] Post-nuclease treatment & final cleanup yields typically ranged from ~10% to ~25% when using single-cell 5’ cDNA samples for Kinnex single-cell RNA library construction.

Example sequencing performance for Kinnex single-cell RNA libraries prepared with human cDNA

Revio system example data[1] – Kinnex single-cell RNA 3' library sample

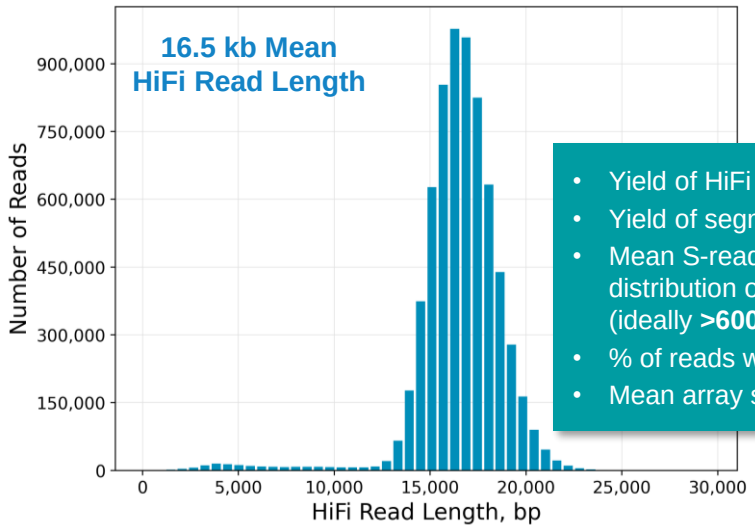
Raw Data Report



Raw Base Yield	1,289 Gb
Mean Polymerase Read Length	73.16 kb
P0	27%
P1	70%
P2	3%

Example sequencing metrics for a human Kinnex single-cell RNA 3' library sample run on a Revio system with Revio polymerase kit / 130 pM on-plate loading concentration (OPLC) / 24-hrs movie time.

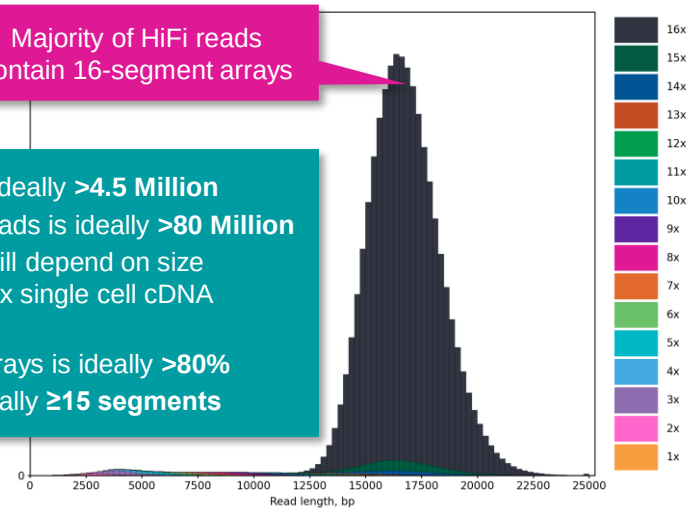
HiFi Read Length



HiFi Reads	6.7 M
HiFi Base Yield	111.24 Gb
Mean HiFi Read Length	16.55 kb
Median HiFi Read Quality	Q28
HiFi Read Mean # of Passes	8

For human Kinnex single-cell RNA libraries, per-Revio SMRT Cell HiFi read counts were typically ~4 – 7 Million depending on the final library insert size and *P1* loading performance.

Read Segmentation Metrics



- Yield of HiFi reads is ideally **>4.5 Million**
- Yield of segmented reads is ideally **>80 Million**
- Mean S-read length will depend on size distribution of input 10x single cell cDNA (ideally **>600 bp**)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **≥15 segments**

Input HiFi Reads	6,673,602
Segmented reads (S-reads)	104,869,257
Mean length of S-reads	1,031 bp
Percent of reads with full arrays	93.89%
Mean array size (concentration factor)	15.71

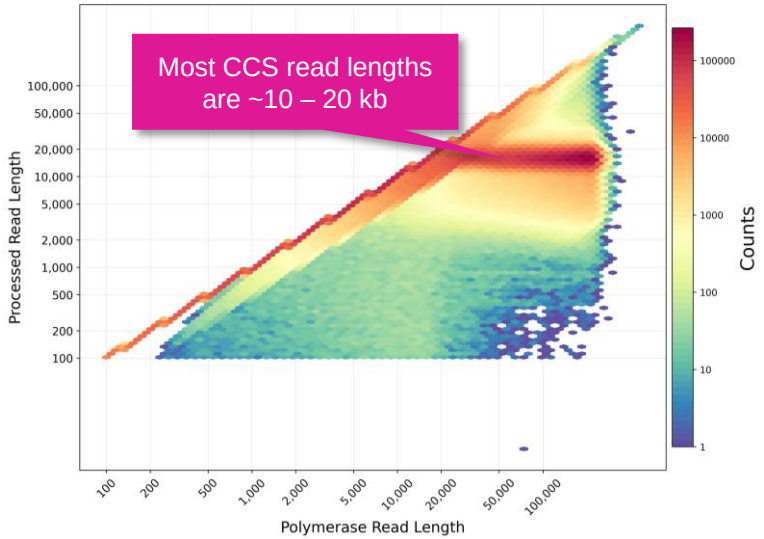
For Kinnex single-cell RNA libraries, per-Revio SMRT Cell segmentation read counts were typically >80 Million.

[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.

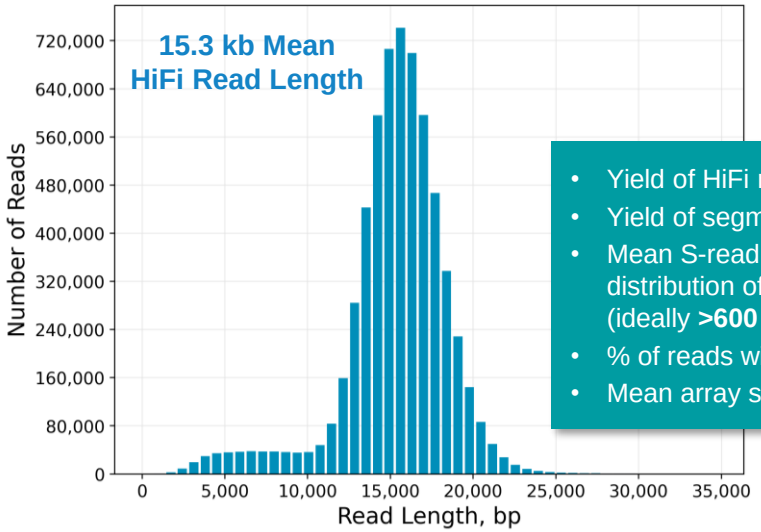
Example sequencing performance for Kinnex single-cell RNA libraries prepared with human cDNA

Revio system example data[1] – Kinnex single-cell RNA 5’ library sample

Raw Data Report

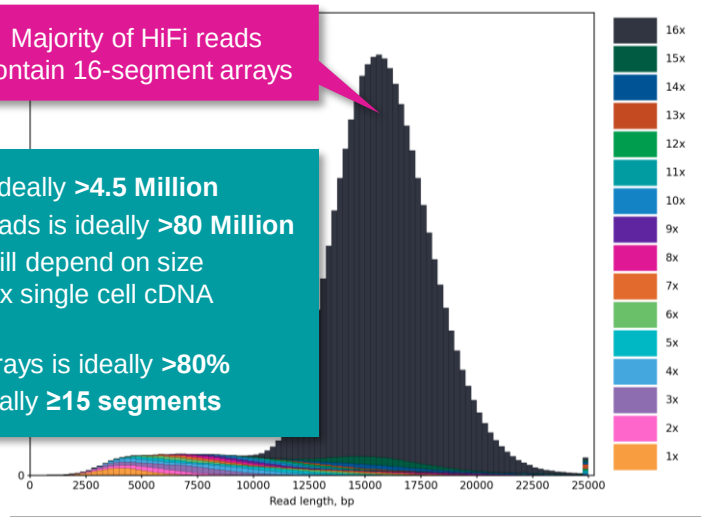


HiFi Read Length



- Yield of HiFi reads is ideally **>4.5 Million**
- Yield of segmented reads is ideally **>80 Million**
- Mean S-read length will depend on size distribution of input 10x single cell cDNA (ideally **>600 bp**)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **≥15 segments**

Read Segmentation Metrics



Raw Base Yield	1,116 Gb
Mean Polymerase Read Length	74,7 kb
P0	40%
P1	59%
P2	1%

Example sequencing metrics for a human Kinnex single-cell RNA 5’ library sample run on a Revio system with Revio polymerase kit / 130 pM on-plate loading concentration (OPLC) / 24-hrs movie time.

HiFi Reads	6.1 M
HiFi Base Yield	93.7 Gb
Mean HiFi Read Length	15.3 kb
Median HiFi Read Quality	Q30
HiFi Read Mean # of Passes	9

For human Kinnex single-cell RNA libraries, per-Revio SMRT Cell HiFi read counts were typically ~4 – 7 Million depending on the final library insert size and *P1* loading performance.

Input HiFi Reads	6,104,086
Segmented reads (S-reads)	91,323,803
Mean length of S-reads	980 bp
Percent of reads with full arrays	87.46%
Mean array size (concentration factor)	14.96

For Kinnex single-cell RNA libraries, per-Revio SMRT Cell segmentation read counts were typically >80 Million.

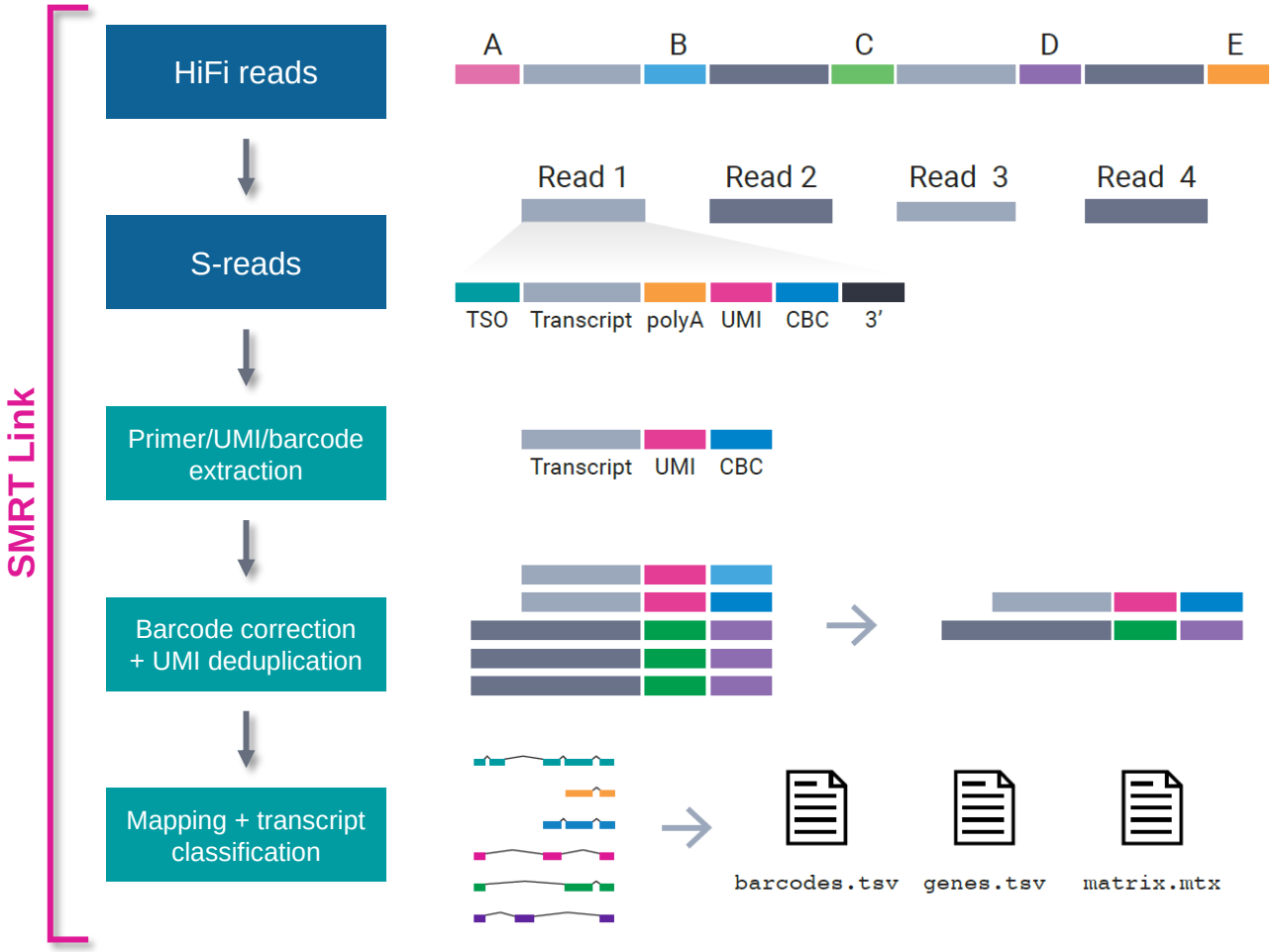
[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time.
Note: Shorter library insert sizes (<15 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result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

Kinnex single-cell RNA data analysis workflow overview

Kinnex single-cell RNA bioinformatics workflow overview

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

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



Read segmentation

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

Primer/UMI/barcode extraction

- Primers and polyA tails are removed, but also used to orient the read into 5' – 3' orientation
- Single-cell barcode and UMI information are extracted

Barcode correction & UMI deduplication

- Cell barcodes are corrected given an expected barcode list.
- Real cells — cell barcodes that represent encapsulated single cells (as opposed to ambient RNA) are also identified at this step.
- Reads are then deduplicated based on cell barcodes and UMIs.

Mapping and transcript classification[2]

- Deduplicated reads are mapped to the reference genome and classified against a transcript annotation (e.g., GENCODE).
- Finally, a gene- and isoform-level single-cell matrix is output for tertiary analysis.

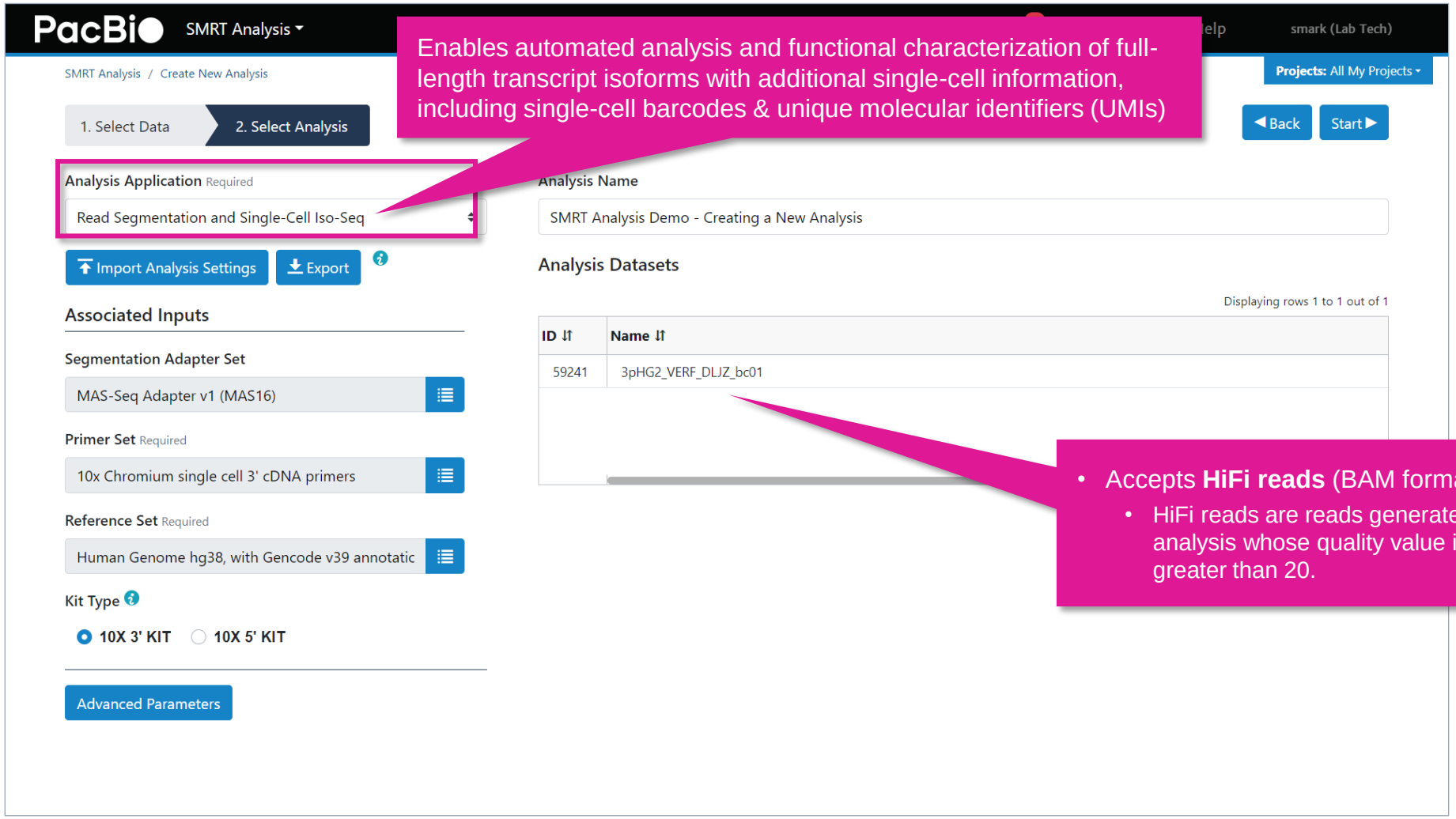
PacBio

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

[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 Single-Cell Iso-Seq analysis application setup

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



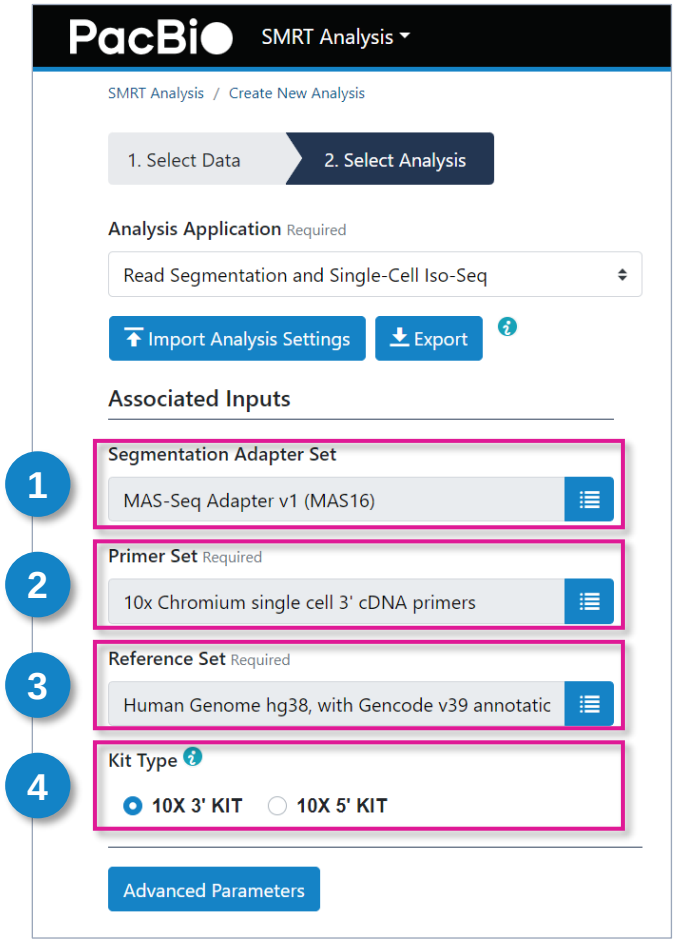
Enables automated analysis and functional characterization of full-length transcript isoforms with additional single-cell information, including single-cell barcodes & unique molecular identifiers (UMIs)

- 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.

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

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

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



1. Segmentation Adapter Set (Required)

- 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)

- 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 Ts and is written in reverse complement.
- 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`.

4. Segmentation Adapter Set (Required)

- Specify the 10x 3' Kit, or 10x 5' Kit. This determines which set of 10x primers and barcode sequences to use, and also affects the UMI and single-cell barcode design settings.

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

Example SMRT Link Read Segmentation data utility processing results[1] for Kinnex single-cell RNA libraries prepared with PBMC single cell cDNA

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

Summary Metrics

Value	Analysis Metric
6,104,086	Reads
91,323,803	Segmented reads (S-reads)
980	Mean length of S-reads
87.46 %	Percent of reads with full arrays
14.96	Mean array size (concatenation factor)

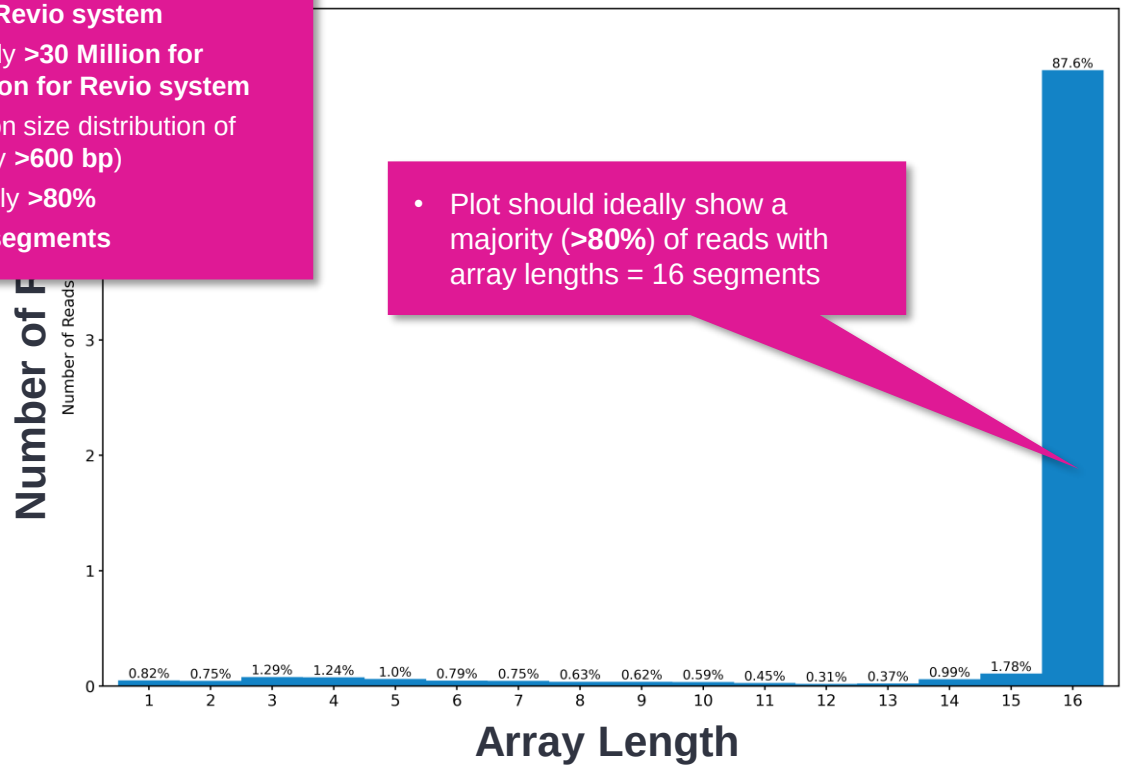
Example Revio system data shown.

With **optimal sample *P1* loading**:

- Yield of HiFi reads is ideally **>1.5 Million for Sequel II/IIe system or >4.5 Million for Revio system**
- Yield of segmented reads is ideally **>30 Million for Sequel II/IIe system or >80 Million for Revio system**
- Mean S-read length will depend on size distribution of input 10x single cell cDNA (ideally **>600 bp**)
- % of reads with full arrays is ideally **>80%**
- Mean array size is ideally **>15.0 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 = 16 segments

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

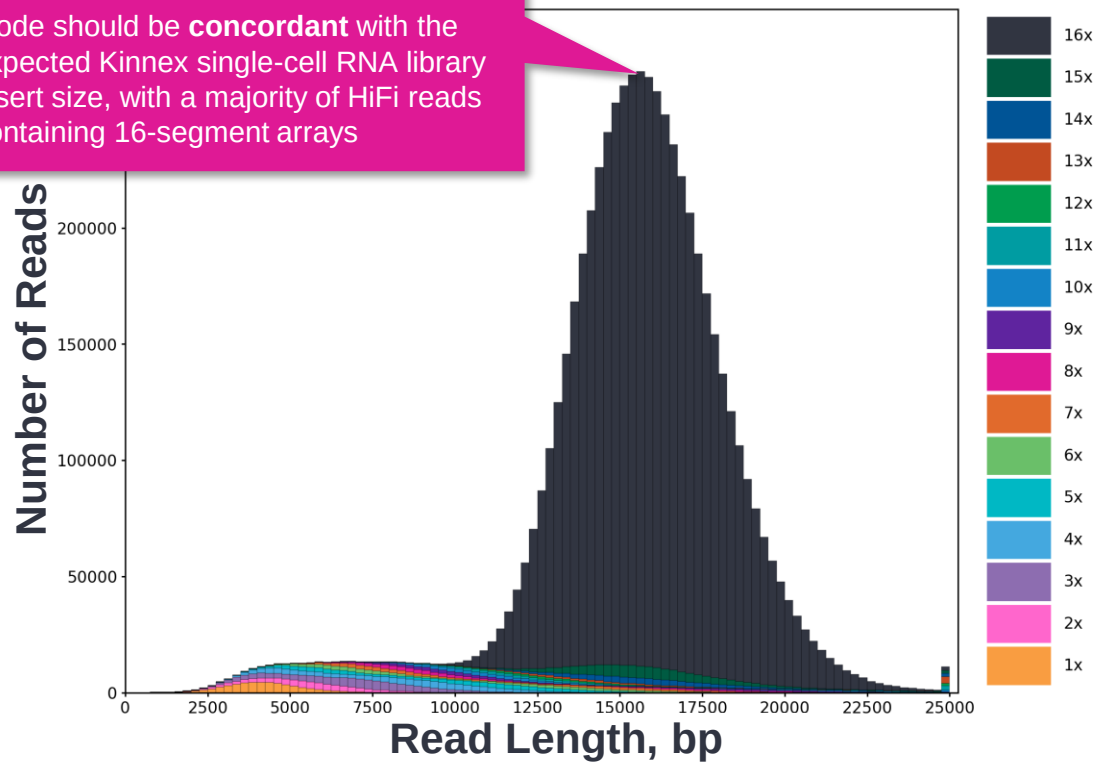
[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Note: Refer to **SMRT Link Kinnex single-cell RNA troubleshooting guide** (102-994-400) for example performance metrics typically achievable with Kinnex single-cell RNA single-cell 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 for Kinnex single-cell RNA libraries prepared with PBMC single cell cDNA (cont.)

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

Length of Reads

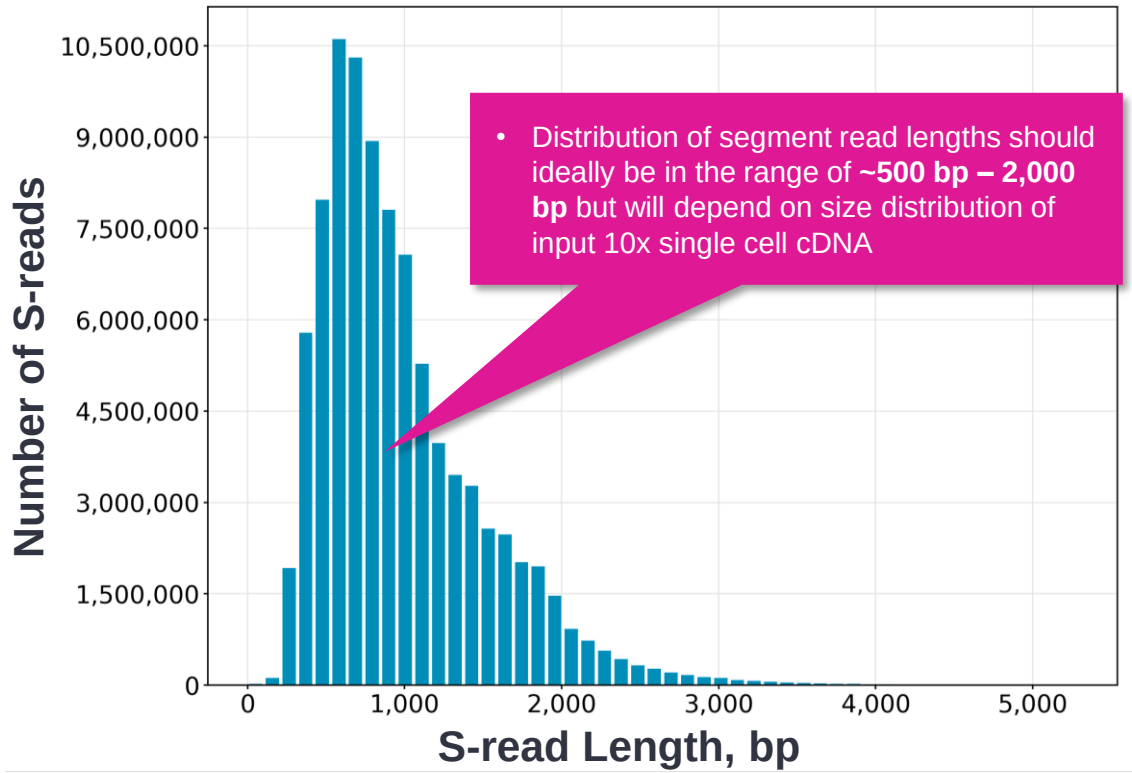
- Mode should be **concordant** with the expected Kinnex single-cell RNA library insert size, with a majority of HiFi reads containing 16-segment arrays



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

S-read Length

- Distribution of segment read lengths should ideally be in the range of **~500 bp – 2,000 bp** but will depend on size distribution of input 10x single cell cDNA



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 Kinnex single-cell RNA troubleshooting guide** (102-994-400) for example performance metrics typically achievable with Kinnex single-cell RNA single-cell 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 Single-Cell Iso-Seq Analysis results for Kinnex single-cell RNA libraries prepared with PBMC single cell cDNA

SMRT Link Single-Cell Iso-Seq Analysis job report – Read Statistics

Summary Metrics

Value	Analysis Metric
91,323,803	Reads
SEGMENT	Read Type
90,598,328	Reads with 5' and 3' Primers with extracted UMIs and Barcodes
89,790,396	Non-Concatamer Reads with 5' and 3' Primers and Poly-A Tail (FLNC reads)
85,414,801	FLNC Reads with Valid Barcodes
88,711,954	FLNC Reads with Valid Barcodes, corrected
44,323,585	Reads after Barcode Correction and UMI Deduplication

Example Revio system data shown.

- **Reads:** Total number of input reads for analysis.
- **Read Type:** Type of input reads - CCS, SEGMENT, or mixed if there are multiple input data sets with mixed data types.
- **Reads with 5' and 3' Primers with extracted UMIs and Barcodes:** The number of reads with 5' and 3' cDNA primers detected, and UMI/cell barcode information extracted. Also known as full-length tagged reads (FLT Reads).
- **Non-Concatemer Reads with 5' and 3' Primers and Poly-A Tail (FLNC Reads):** The number of non-concatemer reads with 5' and 3' primers and polyA tails detected after UMI/cell barcode information has been extracted.
- **FLNC Reads with Valid Barcodes:** Number of full-length non-concatemer reads that include valid single-cell barcodes.
- **FLNC Reads with Valid Barcodes, corrected:** Number of full-length non-concatemer reads that include valid single-cell barcodes, after barcode correction.
- **Reads after Barcode Correction and UMI Deduplication:** Number of deduplicated reads, after barcode correction.

PacBio

[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 Kinnex single-cell RNA troubleshooting guide** (102-994-400) for example performance metrics typically achievable with Kinnex single-cell RNA single-cell 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 Single-Cell Iso-Seq Analysis results for Kinnex single-cell RNA libraries prepared with PBMC single cell cDNA (cont.)

SMRT Link Single-Cell Iso-Seq Analysis job report – Cell Statistics

Summary Metrics

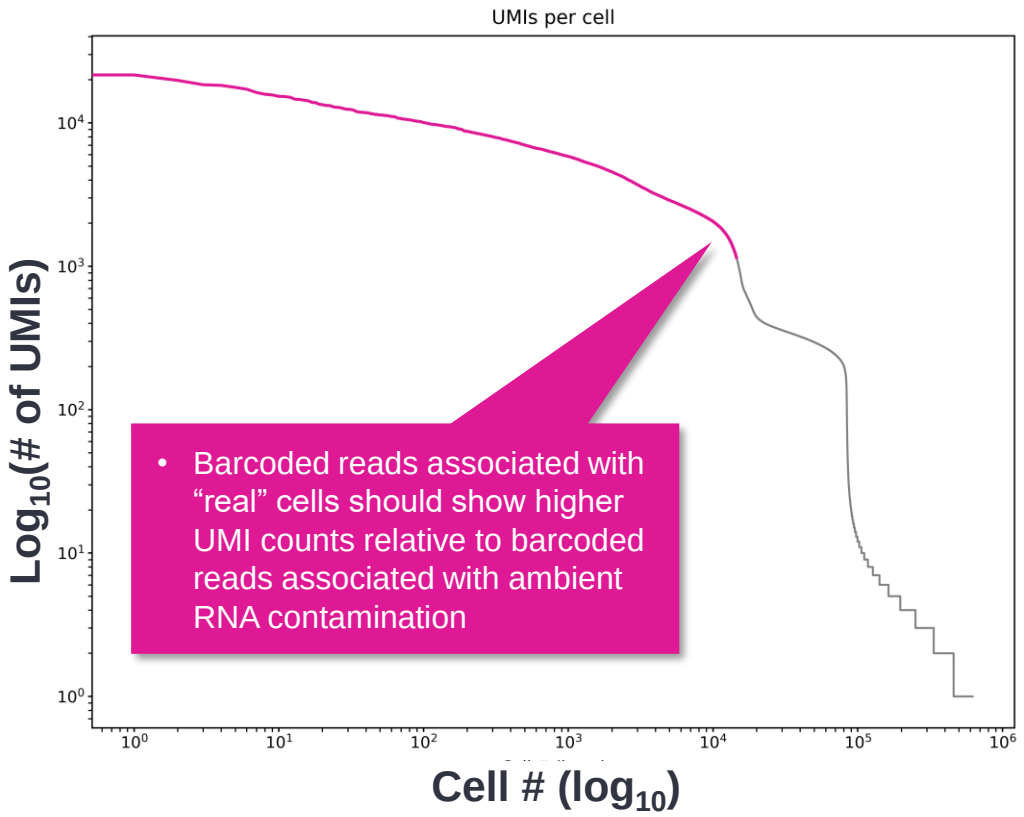
Value	Analysis Metric
13,984	Estimated Number of Cells
64.57%	Reads in Cells
4,146	Mean Reads per Cell
2,498	Median UMIs per Cell

Example Revio system data shown.

- There is no “correct” number of cells – this metric depends on what was specified in the 10x Chromium single cell workflow as the intended target cell recovery

- **Estimated Number of Cells:** The estimated number of cells.
- **Reads in Cells:** The percentage of reads in cells.
- **Mean Reads per Cell:** The mean number of reads per cell.
- **Median UMIs per Cell:** The median number of unique molecular identifiers (UMIs) per cell.

Barcode Rank Plot



- Barcoded reads associated with “real” cells should show higher UMI counts relative to barcoded reads associated with ambient RNA contamination

Displays the distribution of UMI counts and which barcodes were inferred to be associated with cells. The X-axis denotes barcodes ranked in decreasing order by UMI counts mapped to each barcode, and the Y-axis denotes the UMI count for the *N*-th ranked barcode. (Example Revio system data shown.)

Example SMRT Link Single-Cell Iso-Seq Analysis results for Kinnex single-cell RNA libraries prepared with PBMC single cell cDNA (cont.)

SMRT Link Single-Cell Iso-Seq Analysis job report – Transcript Statistics

Summary Metrics

Value	Analysis Metric
46,891,707	FLNC Reads Mapped Confidently to Genome
29,321,577	FLNC Reads Mapped Confidently to Transcriptome
1,211,184	Total Unique Genes
47,270	Total Unique Genes, filtered
32,767	Total Unique Genes, known genes only
25,242	Total Unique Genes, filtered, known genes only
2,554,444	Total Unique Transcripts
505,278	Total Unique Transcripts, filtered
88,764	Total Unique Transcripts, known transcripts only
78,023	Total Unique Transcripts, filtered, known transcripts only

Example Sequel IIe system data shown.

- **FLNC reads mapped confidently to genome:** The number of FLNC reads mapped to the reference genome. This number is calculated first based on the number of deduplicated reads mapped to the genome, then expanded to account for duplicate FLNC reads for each unique molecule.
- **FLNC reads mapped confidently to transcriptome:** The number of FLNC reads mapped to the reference genome in which the read is later associated with a transcript that is classified as one of the following: FSM, ISM, NIC, or NNC.
- **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 genes, known genes only:** The total number of unique genes across all cells in which the gene is annotated in the reference annotation.
- **Total unique genes, filtered, known genes only:** The total number of unique genes (genes annotated in the reference annotation) across all cells, after filtering out reads based on the SQANTI transcript filtering criteria.
- **Total unique transcripts:** The total number of unique transcripts across all cells.
- **Total unique transcripts, filtered:** The total number of unique transcripts across all cells, after filtering out reads based on the SQANTI transcript filtering criteria.
- **Total unique transcripts, known transcripts only:** The total number of unique transcripts across all cells in which the gene the transcript belongs to is annotated in the reference annotation.
- **Total unique transcripts, filtered, known transcripts only:** The total number of unique transcripts across all cells, after filtering out reads based on the SQANTI transcript filtering criteria. Only transcripts associated with known genes (genes annotated in the reference annotation) are included.

[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 Kinnex single-cell RNA troubleshooting guide** (102-994-400) for example performance metrics typically achievable with Kinnex single-cell RNA single-cell 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 Single-Cell Iso-Seq Analysis results for Kinnex single-cell RNA libraries prepared with PBMC single cell cDNA (cont.)

SMRT Link Single-Cell Iso-Seq Analysis job report – Transcript Statistics

Transcript Summary

Value	Analysis Metric
1,170	Median Genes per Cell
917	Median Genes per Cell, known genes only
1,315	Median Transcripts per Cell
714	Median Transcripts per Cell, known transcripts only
1,211,184	Total Unique Genes
32,767	Total Unique Genes, known genes only
2,554,444	Total Unique Transcripts
88,764	Total Unique Transcripts, known transcripts only

Filter out reads based on the SQANTI3 transcript filtering criteria[1]

Example Revio system data shown.

Transcript Summary, Filtered

Value	Analysis Metric
697	Median Genes per Cell
689	Median Genes per Cell, known genes only
772	Median Transcripts per Cell
624	Median Transcripts per Cell, known transcripts only
47,270	Total Unique Genes
25,242	Total Unique Genes, known genes only
505,278	Total Unique Transcripts
78,023	Total Unique Transcripts, known transcripts only

- **Median genes per cell:** The median number of genes per cell.
- **Median genes per Cell, known genes only:** The median number of unique, known genes (genes annotated in the reference annotation) per input cell.
- **Median transcripts per cell:** The median number of transcripts per cell.
- **Median transcripts per cell, known transcripts only:** The median number of transcripts per cell. Only transcripts associated with known genes are included.
- **Total unique genes:** The total number of unique genes across all cells.
- **Total unique genes, known genes only:** The total number of unique, known genes (genes annotated in the reference annotation) across all cells.
- **Total unique transcripts:** The total number of unique transcripts across all cells.
- **Total unique transcripts, known transcripts only:** The total number of unique transcripts across all cells. Only transcripts associated with known genes are included.

[1] Refer to the ***SMRT Link User Guide*** (Documentation) for further details regarding filtering criteria based on the SQANTI3 software.

Example SMRT Link Single-Cell Iso-Seq Analysis results for Kinnex single-cell RNA libraries prepared with PBMC single cell cDNA (cont.)

SMRT Link Single-Cell Iso-Seq Analysis job report – Transcript Statistics

Transcript Classification, filtered (All samples)

Category	Count	CAGE Detected	CAGE Detected, (%)	polyA Detected	polyA Detected, (%)
FSM	130264	84416	64.80%	49485	37.98%
ISM	204318	118023	57.76%	72301	35.38%
NIC	140202	102964	73.43%	59919	42.73%
NNC	245015	170439	69.56%	117602	47.99%
Antisense	7819	1145	14.64%	4416	56.47%
Fusion	8897	5561	62.50%	4718	53.02%
More junctions	123	77	62.60%	70	56.91%
Genic intron	0	0	0.00%	0	0.00%
Genic genomic	6555	3697	56.39%	2920	44.54%
Intergenic	18644	1186	6.36%	11074	59.39%

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

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[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 Single-Cell Iso-Seq Analysis results for Kinnex single-cell RNA libraries prepared with PBMC single cell cDNA (cont.)

SMRT Link Single-Cell Iso-Seq Analysis job report – Transcript Statistics

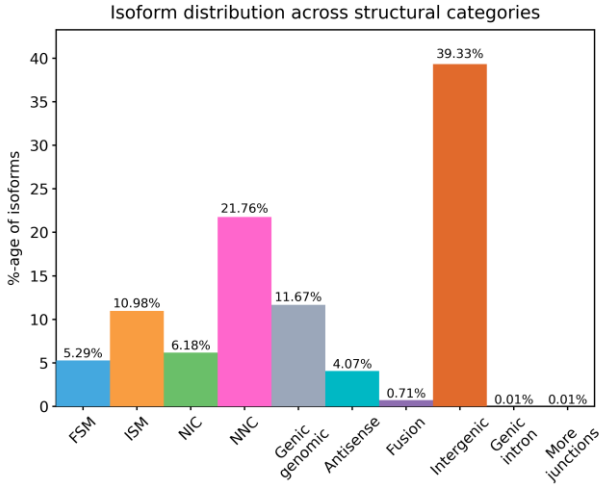
- **Isoform distributions across structural categories:**
  - Distribution of the % of isoforms by structural categories.

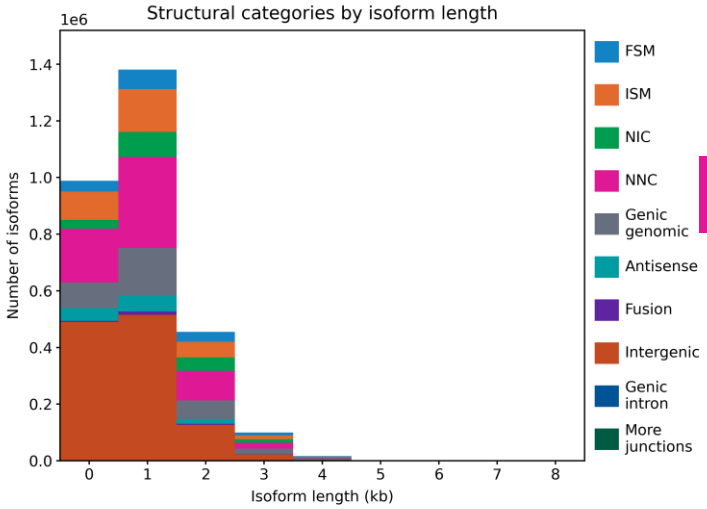
Example Revio system data shown.

- **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.

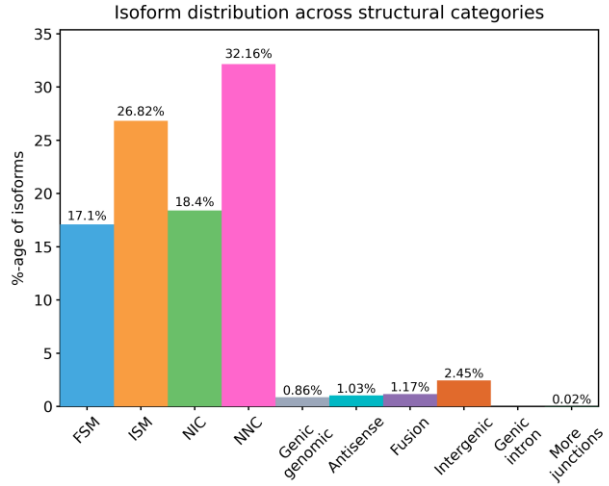
Transcript Classification Plots

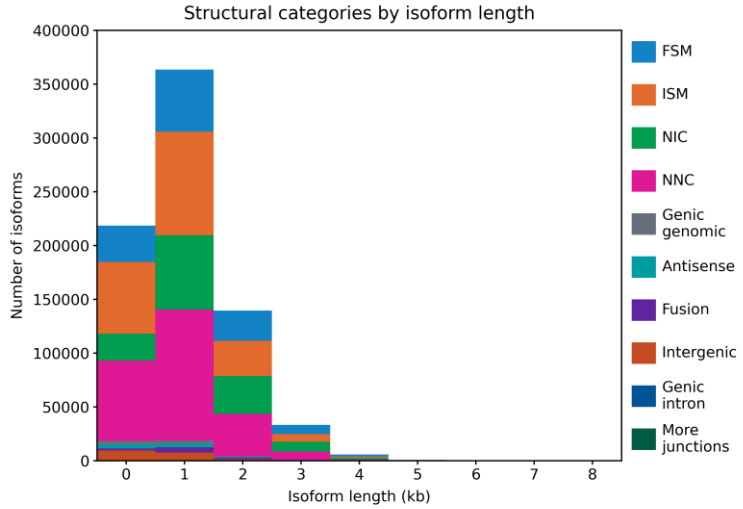




Filter out reads based on the SQANTI3 transcript filtering criteria

Transcript Classification Plots, Filtered





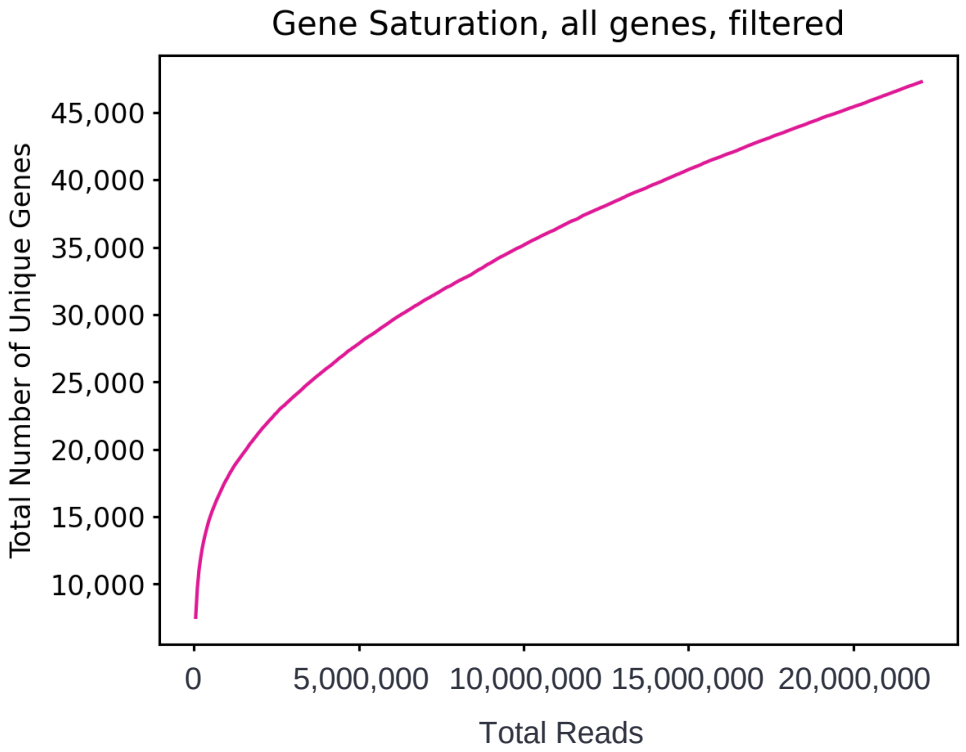
Filter out reads based on the SQANTI3 transcript filtering criteria

PacBio

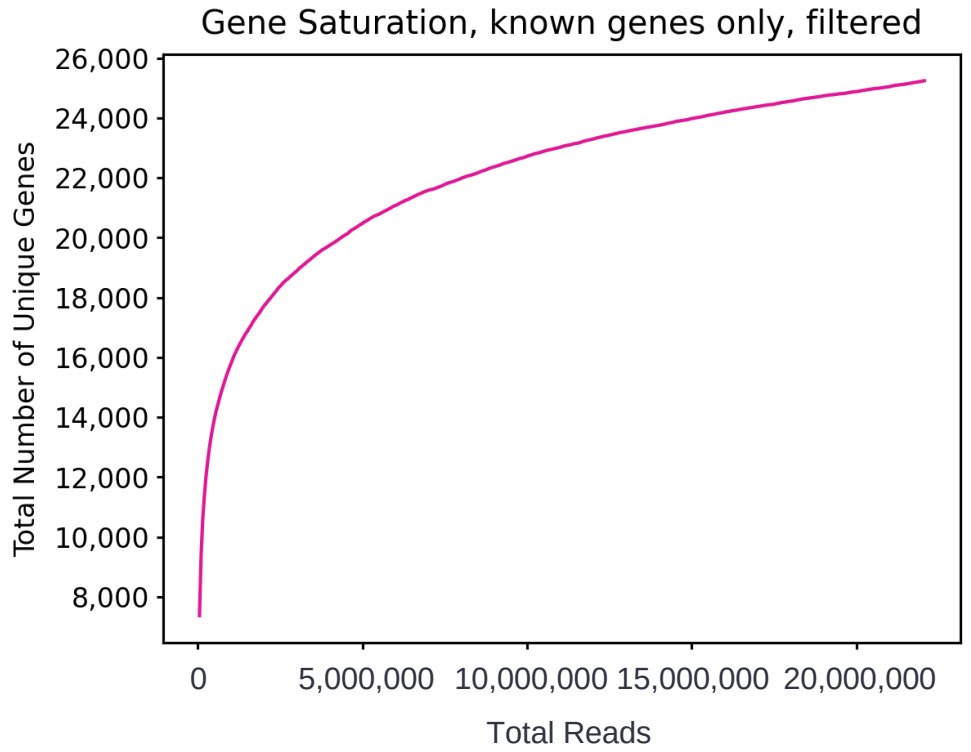
Example SMRT Link Single-Cell Iso-Seq Analysis results for Kinnex single-cell RNA libraries prepared with PBMC single cell cDNA (cont.)

SMRT Link Single-Cell Iso-Seq Analysis job report – Transcript 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 Single-Cell Iso-Seq Analysis results for Kinnex single-cell RNA libraries prepared with PBMC single cell cDNA (cont.)

File downloads tab

Edit Output File Name Prefix **Example:**analysis-Bio Sample 3-78360

File	Size	Type
Non-passing reads, unaligned	4 GB	bam
Report read_segmentation	3 KB	JsonReport
SMRT Link Log	14 KB	log
Segmented Reads, passing, unaligned	57 GB	bam
Single-cell isoform and gene matrix, tar-gzipped	1 GB	tgz
Unique mapped transcripts, GFF	1 GB	gff
Unique mapped transcripts, classification TXT	788 MB	txt
Unique mapped transcripts, filtered, GFF	481 MB	gff
Unique mapped transcripts, filtered, classification TXT	246 MB	txt
Unique mapped transcripts, filtered, junctions TXT	451 MB	txt
Unique mapped transcripts, junctions TXT	785 MB	txt

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.

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

- **Key output file!**
- Gzipped file containing Seurat-compatible isoform and gene matrix 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

Technical documentation & applications support resources

Technical resources for Kinnex single-cell RNA library preparation, sequencing & data analysis

Single-cell cDNA sample preparation literature & other resources

- 10x Genomics Chromium Next GEM Single Cell 3' v3.1 (Single Index) How-to Video [Link]
- 10x Genomics Chromium Single Cell 3' Reagent Kits User Guide – v3.1 (CG000204)
- 10x Genomics Chromium Single Cell 5' Reagent Kits User Guide – v2 Chemistry Dual Index (CG000331)

Kinnex single-cell RNA library preparation literature & other resources

- Application note – Kinnex single-cell RNA for single-cell isoform sequencing (102-326-549)
- Procedure & checklist – Preparing Kinnex libraries using Kinnex single-cell RNA kit (102-254-300)
- 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 single-cell RNA kit (103-344-600)
- Video tutorial – PacBio Kinnex single-cell RNA TSO artifact removal demo for Kinnex single-cell RNA kit [Link]
- Video tutorial – SMRT Link Sample Setup and Run Design setup procedure for Kinnex kits [Link]

Data analysis resources

- Application note – Bioinformatics tools for full length isoform sequencing (102-326-593)
- SMRT Link v12.0 MAS-Seq troubleshooting guide (102-994-400)
- SMRT Link software installation guide [Link]
- SMRT Link user guide [Link]
- SMRT Tools reference guide [Link]

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

Publications

- Al'Khafaji, A.M. et al. (2023) High-throughput RNA isoform sequencing using programmable cDNA concatenation. Nature biotechnology. [Link]

Webinars

- PacBio webinar (2023) – Understanding clonal evolution using game theory and single-cell long-read isoform analysis [Link]
- 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]
- PacBio Iso-Seq Social club webinar (2022) – Single-cell Iso-Seq applications in cancer and neurological disorders [Link]

Example PacBio data sets

Application	Dataset	Data type	PacBio system
Kinnex single-cell RNA sequencing	Homo sapiens - PBMC 10x Chromium Single Cell 5' and 3' libraries [Link]	HiFi long read	Sequel II & Revio systems
	Homo sapiens - HG002 (10x 5') [Link]	HiFi long read	Revio system

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