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

Sequel II and Ile 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-specific best practices guide

Application-specific Procedure & checklist

Application-specific technical overview

Library construction, sequencing & analysis

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

Application Brief: Long-Read RNA sequencing – Best practices (102-193-643)
Summary overview of application-specific sample preparation and data analysis workflow recommendations

Procedure & Checklist – Preparing Iso-Seq libraries 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.

* 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.

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

APPLICATIONS

RNA SEQUENCING

Full-length transcript isoform sequencing (Iso-Seq method)
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

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

*HiFi Read*
PacBio HiFi reads achieve 99.9% accuracy

*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

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.
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

<table>
<thead>
<tr>
<th>Sequel system</th>
<th>Sequel II and Ile systems</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For genome annotation</strong></td>
<td><strong>For genome annotation</strong></td>
</tr>
<tr>
<td>One transcriptome → one SMRT Cell 1M</td>
<td>Up to 12-plex transcriptome* → one SMRT Cell 8M</td>
</tr>
</tbody>
</table>

**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.
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

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.
Iso-Seq SMRTbell library construction workflow overview

Iso-Seq workflow using SPK 3.0
(102-396-000)

- cDNA synthesis (300 ng total RNA input) & 1.3X SMRTbell bead cleanup

**Walk-away time**

- 50 min: cDNA amplification & SB bead cleanup
  - Safe Stop Point: DNA QC (Sizing & Qubit)
  - Optional: Pool if using barcoded primers

- 35 min: DNA repair & A-tailing

- 50 min: Adapter ligation & 1.3X SMRTbell bead cleanup

- 35 min: Nuclease Tx & 1.3X SMRTbell bead cleanup
  - Optional: Pool if using barcoded adaptors & 1.3X SMRTbell bead cleanup
  - Safe Stop Point: DNA QC (Sizing & Qubit)

- 20 min: Sequencing preparation & data collection

**5.5 hrs**

Optional: Can use barcoded cDNA primers for multiplexing

Optional: Can use barcoded adapters for multiplexing

- mRNA
  - Reverse transcription
  - Template switching
  - PCR amplification
  - Optional: Can use barcoded cDNA primers for multiplexing
  - Amplified cDNA
  - DNA repair & A-tailing
  - Adapter ligation & nuclease treatment
  - Optional: Can use barcoded adapters for multiplexing

- SMRTbell library

*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

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TUBE, Iso-Seq Express Template Switching Oligo</td>
</tr>
<tr>
<td>2</td>
<td>TUBE, Iso-Seq Express cDNA PCR Primer</td>
</tr>
</tbody>
</table>

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**
- **NEBNext Single cell/low input cDNA synthesis & amplification module**
- **Iso-Seq Express Template Switching Oligo**
- **Iso-Seq Express cDNA PCR Primer**
- **NEBNext Single Cell RT Primer**
- **NEBNext Single Cell cDNA PCR Primer**

Starting total RNA input (~300 ng) (Contains poly(a)-tailed mRNA)

- **Reverse transcription (1st strand synthesis)**
  - **RT Primer (NEB)**
  - **Oligo-dT VN**

- **Template switching (from mRNA to TS oligo)**
  - **rGrGrG C C C**

- **Second strand synthesis and PCR amplification**
  - **Forward PCR Primer (PB):**
    - **GGG C C C**
  - **Reverse PCR Primer (NEB):**
    - **GGG C C C**

* 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.
Barcoding options for preparing multiplexed Iso-Seq SMRTbell libraries

Two methods are available for barcoding Iso-Seq samples

**Multiplexed Iso-Seq workflow using barcoded cDNA primers**

1. 1st-strand cDNA synthesis & 1.3X SMRTbell bead cleanup
2. cDNA amplification with barcoded cDNA primers & SMRTbell bead cleanup
3. DNA repair & A-tailing
4. Adapter ligation with non-barcoded adapters & 1.3X SMRTbell bead cleanup
5. Nuclease Tx & 1.3X SMRTbell bead cleanup
6. Pool barcoded samples

**Protocol complete**

**Multiplexed Iso-Seq workflow using barcoded adapters**

1. 1st-strand cDNA synthesis & 1.3X SMRTbell bead cleanup
2. cDNA amplification with non-barcoded cDNA primers & SMRTbell bead cleanup
3. DNA repair & A-tailing
4. Adapter ligation with barcoded adapters & 1.3X SMRTbell bead cleanup
5. Nuclease Tx & 1.3X SMRTbell bead cleanup
6. Pool barcoded samples & 1.3X SMRTbell bead cleanup

**Protocol complete**
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)

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Scale</th>
<th>Purification</th>
</tr>
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<tbody>
<tr>
<td>bc1001-R</td>
<td>CACATATCAGTGCGGGAGATGCGGTTGAG</td>
<td>25µm</td>
<td>STD</td>
</tr>
<tr>
<td>bc1001-R</td>
<td>CACATATCAGTGCGGGAGATGCGGTTGAG</td>
<td>25µm</td>
<td>STD</td>
</tr>
<tr>
<td>bc1002-R</td>
<td>CACATATCAGTGCGGGAGATGCGGTTGAG</td>
<td>25µm</td>
<td>STD</td>
</tr>
<tr>
<td>bc1003-R</td>
<td>CACATATCAGTGCGGGAGATGCGGTTGAG</td>
<td>25µm</td>
<td>STD</td>
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<tr>
<td>bc1003-R</td>
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<td>25µm</td>
<td>STD</td>
</tr>
<tr>
<td>bc1004-F</td>
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<td>25µm</td>
<td>STD</td>
</tr>
<tr>
<td>bc1005-F</td>
<td>CACATATCAGTGCGGGAGATGCGGTTGAG</td>
<td>25µm</td>
<td>STD</td>
</tr>
<tr>
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<td>25µm</td>
<td>STD</td>
</tr>
<tr>
<td>bc1006-F</td>
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<td>25µm</td>
<td>STD</td>
</tr>
<tr>
<td>bc1006-R</td>
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<td>25µm</td>
<td>STD</td>
</tr>
<tr>
<td>bc1007-F</td>
<td>CACATATCAGTGCGGGAGATGCGGTTGAG</td>
<td>25µm</td>
<td>STD</td>
</tr>
<tr>
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<td>STD</td>
</tr>
<tr>
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<td>25µm</td>
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<td>bc1008-R</td>
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<td>bc1009-R</td>
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<td>STD</td>
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<tr>
<td>bc1013-F</td>
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<td>bc1013-R</td>
<td>CACATATCAGTGCGGGAGATGCGGTTGAG</td>
<td>25µm</td>
<td>STD</td>
</tr>
</tbody>
</table>

Oligo order sheet for 24 barcoded Iso-Seq primers can be downloaded from PacBio’s Multiplexing website:
- Oligo-Ordering-Sheet-for-IsoSeq.xlsx [Link]
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)

* 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:

<table>
<thead>
<tr>
<th>Workflow</th>
<th>Goal of experiment</th>
<th>SMRTbell cleanup bead volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>Typical transcripts, centered around 2 kb, for high-quality RNA</td>
<td>86 µL</td>
</tr>
<tr>
<td>Short transcripts</td>
<td>Short transcripts &lt;2 kb or degraded samples with RIN &lt; 7</td>
<td>95 µL</td>
</tr>
<tr>
<td>Long transcripts</td>
<td>Enrich for long transcripts &gt;3 kb*</td>
<td>82 µL</td>
</tr>
</tbody>
</table>

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

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

![Graph showing the distribution of full-length transcript lengths with three peaks at ≈2 kb, <2 kb, and Enrich > 3 kb.]
Quantitation of Amplified cDNA Products

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

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

* 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:

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

   * 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-barcode 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-barcode sample. Use the following equation to determine Molarity:

   \[ Concentration \text{ in nM} = \frac{(DNA \text{ Concentration in ng \muL}^{-1}) \times 10^6}{(660 \text{ g mol}^{-1} \times \text{Average Library Size in bp})} \]

   * 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-barcode 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

<table>
<thead>
<tr>
<th>Typical transcripts (centered around ~2 kb)</th>
<th>Recommended binding kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short transcripts &lt;2 kb</td>
<td>Sequel II binding kit 3.1</td>
</tr>
<tr>
<td>Enriched for long transcripts &gt;3 kb</td>
<td>Sequel II binding kit 3.2</td>
</tr>
</tbody>
</table>

- Refer to [Quick reference card – Loading and pre-extension time recommendations for the Sequel II and Ile systems (101-769-100)](https://example.com) 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.

- 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.

- 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

- 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

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

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 dropdown menu in SMRT Link Run Design
- The following fields are auto-populated and highlighted 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.
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 run the Demultiplexing Barcodes utility on-instrument or in SMRT Link first.

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 summary overview

1. SMRT sequencing data
2. Classify reads
3. Determine isoforms
4. Align genome (optional)
5. Map isoform sequences to a reference genome
6. Annotate genome (optional)
7. Cluster FLNC reads at the isoform level and create polished consensus
8. Annotate and compare transcripts using community tools

Iso-Seq analysis in SMRT Link
Iso-Seq analysis workflow details

**Workflow step**

- SMRT sequencing data
- Classify reads
- Determine isoforms
- Align genome (optional)
- Annotate genome (optional)

**HiFi reads (≥Q20 CCS reads)**

- SMRTbell
- Subread elongation
- Full-length (passes)
- Subreads
- Generate consensus read

**Iso-Seq library**

- 5’ primer
- cDNA insert
- polyA tail
- 3’ primer

**HiFi reads**

- (AAA)n
- (TTT)n
- (AAA)n
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
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.)

Workflow step
- SMRT sequencing data
- Classify reads
- Determine isoforms
- Align genome (optional)
- Annotate genome (optional)

Isoform Clusters
- Isoform 1
- Isoform 2
- Isoform 3

Gene A
- Isoform Clusters
- gene structure

Gene B
- Isoform Clusters
- gene structure

Gene C
- Isoform Clusters
- gene structure
Iso-Seq analysis workflow details (cont.)

Workflow step

- SMRT sequencing data
- Classify reads
- Determine isoforms
- Align genome (optional)
- Annotate genome (optional)

Gene A

Gene B

Gene C

SQANTI

TAPPAS

CAT

TAMA
Iso-Seq library example sequencing performance data
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

Raw Data Report

CCS Analysis Report

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

<table>
<thead>
<tr>
<th>Raw Base Yield</th>
<th>402.6 Gb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Polymerase Read Length</td>
<td>55.8 kb</td>
</tr>
<tr>
<td>P0</td>
<td>13.4%</td>
</tr>
<tr>
<td>P1</td>
<td>84.5%</td>
</tr>
<tr>
<td>P2</td>
<td>2.2%</td>
</tr>
</tbody>
</table>

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.

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.

* 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.

Counts
Number of Reads

HiFi Read Length (bp)

Processed Read Length
Pol. RL (bp)

CCS read lengths are typically ~1 – 10 kb

3.7 kb Mean HiFi Read Length

10 kb

1 kb

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

Final Library QC

Raw Data Report

CCS Analysis Report

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

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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-Concatemeter Reads

CCS Analysis Read Classification Summary

<table>
<thead>
<tr>
<th>Value</th>
<th>Analysis Metric</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,082,360</td>
<td>Reads</td>
</tr>
<tr>
<td>3,707,198</td>
<td>Reads with 5’ and 3’ Primers</td>
</tr>
<tr>
<td>3,704,840</td>
<td>Non-Concatemer Reads with 5’ and 3’ Primers</td>
</tr>
<tr>
<td>3,699,995</td>
<td>Non-Concatemer Reads with 5’ and 3’ Primers and Poly-A Tail</td>
</tr>
<tr>
<td>3,655</td>
<td>Mean Length of Full-Length Non-Concatemer Reads</td>
</tr>
<tr>
<td>1</td>
<td>Unique Primers</td>
</tr>
<tr>
<td>3,707,198</td>
<td>Mean Reads per Primer</td>
</tr>
<tr>
<td>3,707,198</td>
<td>Max. Reads per Primer</td>
</tr>
<tr>
<td>3,707,198</td>
<td>Min. Reads per Primer</td>
</tr>
<tr>
<td>375,162</td>
<td>Reads without Primers</td>
</tr>
<tr>
<td>91.61%</td>
<td>Percent Bases in Reads with Primers</td>
</tr>
<tr>
<td>90.81%</td>
<td>Percent Reads with Primers</td>
</tr>
</tbody>
</table>

* 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
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 Ile 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 Ile systems: Data files (102-144-100)

Example PacBio data sets

<table>
<thead>
<tr>
<th>Iso-Seq application</th>
<th>Dataset</th>
<th>Data type</th>
<th>PacBio system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Transcriptome</td>
<td><em>Homo sapiens – Brain with Alzheimer’s Disease</em></td>
<td>HiFi Reads</td>
<td>Sequel II System</td>
</tr>
<tr>
<td>Whole Transcriptome</td>
<td><em>Homo sapiens – Universal Human Reference RNA (UHRR)</em></td>
<td>HiFi Reads</td>
<td>Sequel II System</td>
</tr>
</tbody>
</table>
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
• 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]
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]
- 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.

<table>
<thead>
<tr>
<th>Kit type</th>
<th>Product name</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA isolation</td>
<td>Ambion Poly(A) Purist MAG Kit (<a href="#">Link</a>)</td>
</tr>
<tr>
<td>Total RNA isolation</td>
<td>Qiagen RNeasy Plus Kits (<a href="#">Link</a>)</td>
</tr>
<tr>
<td></td>
<td>Sigma Spectrum Plant Total RNA Kit (<a href="#">Link</a>)</td>
</tr>
<tr>
<td></td>
<td>iNtRON Easy Spin Total RNA (<a href="#">Link</a>)</td>
</tr>
<tr>
<td></td>
<td>TRIzol Reagent can be used to isolate total RNA from tissues or cells, including lipid-rich and difficult samples (<a href="#">Link</a>)</td>
</tr>
<tr>
<td>RNA stabilization &amp; storage</td>
<td>RNALater is an aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA (<a href="#">Link</a>)</td>
</tr>
</tbody>
</table>
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.