Technical overview: Iso-Seq library preparation using SMRTbell prep kit 3.0

Sequel II and IIe Systems ICS v11.0 / SMRT Link v11.0

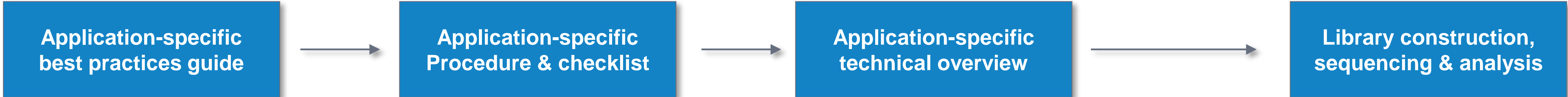
PN 102-393-400 Version 01 (April 2022)

Iso-Seq library preparation using SMRTbell prep kit 3.0

Technical overview

1. Iso-Seq method workflow overview, input RNA sample QC requirements & experimental design considerations
2. Iso-Seq library sample preparation workflow details
3. Iso-Seq library sequencing preparation workflow overview
4. Iso-Seq data analysis workflow overview
5. Iso-Seq library example sequencing performance data
6. Technical documentation & applications support resources
7. Appendix: RNA isolation kit options for Iso-Seq SMRTbell library construction

Full-length transcript isoform sequencing (Iso-Seq method): How to get started

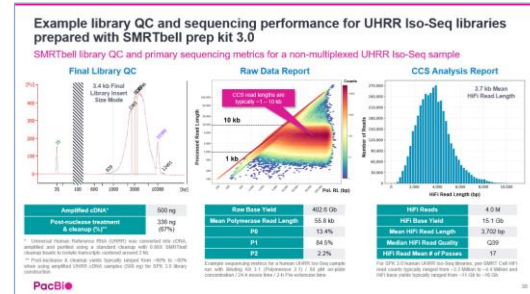


[Application Brief: Long-Read RNA sequencing – Best practices](102-193-643) (102-193-643)

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

Procedure & Checklist – Preparing Iso-Seq libraires using SMRTbell prep kit 3.0 (102-396-000)

Technical documentation containing sample library construction and sequencing preparation protocol details



Technical Overview: Iso-Seq library preparation using SMRTbell prep kit 3.0 (102-393-400)

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.

Total RNA QC and cDNA synthesis

300 ng input total RNA per sample
RIN (RNA integrity number) ≥7.0

Library construction (SMRTbell prep kit 3.0)

Multiplex Iso-Seq samples using SMRTbell barcoded cDNA primers or barcoded adapter plate 3.0

Sequencing (Sequel II and IIe systems)

ABC* with Sequel II binding kit:
Binding kit 3.1 (standard Iso-Seq samples)
Binding kit 3.2 (focus on long transcripts)
24 hr movie collection time

Data analysis (SMRT Link)

Iso-Seq analysis application

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

Iso-Seq method workflow overview, input RNA sample QC requirements & experimental design considerations

Iso-Seq sample preparation procedure description

Procedure & checklist – Preparing Iso-Seq libraries using SMRTbell prep Kit 3.0 (102-396-000) describes a method for constructing SMRTbell libraries that are suitable for generating HiFi reads on the Sequel II and IIe systems for full-length RNA sequencing applications.

SMRTbell prep kit 3.0 (102-182-700)

SMRTbell barcoded adapter plate 3.0 (102-009-200)

Procedure highlights

- Uses SMRTbell prep kit 3.0 (SPK 3.0) for library construction starting from ≥300 ng of high-quality total RNA input (RIN ≥7.0) per sample
    - Third-party reagents are also used for first-strand cDNA synthesis and cDNA amplification reactions
- Multiplexing of up to 12 Iso-Seq samples per SMRT Cell 8M can be performed using SMRTbell barcoded adapter plate 3.0 (102-009-200) or with barcoded cDNA primers
- 8-hr workflow time to process up to 12 samples from cDNA synthesis to purified SMRTbell library

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

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Procedure & checklist

Before you begin

This procedure describes the workflow for constructing Iso-Seq libraries from RNA for sequencing on PacBio systems.

Overview	
Samples	1–12
Workflow time	8 hours [for up to 12 samples]
RNA input	
Quality / size distribution	RIN (RNA integrity number) ≥7.0
Quantity	300ng per library
cDNA Input	
Quantity	≥ 160 ng per library for 1 SMRT Cell 8M

Workflow

1. Input DNA quality control — Safe stop
2. cDNA synthesis + cleanup — Safe stop
3. cDNA amplification + cleanup — Safe stop
4. Repair and a-tailing
5. Adapter ligation + cleanup — Safe stop
6. Nuclease treatment + cleanup

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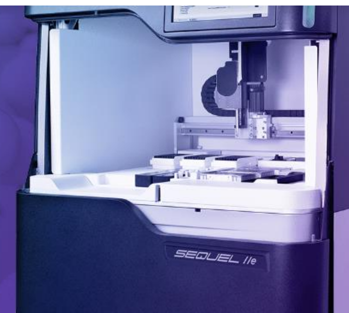
PacBio Documentation (102-396-000)



Iso-Seq sample preparation & sequencing workflow overview

Workflow summary for constructing SMRTbell libraries suitable for sequencing on the Sequel II and IIe systems for full-length RNA sequencing applications

Total RNA QC and cDNA synthesis

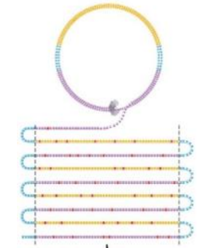
- ≥300 ng of total RNA input per sample (RIN ≥7.0)
- cDNA synthesis with NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module
- Optionally barcode Iso-Seq samples during cDNA amplification using barcoded cDNA primers
- If enriching for long transcripts >3kb, perform additional rounds of cDNA amplification

SMRTbell library construction

- ***Procedure & checklist – Preparing Iso-Seq libraries using SMRTbell prep kit 3.0*** (102-396-000)
- Optionally barcode Iso-Seq samples during SMRTbell library construction using SMRTbell barcoded adapter plate 3.0 (102-009-200).

Sequencing

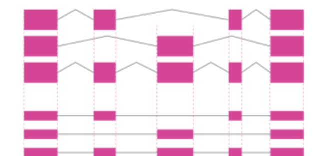
- Follow **SMRT Link Sample Setup** instructions for primer annealing, polymerase binding, complex cleanup and sample loading



PacBio HiFi reads achieve 99.9% accuracy

Data analysis

- Use SMRT Link Iso-Seq analysis application to output high-quality, full-length transcript sequences, with no assembly required

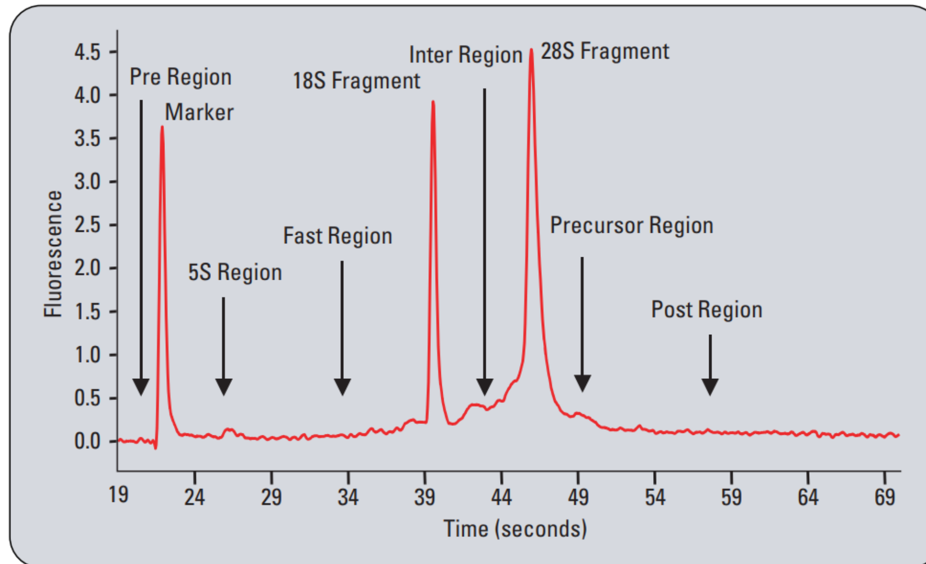


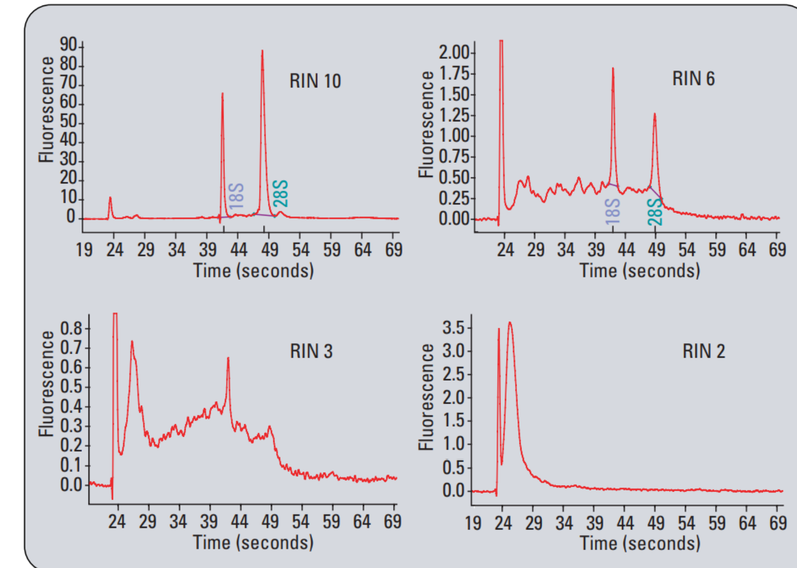
HiFi reads cover the full length of transcripts with *no assembly required*

Iso-Seq sample QC requirements

Evaluation of input total RNA sample integrity

- Sample QC of input total RNA samples should be assessed by measuring the RNA Integrity Number (RIN) using a Bioanalyzer 2100 instrument (Agilent Technology)
- RIN score (1 to 10) is related to the ratio of the area under the 28s and 18s fragment peaks and also takes into account the signal intensity above the baseline in the Inter-Region and Fast Region since this is where degradation products appear
- Higher RIN numbers are correlated with better overall sample quality and lower degradation





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

A RIN ≥7.0 (ideally ≥8.0) is sufficient for the Iso-Seq protocol. Samples with a RIN <7.0 can be processed, but the risk of significant underperformance or even failure is greatly increased.

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Iso-Seq sample QC requirements (cont.)

Evaluation of input total RNA sample purity

- RNA purity can be determined by using a NanoDrop system [Thermo Fisher Scientific] or other spectrophotometer tool
- For pure RNA, A260/280 ratio is typically ~2.0 and A260/230 ratio is ≥2.0
- For samples with ratios that fall outside the expected optimal values, refer to the manufacturer of the RNA isolation kit for additional information regarding protocol optimization and troubleshooting.

A260/A280 Ratio

- A low A260/A280 ratio may be the result of:
  - ❑ Protein
  - ❑ Phenol
  - ❑ Other contaminants that absorb strongly at or near 280 nm
  - ❑ Sometimes it may be caused by a very low concentration of nucleic acid.
- High 260/280 ratios are not indicative of an issue

PacBio recommends only proceeding with RNA samples that have an absorbance **A260/A280 ratio between 1.8 – 2.0** (or higher) and a **A260/A230 between 2.0 – 2.5**.

A260/A230 Ratio

- A low A260/A230 ratio may be the result of:
  - ❑ Protein*
  - ❑ Carbohydrate carryover (often a problem with plants)
  - ❑ Residual phenol from nucleic acid extraction
  - ❑ Residual guanidine (often used in column-based kits)
  - ❑ Glycogen used for precipitation
- A high A260/A230 ratio may be the result of:
  - ❑ Making a blank measurement on a dirty pedestal of a Nanodrop instrument
  - ❑ Using an inappropriate solution for the blank measurement

* See NEB Technical Note: A Practical Guide to Analyzing Nucleic Acid Concentration and Purity with Microvolume Spectrophotometers (2019)

Iso-Seq sample QC requirements (Cont.)

Minimize Genomic DNA Contamination

- It is best to use extraction methods that selectively precipitate RNA and minimize contaminating genomic DNA
- DNase I treatments can be used to remove contaminating DNA, but before performing a treatment we recommend assessing the risk it poses to RNA integrity
  - For example, only use RNase-free DNase and avoid the heat inactivation methods which can degrade RNA in the presence of metal ions
  - If you do use a DNase treatment, PacBio recommends using one of the commercially available kits that includes a purification method that does not involve heat inactivating the DNase I enzyme
- In most circumstances, low-level residual genomic DNA contamination is not problematic for Iso-Seq applications
  - This is because of the use of the oligo-dT primer in combination with the 5' template-switching oligo (TSO) during cDNA synthesis
  - Moreover, the subsequent PCR using primers annealing to the sequences on the 5' TSO and 3' dT primer further selects against any contaminating DNA fragments

Iso-seq express experimental design considerations

Iso-seq express use case recommendations for PacBio systems

Sequel system	Sequel II and IIe systems
For genome annotation One transcriptome → one SMRT Cell 1M	**For genome annotation** Up to 12-plex transcriptome* → one SMRT Cell 8M
	For deep transcriptome profiling One human transcriptome → one SMRT Cell 8M

* Can multiplex up to a total of 12 Iso-Seq samples on one SMRT Cell 8M using either barcoded cDNA primers or barcoded adapters during Iso-Seq library construction.

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Iso-Seq library sample preparation
workflow details

Procedure & checklist – Iso-Seq library preparation using SMRTbell prep kit 3.0 (102-396-000)

Procedure & checklist 102-396-000 describes a method for constructing SMRTbell libraries using SMRTbell prep kit 3.0 (SPK 3.0) and SMRTbell barcoded adapter plate 3.0 that are suitable for generating HiFi reads on the Sequel II and IIe systems for full-length RNA sequencing applications

Procedure & checklist contents

1. General best practices and input RNA QC recommendations.
2. Multiplexing best practices guidance for using either barcoded adapters or barcoded cDNA primers to construct barcoded Iso-Seq libraries.
3. Enzymatic workflow steps for preparation and amplification of cDNA using Iso-Seq express oligo kit (101-737-500) and other third-party reagents
4. Enzymatic workflow steps for preparation of multiplexed Iso-Seq SMRTbell libraries from amplified cDNA products using SMRTbell prep kit 3.0 (102-182-700) and SMRTbell barcoded adapter plate 3.0 (102-009-200).
5. Guidance for pooling barcoded Iso-Seq SMRTbell libraries for multiplexed sequencing on a single SMRT Cell.

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

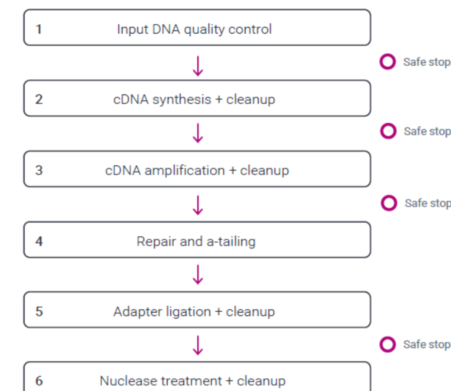
Procedure & checklist

Before you begin

This procedure describes the workflow for constructing Iso-Seq libraries from RNA for sequencing on PacBio systems.

Overview	
Samples	1–12
Workflow time	8 hours [for up to 12 samples]
RNA input	
Quality / size distribution	RIN (RNA integrity number) ≥7.0
Quantity	300ng per library
cDNA Input	
Quantity	≥ 160 ng per library for 1 SMRT Cell 8M

Workflow



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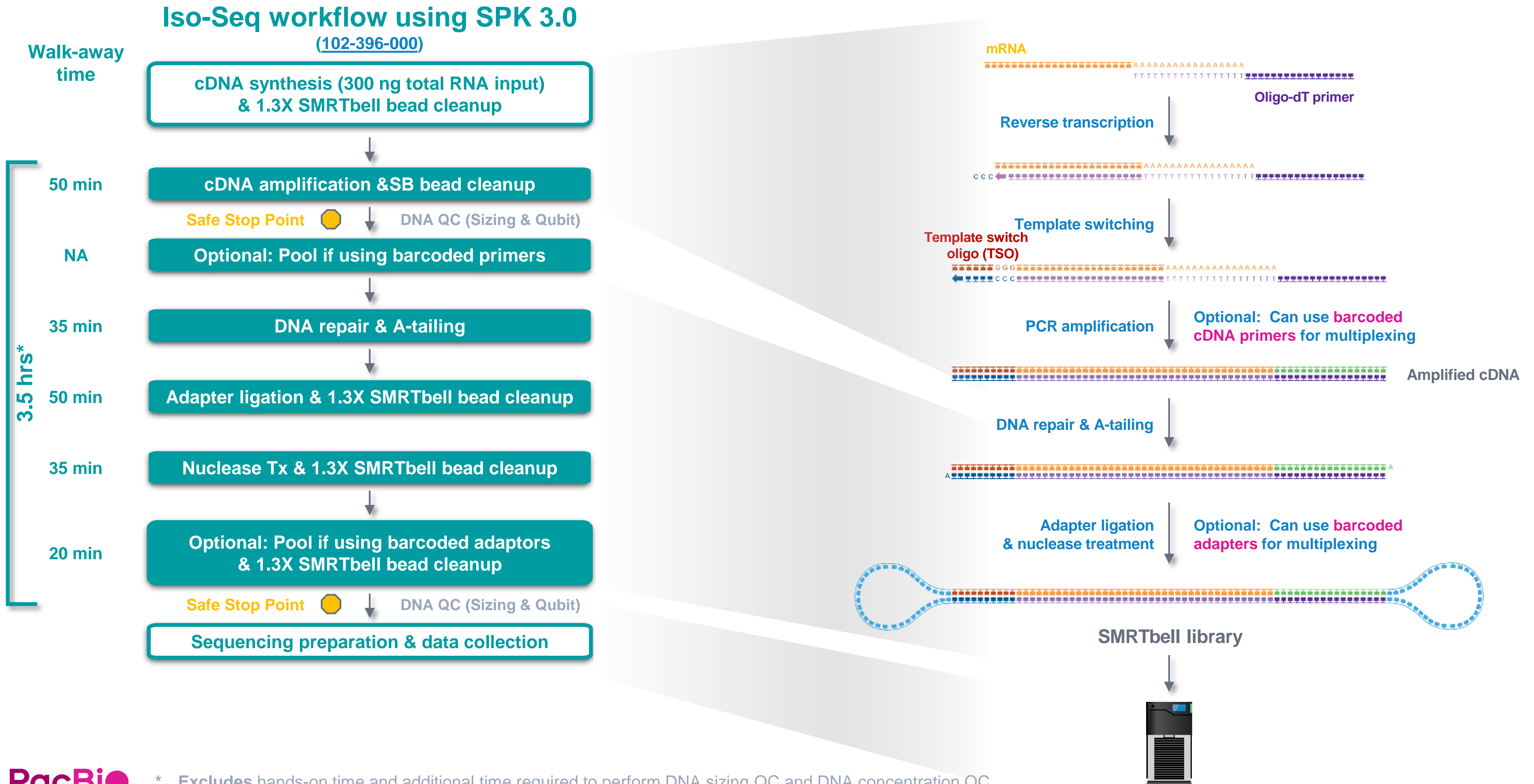
PacBio Documentation (102-396-000)

Iso-Seq SMRTbell library construction workflow overview



* **Excludes** hands-on time and additional time required to perform DNA sizing QC and DNA concentration QC

PacBio Iso-Seq express oligo kit

Accessory kit to support RNA sequencing workflow to characterize isoform diversity in full-length transcriptomes and for genome annotation

Iso-Seq express oligo kit (101-737-500)	
Tube #	**Description**
1	TUBE, Iso-Seq Express Template Switching Oligo
2	TUBE, Iso-Seq Express cDNA PCR Primer

Important:
Store the Iso-Seq express oligo kit at **-70°C to -80°C**

- PacBio Iso-Seq express oligo kit (101-737-500) accessory kit contains the Iso-Seq Express Template Switching Oligo and cDNA PCR Primer to be used in conjunction with the NEB kit below for performing 1st-strand cDNA synthesis and PCR amplification of cDNA products:
    - NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module (NEB PN: E6421S for 24 reactions or E6421L for 96 reactions)

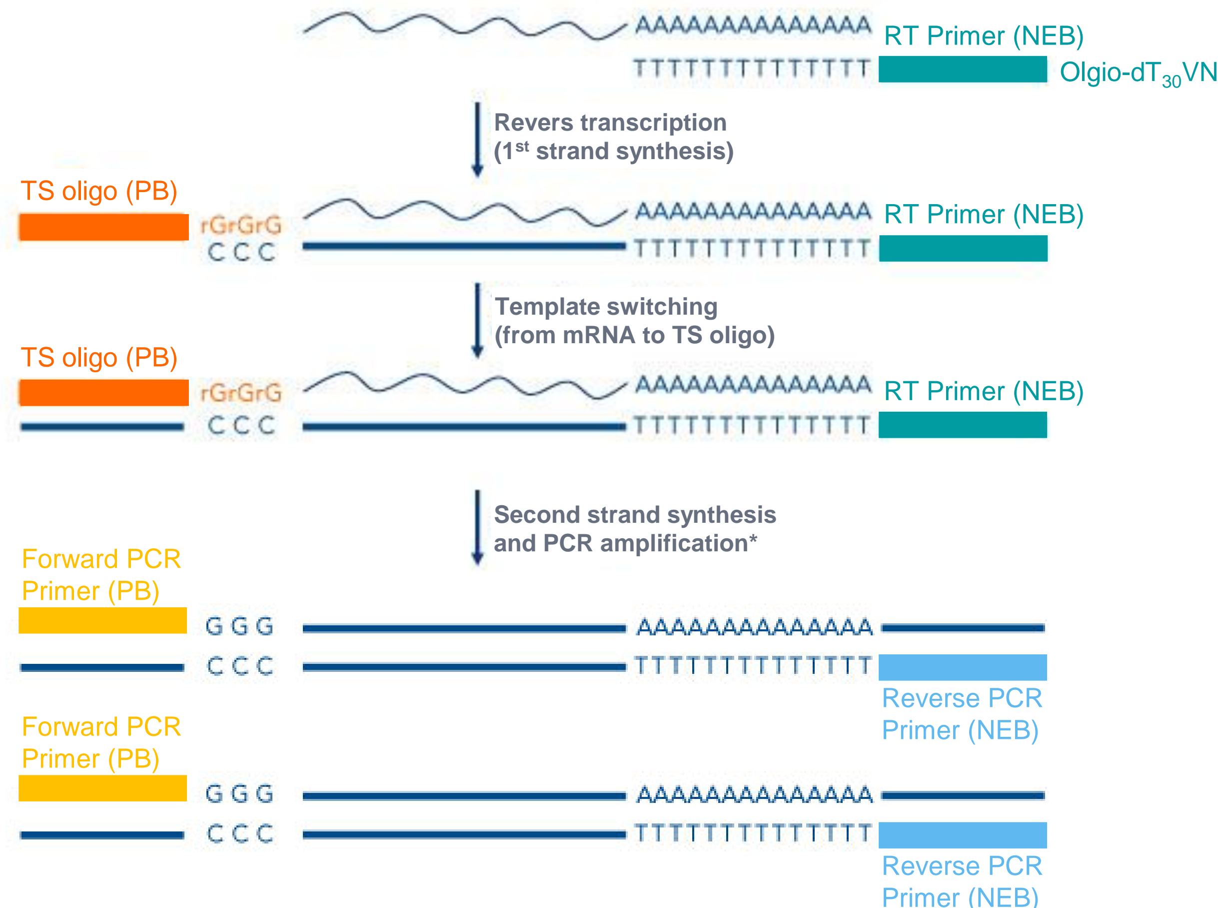
First-strand cDNA synthesis and PCR amplification of cDNA products

Use the NEBNext Single cell/low input cDNA synthesis & amplification module and PacBio Iso-Seq express oligo kit to perform first-strand cDNA synthesis and PCR amplification

PacBio Iso-Seq express oligo kit

Iso-Seq Express Template Switching Oligo

Iso-Seq Express cDNA PCR Primer



NEBNext Single cell/low input cDNA synthesis & amplification module

NEBNext Single Cell RT Primer

NEBNext Single Cell cDNA PCR Primer

* To generate multiplexed Iso-Seq libraries using barcoded cDNA primers (instead of barcoded adapters) **both** the NEBNext Single Cell cDNA PCR Primer **and** the Iso-Seq Express cDNA PCR Primer must be barcoded. See **Appendix 3** in the procedure for sequences that can be ordered from any oligo synthesis company.

General best practices recommendations for preparing Iso-Seq SMRTbell libraries

- Accurately pipette SMRTbell cleanup beads because small changes in volume can significantly alter the size distribution of your sample
- Equilibrate the SMRTbell cleanup beads at room temperature for 30 – 60 mins prior to use
  - Do not use SMRTbell cleanup beads straight out of the refrigerator without allowing sufficient time for the beads to warm up to room temperature
- The Iso-Seq workflow takes ~8 hrs to complete processing of up to 12 RNA samples
  - Plan your experiments so that the entire workflow can be completed within an 8-hour day.
  - If a stop is necessary, refer to workflow for safe stopping points
- When performing SMRTbell cleanup bead purification steps, note that 80% ethanol is hygroscopic and should be prepared fresh to achieve optimal results.
- Using a multi-channel pipettor greatly enhances the ease of processing more than 1 sample
- Measure DNA concentration using a Qubit fluorometer and Qubit dsDNA High Sensitivity (HS) Assay Kit reagents as recommended by the manufacturer

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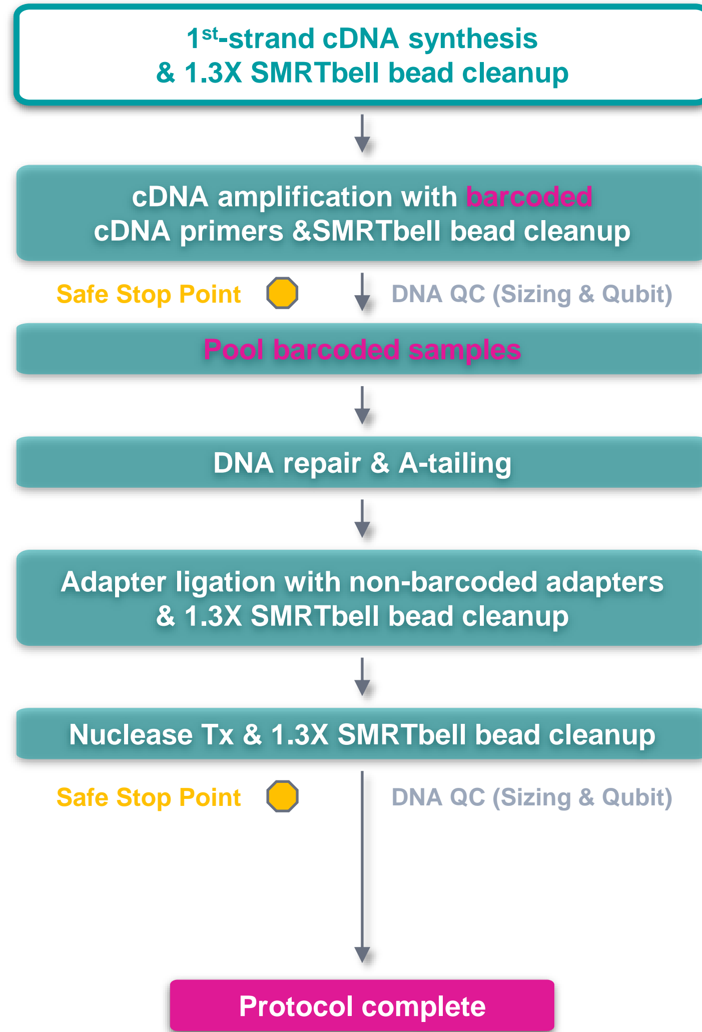
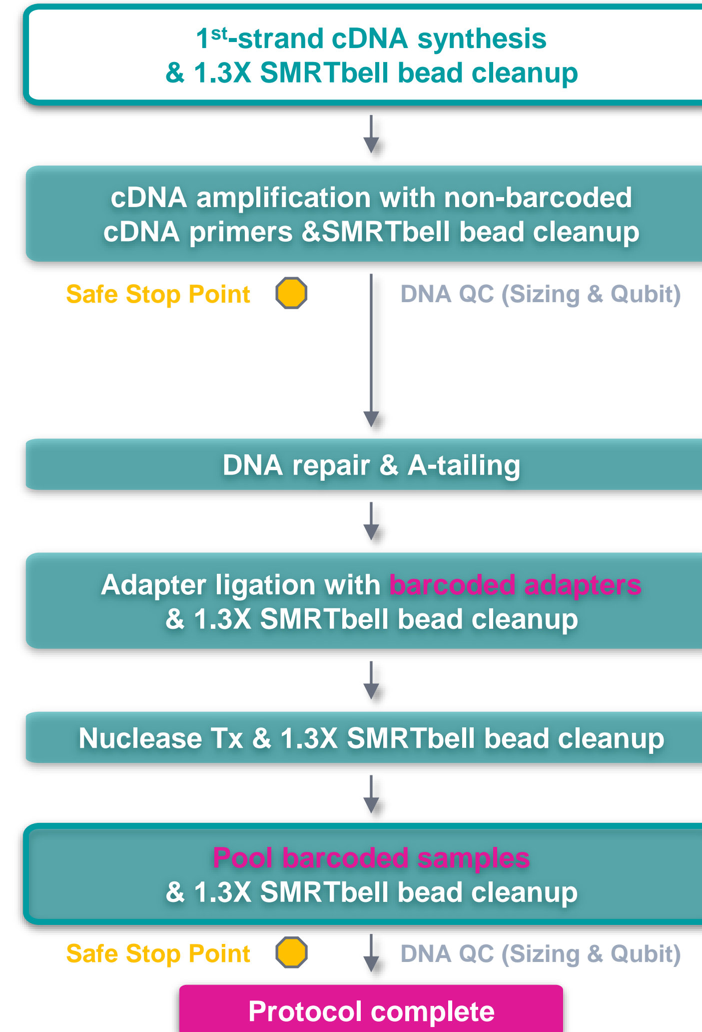
Barcoding options for preparing multiplexed Iso-Seq SMRTbell libraries

Two methods are available for barcoding Iso-Seq samples





Barcoding options for preparing multiplexed Iso-Seq SMRTbell libraries (cont.)

Barcoding Iso-Seq samples using barcoded cDNA primers

- Using barcoded cDNA primers, samples may be barcoded and pooled together prior to construction into a SMRTbell library as a "single" sample
- To multiplex, use barcoded forward and reverse primers (i.e., barcoded NEBNext Single Cell cDNA PCR Primer and barcoded Iso-Seq express cDNA PCR Primer) to amplify cDNA samples
- Once the amplified cDNA samples are barcoded, they are purified using SMRTbell cleanup beads, pooled together and then constructed into a SMRTbell library as a "single" sample (see the end of Step 3 ("cDNA amplification") in the procedure
- There are 24 pairs of barcoded primer sequences published by PacBio for use with Iso-Seq applications (see Appendix 3 of the protocol)
    - The 24 pairs of barcoded cDNA primers support multiplexing of up to 12 Iso-Seq samples per SMRT Cell 8M
- Barcoded forward and reverse primers may be ordered from any oligo synthesis company and standard desalting purification is acceptable
- The oligos must be diluted to 12 µM concentration for use in the "cDNA Amplification" section of the procedure. (Use 10 mM Tris, 0.1 mM EDTA for diluting oligos)

Appendix 3: Recommended barcoded NEBNext single cell cDNA PCR primers and Iso-Seq express cDNA PCR primers

Name	Sequence	Scale	Purification
bc1001-F	CACATATCAGAGTGCGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1001-R	CACATATCAGAGTGCGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1002-F	ACACACAGACTGTGAGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1002-R	ACACACAGACTGTGAGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1003-F	ACACATCTCGTGAGAGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1003-R	ACACATCTCGTGAGAGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1004-F	CACGCACACACGCGCGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1004-R	CACGCACACACGCGCGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1005-F	CACTCGACTCTCGCGTGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1005-R	CACTCGACTCTCGCGTAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1006-F	CATATATATCAGCTGTGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1006-R	CATATATATCAGCTGTAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1008-F	ACAGTCGAGCGCTGCGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1008-R	ACAGTCGAGCGCTGCGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1012-F	ACACTAGATCGCGTGTGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1012-R	ACACTAGATCGCGTGTAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1018-F	TCACGTGCTCACTGTGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1018-R	TCACGTGCTCACTGTGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1019-F	ACACACTCTATCAGATGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1019-R	ACACACTCTATCAGATAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1020-F	CACGACACGACGATGTGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1020-R	CACGACACGACGATGTAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1023-F	CAGAGAGATATCTCTGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1023-R	CAGAGAGATATCTCTGAAGCAGTGGTATCAACGCAGAGT	25nm	STD

Oligo order sheet for 24 barcoded Iso-Seq primers can be downloaded from PacBio's Multiplexing website:

- Oligo-Ordering-Sheet-for-IsoSeq.xlsx [Link]

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Barcoding options for preparing multiplexed Iso-Seq SMRTbell libraries (cont.)

Barcoding Iso-Seq samples using barcoded adapters

- For Sequel II and IIe Systems, SMRTbell barcoded adapter plate 3.0 (102-009-200) is available for multiplexing Iso-Seq samples

- Use barcoded adapters from SMRTbell barcoded adapter plate 3.0 for barcoding Iso-Seq samples at Step 5 (“Adapter ligation”) in the procedure
  - Pooling of barcoded libraries is described in Appendix 2 of the protocol
- SMRTbell barcoded adapter plate 3.0 contains 96 barcoded adapters to support multiplexed SMRTbell library construction for up to 96 samples using SMRTbell prep kit 3.0
  - Each barcoded adapter contains a 5 bp padding sequence for more uniform ligation performance across different barcode sequences
  - Each well on the plate contains a barcoded adapter with a unique 10-base pair PacBio barcode sequence
  - Each barcoded adapter is present in only one well and supports a single reaction
- SMRT Link comes pre-installed with the following barcode set FASTA file containing SMRTbell barcoded adapter plate 3.0 barcode sequences*: `SMRTbell Barcoded Adapter Plate 3.0 (bc2001-bc2096)`

Reagent kit quantities support a **single use** of each of the 96 barcoded adapters in the plate for SMRTbell library preparations.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC 2001	BC 2009	BC 2017	BC 2025	BC 2033	BC 2041	BC 2049	BC 2057	BC 2065	BC 2073	BC 2081	BC 2089
B	BC 2002	BC 2010	BC 2018	BC 2026	BC 2034	BC 2042	BC 2050	BC 2058	BC 2066	BC 2074	BC 2082	BC 2090
C	BC 2003	BC 2011	BC 2019	BC 2027	BC 2035	BC 2043	BC 2051	BC 2059	BC 2067	BC 2075	BC 2083	BC 2091
D	BC 2004	BC 2012	BC 2020	BC 2028	BC 2036	BC 2044	BC 2052	BC 2060	BC 2068	BC 2076	BC 2084	BC 2092
E	BC 2005	BC 2013	BC 2021	BC 2029	BC 2037	BC 2045	BC 2053	BC 2061	BC 2069	BC 2077	BC 2085	BC 2093
F	BC 2006	BC 2014	BC 2022	BC 2030	BC 2038	BC 2046	BC 2054	BC 2062	BC 2070	BC 2078	BC 2086	BC 2094
G	BC 2007	BC 2015	BC 2023	BC 2031	BC 2039	BC 2047	BC 2055	BC 2063	BC 2071	BC 2079	BC 2087	BC 2095
H	BC 2008	BC 2016	BC 2024	BC 2032	BC 2040	BC 2048	BC 2056	BC 2064	BC 2072	BC 2080	BC 2088	BC 2096

Figure illustration of mapping between a specific well location and a unique PacBio barcode sequence on a 96-well plate in the SMRTbell Barcoded Adapter Plate (102-009-200)

Plate Layout (Excel) [Link]

Barcode Sequences (FASTA) [Link]

Product insert: SMRTbell barcoded adapter plate 3.0 (96 barcodes, 96 samples) [Link]

* If your SMRT Link version does not have the required barcode set FASTA file for a specific analysis application, please visit PacBio’s Multiplexing website to download the appropriate FASTA file or contact PacBio Technical Support.

Purification of amplified cDNA products with SMRTbell cleanup beads

The concentration of SMRTbell cleanup beads will influence the size profile of the amplified cDNA

- Use SMRTbell cleanup beads for purification of amplified cDNA products according to the table below:

Workflow	Goal of experiment	SMRTbell cleanup bead volume
Standard	• Typical transcripts, centered around 2 kb, for high-quality RNA	86 µL
Short transcripts	• Short transcripts <2 kb or degraded samples with RIN < 7	95 µL
Long transcripts	• Enrich for long transcripts >3 kb*	82 µL

- After purification, perform a sizing QC by running 1 µL of the purified cDNA products on a Bioanalyzer using a High Sensitivity DNA kit.
- Examining the amplified cDNA on a Bioanalyzer prior to PacBio library construction is an excellent quality control step to ensure that the amplified cDNA material has the expected size distribution.

* **NOTE:** If you want to **enrich for longer transcripts (>3 kb),** additional cDNA amplification after this purification step is **required** (see **Appendix 1**)

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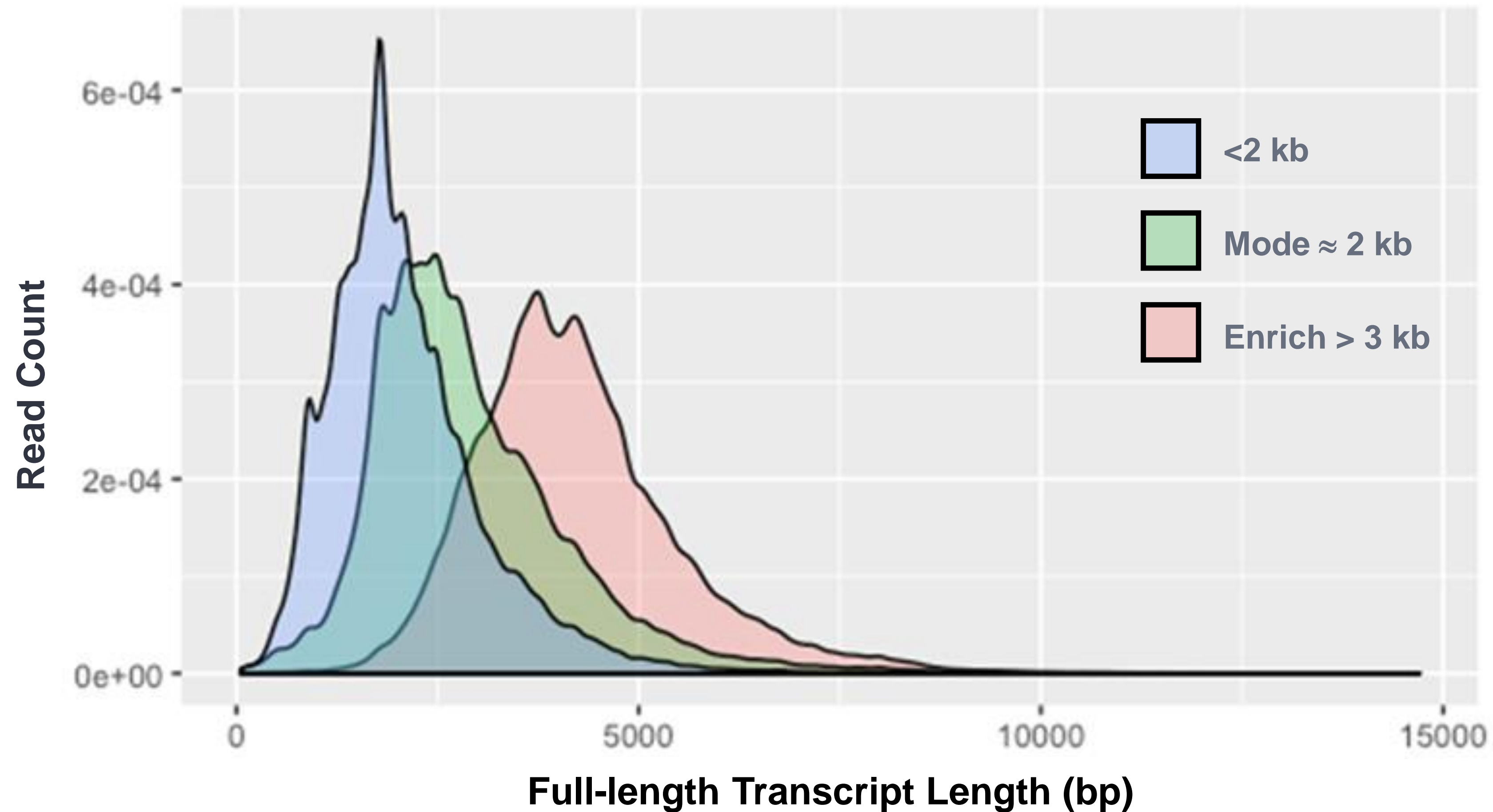
Purification of amplified cDNA products with SMRTbell cleanup beads (cont.)

SMRTbell cleanup bead purification enables modulation of the full-length cDNA transcript size distribution



Quantitation of Amplified cDNA Products

You must have the required mass of purified cDNA to proceed with SMRTbell library construction

Iso-Seq library type	Min. cDNA amount for non-multiplexed library	Min. cDNA amount for multiplexed library	Recommendation for samples With low yield
Non-multiplexed library (single sample)	160-500 ng	N/A	If cDNA mass is <160 ng (<3.5 ng/μL) → Go to **Appendix 1**
Multiplexed library using barcoded cDNA primers	N/A	160-500* ng	If total cDNA mass is <160 ng (<3.5 ng/μL) → Go to **Appendix 1**
Multiplexed library using barcoded adapters	N/A	160-500** ng	If cDNA mass is <160 ng (<3.5 ng/μL) → Go to **Appendix 1**

* For multiplexed libraries using barcoded cDNA primers, this refers to the required **total combined mass** of the **pooled** cDNA samples (not individual samples within the pool). See Step 3.3 ("Pooling barcoded cDNA) section for additional information.

** For multiplexed libraries using barcoded adapters, this refers to the required **mass per individual** cDNA sample.

- **Appendix 1:** Recommendations for additional cDNA amplification by PCR for samples with a lower yield or to enrich for longer transcripts
  - The Sequel II and Sequel IIe systems require >160 ng of amplified cDNA material to proceed with SMRTbell library construction
  - If there is not enough DNA to proceed with library construction, refer to Appendix 1 of the procedure which describes a workflow for enriching cDNA by PCR.
- **Note:** Over-amplification can result in sub-optimal data
  - For high-yield samples with concentrations >40 ng/μL, optimal libraries may be obtained by repeating cDNA generation with less RNA input or by decreasing the number of PCR cycles.

Sample pooling guidance for multiplexed Iso-Seq samples

Pooling recommendations for multiplexed Iso-Seq libraries using barcoded cDNA primers

- Using the concentration reading from the Qubit fluorometer, **pool an equal mass** of each barcoded cDNA sample
- Use the maximum total combined mass possible without exceeding 500 ng and not less than 160 ng in 46 µL
- Store any remaining purified amplified, barcoded cDNA at 4°C for future use
- Assuming transcript size distribution profiles are similar, equal mass pooling should provide a balanced representation.
  - For more sensitive applications, or when the transcript profiles are significantly different, then performing equal molar pooling using the steps below may be more appropriate

OPTIONAL: Equal molar pooling procedure

1. Use the Qubit DNA concentration and average library size* from the Bioanalyzer trace to determine the molarity of each sample. Use the following equation to determine Molarity:

$$Concentration\ in\ nM = \frac{(DNA\ Concentration\ in\ ng\ \mu L^{-1})\ x\ 10^{6}}{(660\ g\ mol^{-1}\ x\ Average\ Library\ Size\ in\ bp)}$$

   * To determine the average library size using a Bioanalyzer System, select the region of interest by defining the start of the smear at 200 bp and the end point at 9500 bp (when using a High Sensitivity DNA assay kit)

2. Pool equal molar quantities of the barcoded cDNA
   - Use the maximum total combined mass possible without exceeding 500 ng in 47.4 µL
   - The total combined mass must >160 ng for Sequel II/IIe to proceed to DNA repair & A-tailing
   - If the volume required to achieve the minimum mass of the pooled cDNA exceeds 47.4 µL, concentrate the pooled cDNA by performing a 1.3X volume of SMRTbell cleanup beads and elute it in 48 µL. To account for potential losses during concentration at this step, start with ≥200 ng of cDNA material.

3. The pooled cDNA can now be constructed into a SMRTbell library as a single sample. Proceed to the DNA repair & A-tailing step.

Sample pooling guidance for multiplexed Iso-Seq samples (cont.)

Pooling recommendations for multiplexed Iso-Seq libraries using barcoded adapters

- Using the final SMRTbell library concentration taken after nuclease treatment, **pool an equal mass** of each adapter-barcoded sample
- Store any remaining barcoded SMRTbell library at 4°C for future use
- Assuming transcript size distribution profiles are similar, equal mass pooling should provide a balanced representation.
  - For more sensitive applications, or when the transcript profiles are significantly different, then performing equal molar pooling using the steps below may be more appropriate

OPTIONAL: Equal molar pooling procedure

1. Use the Qubit DNA concentration and average library size* from the Bioanalyzer trace to determine the molarity of each adapter-barcoded sample. Use the following equation to determine Molarity:

$$Concentration\ in\ nM = \frac{(DNA\ Concentration\ in\ ng\ \mu L^{-1})\ \ x\ 10^6}{(660\ g\ mol^{-1}\ x\ Average\ Library\ Size\ in\ bp)}$$

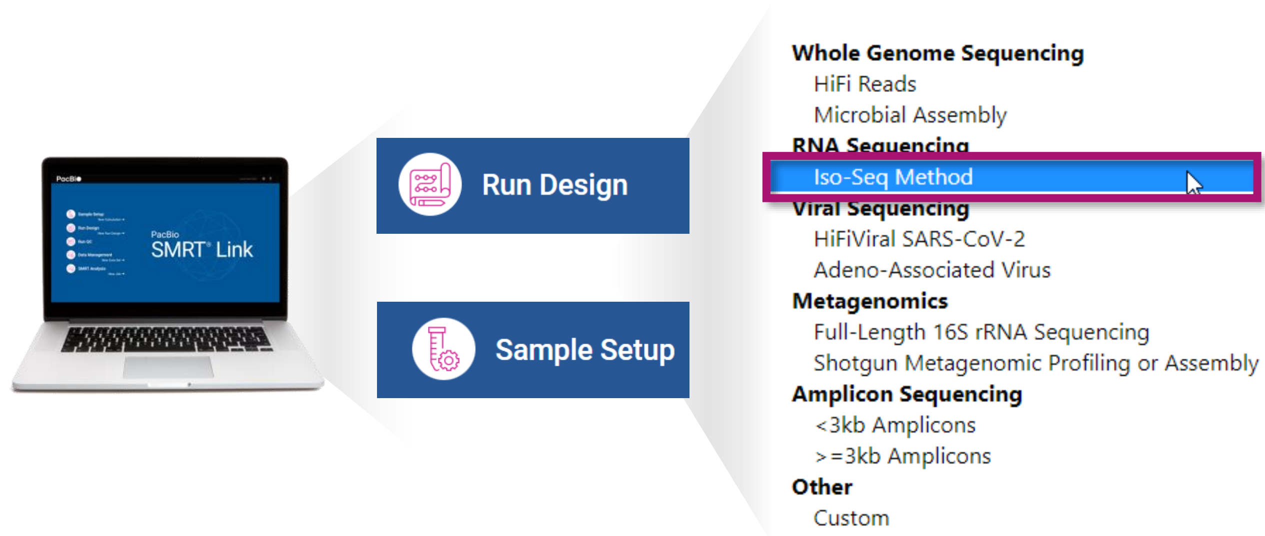
   * To determine the average library size using a Bioanalyzer System, select the region of interest by defining the start of the smear at 200 bp and the end point at 9500 bp (when using a High Sensitivity DNA assay kit)

2. Pool equal molar quantities of the adapter-barcoded sample
3. Purify and concentrate the pooled library sample using 1.3X SMRTbell cleanup beads.
4. Measure the DNA concentration and size distribution of the purified pooled library sample. Proceed to sequencing using SMRT Link Sample Setup.

Iso-Seq library sequencing preparation
workflow overview

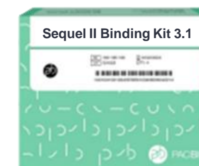
Sample Setup & Run Design recommendations for Iso-Seq libraries

In SMRT Link Sample Setup & Run Design, select 'RNA Sequencing' / 'Iso-Seq Method' for application type



Iso-Seq library type	Recommended binding kit
Typical transcripts (centered around ~2 kb)	Sequel II binding kit 3.1
Short transcripts <2 kb	Sequel II binding kit 3.1
Enriched for long transcripts >3 kb	Sequel II binding kit 3.2

- Refer to Quick reference card – Loading and pre-extension time recommendations for the Sequel II and IIe systems (101-769-100) for updates to annealing / binding / cleanup (ABC) workflow for specific applications



Sequel II binding kit 3.1 & cleanup beads (102-333-400) is recommended for preparing standard Iso-Seq samples for sequencing.

Sequel II binding kit 3.1 & cleanup beads (102-333-400) includes:

- Sequencing primer 3.1
- Sequel II polymerase 2.1
- DNA internal control 3.1 (defined 2 kb template bound to Polymerase 2.1)
- SMRTbell cleanup beads for complex cleanup

Sequel II binding kit 3.2 & cleanup beads (102-333-300) is recommended for preparing Iso-Seq samples with a focus on long transcripts (>3 kb) for sequencing.

Sequel II binding kit 3.2 & cleanup beads (102-333-300) includes:

- Sequencing primer 3.2
- Sequel II polymerase 2.2
- DNA internal control 3.2 (defined 11 kb template bound to Polymerase 2.2)
- SMRTbell cleanup beads for complex cleanup

Iso-Seq library Sample Setup guidance

Use SMRT Link Sample Setup High-Throughput (HT) mode and follow instructions to perform ABC (anneal primer / bind polymerase / clean up complex) using recommended settings for Iso-Seq samples

PacBio Sample Setup smark (Lab Tech) ?

Sample Setup

New Calculation | Edit/Print | Import | Export | Delete

Version: Classic | High-Throughput

Sample Setup HT for Sequel II and Sequel IIe

Name	Date Created	Number of Samples	Comment	Created By	Locked
Example Iso-Seq Sample Setup	2022-04-15, 09:23:23 PM	2	This batch includes Pooled_Iso-Seq_Sample_01 Pooled_Iso-Seq_Sample_02	smark	false

Note: Default binding kit for Iso-Seq samples is Sequel II Binding kit 3.1. For Iso-Seq samples with a focus on **long transcripts (>3 kb)**, we recommend using Sequel II binding kit 3.2

- Sample Setup High-Throughput mode provides a simplified, streamlined workflow to efficiently process either one sample or multiple samples with similar library properties (such as mean insert size and DNA concentration) in parallel
- You can also export the calculated values to a CSV file for laboratory automation

	Sample Group	
Actions	Copy Remove Lock Automate	
Name	Example Iso-Seq Sample Setup	
Comment	This batch includes Pooled_Iso-Seq_Sample_01 Pooled_Iso-Seq_Sample_02	
Application	Iso-Seq Method	
Binding Kit	Sequel® II Binding Kit 3.1	
Number of Samples	2	samples
SMRT Cells per Sample	1	cells
Available Volume per Sample	15	uL
Insert Size	2800	bp
Sample Concentration	10	ng/uL
Cleanup Anticipated Yield	60	%
Recommended Concentration on Plate	40-80 pM	
Specify Concentration on Plate	50	pM
Minimum Pipetting Volume	1	uL
Warnings		

Example Sample Setup HT mode worksheet for a batch comprised of two Iso-Seq samples.

Iso-Seq library Run Design guidance

Follow SMRT Link Run Design instructions to set up a sequencing run using recommended settings for Iso-Seq samples

- Select Iso-Seq Method from the Application field drop-down menu in SMRT Link Run Design
- The following fields are auto-populated and high-lighted in green:
  - Template Prep Kit
  - Binding Kit
  - Sequencing Kit
  - DNA Control Complex
  - Movie Time Per SMRT Cell
  - Pre-Extension Time

Note: By default, all newly created run designs (regardless of application type) will specify to **automatically** perform CCS analysis and output **only** HiFi reads

PacBio Run Design

Run Information

System Type

SEQUEL II
SEQUEL IIe

Run Name

Example_Iso-Seq_Run_Design

Run Comments

Experiment Name

Experiment ID

Estimated Run Duration (hours): 29.4

Run Reagents / Consumables

1 SMRT Cell
1 sequencing reagent plate
1 mineral oil tube
3 boxes of tips
1 mixing plate
1 sample plate

Sample Information

SAMPLE 1: Pooled_Iso-Seq_Sample_01, A01, 24 hour movie, 2800 bp insert

Field	Value
Import from Sample Setup	Select Sample
Application (Required)	Iso-Seq Method
Well Sample Name (Required)	Pooled_Iso-Seq_Sample_01
Bio Sample Name (Required)	
Sample Comment	
Sample Well	A01
Template Prep Kit (Required)	SMRTbell® Prep Kit 3.0
Binding Kit (Required)	Sequel® II Binding Kit 3.1
Sequencing Kit (Required)	Sequel® II Sequencing Plate 2.0 (4 rxn)
DNA Control Complex	Sequel® II DNA Internal Control Complex 3.1
Insert Size (bp) (Required)	2800
Recommended Concentration on Plate (pM)	40-80 pM
On-Plate Loading Concentration (pM) (Required)	50
Movie Time per SMRT Cell (hours)	24
Use Pre-Extension	YES / NO
Pre-Extension Time (hours)	2

CCS Analysis will be performed on-instrument to produce HiFi .bam files.

Example sample information entered into Run Design for sequencing an Iso-Seq sample.

Iso-Seq library Run Design guidance (cont.)

OPTIONAL: Run Design setup procedure for automated demultiplexing of pooled Iso-Seq library samples barcoded with SMRTbell barcoded adapter plate 3.0*

1. Sample is Barcoded: YES
2. Barcode Set: Select 'SMRTbell Barcoded Adapter Plate 3.0 (bc2001-bc2096)'
3. Same Barcodes on Both Ends of Sequence: YES
4. Assign a Biological Sample Name to each barcoded sample using one of two ways: From a (CSV) File or Interactively
5. Specify if barcode demultiplexing is to be performed on-instrument (Sequel IIe system only) or in SMRT Link. (Optionally specify Do Not Generate.)

* **IMPORTANT:** For multiplexed Iso-Seq library samples barcoded with **barcoded cDNA primers** (see Appendix 3 of the Iso-Seq protocol 102-396-000), demultiplex your data set using the SMRT Link Iso-Seq application (specify '`Iso-Seq 12 Barcoded cDNA Primers`' for the Primer Set). **Do not not run the Demultiplexing Barcodes** utility on-instrument or in SMRT Link first.

PacBio Run Design smark (Lab Tech) ?

Run Information

System Type

SEQUEL II

SEQUEL IIe

Run Name

Example_Iso-Seq_Run_Design

Run Comments

Experiment Name

Experiment ID

Estimated Run Duration (hours): 29.4

Run Reagents / Consumables

1 SMRT Cell
1 sequencing reagent plate
1 mineral oil tube
3 boxes of tips
1 mixing plate
1 sample plate

Sample Information

Advanced Options

Barcoded Sample Options

1 Sample Is Barcoded YES NO

2 Barcode Set Required SMRTbell Barcoded Adapter Plate 3.0

3 Same Barcodes on Both Ends of Sequence YES NO

4 Assign Bio Sample Names to Barcodes Required Interactively From a File

5 Demultiplex Barcodes ON INSTRUMENT IN SMRT LINK DO NOT GENERATE

Copy Delete

Add Sample

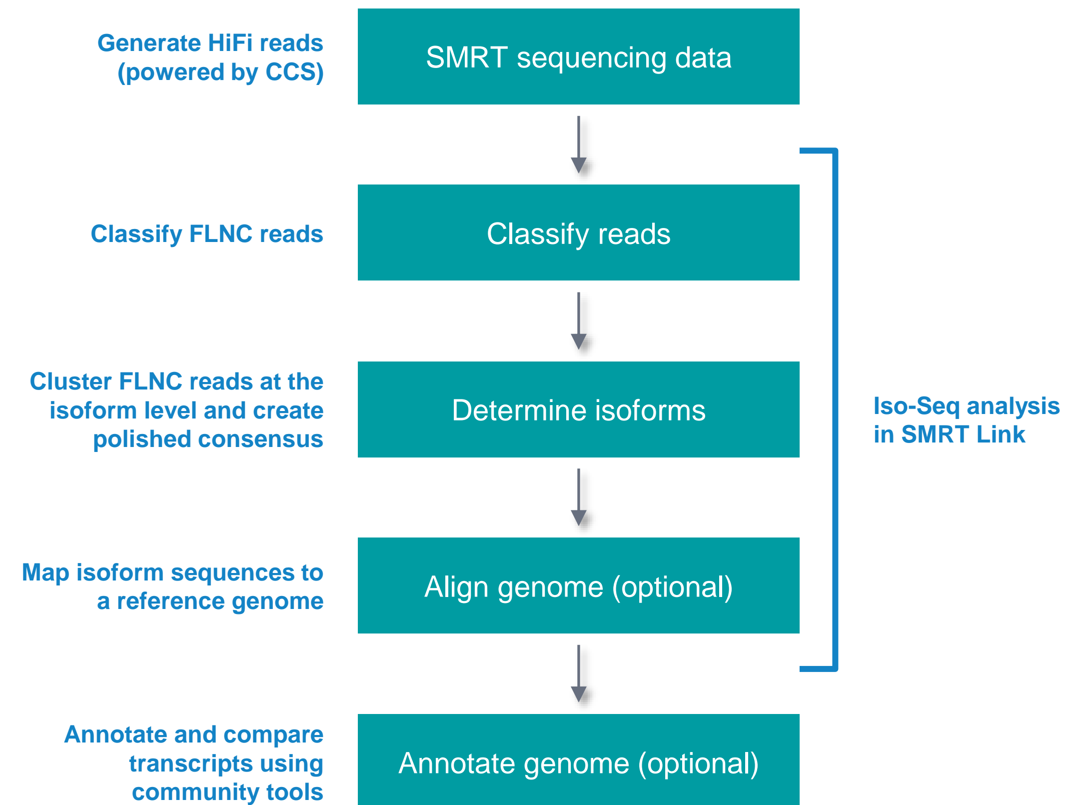
Example barcoding information entered into Run Design for sequencing a pooled Iso-Seq sample barcoded using barcoded adapters from SMRTbell barcoded adapter plate 3.0 (102-009-200).

Iso-Seq data analysis workflow overview

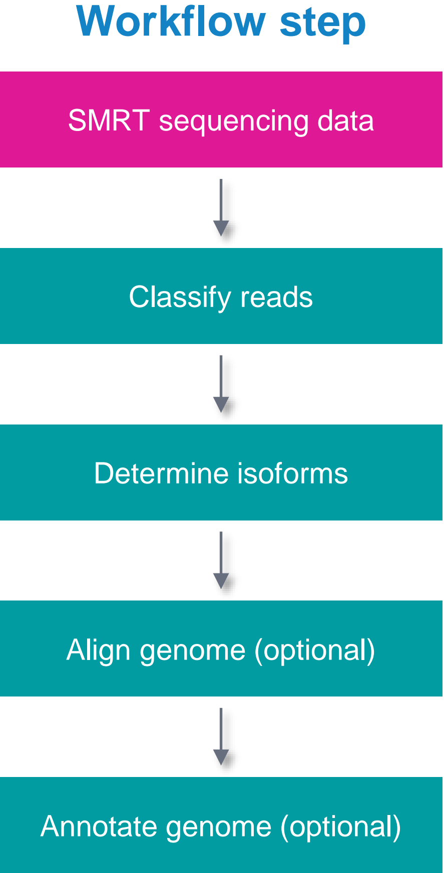
SMRT Link Iso-Seq analysis application

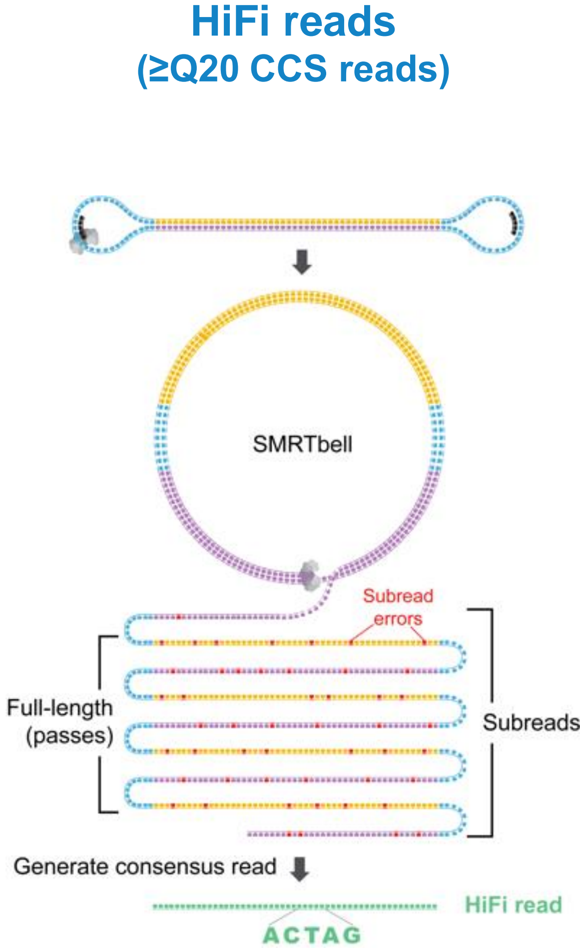
SMRT Link Iso-Seq application enables analysis and functional characterization of transcript isoforms for sequencing data generated on PacBio instruments

- Generate highly accurate long reads (HiFi reads), with single- molecule resolution using circular consensus sequencing (CCS) mode
- Use the **Iso-Seq analysis application** in SMRT Link to output high-quality, full-length transcript FASTA sequences, with no assembly required, to characterize transcripts and splice variants
- Run Iso-Seq analysis with or without a reference genome, and annotate the genome using community tools such as SQANTI, TAMA, and LoReAn

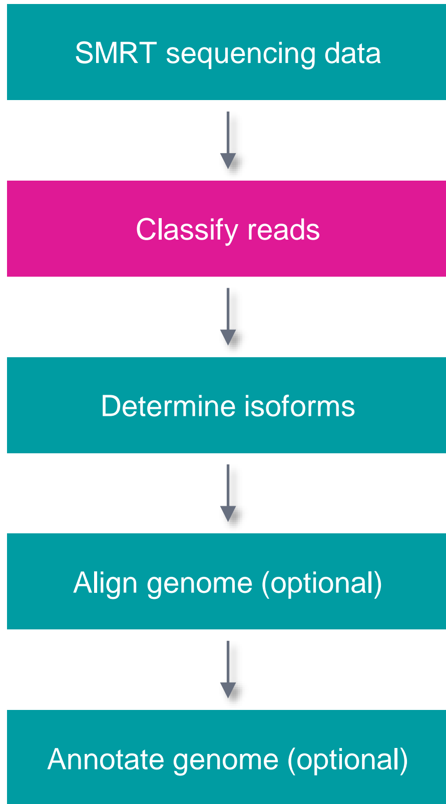


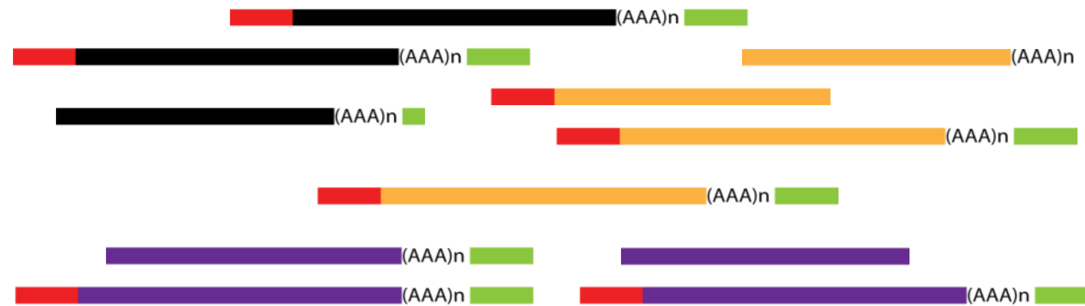
Iso-Seq analysis workflow details





Iso-Seq analysis workflow details (cont.)



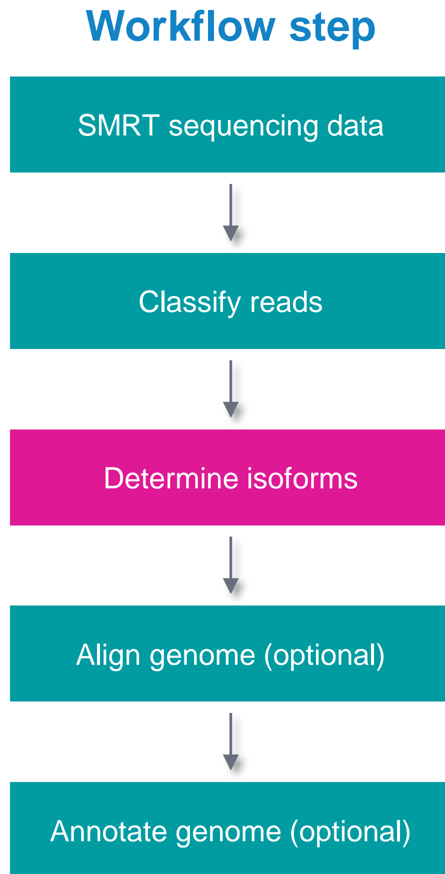


With multiplexed Iso-Seq libraries barcoded using barcoded cDNA primers, the cDNA primer + barcode sequences together are treated as *custom primers*

Full-length transcript reads:

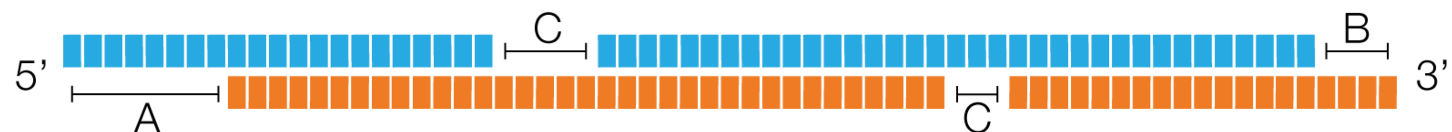
- ❑ 5' cDNA primer sequence* detected
- ❑ 3' cDNA primer sequence* detected
- ❑ polyA tail sequence (>20 bp) detected

Iso-Seq analysis workflow details (cont.)

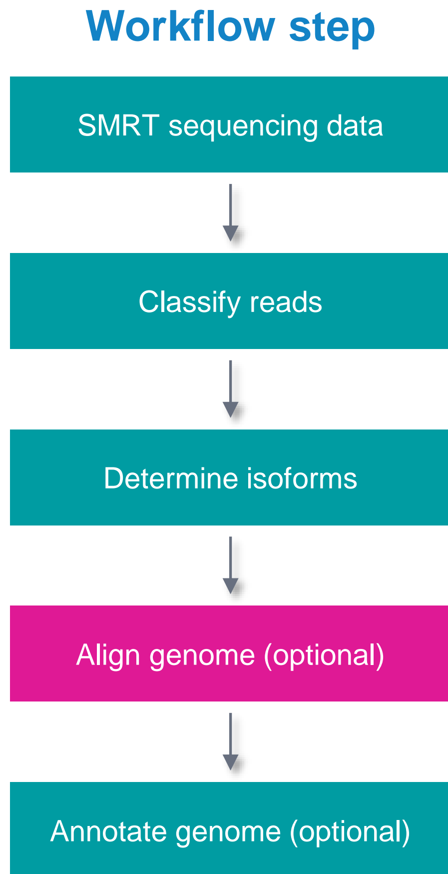


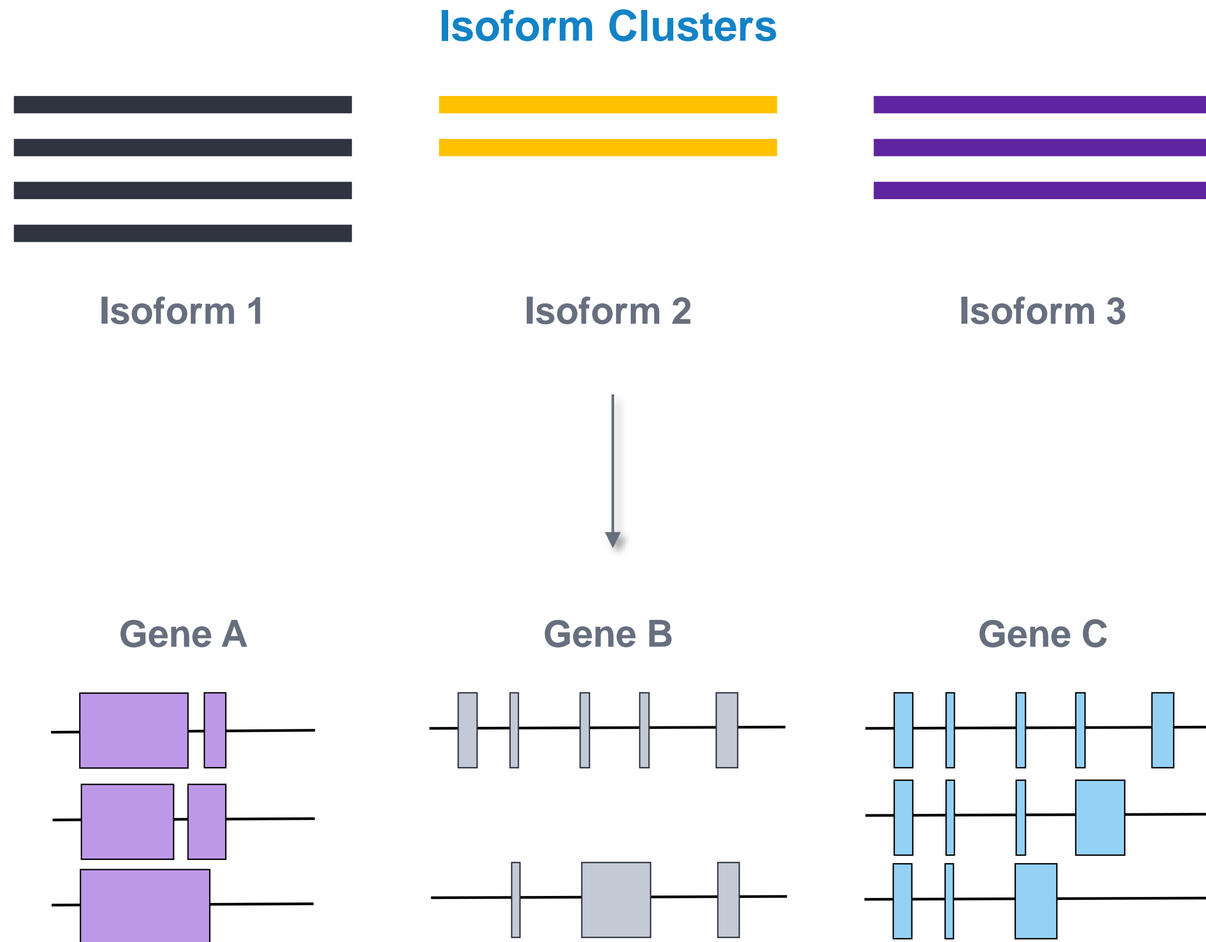
Two full-length transcript reads are considered the same isoform if they are:

A. <100 bp difference in 5’ start
B. <30 bp difference in 3’ end
C. <10 bp in internal gap (exon), no limit on the number of gaps



Iso-Seq analysis workflow details (cont.)





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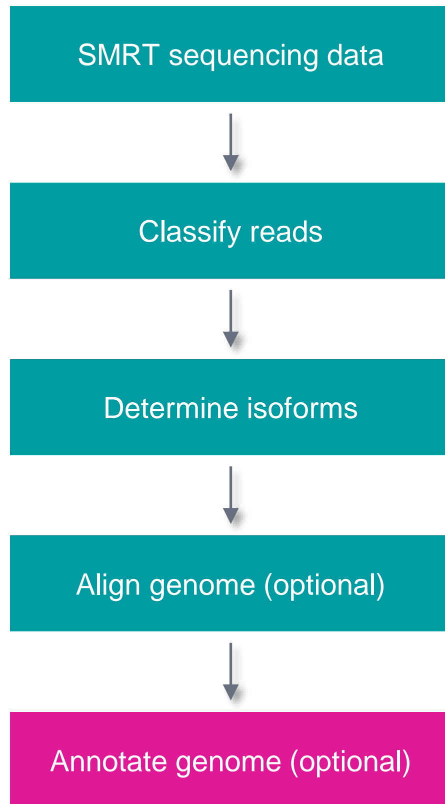
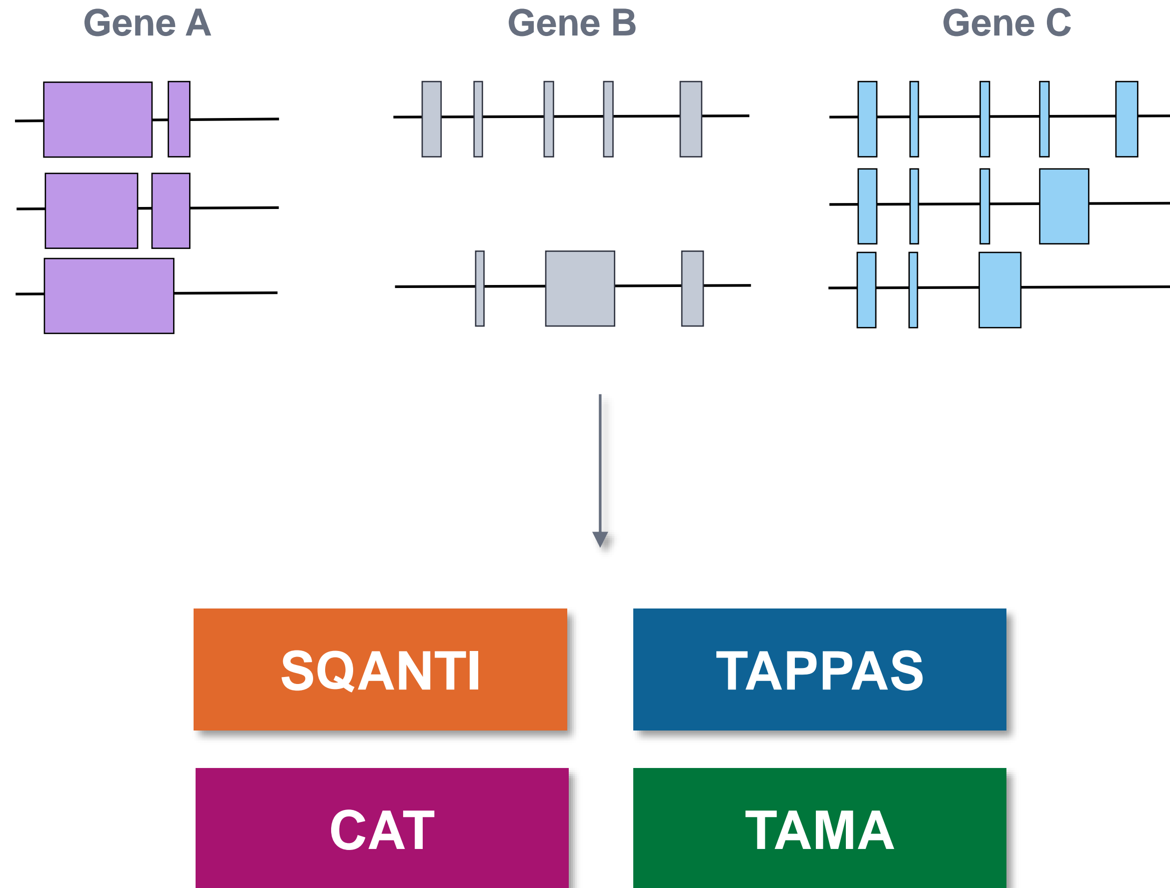
Iso-Seq analysis workflow details (cont.)





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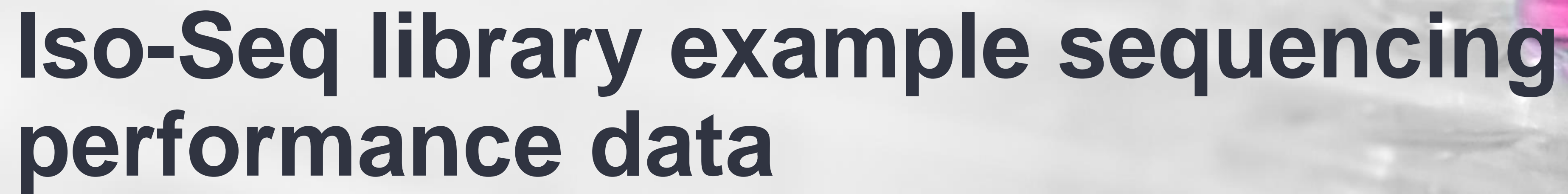


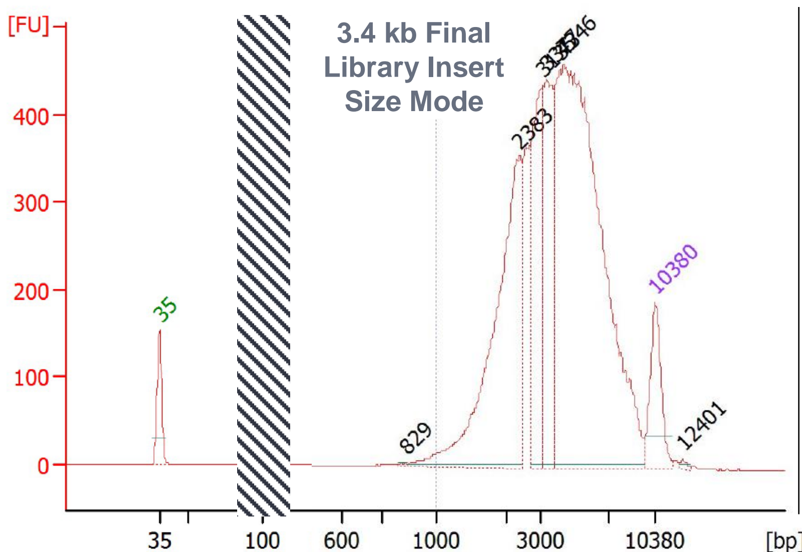
Iso-Seq library example sequencing performance data

PacBio

Example library QC and sequencing performance for UHRR Iso-Seq libraries prepared with SMRTbell prep kit 3.0

SMRTbell library QC and primary sequencing metrics for a non-multiplexed UHRR Iso-Seq sample

Final Library QC

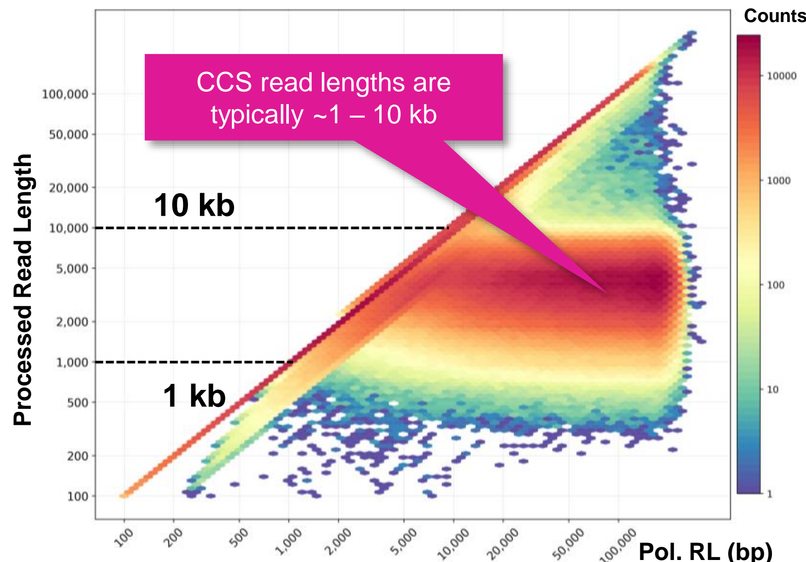


Amplified cDNA*	500 ng
Post-nuclease treatment & cleanup (%)**	336 ng (67%)

* Universal Human Reference RNA (UHRR) was converted into cDNA, amplified and purified using a standard cleanup with 0.86X SMRTbell cleanup beads to isolate transcripts centered around 2 kb.

** Post-nuclease & cleanup yields typically ranged from ~60% to ~80% when using amplified UHRR cDNA samples (500 ng) for SPK 3.0 library construction.

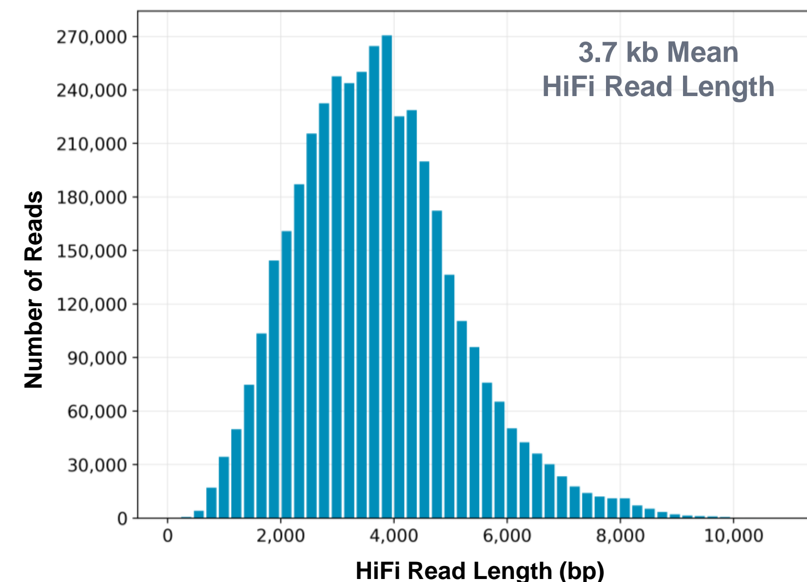
Raw Data Report



Raw Base Yield	402.6 Gb
Mean Polymerase Read Length	55.8 kb
P0	13.4%
P1	84.5%
P2	2.2%

Example sequencing metrics for a human UHRR Iso-Seq sample run with Binding Kit 3.1 (Polymerase 2.1) / 50 pM on-plate concentration / 24-h movie time / 2-h Pre-extension time.

CCS Analysis Report



HiFi Reads	4.0 M
HiFi Base Yield	15.1 Gb
Mean HiFi Read Length	3,702 bp
Median HiFi Read Quality	Q39
HiFi Read Mean # of Passes	17

For SPK 3.0 human UHRR Iso-Seq libraries, per-SMRT Cell HiFi read counts typically ranged from ~3.3 Million to ~4.4 Million and HiFi base yields typically ranged from ~11 Gb to ~16 Gb.

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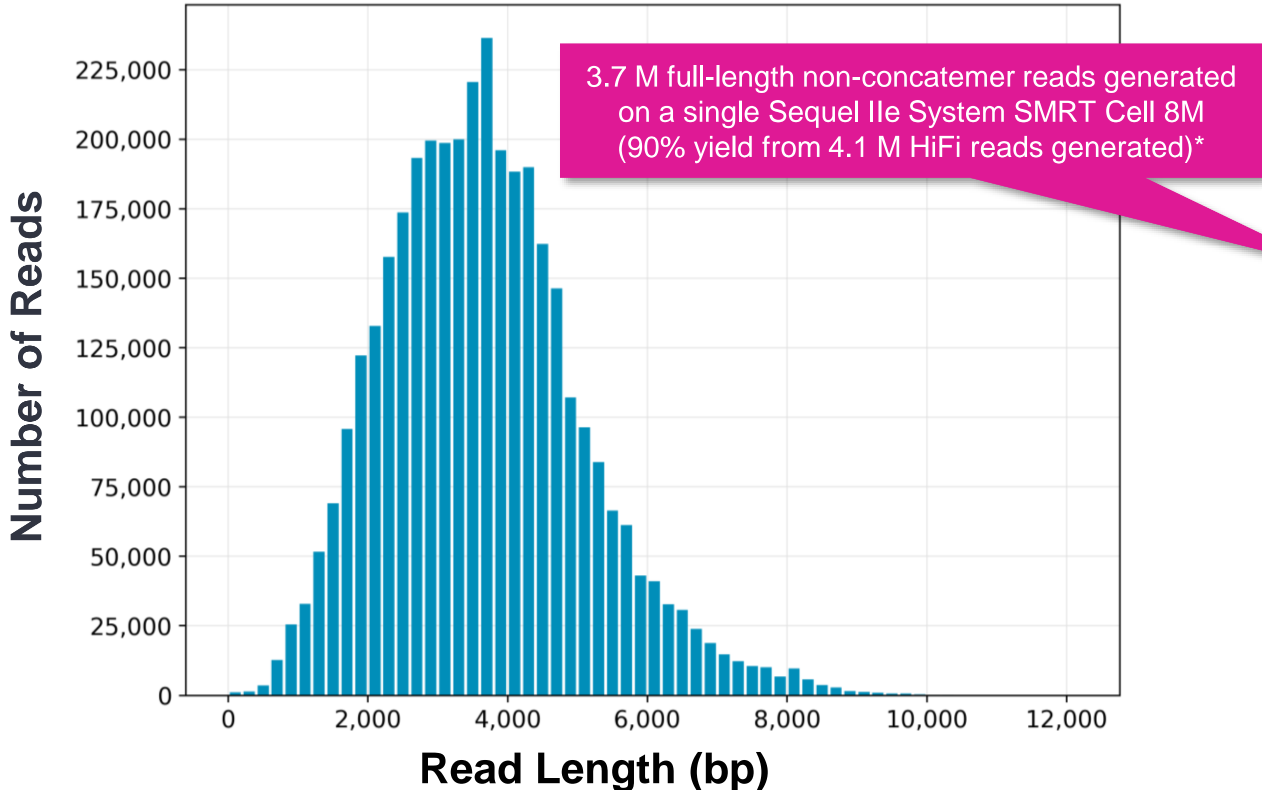
Example Iso-Seq analysis results for UHRR Iso-Seq libraries prepared with SMRTbell prep kit 3.0

CCS analysis read classification report for a non-multiplexed UHRR Iso-Seq sample

Length of Full-length Non-Concatemer Reads



3.7 M full-length non-concatemer reads generated on a single Sequel IIe System SMRT Cell 8M (90% yield from 4.1 M HiFi reads generated)*

CCS Analysis Read Classification Summary

Value	Analysis Metric
4,082,360	Reads
3,707,198	Reads with 5' and 3' Primers
3,704,840	Non-Concatamer Reads with 5' and 3' Primers
3,699,995	Non-Concatamer Reads with 5' and 3' Primers and Poly-A Tail
3,655	Mean Length of Full-Length Non-Concatamer Reads
1	Unique Primers
3,707,198	Mean Reads per Primer
3,707,198	Max. Reads per Primer
3,707,198	Min. Reads per Primer
375,162	Reads without Primers
91.61%	Percent Bases in Reads with Primers
90.81%	Percent Reads with Primers

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* Read lengths, reads/data per SMRT Cell and other sequencing performance results vary based on sample quality/type and insert size.

Technical documentation & applications support resources

PacBio

Technical resources for Iso-Seq library preparation, sequencing & data analysis

Sample preparation literature

- Application Brief: Long-read RNA sequencing – Best practices (102-193-643)
- Overview – Sequel systems application options and sequencing recommendations (101-851-300)
- Procedure & checklist – Preparing Iso-Seq libraries using SMRTbell prep kit 3.0 (102-396-000)
- Quick reference card – Loading and pre-extension recommendations for the Sequel II and IIe systems (101-769-100)
- Technical overview: Iso-Seq library preparation using SMRTbell prep kit 3.0 (102-393-400)

Data analysis resources

- SMRT Link v11.0 user guide (102-278-200)
- SMRT Tools v11.0 reference guide (102-278-500)
- Sequel II and IIe systems: Data files (102-144-100)

Example PacBio data sets

Iso-Seq application	Dataset	Data type	PacBio system
Whole Transcriptome	*Homo sapiens* – Brain with Alzheimer's Disease	HiFi Reads	Sequel II System
Whole Transcriptome	*Homo sapiens* – Universal Human Reference RNA (UHRR)	HiFi Reads	Sequel II System

PacBio

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

Posters

- PacBio ASHG Poster (2021): Towards isoform resolution single-cell transcriptomics for clinical applications using highly accurate long-read sequencing [Link]
- PacBio AGBT Poster (2020): A complete solution for full-length transcript sequencing using the PacBio Sequel II System [Link]
- PacBio PAG Poster (2020): A complete solution for high-quality genome annotation using the PacBio Iso-Seq method [Link]

Publications

- David, J.K. et al. (2022) Retained introns in long RNA-seq reads are not reliably detected in sample-matched short reads. BioRxiv preprint. doi: https://doi.org/10.1101/2022.03.11.484016
- Mahpour, A. and Mullen, A. (2022) Expanded transcriptomic analysis of human hepatic stellate cells links novel coding and noncoding products to human liver fibrosis. BioRxiv preprint. doi: https://doi.org/10.1101/2022.02.01.478715
- Wang, B. et al. (2020) Variant phasing and haplotypic expression from single-molecule long-read sequencing in maize. Commun Biol. 3:78. [Link]
- Ye, Jiabao et al. (2019) A global survey of full-length transcriptome of Ginkgo biloba reveals transcript variants involved in flavonoid biosynthesis. Industrial Crops and Products. 139:111547. [Link]
- Wan, Ying et al. (2019) Systematic identification of intergenic long-noncoding RNAs in mouse retinas using full-length isoform sequencing. BMC Genomics. 20(1):559. [Link]

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

Webinars

- PacBio ASHG Webinar (2021): Allele-specific, isoform-resolution single-cell RNA-seq analysis using long-read sequencing on concatenated single-cell molecules [Link]
- ASHG Webinar (2021): Scalable RNA isoform sequencing using intramolecular multiplexed cDNAs [Link]
- PacBio ASHG CoLab (2020): PacBio HiFi reads for comprehensive characterization of genomes and single-cell isoform expression [Link]
- PacBio ASHG Workshop (2020): Single-cell isoform analysis of the nervous system [Link]
- PacBio ASHG Video Poster (2020): Capture long-read isoform sequencing (Iso-Seq) for uncovering human isoform diversity in the brain and characterizing SARS-CoV2 viral RNAs [Link]
- PacBio ESHG Video Poster (2020): Full-length RNA sequencing of Alzheimer brain sample using long reads reveals complex alternative splicing patterns [Link]
- SMRT Leiden Presentation (2020): Iso-Seq Analysis and beyond! How non-standard analyses of Iso-Seq data can provide insights into your species [Link]

APPENDIX: RNA isolation kit options for
Iso-Seq SMRTbell library construction

RNA extraction kit options for Iso-Seq SMRTbell library preparation

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

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

Special handling recommendations during isolation of total RNA for Iso-Seq library preparation

Some important considerations to bear in mind when isolating total RNA for Iso-Seq analysis include the following:

- RNA sample has not been exposed to high temperatures (e.g.: >65°C for 1 hour can cause a detectable decrease in sequence quality) or pH extremes (<6 or >9).
- RNA sample has an OD260/OD280 ratio ~2.0.
- RNA sample has an OD260/OD230 ratio ≥2.0
- RNA sample has a RIN number ≥7.0 (ideally recommend ≥8.0)
- RNA sample has not been exposed to intercalating fluorescent dyes or ultraviolet radiation. SYBR dyes are not RNA damaging, but do avoid ethidium bromide.
- RNA sample does not contain denaturants (e.g., guanidinium salts or phenol) or detergents (e.g., SDS or Triton-X100).
- RNA sample does not contain carryover contamination from the original organism / tissue (e.g., heme, humic acid, polyphenols, etc.).
- Only use RNase-free water supplied in the reagent kit or other suppliers
- Make aliquots of the RNA sample and TSO to avoid excessive freeze-thaw cycles
- Thaw RNA samples and TSO on ice before use – DO NOT leave on the benchtop
- Avoid excessive pipetting and vortexing when working with RNA
- Note: RNA samples should only be shipped with dry ice.

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