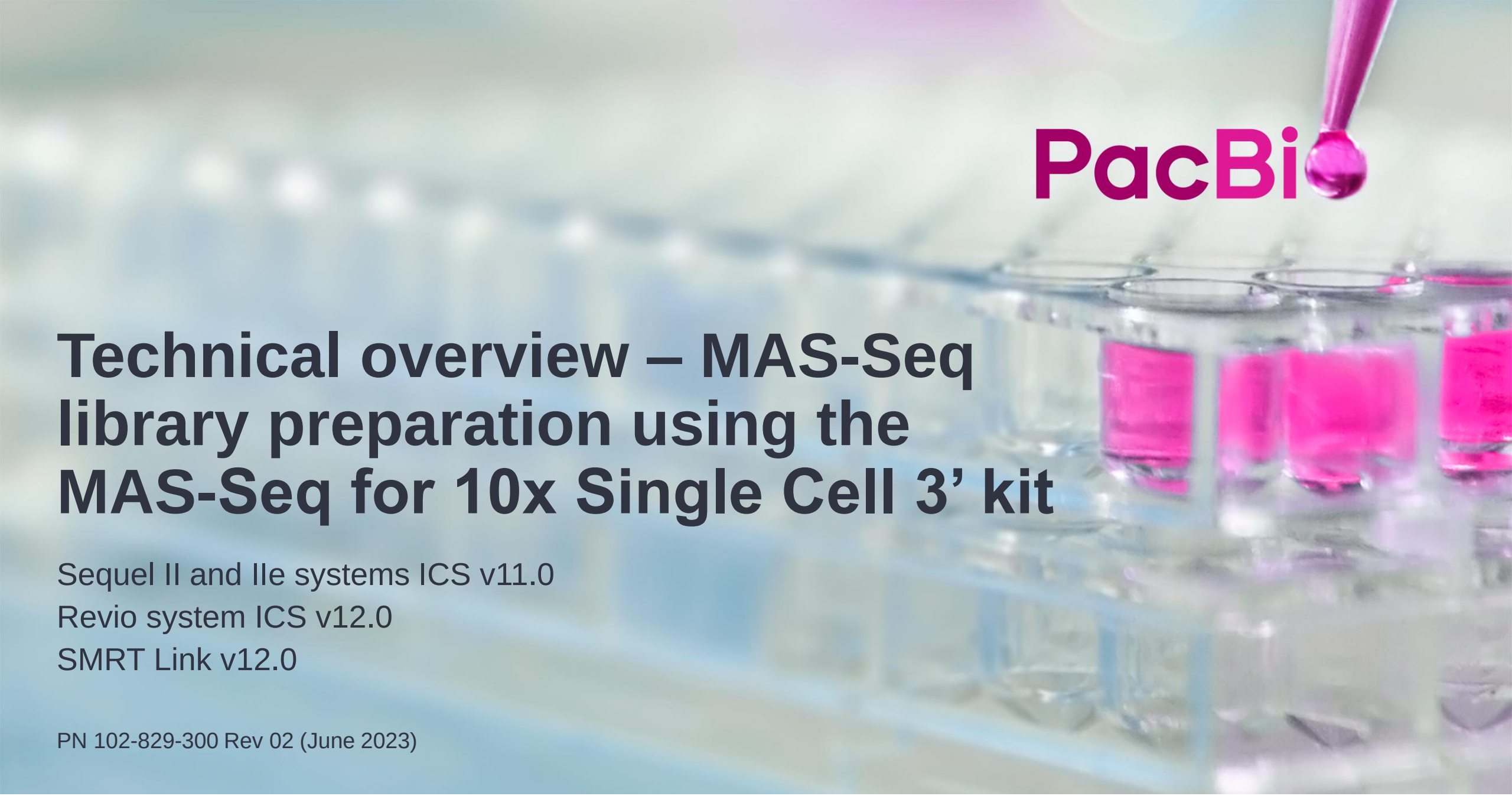


Technical overview – MAS-Seq library preparation using the MAS-Seq for 10x Single Cell 3’ kit

Sequel II and IIe systems ICS v11.0
Revio system ICS v12.0
SMRT Link v12.0

PN 102-829-300 Rev 02 (June 2023)

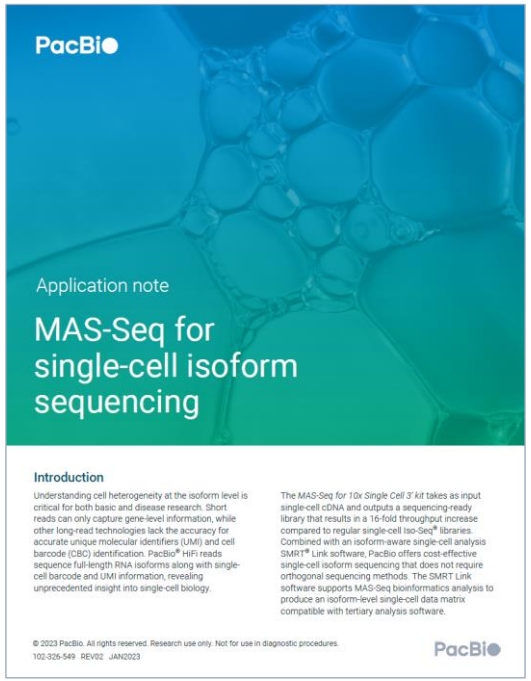
MAS-Seq library preparation using the MAS-Seq for 10x Single Cell 3’ kit

Technical Overview

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

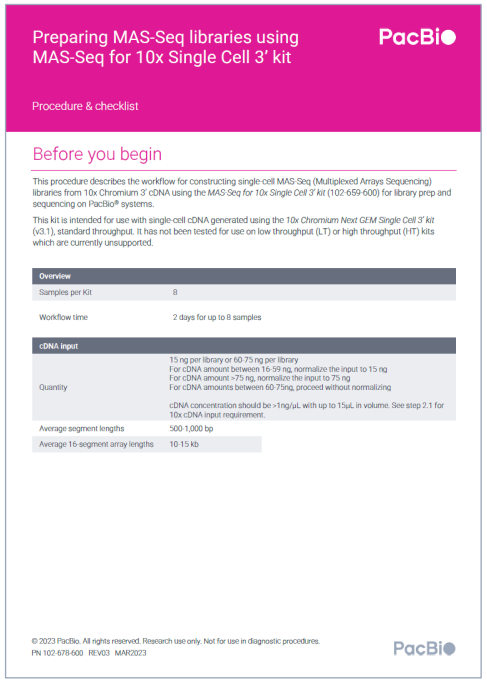
MAS-Seq library preparation using the MAS-Seq for 10x Single Cell 3’ kit: Getting started

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



Application Note - MAS-Seq for single-cell isoform sequencing (102-326-549)

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



Procedure & checklist – Preparing MAS-Seq libraries using MAS-Seq for 10x Single Cell 3’ kit (102-678-600)

Technical documentation containing application-specific library preparation protocol details.

Technical Overview: Single-cell MAS-Seq library preparation using the MAS-Seq for 10x Single Cell 3’ kit (102-829-300)

Technical overview presentations describe sample preparation details for constructing 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 single-cell cDNA synthesis (3,000 – 10,000 cells input)

SMRTbell library preparation (MAS-Seq for 10x 3’ kit)

Use ≥15 ng of 10x Chromium 3’ single-cell cDNA input to generate a 10 – 15 kb MAS-Seq library containing full-length 16-segment arrays

SMRT sequencing (Sequel II/IIe & Revio systems)

Perform ABC* and sequence MAS-Seq single-cell 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 & annotate unique transcript isoforms

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

MAS-Seq method overview

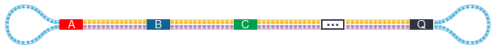
MAS-Seq library preparation procedure description

Procedure & checklist – Preparing MAS-Seq libraries using MAS-Seq for 10x Single Cell 3’ kit (102-678-600) describes the workflow for constructing single-cell MAS-Seq (Multiplexed Arrays Sequencing) libraries from 10x Chromium 3’ single cell cDNA using the **MAS-Seq for 10x Single Cell 3’ kit*** for library preparation and sequencing on PacBio Sequel II/IIe & Revio systems.

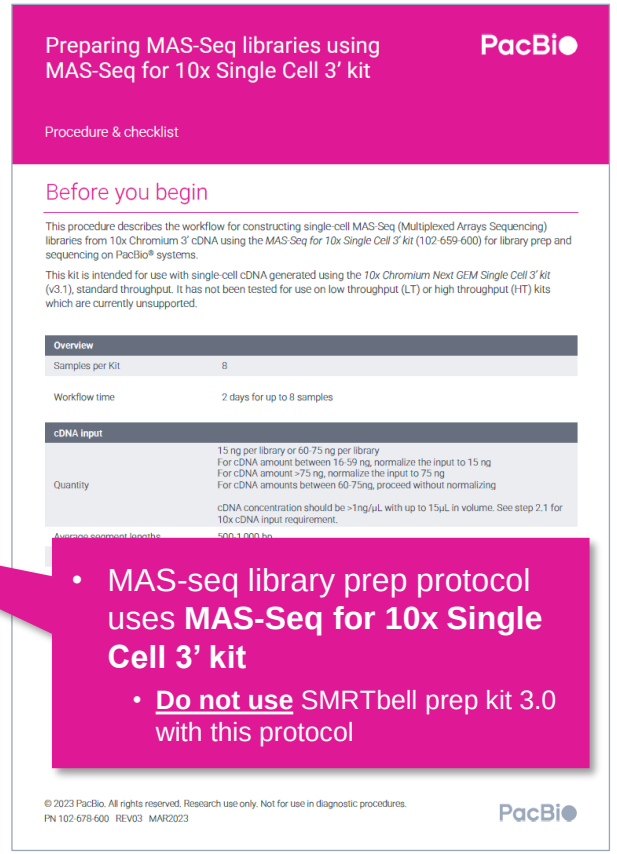
Overview	
Samples per Kit	8
Workflow time	2 days for up to 8 samples

cDNA input	
Quantity	15 ng per library or 60-75 ng per library For cDNA amount between 16-59 ng, normalize the input to 15 ng For cDNA amount >75 ng, normalize the input to 75 ng For cDNA amounts between 60-75ng, proceed without normalizing cDNA concentration should be >1ng/µL with up to 15µL 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

MAS-Seq for 10x Single Cell 3’ kit (102-659-600)



MAS-Seq library template (~10 – 15 kb) containing SMRTbell adapters at both ends


Preparing MAS-Seq libraries using MAS-Seq for 10x Single Cell 3’ kit

PacBio

Procedure & checklist

Before you begin

This procedure describes the workflow for constructing single-cell MAS-Seq (Multiplexed Arrays Sequencing) libraries from 10x Chromium 3’ cDNA using the *MAS-Seq for 10x Single Cell 3’ kit* (102-659-600) for library prep and sequencing on PacBio® systems.

This kit is intended for use with single-cell cDNA generated using the *10x Chromium Next GEM Single Cell 3’ kit* (v3.1), 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	8
Workflow time	2 days for up to 8 samples

cDNA input	
Quantity	15 ng per library or 60-75 ng per library For cDNA amount between 16-59 ng, normalize the input to 15 ng For cDNA amount >75 ng, normalize the input to 75 ng For cDNA amounts between 60-75ng, proceed without normalizing cDNA concentration should be >1ng/µL with up to 15µL in volume. See step 2.1 for 10x cDNA input requirement.

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PacBio

- MAS-seq library prep protocol uses **MAS-Seq for 10x Single Cell 3’ kit**
  - **Do not use** SMRTbell prep kit 3.0 with this protocol

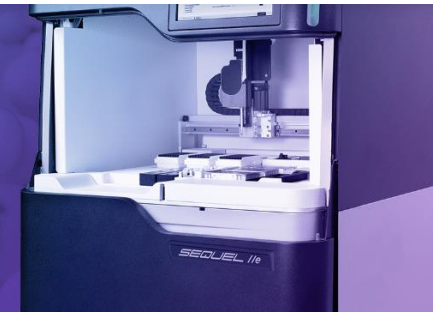
PacBio Documentation (102-678-600)

* Please contact PacBio Technical Support for questions about compatibility of the MAS-Seq library preparation workflow with other 10x Chromium kits.

APPLICATIONS

RNA SEQUENCING

Single-cell transcript isoform sequencing (MAS-Seq method)

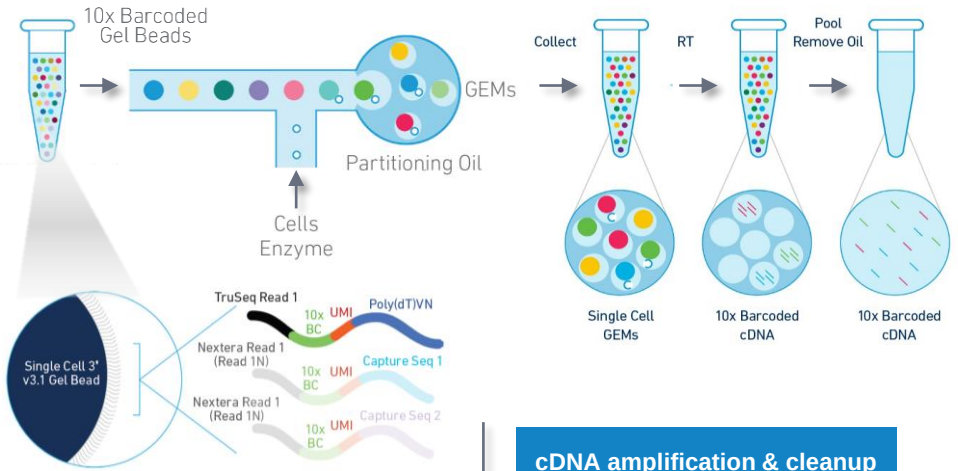


MAS-Seq method overview

Single-cell cDNA sample preparation*

Chromium Controller/X/iX

Chromium Next GEM Single Cell 3' Kit



cDNA amplification & cleanup

Full-length 10x barcoded single-cell cDNA for input into PacBio MAS-Seq library construction

PacBio

Procedure & checklist (102-678-600)

MAS-Seq library prep, sequencing & analysis

PacBio

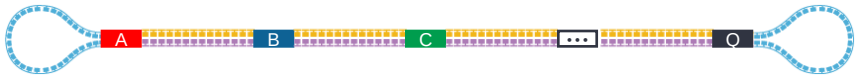
MAS-Seq for 10x Single Cell 3' kit (102-659-600)

10x single-cell cDNA input

Template switch oligo (TSO) artifact removal

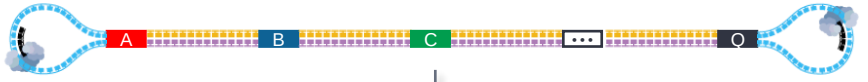
cDNA with expected Fwd & Rev primer sequences

MAS segmentation adapter incorporation & array formation



16-segment MAS array construct containing SMRTbell adapters at both ends

DNA damage repair & nuclease treatment / ABC**



PacBio long-read systems

Sequel II/IIe system
Revio system

SMRT Link

Read segmentation & single-cell Iso-Seq analysis

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

** ABC = Anneal sequencing primer / Bind polymerase / Complex cleanup

Single-cell cDNA sample preparation*

10x GENOMICS

Poly(dT) Primer for 1st-strand Synthesis

10x cell barcode (CBC) = 16 nt
Unique molecular identifier (UMI) = 12 nt
Poly(dT) sequence = 30 nt

5' [CBC][UMI]TTTTTTTTTT 3'
3' AAAAAAAAAA 5'

Poly(A)+ mRNA template

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'

Full-length 10x barcoded single-cell cDNA for input into PacBio MAS-Seq library construction

10x & PacBio key protocol steps

PacBio

Procedure & checklist (102-678-600)

MAS-Seq library prep, sequencing & analysis

PacBio

F R

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

TSO artifact removal

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

MAS PCR

PCR 1 | PCR 2 | PCR 3… | …PCR (*n*)

A B' | B C' | C D'

Pooling & MAS array formation

A B C … Q
A B C D
O P Q

DNA damage repair & nuclease treatment

ABC

Purified polymerase-bound 16-segment MAS-Seq library

PacBio long-read sequencing

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

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

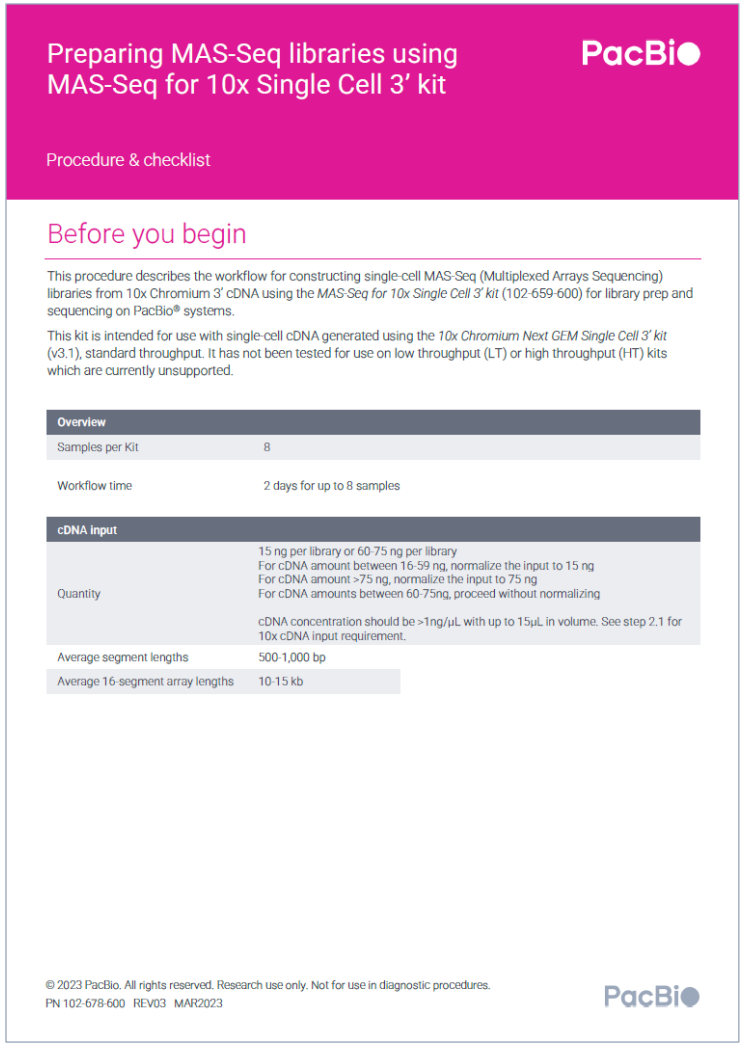
MAS-Seq library preparation workflow details

Procedure & checklist – Preparing MAS-Seq libraries using MAS-Seq for 10x Single Cell 3’ kit

Procedure & checklist 102-678-600 describes the workflow for constructing MAS-Seq single-cell libraries from 10x Chromium 3’ cDNA using the **MAS-Seq for 10x Single Cell 3’ kit** for library preparation and sequencing on PacBio long-read systems.*

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 TSO artifacts from input 10x single cell cDNA samples.
3. Enzymatic workflow steps for construction of 16-segment MAS arrays from 10x single cell cDNA.
4. Enzymatic workflow steps for DNA damage repair & nuclease treatment of MAS-Seq SMRTbell libraries.
5. Workflow steps for final cleanup of MAS-Seq SMRTbell libraries using SMRTbell cleanup beads.



Preparing MAS-Seq libraries using MAS-Seq for 10x Single Cell 3’ kit

PacBio

Procedure & checklist

Before you begin

This procedure describes the workflow for constructing single-cell MAS-Seq (Multiplexed Arrays Sequencing) libraries from 10x Chromium 3’ cDNA using the *MAS-Seq for 10x Single Cell 3’ kit* (102-659-600) for library prep and sequencing on PacBio® systems.

This kit is intended for use with single-cell cDNA generated using the *10x Chromium Next GEM Single Cell 3’ kit* (v3.1), 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	8
Workflow time	2 days for up to 8 samples

cDNA input	
Quantity	15 ng per library or 60-75 ng per library For cDNA amount between 16-59 ng, normalize the input to 15 ng For cDNA amount >75 ng, normalize the input to 75 ng For cDNA amounts between 60-75ng, proceed without normalizing cDNA concentration should be >1ng/µL with up to 15µL 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

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PacBio

PacBio Documentation (102-678-600)

PacBio

* Refer to **SMRT Link Sample Setup** for recommended sample setup conditions for sequencing MAS-Seq single-cell library samples on PacBio Sequel II/IIe & Revio systems.

MAS-Seq for 10x Single Cell 3’ kit

Application kit for generating MAS-Seq libraries from 10x Chromium single cell 3’ cDNA

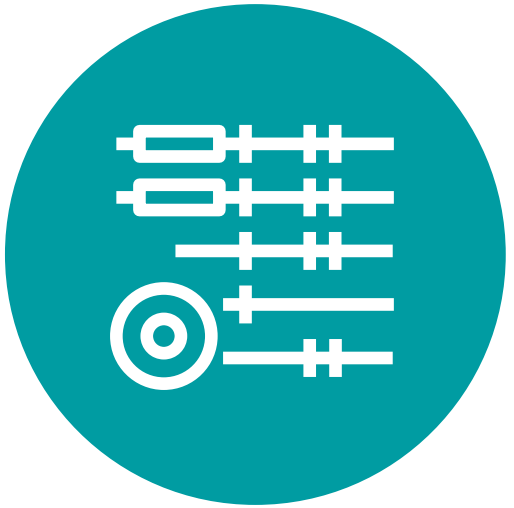
MAS-Seq for 10x Single Cell 3’ kit (102-659-600) product description

The MAS-Seq for 10x Single Cell 3’ kit contains oligos and reagents for generating a MAS-Seq library from cDNA produced by the 10x Chromium Single Cell 3’ kit (v3.1 chemistry). The kit contains MAS-Seq-specific adapters that produces a library ready for sequencing on PacBio Sequel II/IIe & Revio systems.

MAS-Seq for 10x Single Cell 3’ kit bundle (102-659-600)

Part	Packaging	Description
1		**MAS capture beads kit** • Reagents for TSO artifact removal
2		**MAS-Seq for 10x 3' concatenation kit** • Reagents for MAS PCR to incorporate segmentation adapters • Reagents for MAS array formation and SMRTbell template construction
3		**SMRTbell cleanup beads** • Reagent for routine DNA cleanup

- MAS-Seq kit supports cDNA generated from **10x Chromium Next GEM Single Cell 3’ kit (v3.1) [standard kit only** and not HT or LT**]***
- 8 reactions per MAS-Seq kit
- Go from 10X cDNA to sequencing-ready in ~2 days



* Please contact PacBio Technical Support for questions about compatibility of the MAS-Seq library preparation workflow with other 10x Chromium kits.

General best practices recommendations for preparing MAS-Seq 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 MAS-Seq 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.

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

- 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)

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 MAS-Seq libraries (cont.)

Reagent and sample handling

- Thaw repair buffer, nuclease buffer, and elution buffer at room temperature.
- Briefly vortex reagent buffers & MAS 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.
- When resuspending MAS capture beads, use wide-bore pipette tips to help minimize foaming. After MAS array formation, use wide-bore tips for pipette-mixing to minimize damage to array constructs.
- Pipette mix all library prep reactions by pipetting up and down 10 times.
- Samples can be stored at 4°C at all safe stopping points listed in the protocol.
- 1.5X SMRTbell cleanup is recommended before MAS array formation.
    - If the cDNA contains smaller fragments <200 bp, it is recommended to increase the SMRTbell cleanup bead ratio to 1.8 – 2.0X.

Temperature-sensitive reagents		
Step used	**Tube**	**Reagent**
TSO PCR and MAS PCR	Green	MAS PCR mix
	Yellow	MAS capture primers (Fwd and Rev)
	Orange	MAS primers premix (A-P)
MAS array formation	Light green	MAS enzyme
	Yellow	MAS ligase
	White	MAS ligase buffer
	Blue	MAS adapters
DNA damage repair Nuclease treatment	Green	DNA repair mix
	Purple	Repair buffer
	Light green	Nuclease mix
	Light purple	Nuclease buffer

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

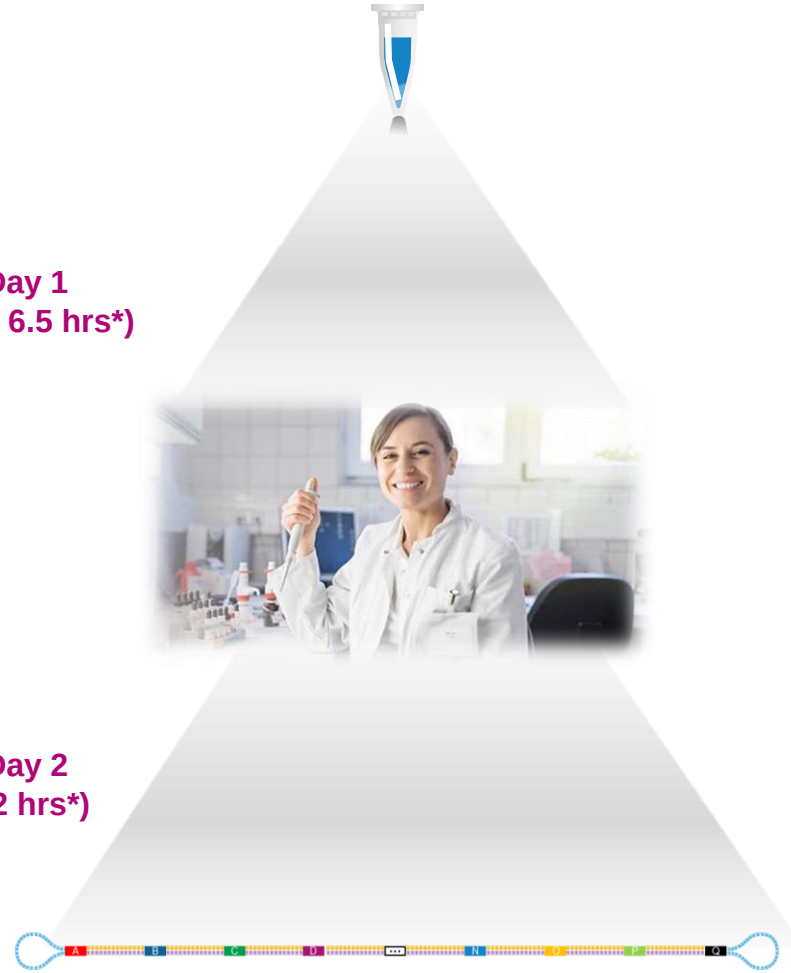
MAS-Seq library construction workflow overview

Procedure & checklist – Preparing MAS-Seq libraries using MAS-Seq for 10x Single Cell 3’ kit (102-678-600)

	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 MAS PCR	1.5X SMRTbell cleanup beads	Qubit HS assay	1.5 – 2.0 hrs
Safe stop			
5 MAS 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 treatment	N/A	N/A	1 hr
8 Final cleanup	1.2X SMRTbell cleanup beads	Qubit HS assay Femto Pulse gDNA 165 kb kit	30 min

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.

Input cDNA quality control

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

- We recommend using an optimal range of 3,000 – 10,000 cells input into 10x Chromium 3' single cell workflow[1]
- Protocol requires a **minimum of 15 ng** of 10x Chromium 3' 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 3' 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 MAS PCR

Safe stop

5 MAS array formation

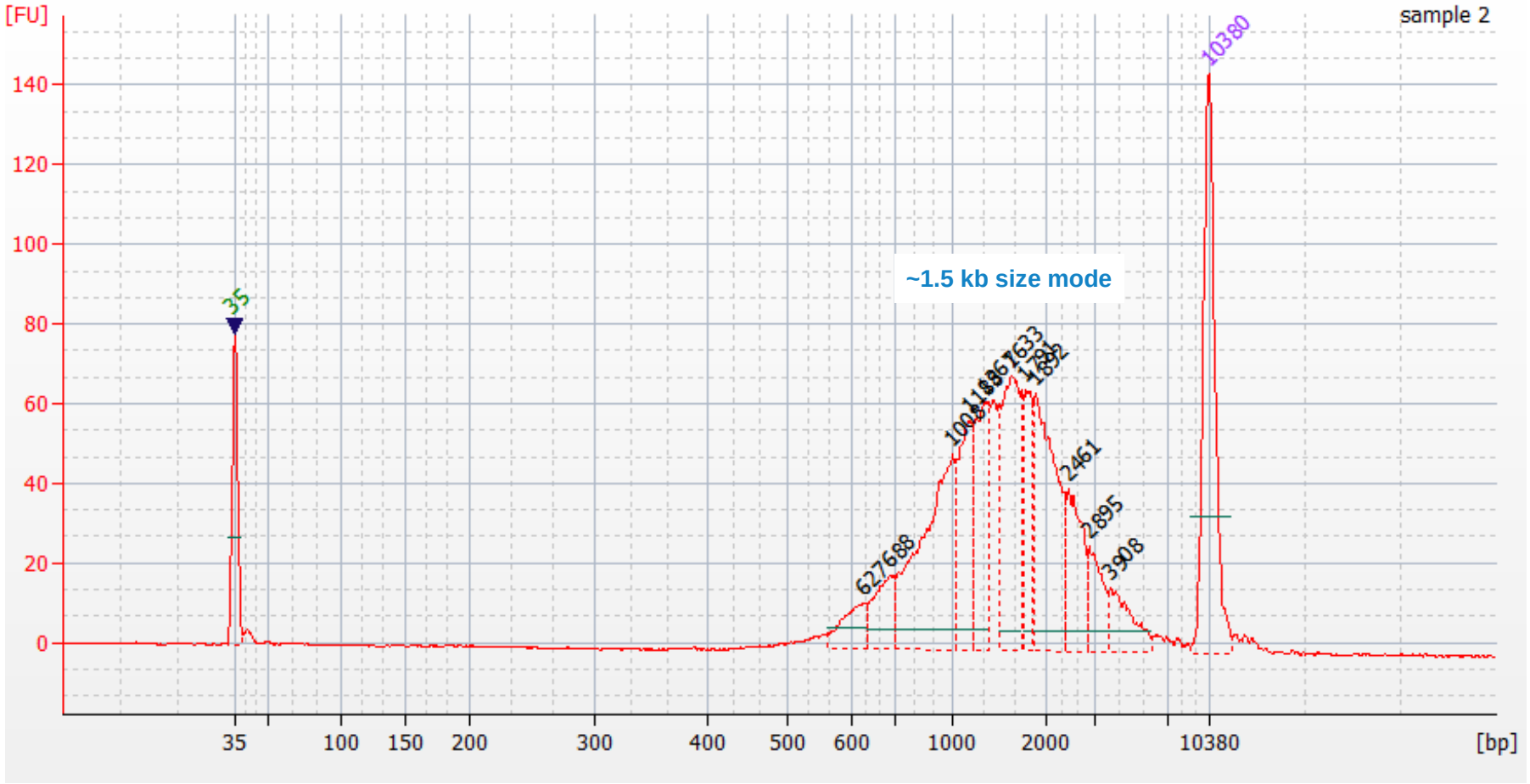
Safe stop

6 DNA damage repair

Safe stop

7 Nuclease treatment

8 Final cleanup



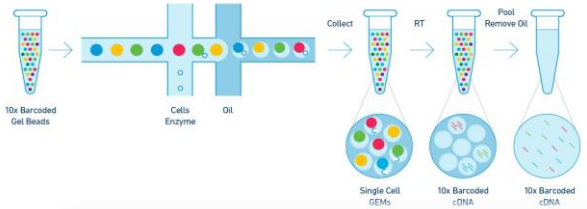
Bioanalyzer DNA sizing QC analysis results for a 10x Chromium single cell 3’ cDNA sample prepared from a human GM12878 cell line.

TSO PCR & TSO artifact removal

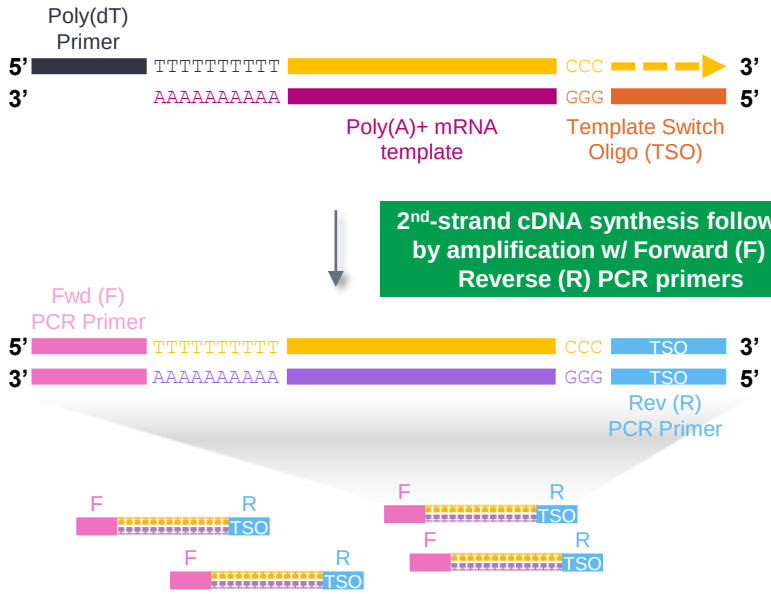
Perform Steps 2 & 3 to remove template switch oligo (TSO) priming artifacts generated during 10x cDNA synthesis

1 Input cDNA quality control
2 **TSO PCR**
Safe stop
3 **TSO artifact removal**
Safe stop
4 MAS PCR
Safe stop
5 MAS array formation
Safe stop
6 DNA damage repair
Safe stop
7 Nuclease treatment
8 Final cleanup

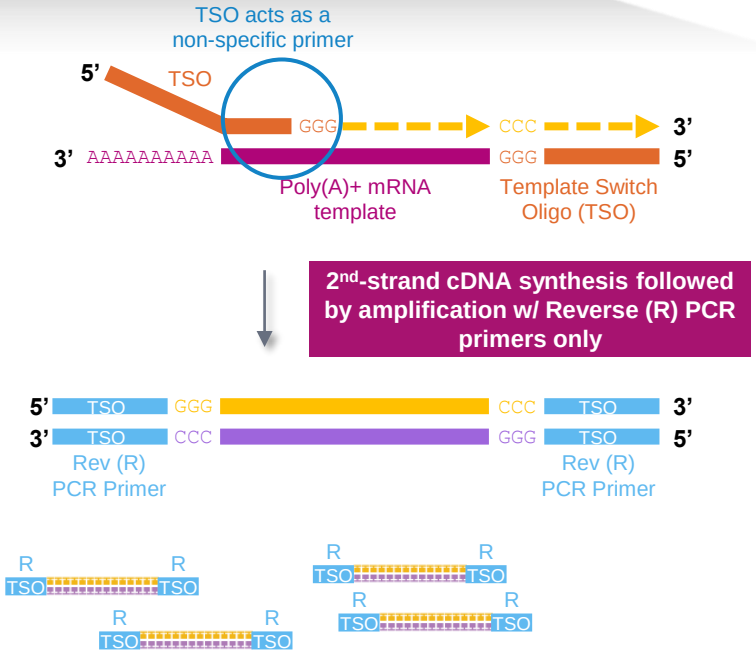
TSO priming artifacts can occur if the TSO acts as a nonspecific primer on poly(A)+ mRNA



- Up to ~50% of cDNA products from the 10x Chromium single cell 3' cDNA preparation workflow may contain a TSO priming artifact instead of the correct structure



Amplified (full-length) 10x cDNA products with correct structure (TSO sequence at <u>one</u> end)



Amplified (non-full length) 10x cDNA products with TSO priming artifacts (TSO sequences at <u>both</u> ends)

TSO PCR & TSO artifact removal (cont.)

Role of MAS capture primers and MAS capture beads

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

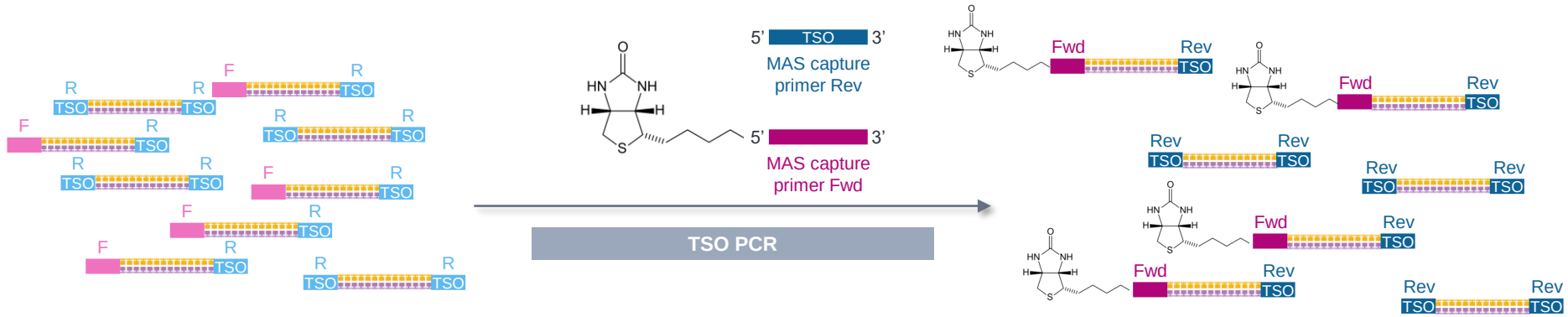
6 DNA damage repair

Safe stop

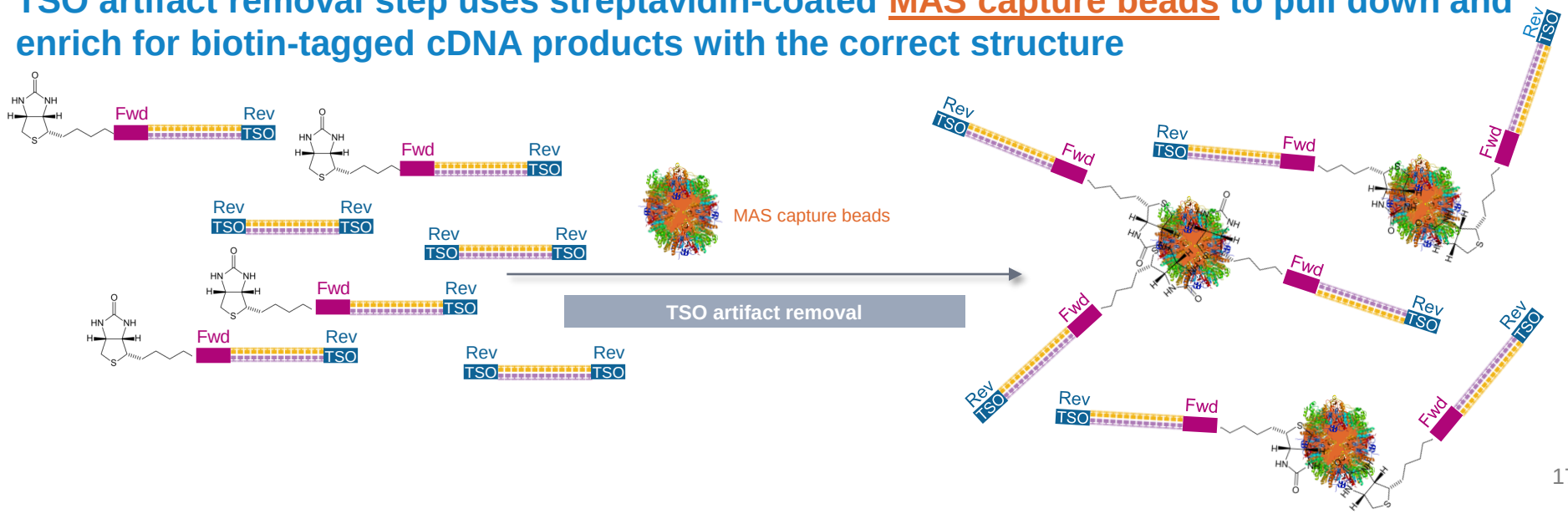
7 Nuclease treatment

8 Final cleanup

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



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



TSO PCR & TSO artifact removal (cont.)

TSO PCR 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 treatment
8. Final cleanup

✔	Step	Instructions
		cDNA amplification with MAS capture primers
		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.
		Set up the following PCR reaction on ice (**RM1**).

Reaction Mix 1 (RM1)

✔	Tube	Component	Volume
2.1		Nuclease-free water	Make up volume
	Green	MAS PCR mix (2X) 102-692-800	25 µL
	Yellow	MAS capture primer Fwd 102-693-300	5 µL
	Yellow	MAS capture primer Rev 102-693-900	5 µL
		10x 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.	

- If needed, **normalize** cDNA sample input amounts to 15 ng or 75 ng

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

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		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

- For **lower** cDNA sample inputs (15 ng), use a **higher** number of PCR cycles (5)

- After **TSO PCR** step, perform cleanup with 1.5X SMRTbell cleanup beads and proceed to **TSO artifact removal** (Step 3)

PacBio

TSO PCR & TSO artifact removal (cont.)

TSO artifact removal 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

8 Final cleanup

- Add MAS enzyme to **cleave** captured cDNA products from MAS capture beads

✔	Step	Instructions
	3.1	Bring MAS capture bead kit to room temperature. Resuspend the beads by vortexing.
	3.2	Transfer **10 µL resuspended MAS capture beads** 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 Lo-bind tube instead of PCR tube.
	3.3	Place the tube on the magnet until 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 MAS 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. • Scale up the volume of MAS bead binding buffer accordingly, if preparing more than 40 µL of beads.
	3.6	Place the tube on the magnet until beads separate fully from the solution and remove the supernatant.
	3.7	• Resuspend the beads in **40 µL MAS bead binding buffer** by pipetting slowly 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.** • Scale up the volume of MAS bead binding buffer accordingly, if preparing more than 40 µL of beads. • Distribute 40 µL of resuspended MAS capture beads into appropriate number of PCR tubes before proceeding to **Step 3.8**.
	3.8	Add **40 µL** of a solution containing the **biotinylated DNA-fragments** (from **Step 2.18**) to the resuspended beads. Mix carefully using wide bore tips to avoid foaming of the solution.
	3.9	Incubate the tube at **room temperature** for **15 minutes** on a rotator to keep the beads in suspension. Quick spin the tube in a microcentrifuge to collect liquid.
	3.10	Place the tube on the magnet until beads separate fully from the solution and remove the supernatant.
	3.11	Resuspend the MAS capture beads/DNA-complex in **80 µL MAS bead washing buffer** by pipet… until evenly distributed.
	3.12	Place the tube on the magnet until beads sep…
	3.13	Remove the tube from the magnet. Resuspe… **washing buffer** by pipette mixing or until even…
	3.14	Place the tube on the magnet until beads sep…
	3.15	Remove the tube from the magnet. Resuspe… **water** by pipette mixing or until evenly distributed.
	3.16	Place the tube on the magnet until beads separate fully from the solution and remove the supernatant.
		Resuspend the capture beads/DNA-complex in 40 µL of elution buffer by pipette mixing or until evenly …tributed.
	3.18	Add 2 µL **MAS enzyme** to cleave the captured DNA products from MAS capture beads.
	3.19	Pipette mix each sample and quick spin in a microcentrifuge to collect liquid.

- Keep the **supernatant** after treatment with MAS enzyme and placement on the magnet

- Bring MAS capture beads to **room temperature** and resuspend by vortexing

- **Critical step! For all MAS 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

TSO artifact removal program

Heated lid set at 47°C

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

3.20	Run the **TSO artifact removal program**.
3.21	Place the tube on the magnet for **1 minute** and move the supernatant containing the library to a fresh tube.

Cleanup with 1.5X SMRTbell cleanup beads

- After **TSO artifact removal** step, perform cleanup with 1.5X SMRTbell cleanup beads and proceed to **MAS PCR** (Step 4) if sample quantity is acceptable (**maximum 50 ng**[1])
  - Note: If total cDNA amount is <50 ng, perform additional PCR cycles as described in Step 4
  - If cDNA amount is >50 ng, dilute cDNA to 50 ng using elution buffer in a total volume of 45 µL. Do not proceed with MAS PCR with cDNA amount >50 ng as this may lead to PCR artifacts and chimera formation.

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. MAS PCR
   - Safe stop
5. MAS array formation
   - Safe stop
6. DNA damage repair
   - Safe stop
7. Nuclease treatment
8. Final cleanup

MAS PCR

Perform Step 4 step to 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 MAS PCR

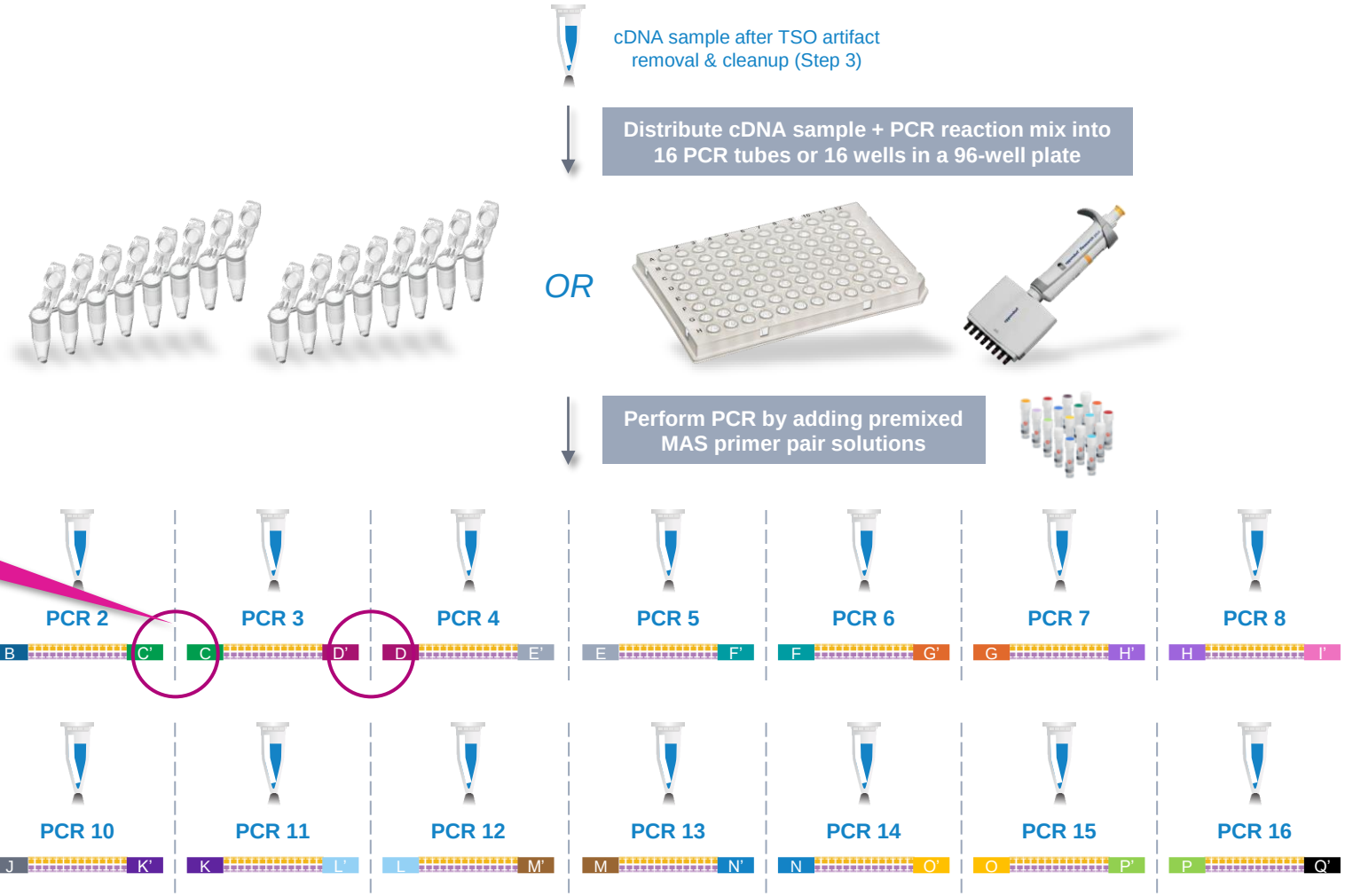
Safe stop

7 Nuclease treatment

8 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 MAS primers to generate amplified cDNA products containing programmable sequences at both ends.



MAS PCR (cont.)

MAS PCR 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 treatment
8. Final cleanup

✔	Step	Instructions
		cDNA amplification with MAS primers (16 reactions per sample)
		Set up the following PCR reaction mix per sample **on ice** (**RM2**).
	4.1	Reaction Mix 2 (RM2)

✔	Tube	Component	Volume
		Nuclease-free water	125 µL
	Green	MAS PCR mix (2X)	212.5 µL
		Purified cDNA from **Step 3.35**	45 µL
		Total volume	**382.5 µL**

4.2	Pipette mix **RM2**.
4.3	Quick spin **RM2** in a microcentrifuge to collect liquid.
4.4	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 **MAS primers premix** into each of 16 PCR tubes **on ice** according to the table below.

1	MAS primers premix A
2	MAS primers premix B
3	MAS primers premix C
4	MAS primers premix D
5	MAS primers premix E
6	MAS primers premix F
7	MAS primers premix G
8	MAS primers premix H
9	MAS primers premix I
10	MAS primers premix J
11	MAS primers premix K
12	MAS primers premix L
13	MAS primers premix M
14	MAS primers premix N
15	MAS primers premix O
16	MAS primers premix P

4.5	
4.6	Pipette mix each sample. The total volume of each tube should be 25.0 µL.
4.7	Quick spin the strip tubes in a microcentrifuge to collect liquid.
4.8	Run the MAS PCR program. Reactions can be held overnight in the cycler.
	Note: If the total sample quantity is less than 50 ng, follow the table below for cycle number recommendations.

cDNA input amount	Cycle number
30 - 50 ng	9
12.5 - 29.9 ng	10

Cleanup with 1.5X SMRTbell cleanup beads

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

- Default MAS PCR program uses 9 cycles. **Add 1 additional PCR cycle** if input cDNA amount recovered after TSO artifact removal (Step 3) is **<50 ng**
  - Failure to do so will result in **insufficient** PCR amplicon products for MAS array formation (Step 5)

MAS 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

* Note: If the total sample quantity is less than 50 ng, follow the table in Step 4.8 for cycle number recommendations.

- After **MAS 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.

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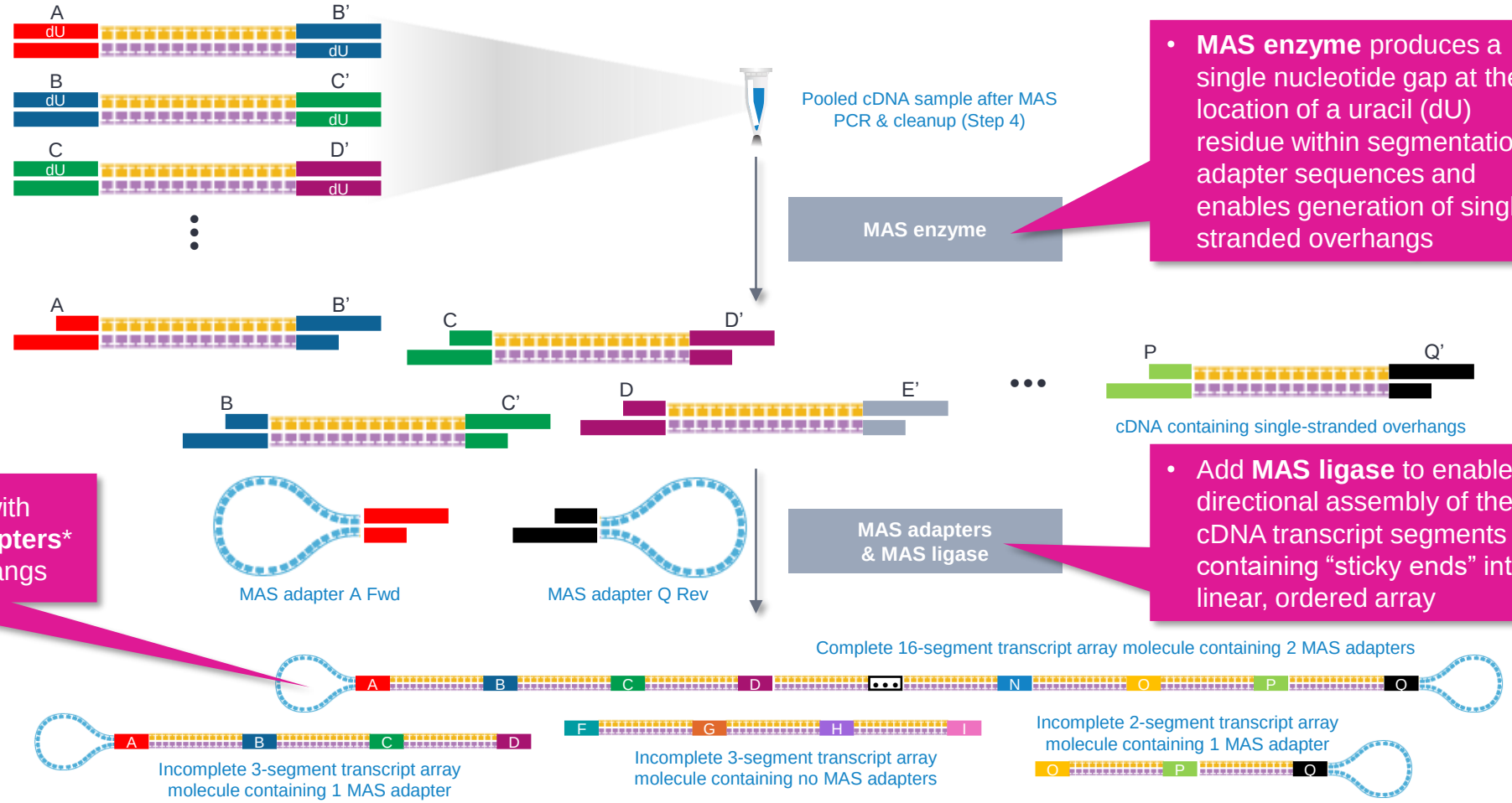
MAS array formation

Perform Step 5 to 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 MAS PCR
Safe stop
5 MAS array formation
Safe stop
6
7
8 Final cleanup

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



- **MAS enzyme** produces a single nucleotide gap at the location of a uracil (dU) residue within segmentation adapter sequences and enables generation of single-stranded overhangs

- Add **MAS ligase** to enable directional assembly of the cDNA transcript segments containing “sticky ends” into a linear, ordered array

- Full-length arrays are capped with MAS-Seq kit-specific **MAS adapters*** compatible with terminal overhangs

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* **Note:** Single-cell MAS-Seq library prep workflow described in this **Procedure & checklist** (102-678-600) 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.

MAS array formation (cont.)

MAS array formation 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 treatment
8. Final cleanup

Step	Instructions
5.1	In a 0.2 mL PCR tube, add **10 µg of sample** from **Step 4.23**, in **47 µL** of volume. Dilute with elution buffer adding into this step if sample is too concentrated.
5.2	Add **10 µL** of **MAS 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 MAS primer digestion program.
5.5	Add **1.5 µL** of each **MAS adapter** (A Fwd and Q Rev) and **20µL** of **MAS ligation additive** to each sample.
5.6	Pipette mix each sample.
5.7	Add the following components in the order and volume listed below to a new microcentrifuge tube. A component volumes for the number of samples being prepared, plus 10% overage. For individual pre components directly to each sample in the order and volume listed below.
5.8	Pipette mix **RM3** with wide bore tips.
5.9	Quick spin **RM3** in a microcentrifuge to collect liquid.
5.10	Add **20 µL** of **RM3** to each sample.
5.11	Pipette mix each sample with wide bore tips.
5.12	Run the MAS array ligation program.

Tube	Component	Volume
Blue	MAS adapter A Fwd	1.5 µL
Blue	MAS adapter Q Rev	1.5 µL
Red	MAS ligation additive	20 µL
	Total volume	**23 µL**

Reaction Mix 3 (RM3)

Tube	Component	Volume
White	MAS ligase buffer	10 µL
Yellow	MAS ligase	10 µL
	Total volume	**20 µL**

Cleanup with 1.2X SMRTbell cleanup beads

- Recommended minimum input requirement to proceed with MAS array formation is **10 µg** of MAS PCR amplicons (from Step 4)
  - Proceeding with <8 µg is **not recommended** since lower input amounts may lead to insufficient final library yields to enable optimal sequencing results

MAS primer digestion program

Heated lid set at 47°C

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

- **Critical step!** Always add MAS adapters and MAS ligation additive to the sample **BEFORE** adding MAS ligase

MAS array ligation program

Heated lid set at 52°C

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

- After **MAS array formation** step, perform cleanup with 1.2X SMRTbell cleanup beads using **wide bore pipette tips** and proceed to **DNA damage repair** (Step 6)

PacBio

DNA damage repair & nuclease treatment

Perform Steps 6 & 7 to repair nicked/damaged DNA sites and remove incomplete SMRTbell template arrays

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

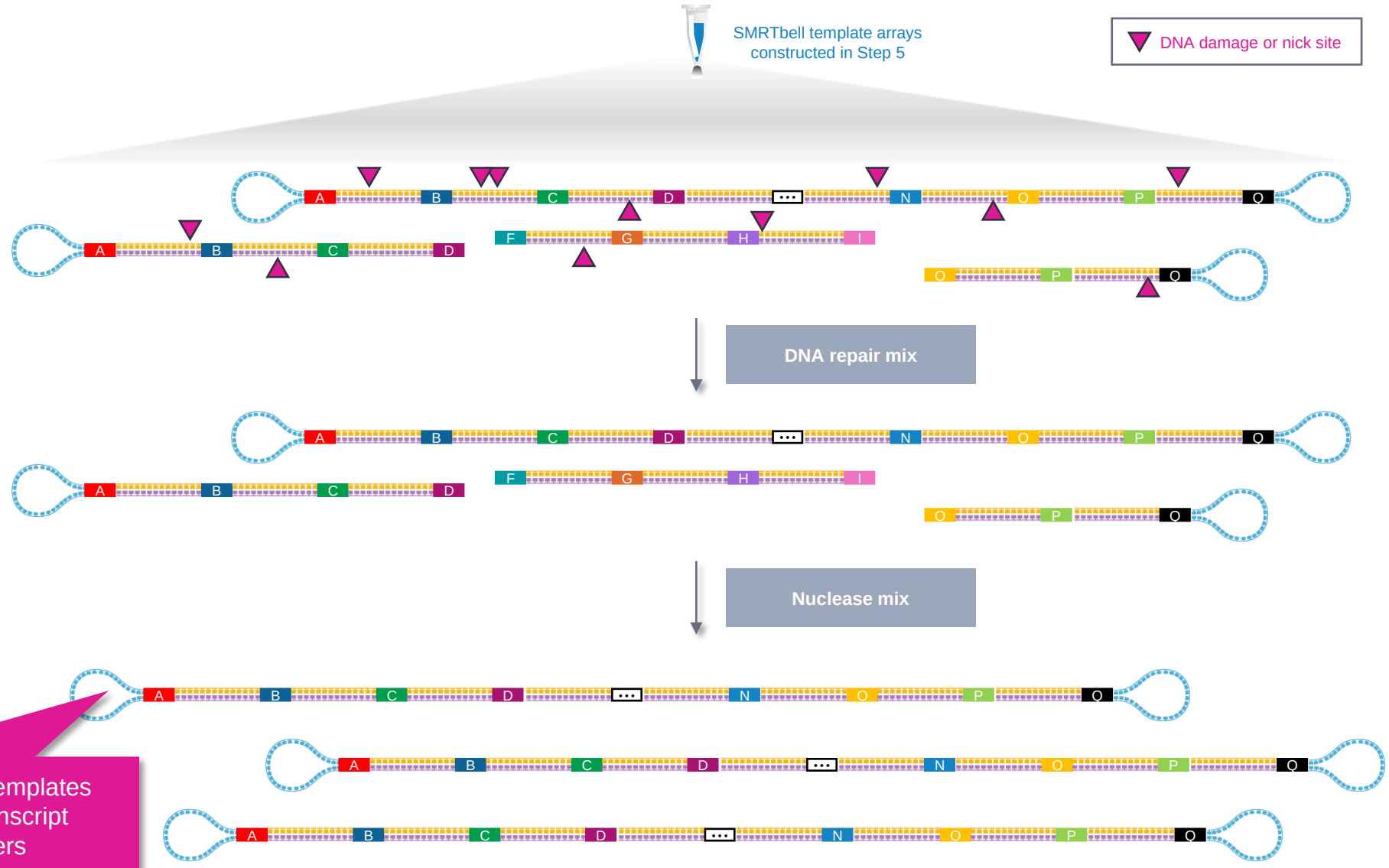
Safe stop

6 DNA damage repair

Safe stop

7 Nuclease treatment

8 Final cleanup



- After Step 7, most remaining SMRTbell templates are complete (full-length) 16-segment transcript array molecules capped with MAS adapters

DNA damage repair & nuclease treatment (cont.)

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 treatment
8. Final cleanup

✔	Step	Instructions
	6.1	In a new PCR strip tube, add **5 µg** of MAS array (in **42 µL** of **elution buffer**) from **Step 5.26.**
	6.2	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample in the order and volume listed below.

Reaction Mix 4 (RM4)

Tube	Component	Volume
Purple	Repair buffer	6 µL
Green	DNA repair mix	2 µL
	Total volume	**8 µL**

	6.3	Pipette mix **RM4** with wide bore tips.
	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 [illegible] µL.
	6.6	Pipette mix each sample with wide bore tips.
	6.7	Quick spin the strip tube in a microcentrifuge to collect liquid.
	6.8	Run the DNA damage repair program.
		Cleanup with 1.2X SMRTbell cleanup beads

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

- Pipette mix with **wide bore tips** to minimize DNA shearing damage to full-length SMRTbell template arrays

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 using **wide bore pipette tips** and proceed to **Nuclease treatment** (Step 7)

DNA damage repair & nuclease treatment (cont.)

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 treatment
↓
8 Final cleanup

✓	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	Component	Volume
	Light purple	Nuclease buffer	5 µL
	Light green	Nuclease mix	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 with wide bore tips.
	7.6	Quick spin the strip tube in a microcentrifuge to collect liquid.
	7.7	Run the nuclease treatment program.

- Pipette mix with **wide bore tips** to minimize DNA shearing damage to full-length SMRTbell template arrays

- **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 using **wide bore pipette tips** (Step 8)

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Final cleanup with SMRTbell cleanup beads

Final cleanup 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 treatment
8. **Final cleanup**

✔	Step	Instructions for cleanup with 1.2X (v/v) SMRTbell cleanup beads
	8.1	Add **60 µL SMRTbell cleanup beads** to each sample from the previous step. Using wide bore tips, pipette mix the beads until evenly distributed.
	8.2	Quick spin the tube strip in a microcentrifuge to collect all liquid.
	8.3	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	8.4	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	8.5	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.
	8.6	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	8.7	Repeat the previous step.
	8.8	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.
	8.9	Remove tube strip from the magnetic rack. **Immediately** add **20 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed with wide bore tips.
	8.10	Quick spin the tube strip in a microcentrifuge to collect liquid.
	8.11	Leave at **room temperature** for **5 minutes** to elute DNA.
	8.12	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	8.13	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer s…atant to a **new tube strip** using wide bore tips. Discard old tube strip with beads.
		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.
	8.14	**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.
		If a Femto Pulse system is unavailable, a Bioanalyzer system may also be used for DNA sizi… SMRTbell library - but note that the sample electropherogram trace may partially overlap with the … marker.
	8.15	Proceed to SMRT Link Sample Setup to prepare the SMRTbell library for sequencing.
	8.16	Store SMRTbell libraries at 4°C if sequencing within the week. Long-term storage should be at -20°C. Minimize freeze-thaw cycles when handling SMRTbell libraries.

PROTOCOL COMPLETE

- Pipette mix with **wide bore tips** to minimize DNA shearing damage to full-length SMRTbell template arrays

- Perform **DNA concentration QC** on final purified MAS-Seq library using a Qubit dsDNA HS assay
  - Typical final SMRTbell library yield from 5 µg of input DNA into DDR 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 MAS-Seq library using a Femto Pulse system (expected final library insert size is ~10 – 15 kb)
- If a Femto Pulse system in not available, can optionally perform DNA sizing QC using a Bioanalyzer system
  - Note: Bioanalyzer electropherogram profile of final library may partially overlap with the 17-kb upper marker

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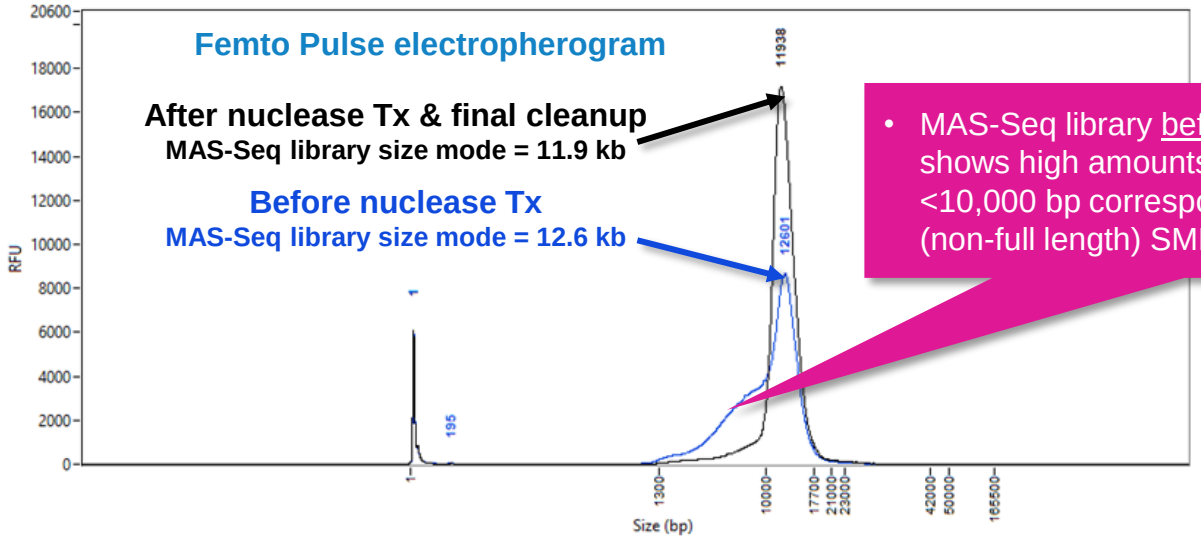
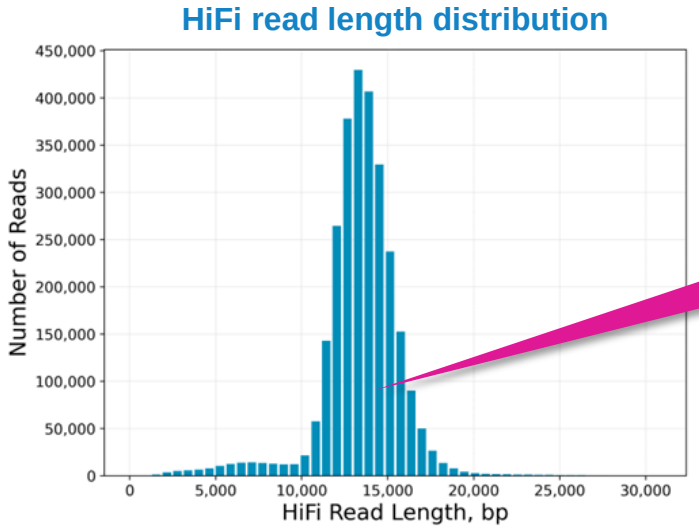
Final cleanup with SMRTbell cleanup beads (cont.)

Example Femto Pulse DNA sizing QC results for MAS-Seq 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 treatment
8. **Final cleanup**



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

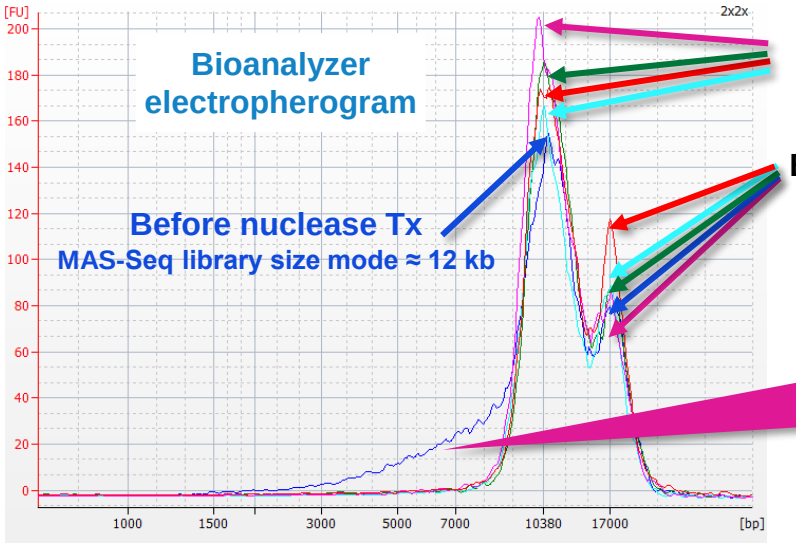


- HiFi read length mode is consistent with expected final library insert size

Final cleanup with SMRTbell cleanup beads (cont.)

Example Bioanalyzer DNA sizing QC results for MAS-Seq 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 treatment
↓
8 Final cleanup

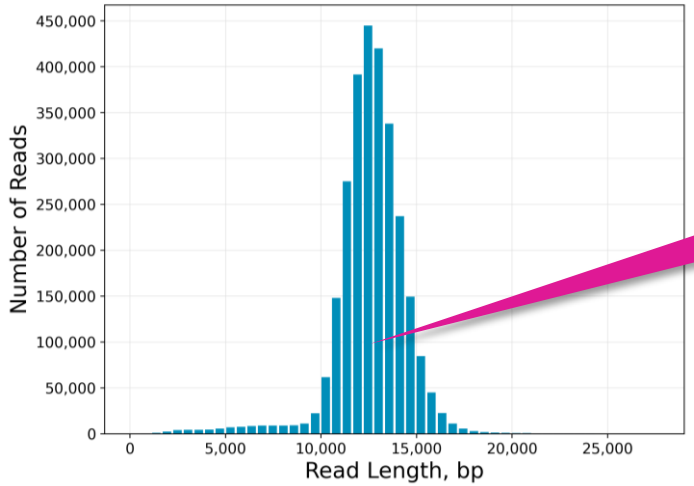


After nuclease Tx & final cleanup
MAS-Seq library size mode = 12 – 13 kb

Bioanalyzer 17 kb upper marker

- MAS-Seq 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 distribution



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

MAS-Seq library preparation expected step yields

Procedure & checklist – Preparing MAS-Seq libraries using MAS-Seq for 10x Single Cell 3’ kit (102-678-600)

	Cleanup	DNA QC	Step input	Step output / Yield (%)
1 Input cDNA quality control	N/A	Qubit HS assay Bioanalyzer HS DNA kit	10x Chromium single cell 3’ cDNA	N/A
2 TSO PCR	1.5X SMRTbell cleanup beads	Qubit HS assay	15 ng or 75 ng of normalized 10x 3’ cDNA input	~200 ng – 1 μg of amplified cDNA after bead cleanup
Safe stop				
3 TSO artifact removal	1.5X SMRTbell cleanup beads	Qubit HS assay	Min. input = 150 ng of TSO PCR products Max. input = 1 μg of TSO PCR products	≥50 ng of TSO artifact-depleted cDNA (~20 – 30% yield) after bead cleanup
Safe stop				
4 MAS PCR	1.5X SMRTbell cleanup beads	Qubit HS assay	Min. input = 50 ng* of TSO artifact-depleted cDNA (split across 16 MAS PCR reactions)	≥10 μg of amplified cDNA products after pooling 16 MAS PCR Rx and bead cleanup
Safe stop				
5 MAS array formation	1.2X SMRTbell cleanup beads	Qubit HS assay	10 μg of pooled MAS PCR products	≥5 μg of MAS array products (≥50% yield) after bead cleanup
Safe stop				
6 DNA damage repair	1.2X SMRTbell cleanup beads	N/A	5 μg of MAS array products	40 μL of DDR reaction products after bead cleanup
Safe stop				
7 Nuclease treatment	N/A	N/A	40 μL of DDR reaction products	50 μL of nuclease treatment reaction products
8 Final cleanup	1.2X SMRTbell cleanup beads	Qubit HS assay Femto Pulse gDNA 165 kb kit	50 μL of nuclease treatment reaction products	20 μL of final purified MAS-Seq SMRTbell library (~10 – 25% yield*) after bead cleanup

PacBio

* Add 1-2 additional PCR cycles for MAS PCR (Step 4) if input cDNA amount recovered after TSO artifact removal (Step 3) is <50 ng.

** Aim for a **minimum MAS-Seq SMRTbell library yield = 5%** to ensure a sufficient amount of library material to run at least one SMRT Cell 8M using recommended OPLC range.

MAS-Seq library sequencing workflow details

Sample Setup recommendations for MAS-Seq single-cell libraries

Follow SMRT Link Sample Setup instructions to prepare MAS-Seq single-cell libraries for sequencing

Sample Setup

Whole Genome Sequencing
- HiFi Reads
- Microbial Assembly

RNA Sequencing
- Iso-Seq Method
- MAS-Seq single cell

Viral Sequencing
- HiFiViral SARS-CoV-2
- Adeno-Associated Virus

Metagenomics
- Full-Length 16S rRNA Sequencing
- Shotgun Metagenomic Profiling or Assembly

Amplicon Sequencing
- <3kb Amplicons
- >=3kb Amplicons

Other
- Custom

- Select **MAS-Seq single cell** in application type drop-down menu

Sequel II binding kit 3.2 & cleanup beads (102-333-300) is recommended for preparing MAS-Seq single-cell libraries for sequencing on PacBio Sequel II and IIe systems.

- After specifying your application type, **SMRT Link** auto-fills selected Sample Setup parameter fields with default recommended values

Application type	PacBio system	Recommended binding/polymerase kit
MAS-Seq single cell	Sequel II/IIe system	Sequel II binding kit 3.2
	Revio system	Revio polymerase kit

Revio polymerase kit (102-817-600) includes SMRTbell cleanup beads and is recommended for preparing MAS-Seq single-cell libraries for sequencing on PacBio Revio systems.

SMRT Link Sample Setup guidance for Sequel II/IIe systems

Use SMRT Link Sample Setup High-Throughput (HT) mode and follow instructions to perform ABC (anneal primer / bind polymerase / complex cleanup) using recommended settings

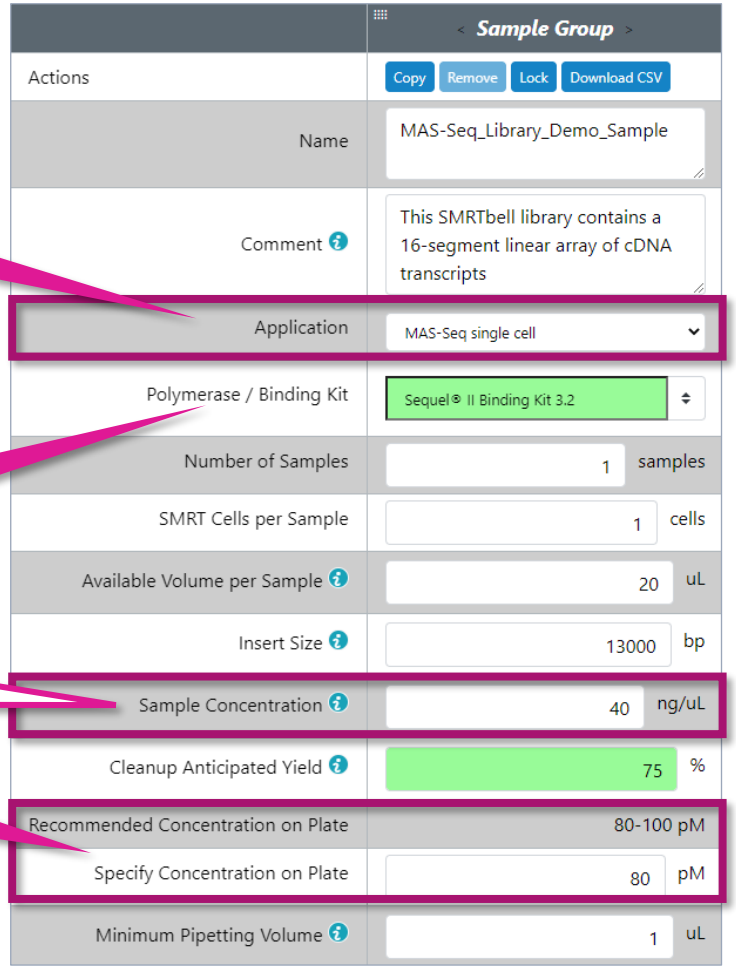
- Select MAS-Seq single cell from the **Application** field drop-down menu
- The following fields are auto-populated with default recommended values and high-lighted in green:
  - ❑ **Binding Kit**
    - → Sequel II binding kit 3.2
  - ❑ **Cleanup Anticipated Yield**
    - → 75%

Select **MAS-Seq single cell** in application type drop-down menu

Recommended binding kit for MAS-Seq samples is **Sequel II binding kit 3.2**

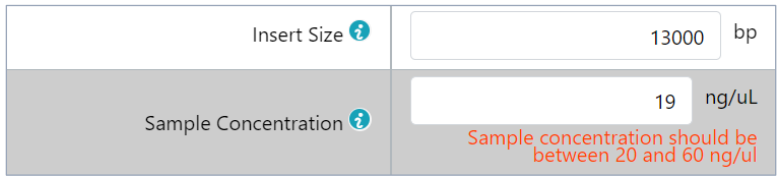
Recommended starting OPLC range[2] is **80 – 100 pM** for Sequel II/IIe systems

Recommended target *P1* loading range for Sequel II/IIe systems is **~60 – 80%**



Example Sample Setup worksheet for a MAS-Seq library sample to be run on one Sequel II SMRT Cell 8M.

- **Note:** If SMRTbell library construction yield is **low**, sample concentration may fall **outside** the recommended range.
  - If sample concentration is too low and a warning appears, can try **reconcentrating** sample using SMRTbell cleanup beads
  - If sample cannot be reconcentrated, you can still proceed with sample setup and sequencing if sample is just slightly below the target concentration range (e.g., 18 – 19 ng/µL[1])



1 Using input library sample concentrations **outside** the recommended ranges may lead to lower-than-expected sequencing performance.

2 OPLC = On-plate loading concentration. Optimal OPLC to achieve ~60 – 80% *P1* loading on a Sequel II/IIe system for a given MAS-Seq sample may vary from ~80 pM to >100 pM.

SMRT Link Sample Setup guidance for Revio system

Use SMRT Link Sample Setup High-Throughput (HT) mode and follow instructions to perform ABC (anneal primer / bind polymerase / complex cleanup) using recommended settings

- Select MAS-Seq single cell from the **Application** field drop-down menu
- Specify the information below in the fields high-lighted in green:
  - ❑ **Binding Kit**
    - → Revio polymerase kit
  - ❑ **Cleanup Anticipated Yield**
    - → 75%

- **Note:** If SMRTbell library construction yield is **low**, sample concentration may fall **outside** the recommended range.
  - If sample concentration is too low and a warning appears, can try **reconcentrating** sample using SMRTbell cleanup beads
  - If sample cannot be reconcentrated, you can still proceed with sample setup and sequencing if sample is just slightly below the target concentration range (e.g., 18 – 19 ng/µL[1])

Insert Size	13000	bp
Sample Concentration	19	ng/uL Sample concentration should be between 20 and 60 ng/ul

Select **MAS-Seq single cell** in application type drop-down menu

Recommended binding kit for MAS-Seq samples is **Revio polymerase kit**

Recommended starting OPLC range[2] is **200 – 250 pM** for Revio system

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

	Sample Group
Actions	Copy / Remove / Lock / Download CSV
Name	MAS-Seq_Library_Demo_Sample
Comment	This SMRTbell library contains a 16-segment linear array of cDNA transcripts
Application	MAS-Seq single cell
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	13000 bp
Sample Concentration	40 ng/uL
Cleanup Anticipated Yield	75 %
Recommended Concentration on Plate	200-250 pM
Specify Concentration on Plate	200 pM
Minimum Pipetting Volume	1 uL

Example Sample Setup worksheet for a MAS-Seq library sample to be run on one Revio SMRT Cell.

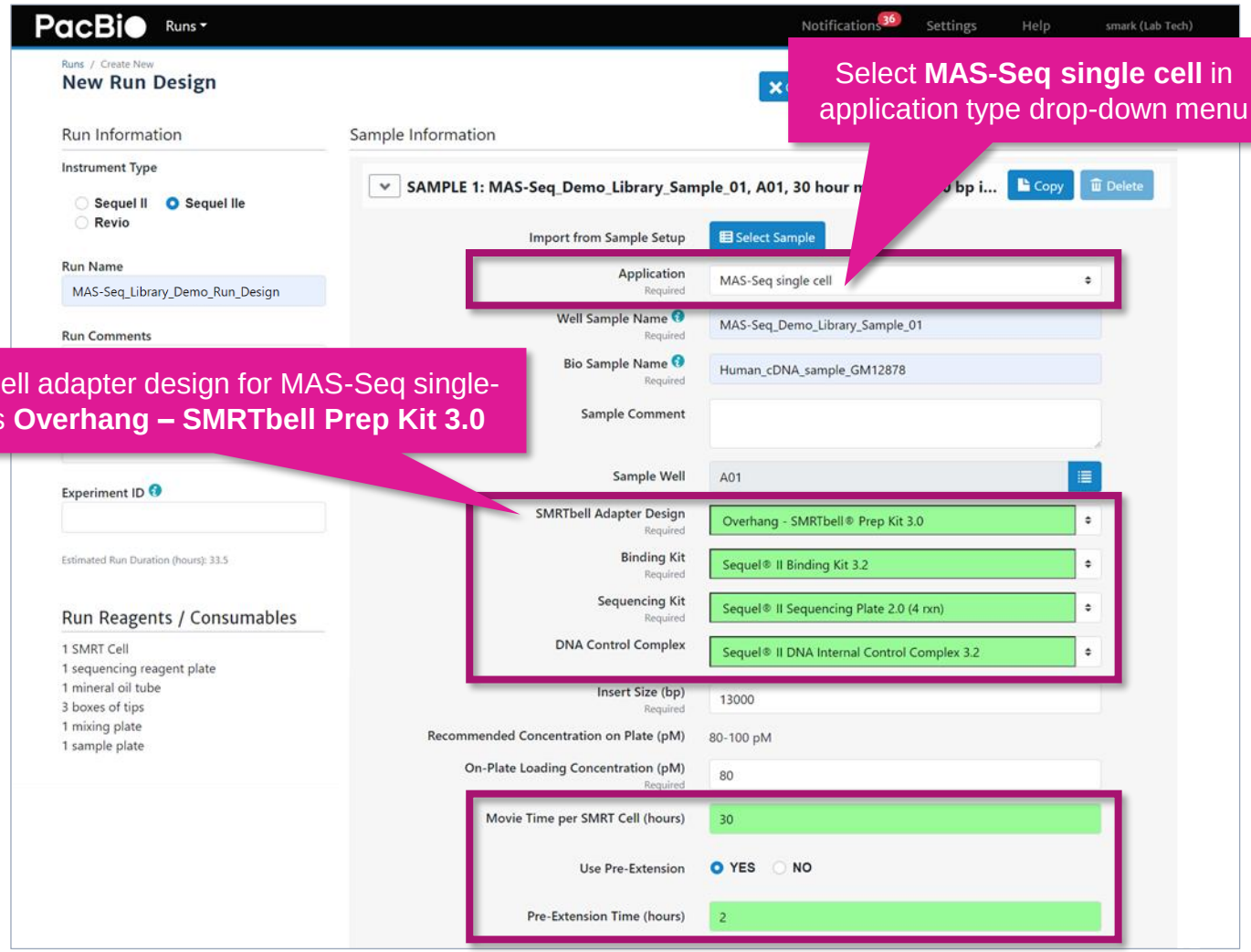
1 Using input library sample concentrations **outside** the recommended ranges may lead to lower-than-expected sequencing performance.
2 OPLC = On-plate loading concentration. Optimal OPLC to achieve ~50 – 70% *P1* loading on a Revio system for a given MAS-Seq sample may vary from ~100 pM to >200 pM.

SMRT Link Run Design guidance for Sequel II/IIe systems

Follow SMRT Link Run Design instructions to set up a sequencing run using recommended settings

- Select MAS-Seq single cell 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**
    - → Overhang – SMRTbell Prep Kit 3.0/MAS-Seq
  - **Binding Kit**
    - → Sequel II Binding Kit 3.2
  - **Sequencing Kit**
    - → Sequel II Sequencing Plate 2.0 (4 rxn)
  - **DNA Control Complex**
    - → Sequel II DNA Internal Control Complex 3.2
  - **Movie Time per SMRT Cell**
    - → 30 hrs
  - **Pre-Extension Time**
    - → 2 hrs

Leave **Advanced Options** at their **default** settings:
- Use Adaptive Loading = **YES**
- Loading Target (P1 + P2) = **0.85**
- Maximum Loading Time = **2 hours**

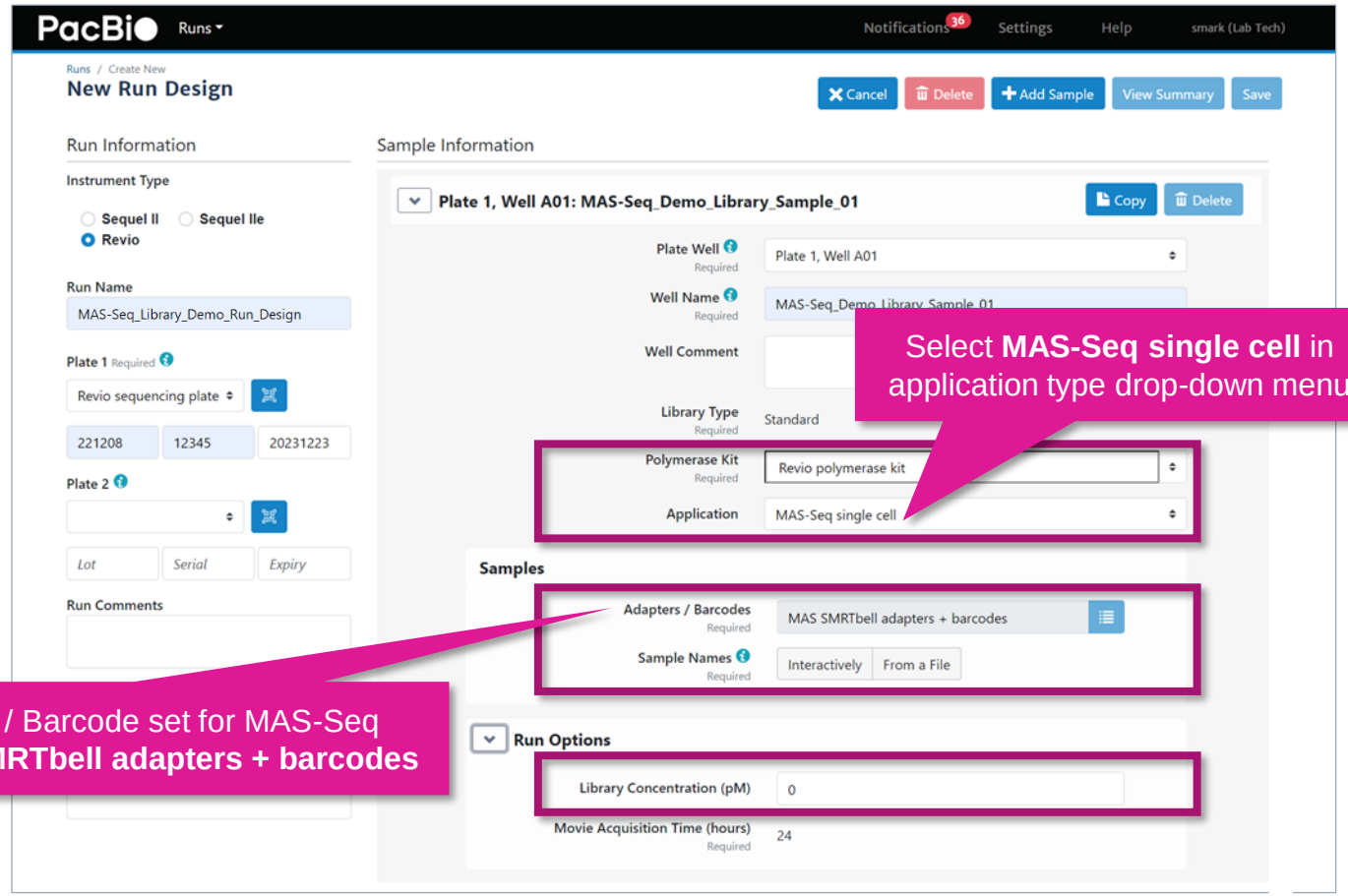


Example sample information entered into a Sequel IIe system run design worksheet for a MAS-Seq single-cell library sample.

SMRT Link Run Design guidance for Revio systems

Follow SMRT Link Run Design instructions to set up a sequencing run using recommended settings

- Select MAS-Seq single cell from the **Application** field drop-down menu
- Specify the information below in the appropriate fields:
  - ❑ **Polymerase Kit**
    - → Revio polymerase kit
  - ❑ **Adapters / Barcodes**
    - → MAS SMRTbell adapters + barcodes
  - ❑ **Sample Names**
    - → Select the 'default--default' barcode and enter in a Bio Sample name for the MAS-Seq single-cell library
  - ❑ **Library Concentration**
    - → Optionally enter in the sample on-plate loading concentration (OPLC)



Example sample information and run options settings entered into a Revio system run design worksheet for a MAS-Seq single-cell library sample.

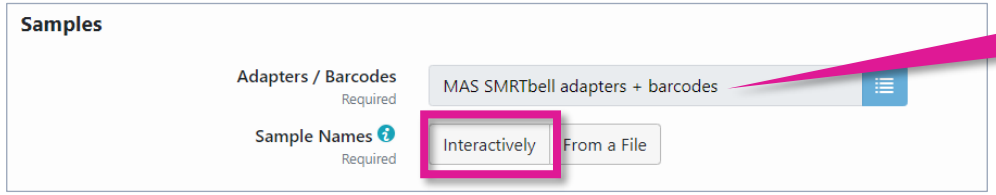
Note: Movie acquisition time is locked at **24 hrs** for all applications run on the Revio system

SMRT Link Run Design guidance for the Revio system (cont.)

SMRTbell adapter / barcode info is **required** for all multiplexed **and** non-multiplexed Revio system samples

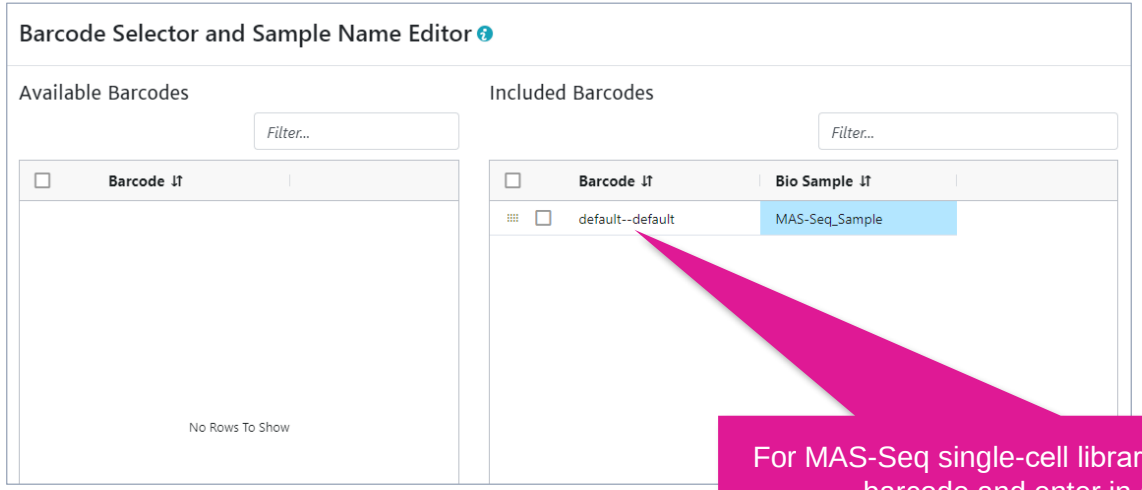
Example interactive barcode specification for MAS-Seq single-cell library samples

'**MAS SMRTbell adapters + barcodes**' barcode set contains one **'default--default'** barcode sequence to use for all **MAS-Seq single-cell library** sample types



For MAS-Seq single-cell libraries, select '**MAS SMRTbell adapters + barcodes**'

For MAS-Seq single-cell library samples:



For MAS-Seq single-cell libraries, select '**default--default**' barcode and enter in a Bio Sample name

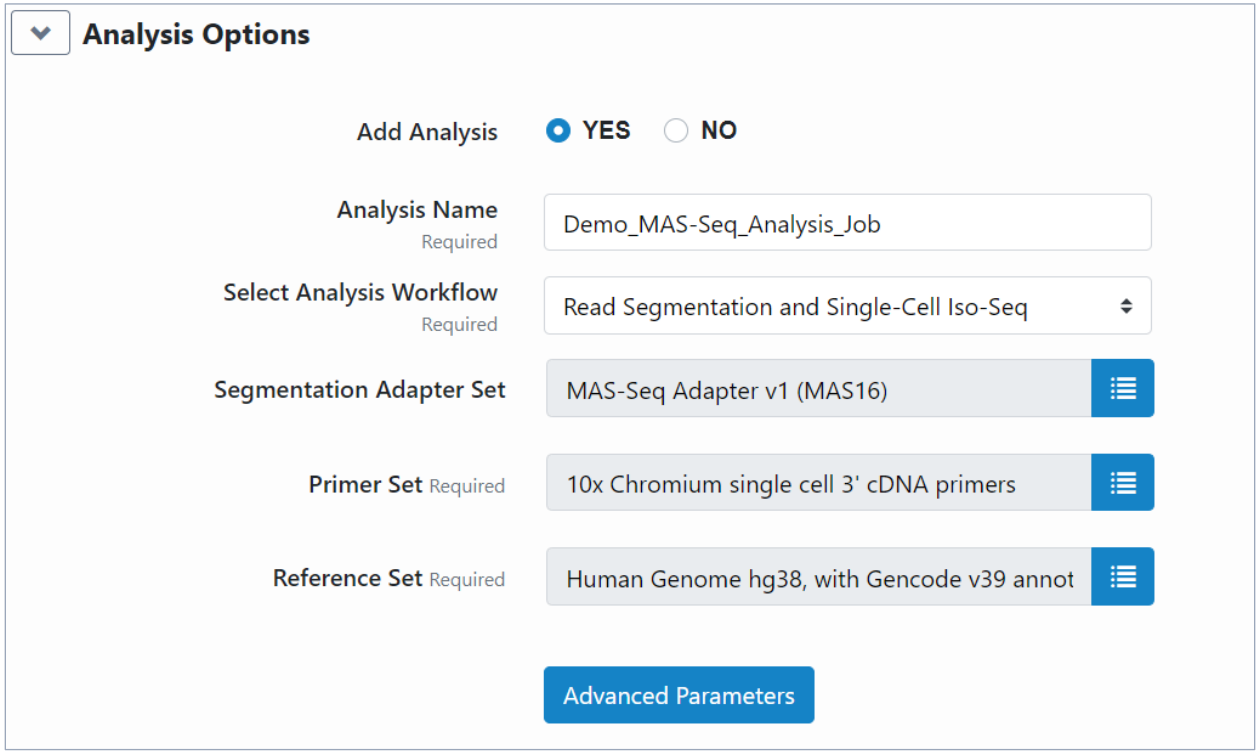
PacBio

* SMRT Link v12.0 Run Design only supports **non-multiplexed** MAS-Seq single-cell library samples.

SMRT Link Run Design analysis options for MAS-Seq single-cell samples

SMRT Link Run Design includes options to enable automated splitting of arrayed cDNA HiFi read segments and single-cell Iso-Seq analysis

- If MAS-Seq single cell is selected as the application in Run Design, the following **Analysis Options** fields display:
  - ❑ **Add Analysis**
    - → Default = YES
  - ❑ **Analysis Name**
    - → Specify a name for the analysis job.
  - ❑ **Select Analysis Workflow**
    - → Default analysis application = Read Segmentation and Single-Cell Iso-Seq
      - → Split arrayed HiFi reads at MAS adapter positions, generating segmented reads (S-reads), then perform analysis and functional characterization of full-length transcript isoforms with additional single-cell information (i.e., single-cell barcodes and unique molecular identifiers (UMIs)).
  - ❑ **Segmentation Adapter Set**
    - → Default = MAS-Seq Adapter v1 (MAS16)
  - ❑ **Primer Set**
    - → Default = 10x Chromium single cell 3’ cDNA primers
  - ❑ **Reference Set**
    - → Default = Human Genome hg38, with Gencode v39 annotations

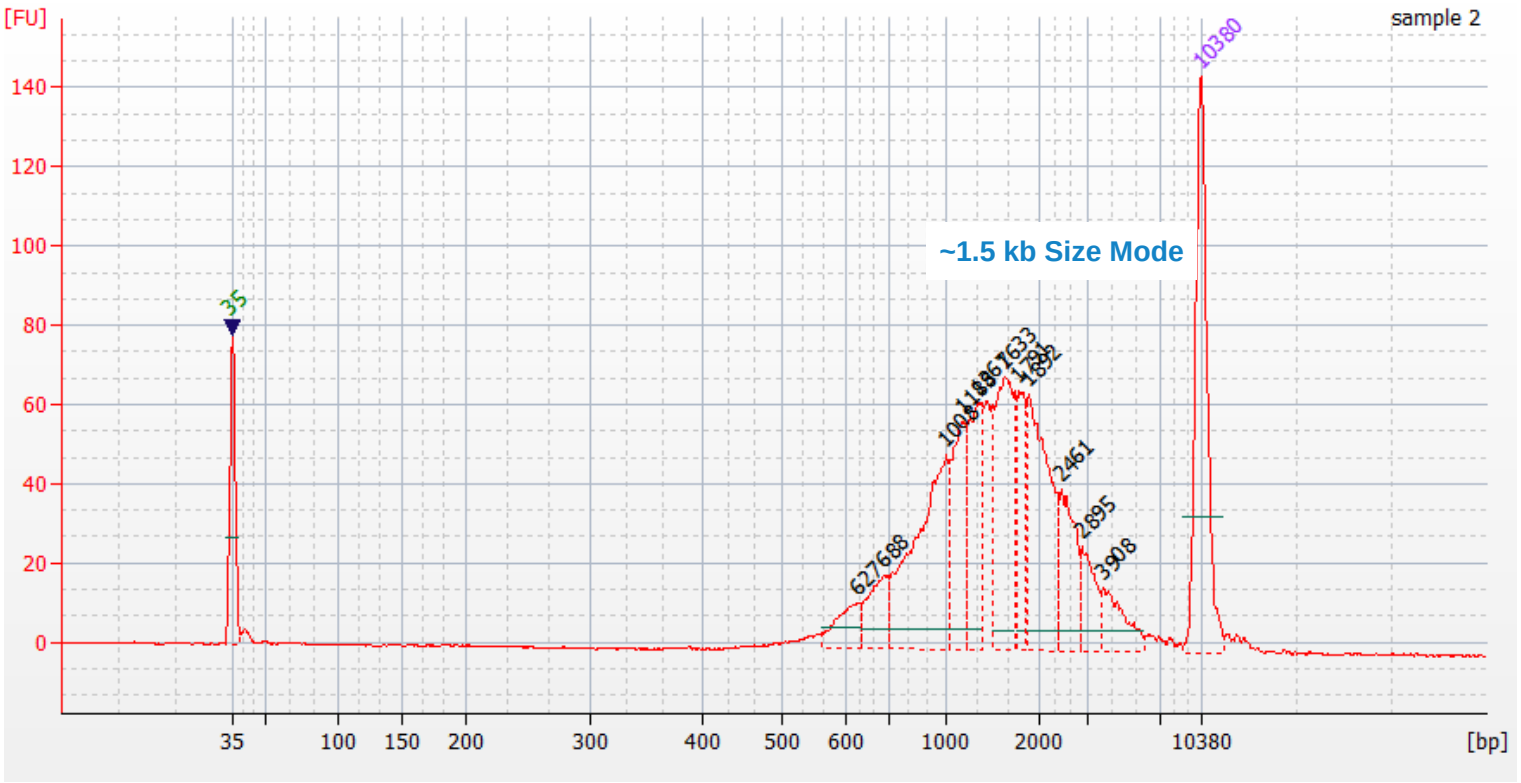


MAS-Seq library example sequencing performance data

Example MAS-Seq single-cell library preparation QC results

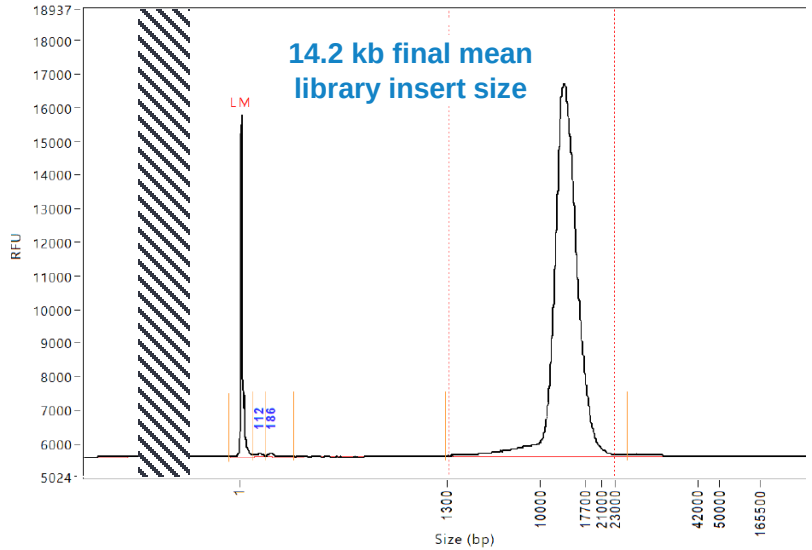
MAS-Seq single-cell library prepared with human cDNA

Input cDNA QC



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

Final MAS-Seq single-cell library QC



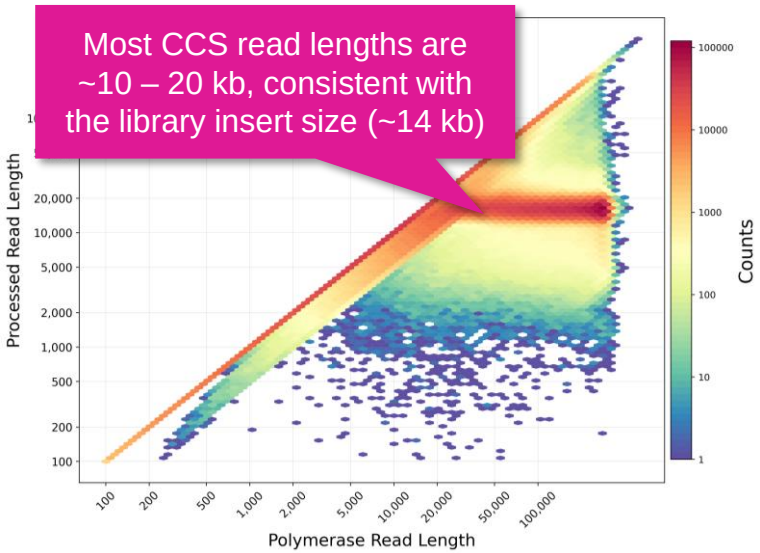
10x single cell 3’ cDNA input	15 ng
MAS array input for DDR	5000 ng
Post-nuclease treatment & final library cleanup yield (%)*	1198 ng (23.4%)

* Post-nuclease Tx & final cleanup yields typically ranged from ~10% to ~25% when using human single-cell cDNA samples for MAS-Seq library construction.

Example sequencing performance for MAS-Seq single-cell libraries prepared with human cDNA

Sequel IIe system example data[1]

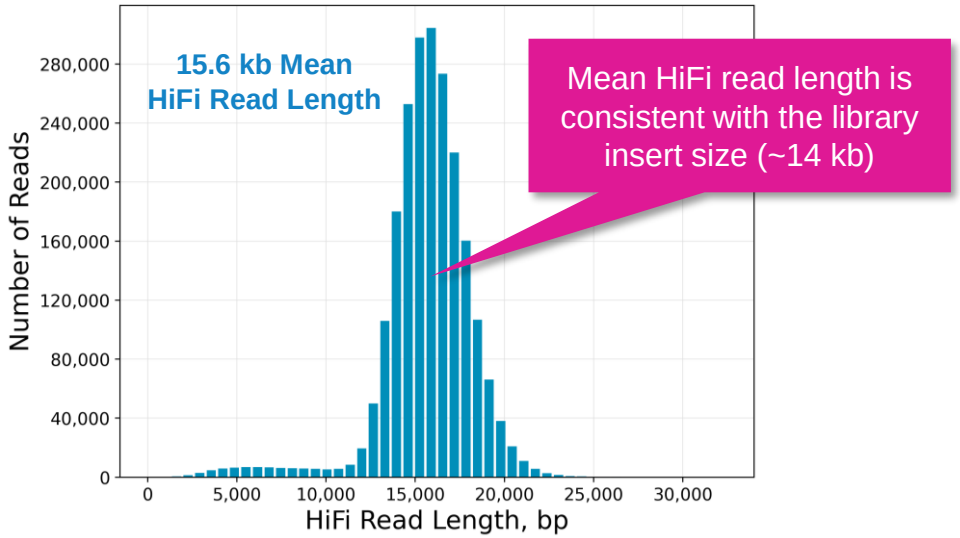
Raw Data Report



Raw Base Yield	562.43 Gb
Mean Polymerase Read Length	93.2 kb
P0	22.0%
P1	75.3%
P2	2.8%

Example sequencing metrics for a human MAS-Seq single-cell library sample run on a Sequel IIe system with Binding Kit 3.2 (Polymerase 2.2) / 80 pM on-plate loading concentration (OPLC) / 30-hrs movie time / 2-hrs pre-extension time.

HiFi Read Length



HiFi Reads	2.2 M
HiFi Base Yield	34.5 Gb
Mean HiFi Read Length	15,687 bp
Median HiFi Read Quality	Q31
HiFi Read Mean # of Passes	11

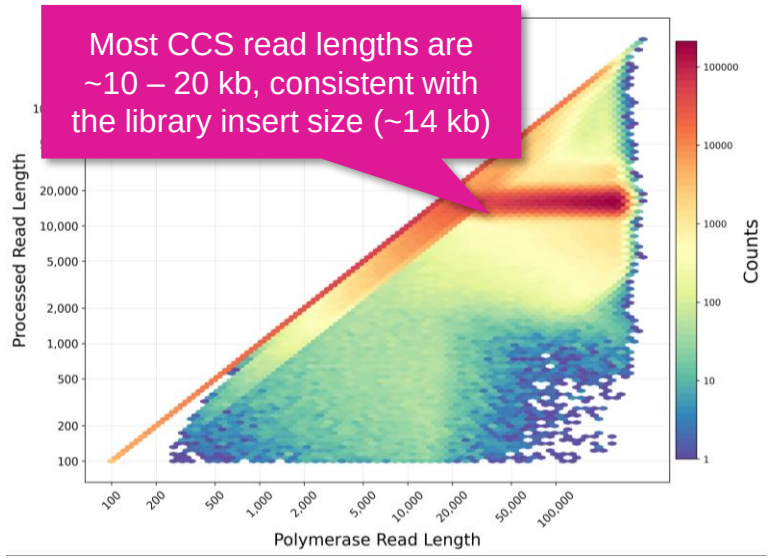
For human MAS-Seq single-cell libraries, per-SMRT Cell 8M HiFi read counts typically ranged from ~1.6 Million to ~2.5 Million and HiFi base yields typically ranged from ~26 Gb to ~36 Gb depending on the final library insert size.

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

Example sequencing performance for MAS-Seq single-cell libraries prepared with human cDNA (cont.)

Revio system example data[1]

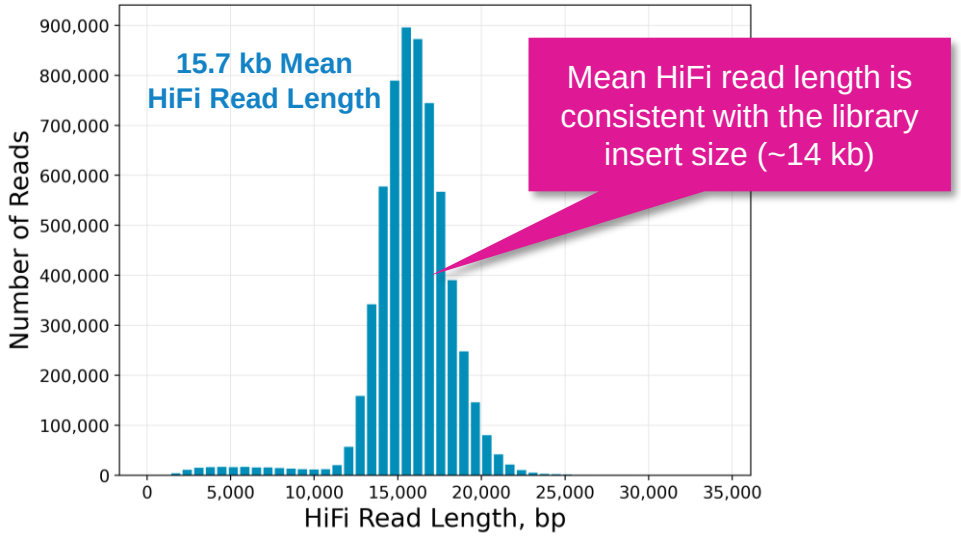
Raw Data Report



Raw Base Yield	1258 Gb
Mean Polymerase Read Length	79.8 kb
P0	47.3%
P1	51.8%
P2	0.9%

Example sequencing metrics for a human MAS-Seq single-cell library sample run on a Revio system with Revio polymerase kit / 115 pM on-plate loading concentration (OPLC) / 24-hrs movie time.

HiFi Read Length



HiFi Reads	6.1 M
HiFi Base Yield	97.3 Gb
Mean HiFi Read Length	15,787 bp
Median HiFi Read Quality	Q30
HiFi Read Mean # of Passes	10

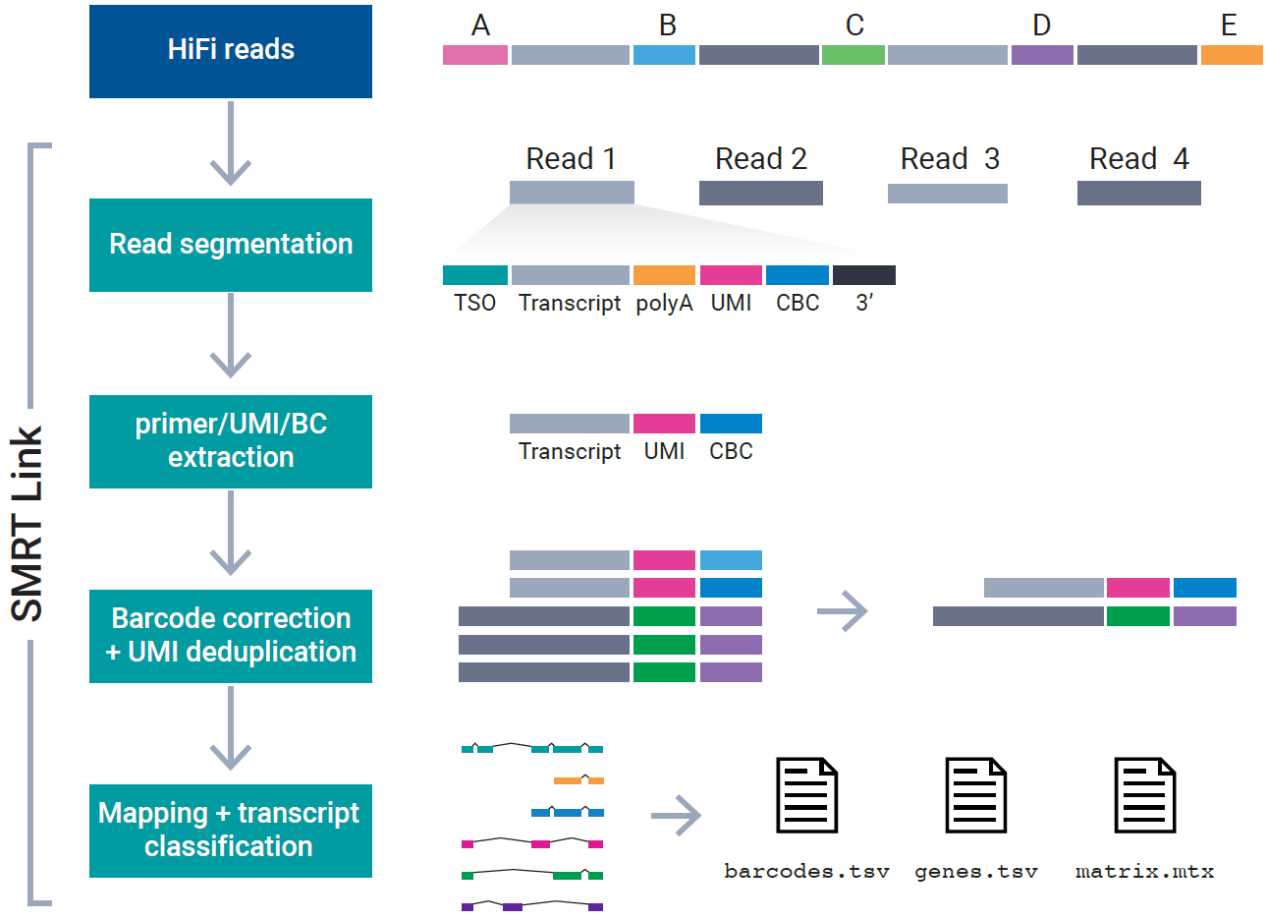
For human MAS-Seq libraries, per-Revio SMRT Cell HiFi read counts were typically ~6 Million and HiFi base yields typically ranged from 88~ Gb to ~98 Gb depending on the final library insert size.

[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.

MAS-Seq data analysis workflow overview

MAS-Seq bioinformatics workflow overview

The **SMRT Link Read Segmentation and Single-cell Iso-Seq** workflow processes HiFi reads generated from a MAS-Seq library to produce gene- and isoform-level count matrices that are compatible with tertiary single-cell analysis tools



Read segmentation

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

Primer/UMI/BC 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

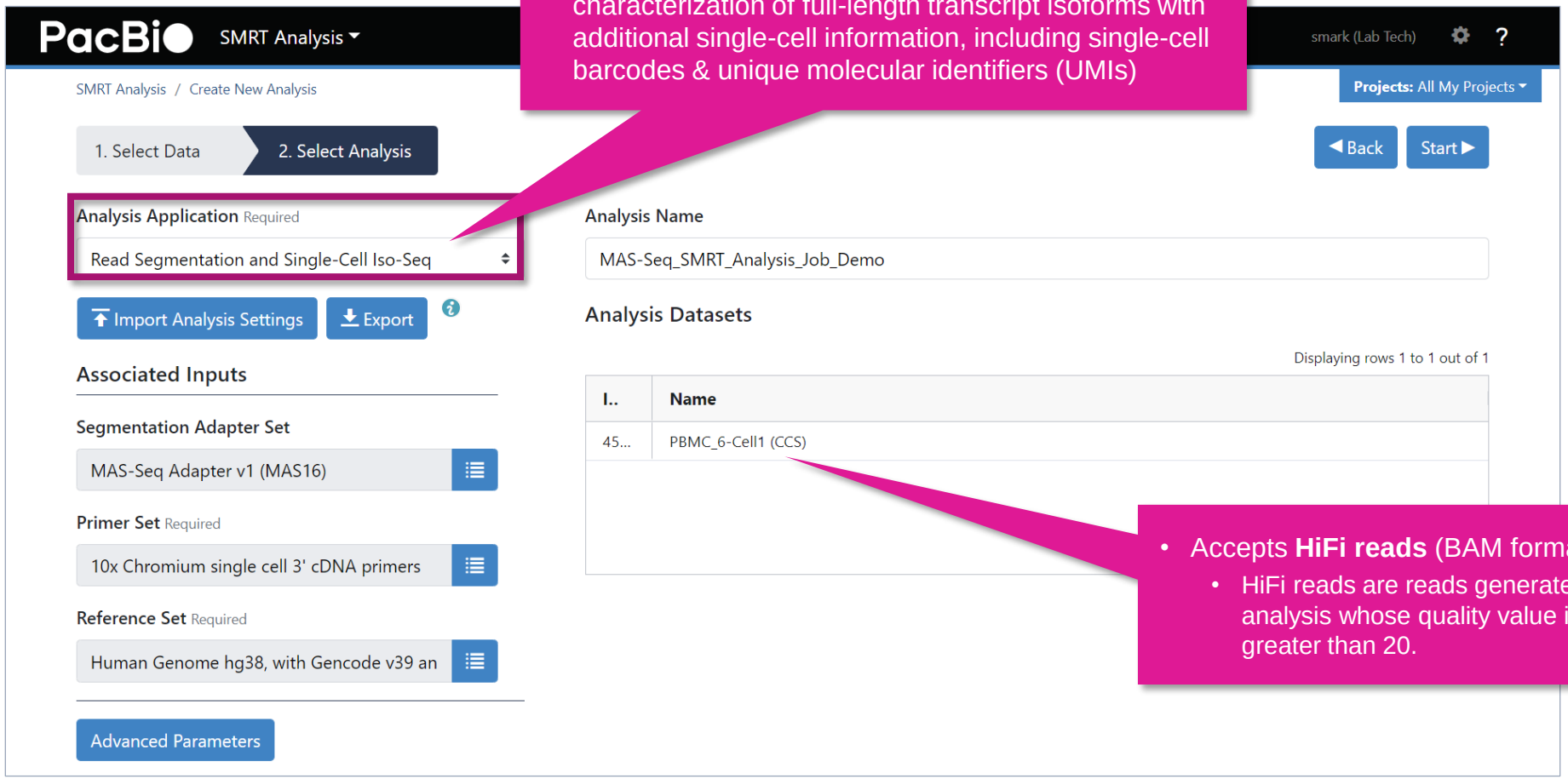
- Deduplicated reads are mapped to the reference genome and classified against a transcript annotation (e.g., GENCODE). Finally, a gene- and isoform-level single-cell matrix is output for tertiary analysis.

* Refer to the **SMRT Link User Guide** (102-877-300) for detailed descriptions of required parameters for Read Segmentation and Single-cell Iso-Seq analysis application.

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

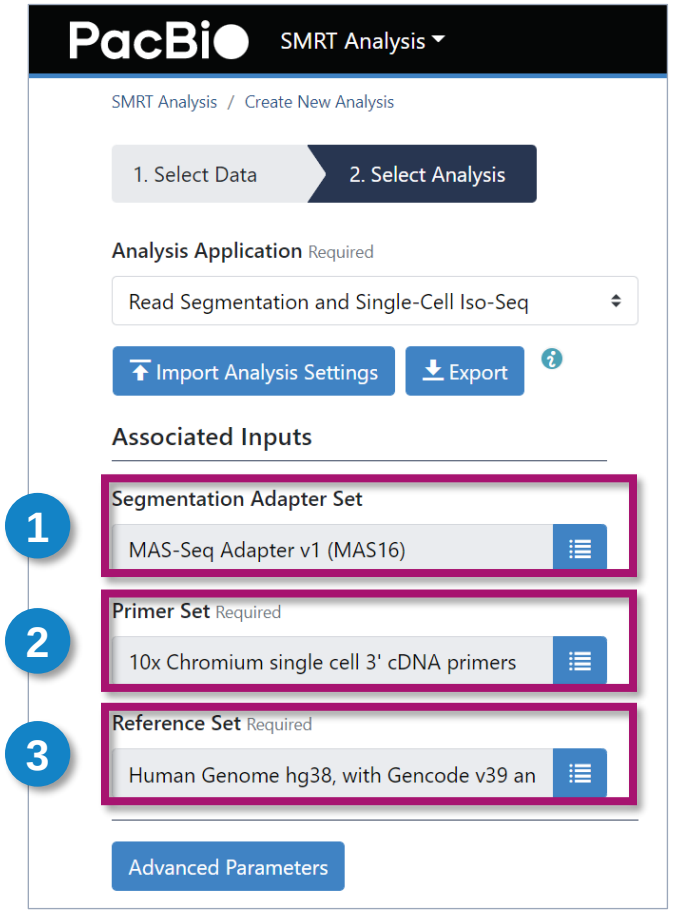
- Enables automated analysis and functional characterization of full-length transcript isoforms with additional single-cell information, including single-cell barcodes & unique molecular identifiers (UMIs)



- 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.

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. 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 table below.

2. Primer Set (Required) (Default = 10x Chromium single cell 3' cDNA primers)

- Specify a primer sequence file in FASTA format to identify cDNA primers for removal. The primer sequence includes the 5’ and 3’ cDNA primers.
- Primer IDs must be specified using the suffix `_5p` to indicate 5’ cDNA primers and the suffix `_3p` to indicate 3’ cDNA primers. The 3’ cDNA primer should not include the Ts and is written in reverse complement. (See the example below.)
- 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`.

Example SMRT Link Read Segmentation data utility processing results[1] for MAS-Seq libraries prepared with human single cell cDNA

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

Summary Metrics

Value	Analysis Metric
2,205,126	Reads
33,991,758	Segmented reads (S-reads)
985	Mean length of S-reads
87.47 %	Percent of reads with full arrays
15.41	Mean array size (concatenation factor)

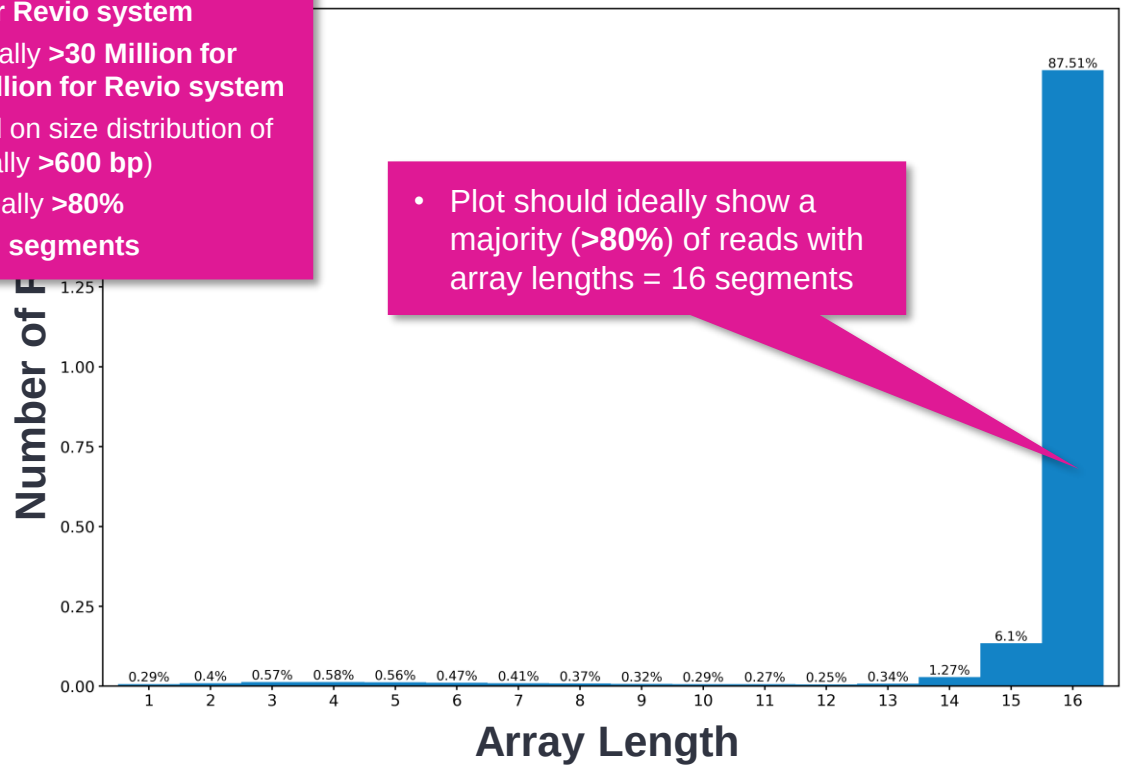
Example Sequel IIe system data shown.

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 Sequel IIe system data shown.)

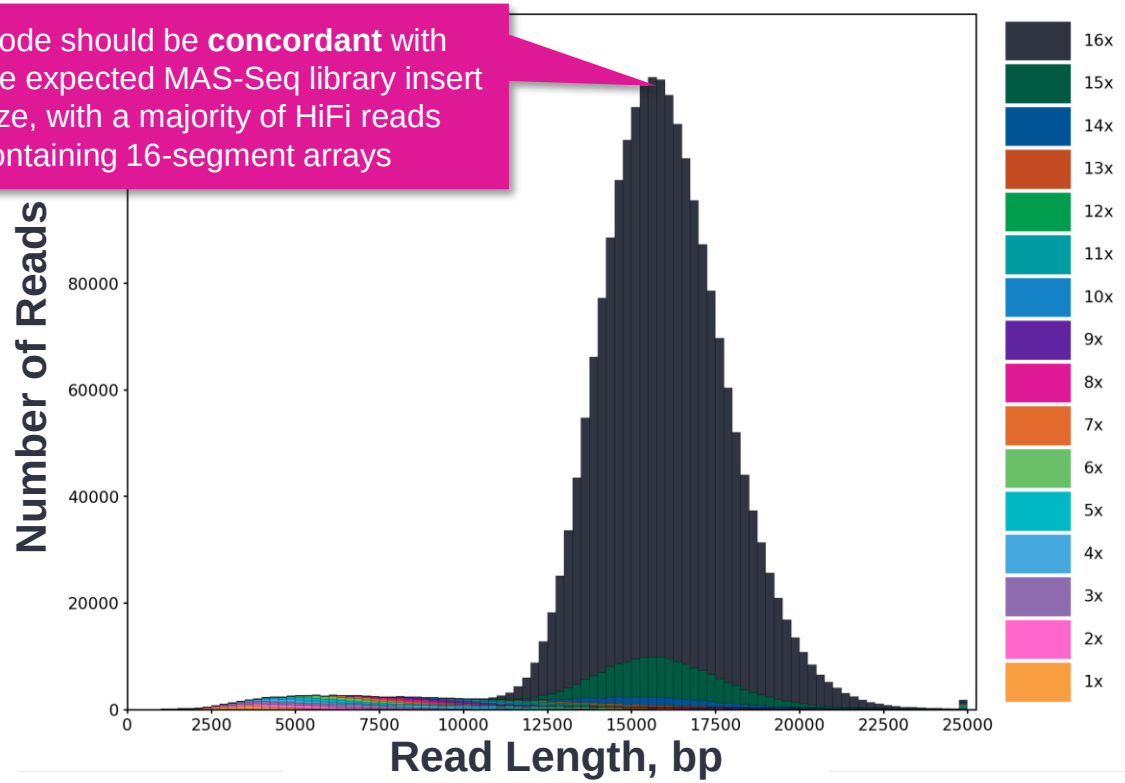
[1] HiFi read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Note: Refer to **SMRT Link MAS-Seq troubleshooting guide** (102-994-400) for example performance metrics typically achievable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

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

Length of Reads

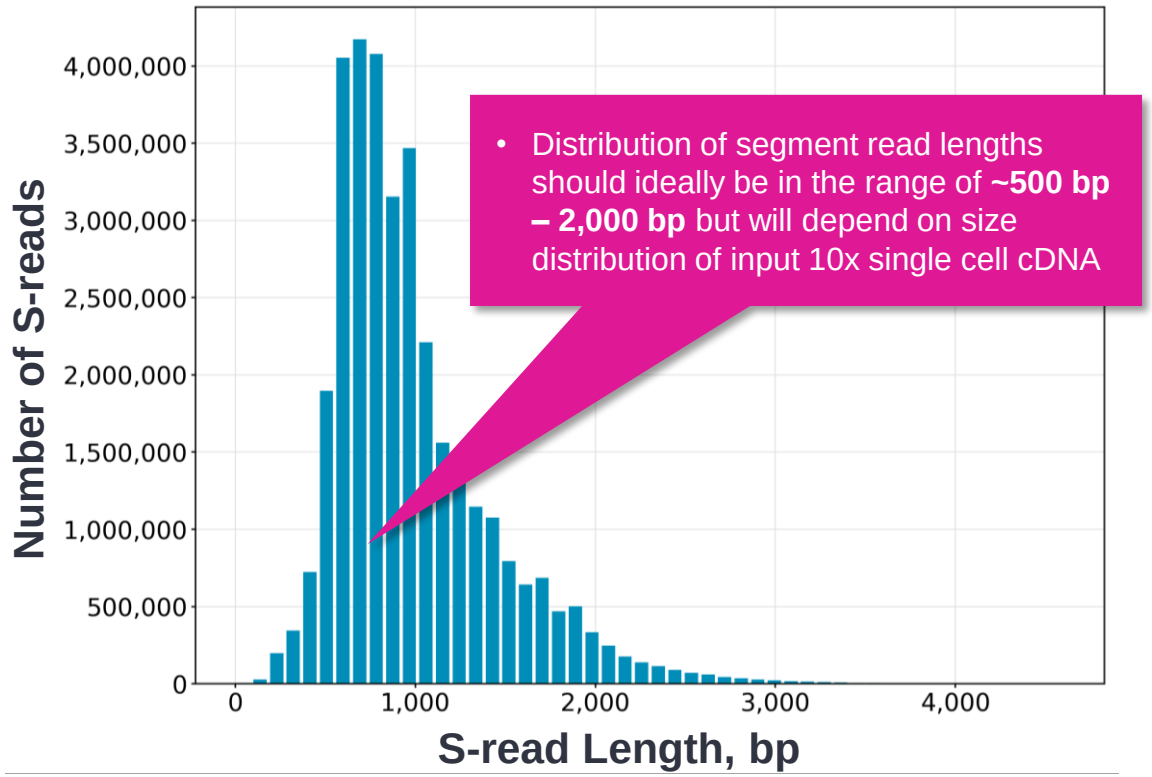
- Mode should be **concordant** with the expected MAS-Seq library insert size, with a majority of HiFi reads containing 16-segment arrays



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

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

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

Summary Metrics

Value	Analysis Metric
33,991,758	Reads
SEGMENT	Read Type
33,742,391	Reads with 5' and 3' Primers with extracted UMIs and Barcodes
33,005,125	Non-Concatamer Reads with 5' and 3' Primers and Poly-A Tail (FLNC reads)
31,980,352	FLNC Reads with Valid Barcodes
32,960,590	FLNC Reads with Valid Barcodes, corrected
30,048,062	Reads after Barcode Correction and UMI Deduplication

Example Sequel IIe 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:** Number of reads with 5' and 3' cDNA primers detected.
- **Non-Concatemer Reads with 5' and 3' Primers and Poly-A Tail (FLNC reads):** Number of non-concatemer reads with 5' and 3' primers and polyA tails detected. This is usually the number for full-length, non-concatemer (FLNC) reads, unless polyA tails are not present in the sample.
- **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.

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

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

Summary Metrics

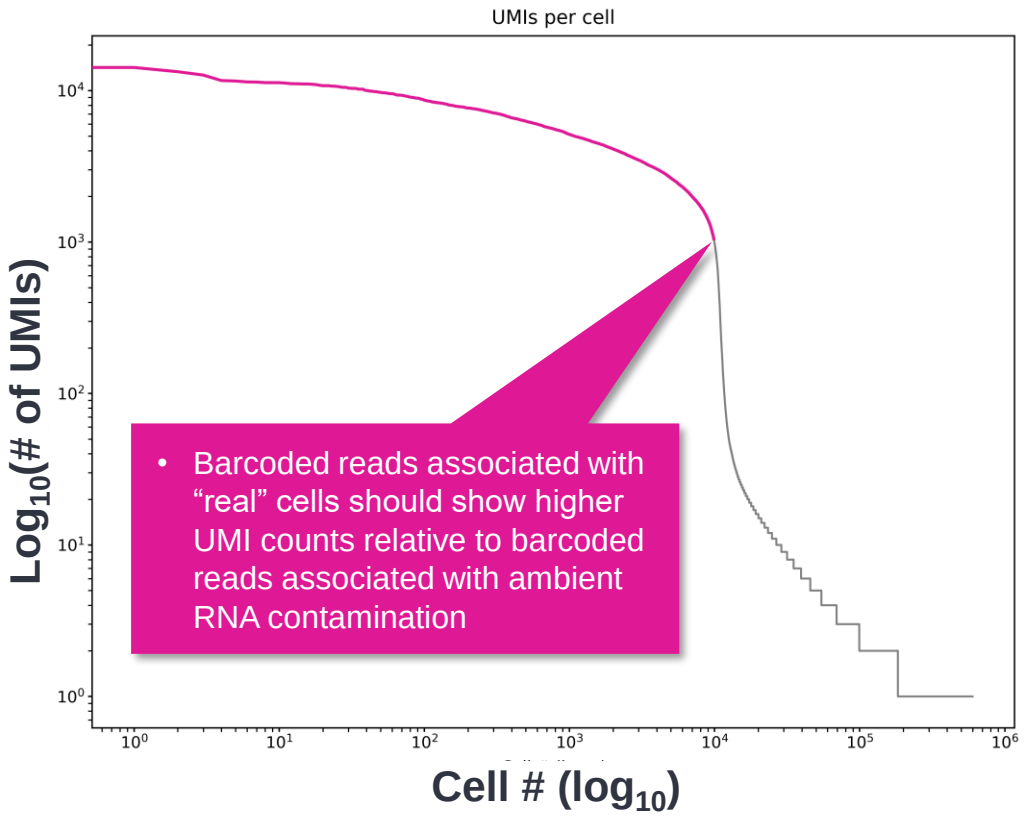
Value	Analysis Metric
9,857	Estimated Number of Cells
93.35%	Reads in Cells
3,125	Mean Reads per Cell
2,668	Median UMIs per Cell

Example Sequel IIe 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 Sequel IIe system data shown.)

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

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

Summary Metrics

Value	Analysis Metric
29,982,592	Deduplicated Reads Mapped to Genome
1,235,244	Total Unique Genes
36,935	Total Unique Genes, filtered
2,452,594	Total Unique Transcripts
436,173	Total Unique Transcripts, filtered

Example Sequel IIe system data shown.

- **Deduplicated Reads Mapped to Genome:** The number of deduplicated reads mapped to the reference genome.
- **Total Unique Genes:** The total number of unique genes across all cells.
- **Total Unique Genes, filtered:** The total number of unique genes across all cells, after transcript filtering.
- **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 transcript filtering.

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

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

Transcript Summary

Value	Analysis Metric
1,461	Median Genes per Cell
1,626	Median Transcripts per Cell
1,235,244	Total Unique Genes
2,452,594	Total Unique Transcripts

Example Sequel IIe system data shown.

Transcript Summary, Filtered

Value	Analysis Metric
770	Median Genes per Cell
858	Median Transcripts per Cell
36,935	Total Unique Genes
436,173	Total Unique Transcripts

- **Median Genes per Cell:** The median number of unique genes per input cell.
- **Median Transcripts per Cell:** The median number of transcripts per input cell.
- **Total Unique Genes:** The total number of unique genes across all input cells.
- **Total Unique Transcripts:** The total number of unique transcripts across all input cells.

Filter out reads based on the SQANTI3 transcript filtering criteria*

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* Refer to the **SMRT Link User Guide** (102-877-300) for further details regarding filtering criteria based on the SQANTI3 software.

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

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

Transcript Classification

Category	Count	CAGE Detected	CAGE Detected, (%)	polyA Detected	polyA Detected, (%)
FSM	96449	55161	57.19%	32134	33.31%
ISM	230786	99018	42.90%	69507	30.11%
NIC	263499	89109	33.81%	86009	32.64%
NNC	432134	249680	57.77%	182369	42.20%
Antisense	144495	2847	1.97%	35615	24.64%
Fusion	8771	5387	61.41%	3820	43.55%
More junctions	94	42	44.68%	49	52.12%
Genic intron	2055	8	0.38%	578	28.12%
Genic genomic	91561	23486	25.65%	26126	28.53%
Intergenic	1182750	5764	0.48%	364928	30.85%

Transcript Classification, Filtered*

Category	Count	CAGE Detected	CAGE Detected, (%)	polyA Detected	polyA Detected, (%)
FSM	81545	48368	59.31%	32134	39.40%
ISM	144272	74464	51.61%	52811	36.60%
NIC	71654	48920	68.27%	31901	44.52%
NNC	115709	68704	59.37%	57048	49.30%
Antisense	5448	688	12.62%	3067	56.29%
Fusion	2920	1621	55.51%	1491	51.06%
More junctions	59	28	47.45%	40	67.79%
Genic intron	0	0	0.00%	0	0.00%
Genic genomic	2563	1138	44.40%	1199	46.78%
Intergenic	12003	980	8.16%	7541	62.82%

Example Sequel IIe system data shown.

- **Category:** Transcript classification** assigned by the classification and filtering tool pigeon, based on the SQANTI3 software.
- **Count:** The number of transcripts 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 Detected:** The number of transcripts where a known polyA motif is detected upstream of the transcription end site.
- **polyA Detected, (%):** The percentage of transcripts where a known polyA motif is detected upstream of the transcription end site.

* Transcript classification after filtering out reads based on the SQANTI3 transcript filtering criteria.
** Refer to the **SMRT Link User Guide** (102-877-300) 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 MAS-Seq libraries prepared with human single cell cDNA (cont.)

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

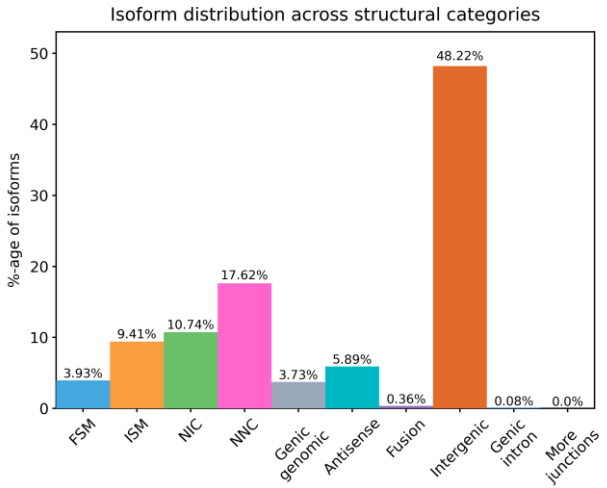
- **Isoform distributions across structural categories:**
  - Distribution of the % of isoforms by structural categories.

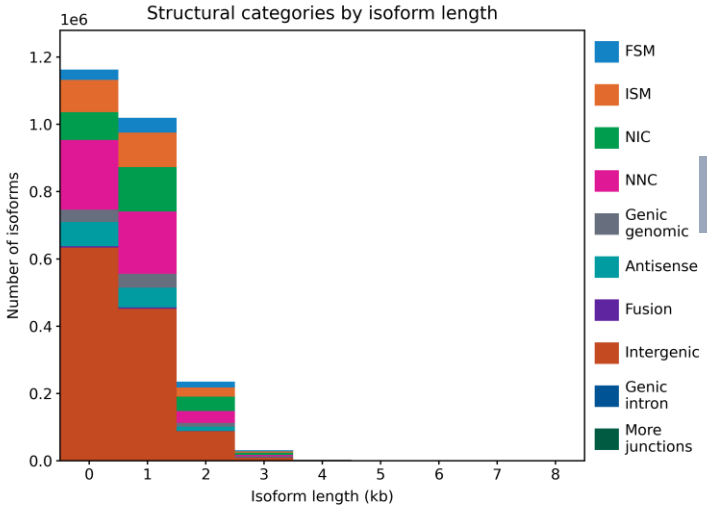
Example Sequel IIe system data shown.

- **Structural categories by isoform lengths:**
  - Histogram display of the number of isoforms by their length in kb and their structural category.

Example Sequel IIe system data shown.

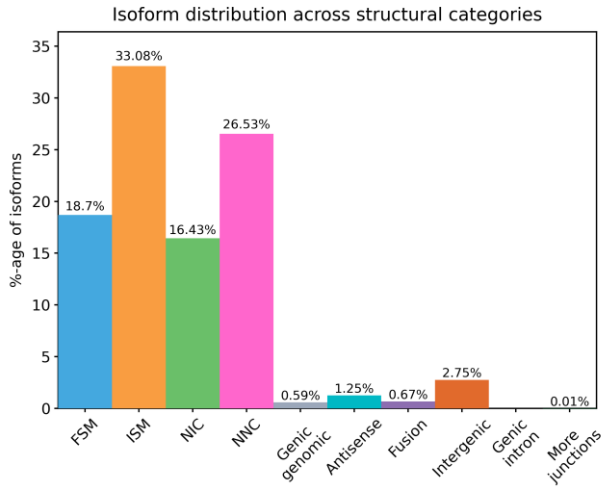
Transcript Classification Plots

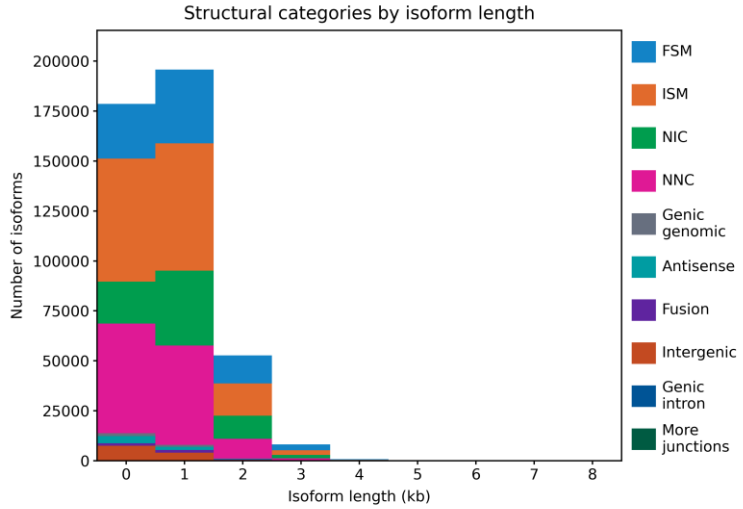




Filter out reads based on the SQANTI3 transcript filtering criteria

Transcript Classification Plots, Filtered





Filter out reads based on the SQANTI3 transcript filtering criteria

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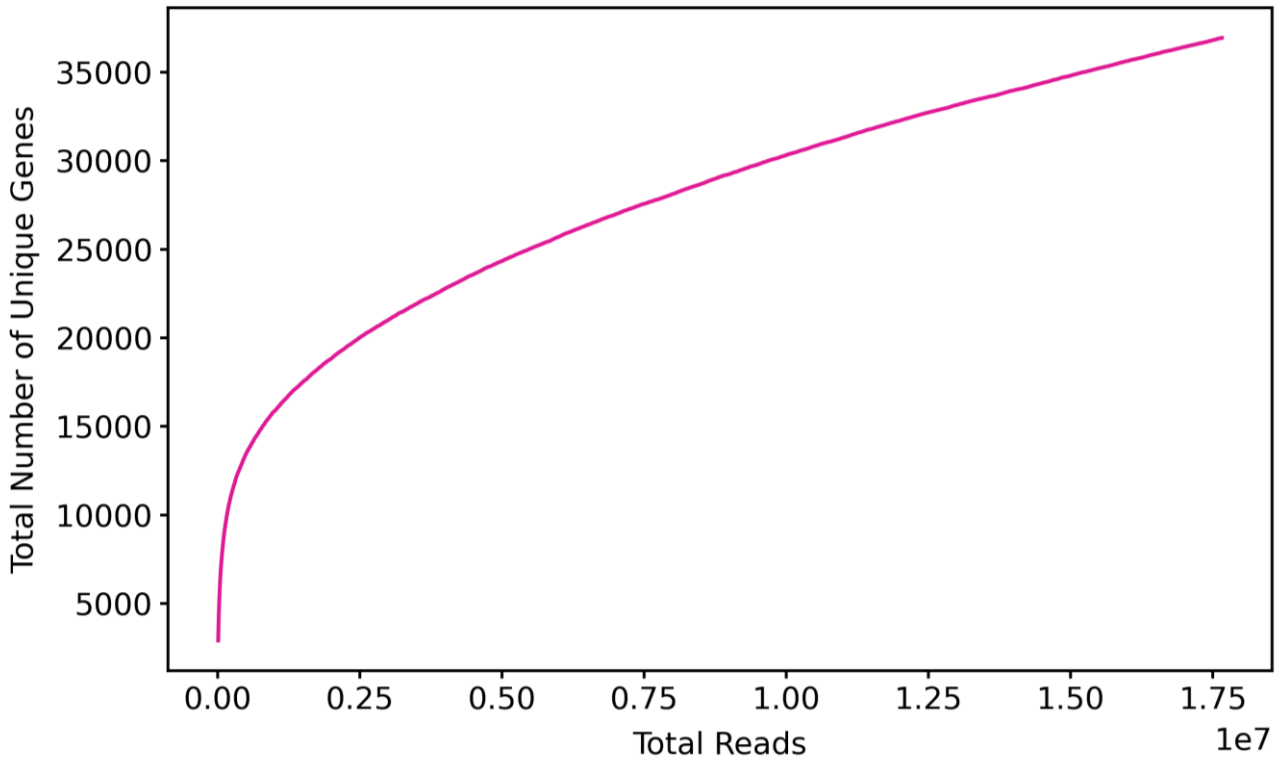
Example SMRT Link Single-Cell Iso-Seq Analysis results for MAS-Seq libraries prepared with human single cell cDNA (cont.)

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

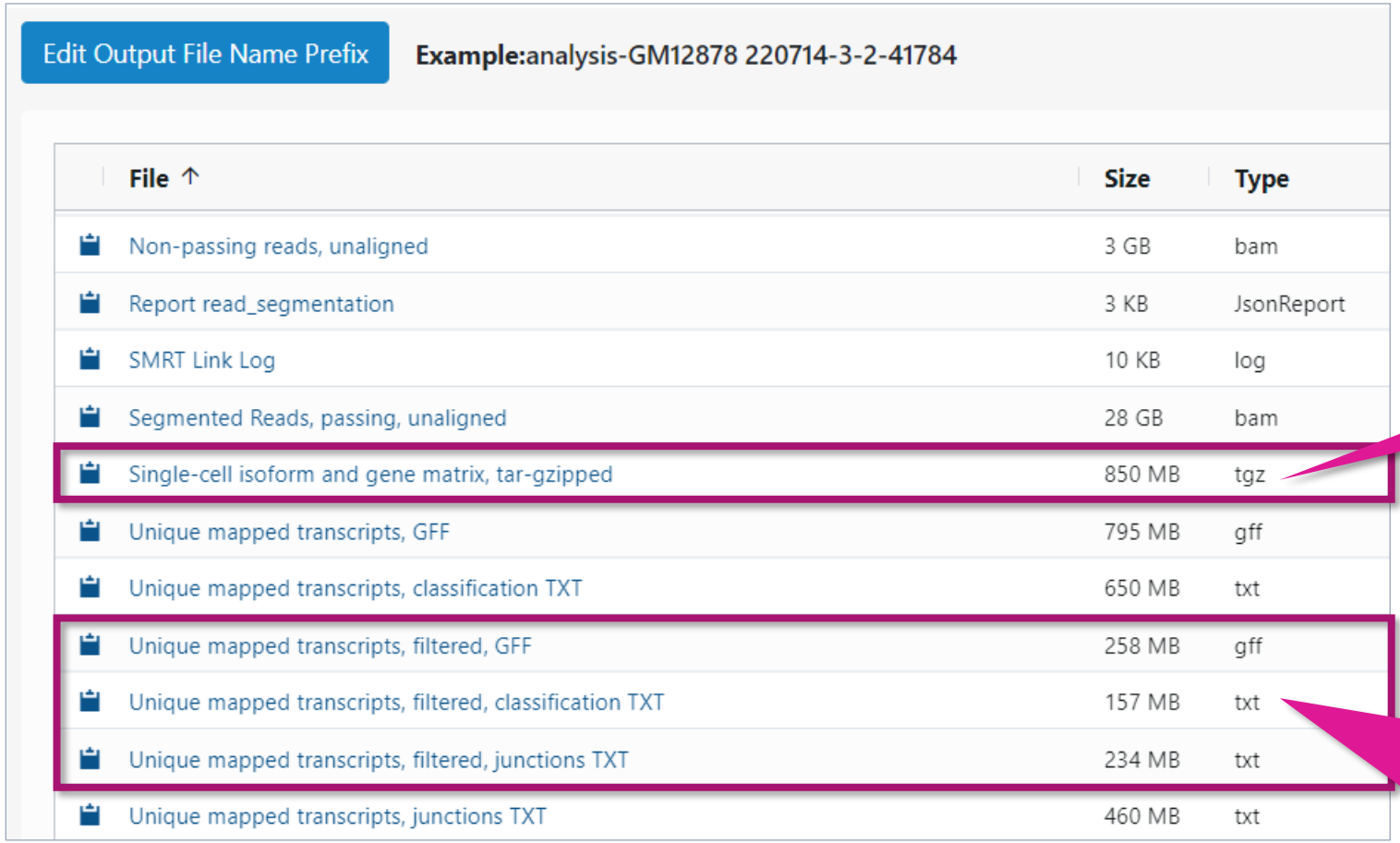
Gene Saturation



Saturation plot showing the level of gene saturation based on the number of subsampled transcript reads. (Example Sequel IIe system data shown.)

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

File downloads tab



File	Size	Type
Non-passing reads, unaligned	3 GB	bam
Report read_segmentation	3 KB	JsonReport
SMRT Link Log	10 KB	log
Segmented Reads, passing, unaligned	28 GB	bam
Single-cell isoform and gene matrix, tar-gzipped	850 MB	tgz
Unique mapped transcripts, GFF	795 MB	gff
Unique mapped transcripts, classification TXT	650 MB	txt
Unique mapped transcripts, filtered, GFF	258 MB	gff
Unique mapped transcripts, filtered, classification TXT	157 MB	txt
Unique mapped transcripts, filtered, junctions TXT	234 MB	txt
Unique mapped transcripts, junctions TXT	460 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.

- **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 MAS-Seq 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)

MAS-Seq library preparation literature & other resources

- Application brief – A more complete cancer transcriptome with the Iso-Seq method – Single-cell and bulk RNA sequencing (102-326-538)
- Application note – MAS-Seq for single-cell isoform sequencing (102-326-549)
- Overview – Sequel systems application options and sequencing recommendations (101-851-300)
- Procedure & checklist – Preparing MAS-Seq libraries using MAS-Seq for 10x Single Cell 3’ kit (102-678-600)
- Technical overview – MAS-Seq library preparation using the MAS-Seq for 10x Single Cell 3’ kit (102-829-300)
- Video tutorial – PacBio MAS-Seq TSO artifact removal demo for MAS-Seq for 10x Single Cell 3’ kit [Link]

Data analysis resources

- SMRT Link v12.0 MAS-Seq troubleshooting guide (102-994-400)
- SMRT Link v12.0 software installation guide (102-878-100)
- SMRT Link v12.0 user guide (102-877-300)
- SMRT Tools v12.0 reference guide (102-978-000)

Technical resources for MAS-Seq library preparation, sequencing & data analysis (cont.)

Publications

- Al'Khafaji, A.M. et al. (2021) High-throughput RNA isoform sequencing using programmable cDNA concatenation. BioRxiv preprint. [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) – Bioinformatics tools for Iso-Seq and single-cell Iso-Seq 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
MAS-Seq single-cell isoform sequencing	PBMC single cell 3' cDNA [Link]	HiFi Reads	Sequel IIe system
	PBMC single cell 3' cDNA [Link]	HiFi Reads	Revio system

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