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Technical Overview: Iso-Seq Express Library Preparation Using SMRTbell Express Template Prep Kit 2.0

Sequel System ICS v8.0 / Sequel Chemistry 3.0 / SMRT Link v9.0
Sequel II Systems ICS v9.0 / Sequel II Chemistry 2.0 / SMRT Link v9.0
Sequel IIe System ICS v10.0 / Sequel II Chemistry 2.0 / SMRT Link v10.0

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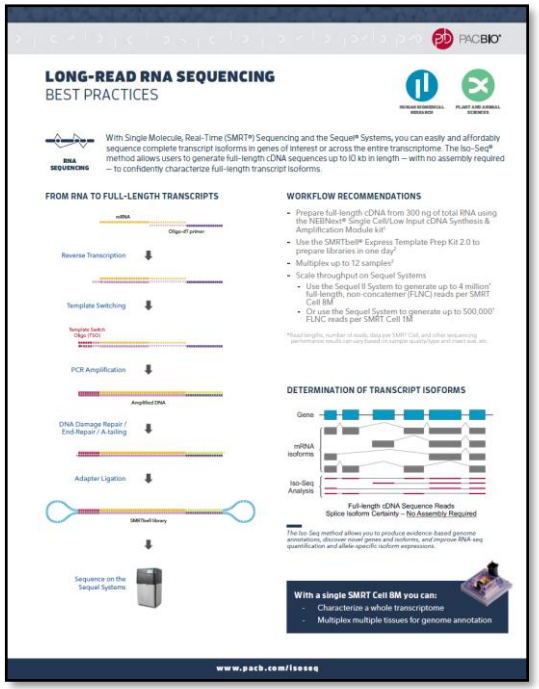
PN 101-814-400 Ver 2021-02-01-A (February 2021)

Iso-Seq Express Library Preparation Using SMRTbell Express Template Prep Kit 2.0

1. Iso-Seq Express Workflow Overview, Input RNA Sample QC Requirements & Experimental Design Considerations
2. Iso-Seq Express Library Sample Preparation Workflow Details
3. Iso-Seq Express Library Sequencing Workflow Details
4. Iso-Seq Express Library Example Sequencing Performance Data
5. Iso-Seq Data Analysis Workflow Overview (SMRT Link GUI)
6. Technical Documentation & Applications Support Resources
7. Appendix: RNA Isolation Kit Options for Iso-Seq Express SMRTbell Library Construction

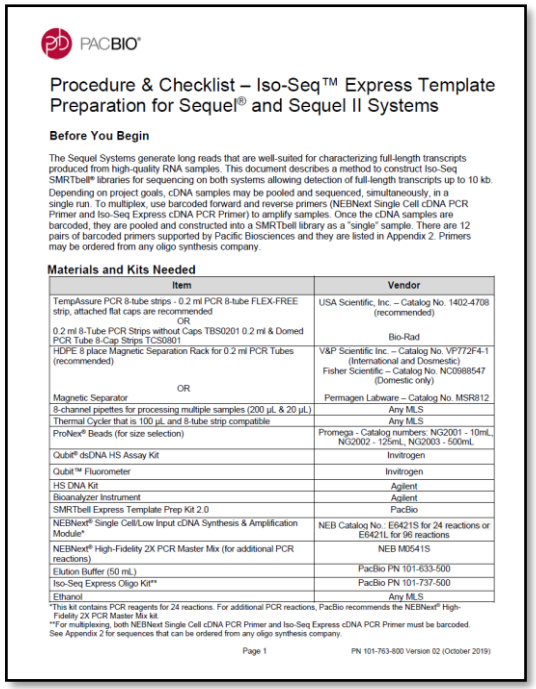
FULL-LENGTH TRANSCRIPT ISOFORM SEQUENCING (ISO-SEQ METHOD): HOW TO GET STARTED

Application-Specific Best Practices Guide → **Application-Specific Procedure & Checklist** → **Application Consumable Bundle Purchasing Guide** → **Library Construction, Sequencing & Analysis**



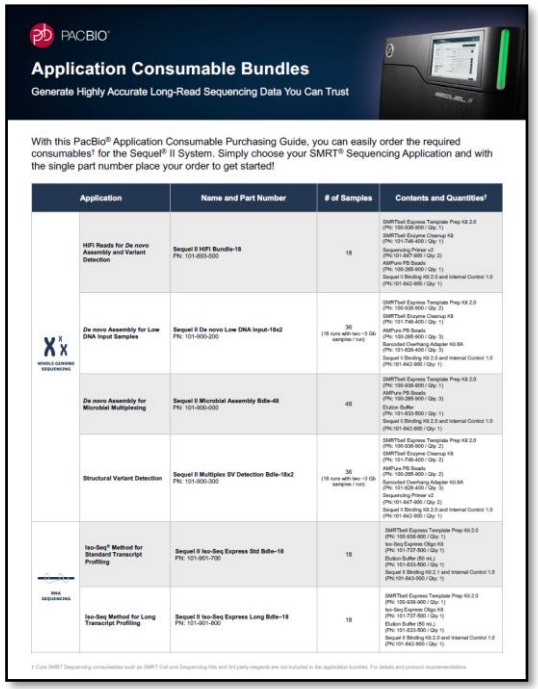
[Application Brief: Long-Read RNA Sequencing – Best Practices](#) (BP103-062619)

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



[Procedure & Checklist – Iso-Seq Express Template Preparation for Sequel® and Sequel II Systems](#) (101-763-800)

Technical documentation containing sample library construction and sequencing preparation protocol details



[PacBio Application Consumable Bundle Purchasing Guide](#) (PG100-082620)

Purchasing Guide enables users to easily order required consumables needed to prepare a SMRTbell library to run a specific type of application on the Sequel II and IIe Systems*

Total RNA QC and cDNA Synthesis

Use Barcoded F/R cDNA Amplification Primers for Multiplexing

Library Construction (SMRTbell Express TPK 2.0)

Purify Iso-Seq Library Using ProNex Beads

HiFi Sequencing

Generate Up To 4 Million Full-length, Non-concatemer (FLNC) reads per SMRT Cell 8M

Data Analysis (SMRT Link)

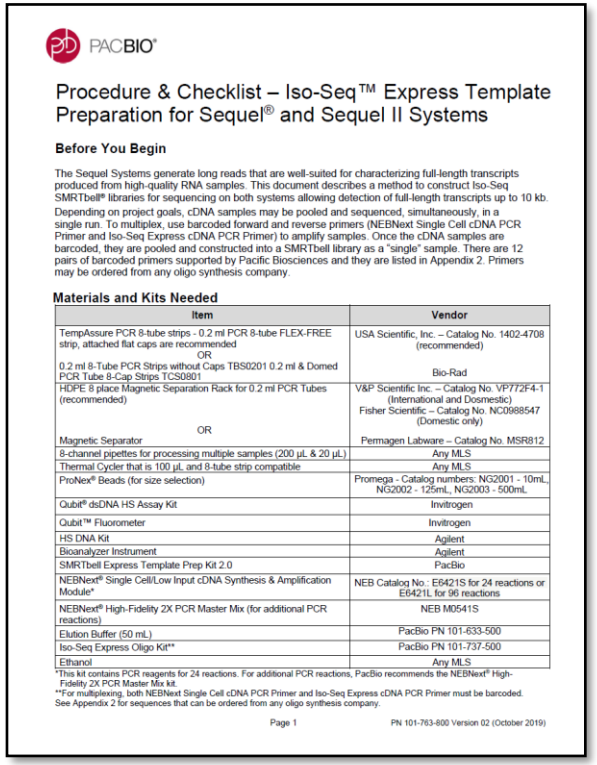
Iso-Seq Analysis Application

* Application Consumable Bundles include reagents for library construction, primer annealing and polymerase binding. Core PacBio-branded SMRT Sequencing consumables (SMRT Cells, Sequencing Kits & SMRT Oil), plastics and other 3rd-party reagents are not included in the application bundles

Iso-Seq Express Workflow Overview, Input RNA Sample QC Requirements & Experimental Design Considerations

ISO-SEQ SAMPLE PREPARATION PROCEDURE DESCRIPTION

- Procedure & Checklist – Iso-Seq Express Template Preparation for Sequel and Sequel II Systems (PN 101-763-800) PacBio protocol document describes a method to construct Iso-Seq SMRTbell libraries for sequencing on Sequel, Sequel II and Sequel IIe Systems allowing detection of full-length transcripts up to 10 kb.
- Multiple cDNA samples may be pooled and simultaneously sequenced on a single SMRT Cell
  - To multiplex, use barcoded forward and reverse primers to amplify samples. (Up to 12 pairs of barcoded primers are supported by Pacific Biosciences.)
  - Once the cDNA samples are barcoded, they are pooled and constructed into a SMRTbell library as a “single” sample using SMRTbell Express Template Prep Kit 2.0.



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Procedure & Checklist – Iso-Seq™ Express Template Preparation for Sequel® and Sequel II Systems

Before You Begin

The Sequel Systems generate long reads that are well-suited for characterizing full-length transcripts produced from high-quality RNA samples. This document describes a method to construct Iso-Seq SMRTbell® libraries for sequencing on both systems allowing detection of full-length transcripts up to 10 kb. Depending on project goals, cDNA samples may be pooled and sequenced, simultaneously, in a single run. To multiplex, use barcoded forward and reverse primers (NEBNext Single Cell cDNA PCR Primer and Iso-Seq Express cDNA PCR Primer) to amplify samples. Once the cDNA samples are barcoded, they are pooled and constructed into a SMRTbell library as a “single” sample. There are 12 pairs of barcoded primers supported by Pacific Biosciences and they are listed in Appendix 2. Primers may be ordered from any oligo synthesis company.

Materials and Kits Needed

Item	Vendor
TempAssure PCR 8-tube strips - 0.2 ml PCR 8-tube FLEX-FREE strip, attached flat caps are recommended OR 0.2 ml 8-Tube PCR Strips without Caps TBS0201 0.2 ml & Domed PCR Tube 8-Cap Strips TCS0801	USA Scientific, Inc. – Catalog No. 1402-4708 (recommended) Bio-Rad
HDPE 8 place Magnetic Separation Rack for 0.2 ml PCR Tubes (recommended) OR Magnetic Separator	V&P Scientific Inc. – Catalog No. VP772F4-1 (International and Dosmestic) Fisher Scientific – Catalog No. NC0988547 (Domestic only) Permagen Labware – Catalog No. MSR812
8-channel pipettes for processing multiple samples (200 µL & 20 µL)	Any MLS
Thermal Cycler that is 100 µL and 8-tube strip compatible	Any MLS
ProNex® Beads (for size selection)	Promega - Catalog numbers: NG2001 - 10mL, NG2002 - 125mL, NG2003 - 500mL
Qubit® dsDNA HS Assay Kit	Invitrogen
Qubit™ Fluorometer	Invitrogen
HS DNA Kit	Agilent
Bioanalyzer Instrument	Agilent
SMRTbell Express Template Prep Kit 2.0	PacBio
NEBNext® Single Cell/Low Input cDNA Synthesis & Amplification Module*	NEB Catalog No.: E6421S for 24 reactions or E6421L for 96 reactions
NEBNext® High-Fidelity 2X PCR Master Mix (for additional PCR reactions)	NEB M0541S
Elution Buffer (50 mL)	PacBio PN 101-633-500
Iso-Seq Express Oligo Kit**	PacBio PN 101-737-500
Ethanol	Any MLS

*This kit contains PCR reagents for 24 reactions. For additional PCR reactions, PacBio recommends the NEBNext® High-Fidelity 2X PCR Master Mix kit.
**For multiplexing, both NEBNext Single Cell cDNA PCR Primer and Iso-Seq Express cDNA PCR Primer must be barcoded. See Appendix 2 for sequences that can be ordered from any oligo synthesis company.

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https://www.pacb.com/support/documentation/

APPLICATIONS

RNA SEQUENCING

Full-Length Transcript Isoform Sequencing (Iso-Seq Method)

ISO-SEQ LIBRARY SAMPLE PREPARATION & SEQUENCING WORKFLOW

Workflow summary for constructing Iso-Seq SMRTbell libraries suitable for full-length transcript isoform sequencing applications using the Sequel, Sequel II and Sequel IIe Systems

Total RNA QC (0.5 h)

- ≥300 ng of Total RNA input recommended
- RNA integrity number ≥7.0 (ideally ≥8.0)

First Stand cDNA Synthesis, cDNA Amplification & Pooling (3 hrs)

- NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module
- Multiplex up to 12 cDNA samples per SMRT Cell
- Purify amplified cDNA samples with ProNex beads followed by equimolar pooling

SMRTbell Library Construction (4 hrs)

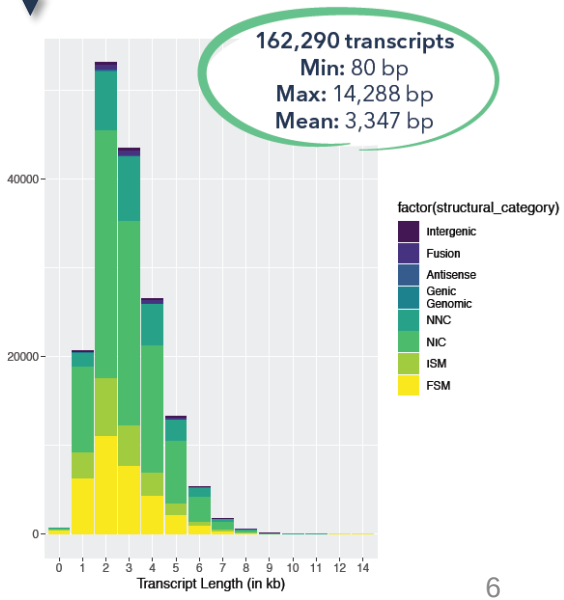
- ***Procedure & Checklist – Iso-Seq Express Template Preparation for Sequel and Sequel II Systems*** (PN 101-763-800)

HiFi Sequencing

- Follow ***Quick Reference Cards*** for primer annealing, polymerase binding, complex cleanup and sample loading
- Generate Up To 4 Million Full-length, Non-concatemer (FLNC) reads per SMRT Cell 8M

Data Analysis

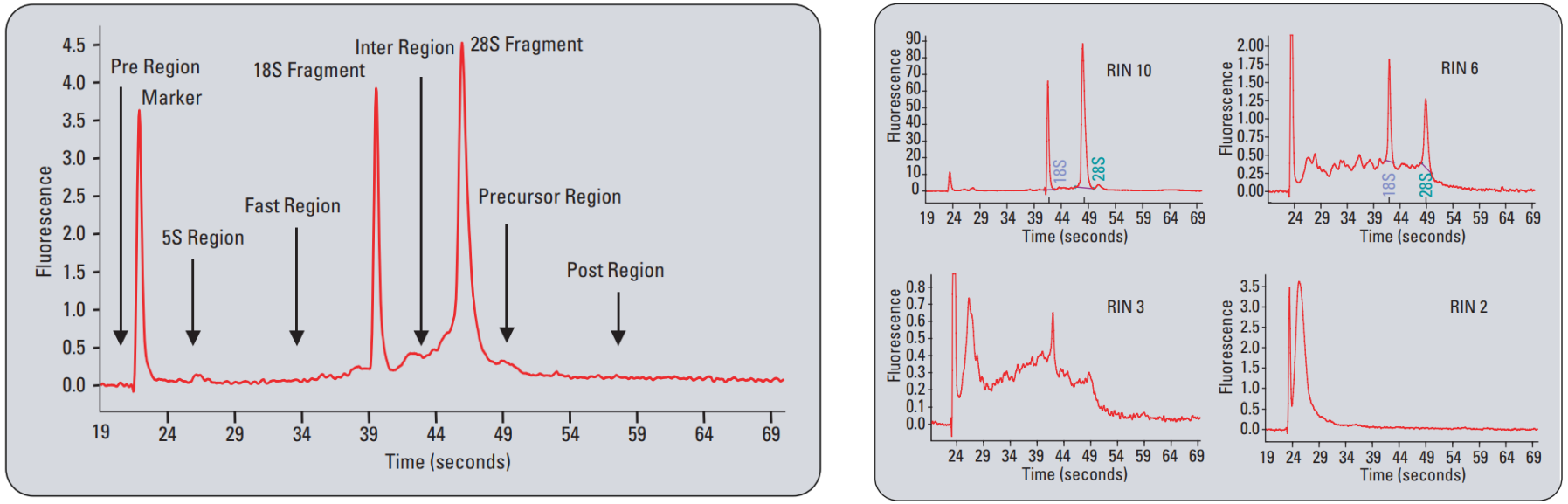
- Use SMRT Link Iso-Seq analysis application to output high-quality, full-length transcript sequences, with no assembly required



ISO-SEQ EXPRESS 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 (https://www.agilent.com/cs/library/applications/5989-1165EN.pdf)*

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.*

ISO-SEQ EXPRESS SAMPLE QC REQUIREMENTS (CONT.)

Evaluation of Input Total RNA Sample Purity

- RNA purity can be assessed through UV-spectrophotometry using a Nanodrop spectrophotometer (Thermo Scientific)
- 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.

260/280 Ratio

- A low A260/A280 ratio may indicate the presence of protein, phenol, or 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.

260/230 Ratio

- A low A260/A230 ratio may be the result of:
  - 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.

*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**.*

ISO-SEQ EXPRESS 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 the Iso-Seq application.
  - 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 Sequel, Sequel II and Sequel IIe Systems

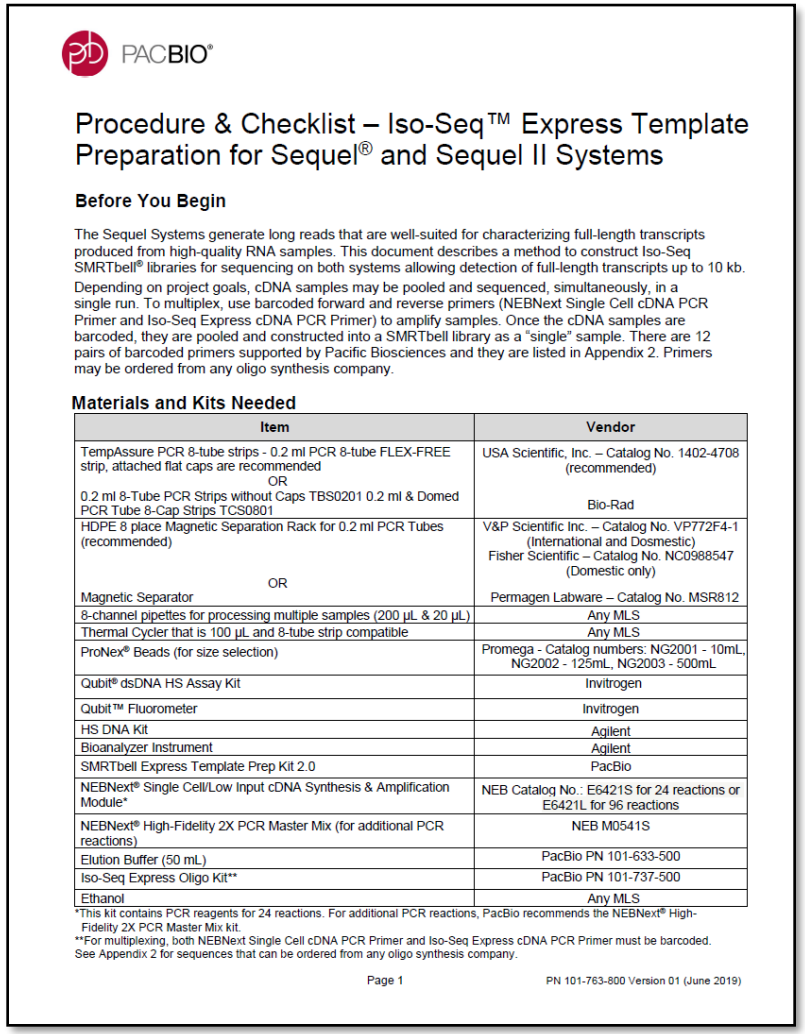
SEQUEL SYSTEM	SEQUEL II AND IIe SYSTEMS
For Genome Annotation One Transcriptome → One SMRT Cell 1M	**For Genome Annotation** Up to 8-plex Transcriptome* → One SMRT Cell 8M
	For Deep Transcriptome Profiling One Human Transcriptome → One SMRT Cell 8M

* Can multiplex up to a total of 12 Iso-Seq library samples on one SMRT Cell 8M

Iso-Seq Express Library Sample Preparation Workflow Details

PROCEDURE & CHECKLIST – ISO-SEQ EXPRESS TEMPLATE PREPARATION FOR SEQUEL AND SEQUEL II SYSTEMS

- This protocol (PN 101-763-800) a method to construct Iso-Seq SMRTbell libraries for sequencing on Sequel, Sequel II and Sequel IIe Systems (Sequel Systems) allowing detection of full-length transcripts up to 10 kb
- Streamlined and accelerated workflow for constructing Iso-Seq libraries in 1 Day
- For multiplexed Iso-Seq analysis, barcoded cDNA samples may be pooled and constructed into a SMRTbell library as a 'single' sample. The pooled library sample can then be sequenced on a single SMRT Cell.
- Protocol document contains:
  1. General laboratory best practices and input RNA QC recommendations
  2. Instructions for performing first-strand cDNA synthesis and amplification of cDNA products prior to SMRTbell library construction
  3. Instructions for constructing SMRTbell libraries using amplified cDNA products and SMRTbell Express Template Prep Kit 2.0
  4. Sample setup guidance for preparing Iso-Seq SMRTbell libraries for sequencing on the Sequel Systems



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Procedure & Checklist – Iso-Seq™ Express Template Preparation for Sequel® and Sequel II Systems

Before You Begin

The Sequel Systems generate long reads that are well-suited for characterizing full-length transcripts produced from high-quality RNA samples. This document describes a method to construct Iso-Seq SMRTbell® libraries for sequencing on both systems allowing detection of full-length transcripts up to 10 kb. Depending on project goals, cDNA samples may be pooled and sequenced, simultaneously, in a single run. To multiplex, use barcoded forward and reverse primers (NEBNext Single Cell cDNA PCR Primer and Iso-Seq Express cDNA PCR Primer) to amplify samples. Once the cDNA samples are barcoded, they are pooled and constructed into a SMRTbell library as a "single" sample. There are 12 pairs of barcoded primers supported by Pacific Biosciences and they are listed in Appendix 2. Primers may be ordered from any oligo synthesis company.

Materials and Kits Needed

Item	Vendor
TempAssure PCR 8-tube strips - 0.2 ml PCR 8-tube FLEX-FREE strip, attached flat caps are recommended OR 0.2 ml 8-Tube PCR Strips without Caps TBS0201 0.2 ml & Domed PCR Tube 8-Cap Strips TCS0801	USA Scientific, Inc. – Catalog No. 1402-4708 (recommended) Bio-Rad
HDPE 8 place Magnetic Separation Rack for 0.2 ml PCR Tubes (recommended) OR Magnetic Separator	V&P Scientific Inc. – Catalog No. VP772F4-1 (International and Dosmestic) Fisher Scientific – Catalog No. NC0988547 (Domestic only) Permagen Labware – Catalog No. MSR812
8-channel pipettes for processing multiple samples (200 µL & 20 µL)	Any MLS
Thermal Cycler that is 100 µL and 8-tube strip compatible	Any MLS
ProNex® Beads (for size selection)	Promega - Catalog numbers: NG2001 - 10mL, NG2002 - 125mL, NG2003 - 500mL
Qubit® dsDNA HS Assay Kit	Invitrogen
Qubit™ Fluorometer	Invitrogen
HS DNA Kit	Agilent
Bioanalyzer Instrument	Agilent
SMRTbell Express Template Prep Kit 2.0	PacBio
NEBNext® Single Cell/Low Input cDNA Synthesis & Amplification Module*	NEB Catalog No.: E6421S for 24 reactions or E6421L for 96 reactions
NEBNext® High-Fidelity 2X PCR Master Mix (for additional PCR reactions)	NEB M0541S
Elution Buffer (50 mL)	PacBio PN 101-633-500
Iso-Seq Express Oligo Kit**	PacBio PN 101-737-500
Ethanol	Any MLS

*This kit contains PCR reagents for 24 reactions. For additional PCR reactions, PacBio recommends the NEBNext® High-Fidelity 2X PCR Master Mix kit.
**For multiplexing, both NEBNext Single Cell cDNA PCR Primer and Iso-Seq Express cDNA PCR Primer must be barcoded. See Appendix 2 for sequences that can be ordered from any oligo synthesis company.

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ISO-SEQ EXPRESS WORKFLOW DETAILED OVERVIEW

The entire workflow from cDNA synthesis to completion of SMRTbell library preparation takes approx. 8 hours to complete. Plan your experiments so that the entire workflow can be completed within an 8-h day. If a stop is necessary, it is safe to pause after PCR amplification of cDNA and after SMRTbell library construction.

1. Input RNA QC

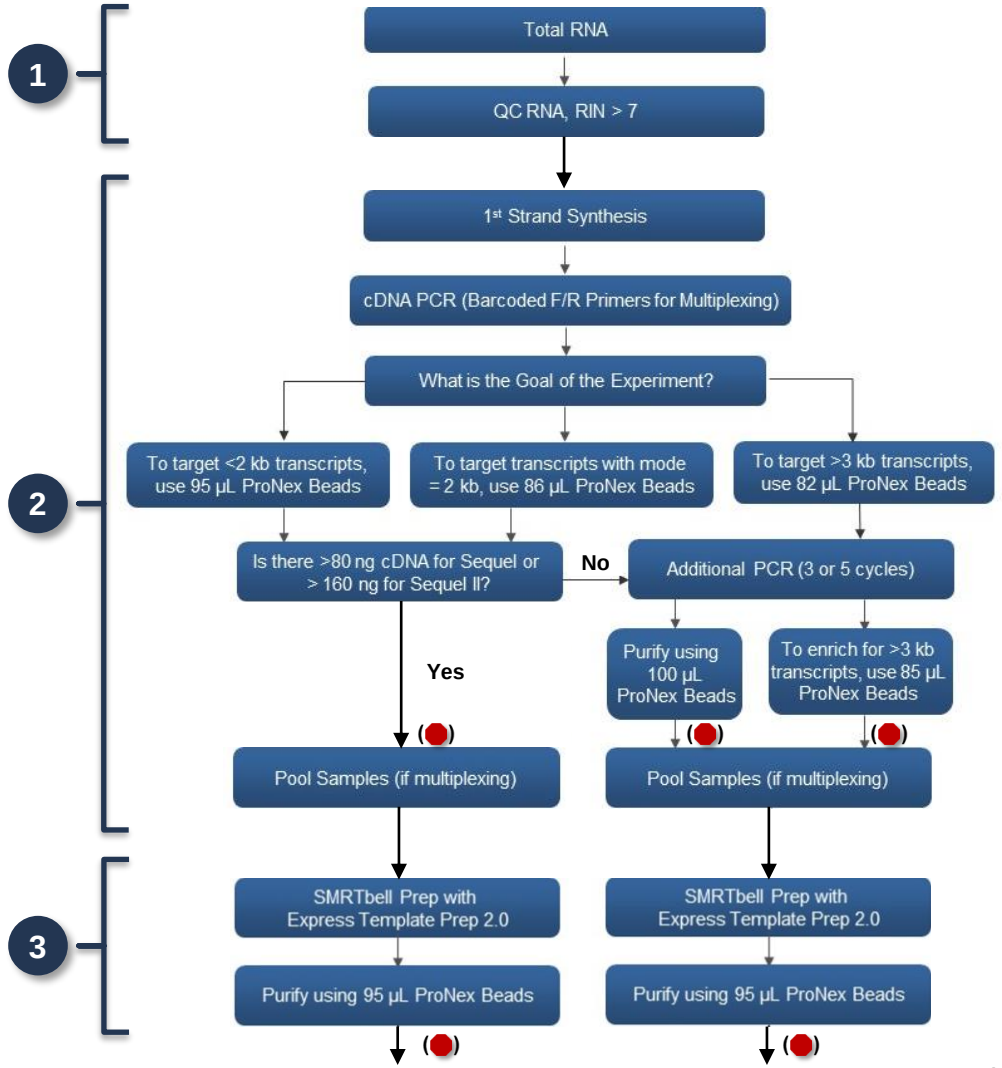
- ≥300 ng of Total RNA input recommended
- RNA integrity number ≥7.0 (ideally ≥8.0)

2. First Stand cDNA Synthesis, cDNA Amplification & Pooling

- NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module
- Multiplex up to 12 samples using barcoded F/R primers to amplify cDNA samples
- Purify amplified cDNA samples with ProNex beads
- Perform equimolar pooling for barcoded samples
- To target >3 kb transcripts, perform additional PCR cycles
- ~3.5 hours

3. SMRTbell Express 2.0 Library Construction

- Single-tube, addition-only reactions
- No size selection required
- Use ProNex beads for purification steps
- Typical library yield >50%
- ~4 hours

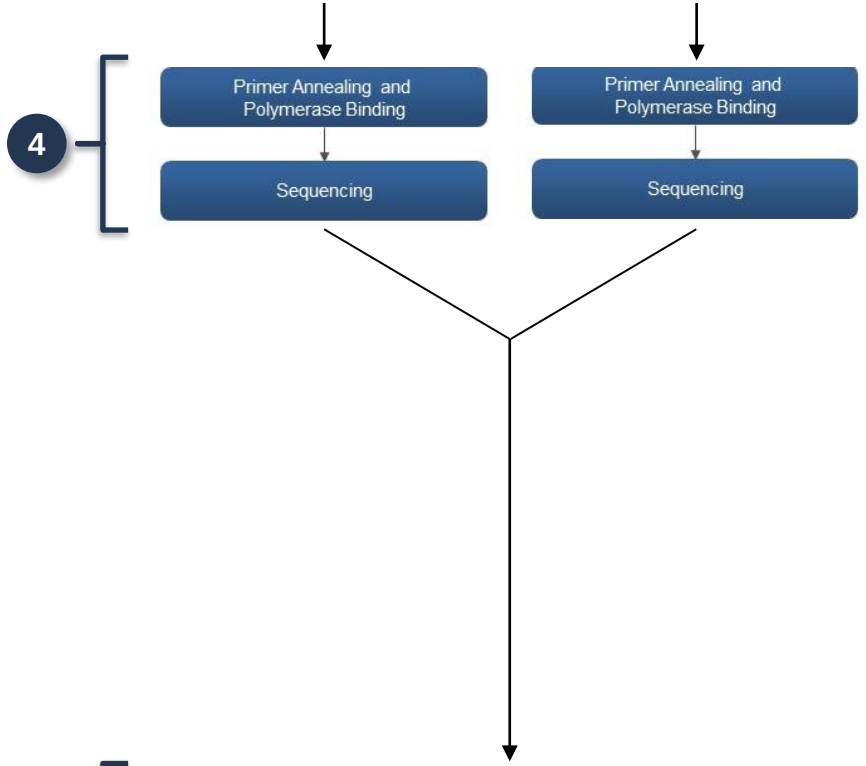


(●) = Optional Stop Point

ISO-SEQ EXPRESS WORKFLOW DETAILED OVERVIEW (CONT.)

4. Sequencing Preparation

- Anneal sequencing primer, bind polymerase, and perform ProNex bead complex cleanup
- Number of SMRT Cells supported per library prep:
  - >3 SMRT Cell 1M (Sequel System)
  - >1 SMRT Cell 8M (Sequel II and IIe Systems)
- Pre-extension Time:
  - 4 h (Sequel System Chemistry 3.0)
  - 2 h (Sequel II or IIe System Chemistry 2.0)
- Movie collection time
  - 20 h (Sequel System Chemistry 3.0)
  - 24 h (Sequel II or IIe System Chemistry 2.0)



5. Analyze

- 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

PacBio highly recommends upgrading to **SMRT Link v9.0** or higher to perform de-multiplexing and analysis of your Iso-Seq data sets

LIST OF REQUIRED MATERIALS AND EQUIPMENT FOR FIRST-STRAND cDNA SYNTHESIS AND SMRTBELL LIBRARY CONSTRUCTION

ITEM	VENDOR	PROTOCOL STEP(S)
NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module*	NEB PN: E6421S for 24 reactions or E6421L for 96 reactions	First-Strand cDNA Synthesis (use Template Switch Oligo from PacBio Iso-Seq Express Oligo Accessory Kit)
NEBNext High-Fidelity 2X PCR Master Mix (for additional PCR reactions)	NEB PN M0541S	First-Strand cDNA Synthesis
Iso-Seq Express Oligo Kit**	PacBio PN 101-737-500	First-Strand cDNA Synthesis
HDPE 8 place Magnetic Separation Rack for 0.2 ml PCR Tubes (recommended); **OR** 0.2 mL PCR Strip Magnetic Separator 8 or 12 Strip	V&P Scientific Inc. – PN VP772F4-1 (International and Domestic) or Fisher Scientific – PN NC0988547 (Domestic only) Permagen Labware – PN MSR812	ProNex Bead Purification
ProNex beads (for size selection)	Promega - PNs: NG2001 - 10mL, NG2002 - 125mL, NG2003 - 500mL	ProNex Bead Purification
Elution Buffer (50 mL)	PacBio PN 101-633-500	ProNex Bead Purification
Ethanol	Any MLS	ProNex Bead Purification
Qubit® dsDNA HS Assay Kit	Invitrogen	DNA quantitation QC
Qubit™ Fluorometer	Invitrogen	DNA quantitation QC
HS DNA Kit	Agilent	DNA quantitation QC
Bioanalyzer Instrument	Agilent	DNA sizing QC
TempAssure PCR 8-tube strips - 0.2 ml PCR 8-tube FLEX-FREE strip, attached flat caps are recommended; **OR** 0.2 ml 8-Tube PCR Strips without Caps 0.2 ml & Domed PCR Tube 8-Cap Strips	USA Scientific, Inc. – PN 1402-4708 (recommended) Bio-Rad – PN TBS0201, TCS0801	First-Strand cDNA Synthesis; ProNex Bead Purification, SMRTbell Library Construction
8-channel pipettes for processing multiple samples (200 µL & 20 µL)	Any MLS	First-Strand cDNA Synthesis; ProNex Bead Purification, SMRTbell Library Construction
Thermal Cycler that is 100 µL and 8-tube strip compatible	Any MLS	First-Strand cDNA Synthesis; ProNex Bead Purification, SMRTbell Library Construction
SMRTbell Express Template Prep Kit 2.0	Pacific Biosciences	SMRTbell Library Construction

*This kit contains PCR reagents for 24 reactions. For additional PCR reactions, PacBio recommends the NEBNext High-Fidelity 2X PCR Master Mix kit.

For multiplexing, both the NEBNext Single Cell cDNA PCR Primer and the Iso-Seq Express cDNA PCR Primer must be barcoded. See **Appendix 2 for sequences that can be ordered from any oligo synthesis company.

PACBIO ISO-SEQ EXPRESS OLIGO KIT AND SMRTBELL EXPRESS TEMPLATE PREP KIT 2.0 REAGENT HANDLING RECOMMENDATIONS

- Several reagents in the kit are sensitive to temperature and vortexing
- PacBio highly recommends:
  - Never leaving reagents at room temperature
  - Working on ice at all times when preparing master mixes
  - Finger tapping followed by a quick-spin prior to use

SMRTbell Express TPK 2.0

PacBio Iso-Seq Express Oligo Kit

LIST OF TEMPERATURE-SENSITIVE REAGENTS INCLUDED IN PACBIO ISO-SEQ EXPRESS OLIGO KIT AND SMRTBELL EXPRESS TPK 2.0

PACBIO KIT	REAGENT	WHERE USED
PacBio Iso-Seq Express Oligo Kit (PN 101-737-500) Store at -70°C to -80°C	TUBE, Iso-Seq Express Template Switching Oligo	cDNA Synthesis
	TUBE, Iso-Seq Express cDNA PCR Primer	cDNA Amplification
SMRTbell Express Template Prep Kit 2.0 (PN 100-938-900) Store at -20°C	DNA Prep Additive	Remove Single-Strand Overhangs
	DNA Prep Enzyme	Remove Single-Strand Overhangs
	DNA Damage Repair Mix v2	DNA Damage Repair
	End Prep Mix	End-Repair/A-tailing
	Overhang Adapter v3	Ligation
	Barcoded Overhang Adapters	Ligation
	Ligation Mix	Ligation
	Ligation Additive	Ligation
	Ligation Enhancer	Ligation

PACBIO ISO-SEQ EXPRESS OLIGO KIT

ISO-SEQ EXPRESS OLIGO KIT (PN 101-737-500)	
Tube Image #	**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 (PN 101-737-500) accessory kit contains **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)

SPECIAL HANDLING RECOMMENDATIONS FOR PREPARING ISO-SEQ EXPRESS SMRTBELL LIBRARIES

1. Always set your heat blocks or thermocyclers to the appropriate temperature for incubations **before** proceeding with the procedure.
2. ProNex beads:
   a. Equilibrate the ProNex beads at room temperature for 30 mins prior to use.
   b. It is critical to **accurately pipette** ProNex beads because small changes in volume can significantly alter the size distribution of your sample.
3. When performing ProNex bead purification steps, note that 80% ethanol is **hygroscopic** and should be prepared FRESH to achieve optimal results.
4. Using a multi-channel pipettor greatly enhances the ease of processing more than 1 sample
5. Measure DNA concentration using a **Qubit fluorometer** and **Qubit dsDNA High Sensitivity (HS) Assay Kit** reagents as recommended by the manufacturer.

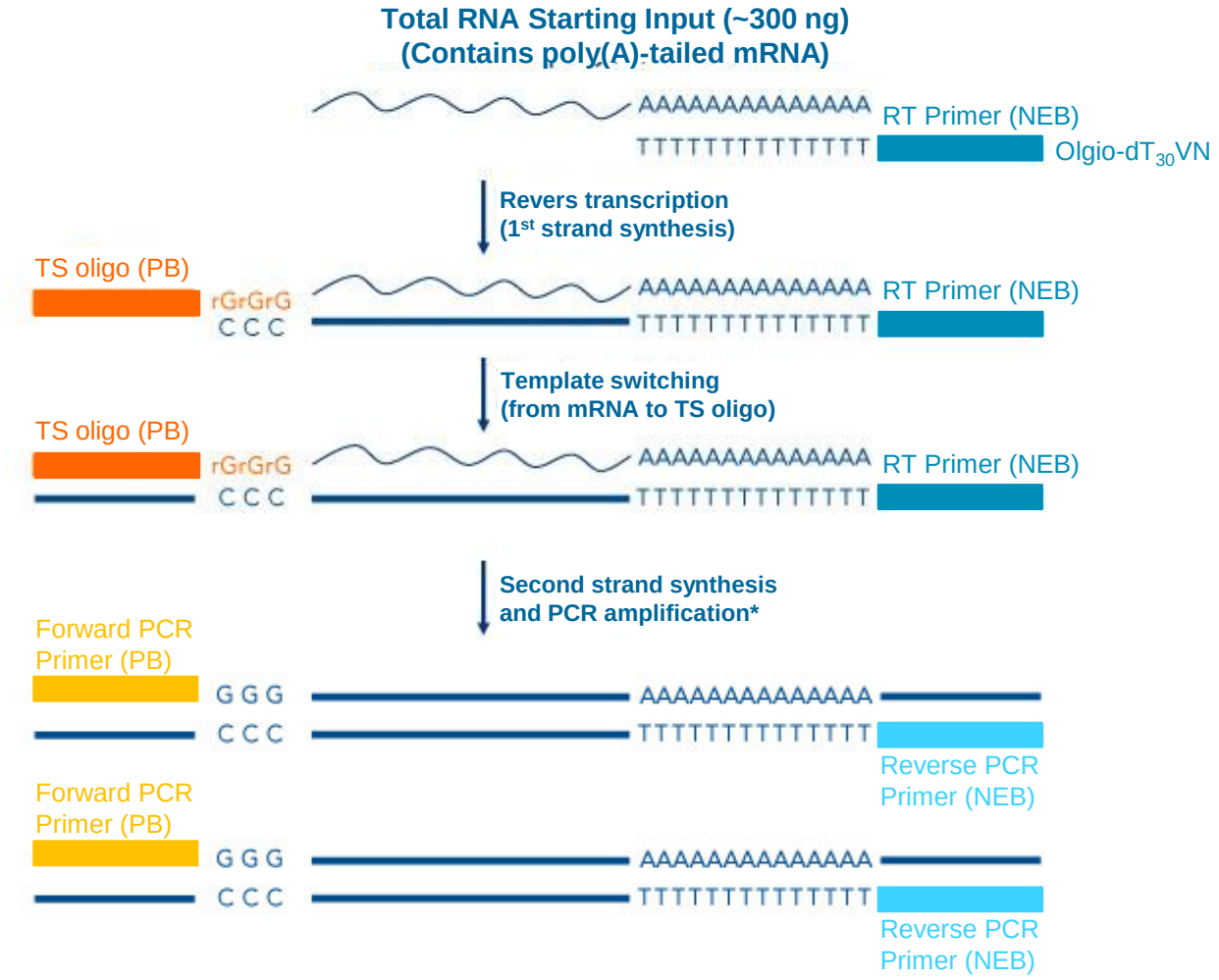
FIRST STRAND CDNA SYNTHESIS AND PCR AMPLIFICATION OF CDNA PRODUCTS

Use the **NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module** and **PacBio Iso-Seq Express Oligo Kit** to perform first-strand cDNA synthesis (reverse transcription and template switching) 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, **both** the NEBNext Single Cell cDNA PCR Primer **and** the Iso-Seq Express cDNA PCR Primer must be barcoded. See **Appendix 2** for sequences that can be ordered from any oligo synthesis company.

BARCODING SAMPLES FOR MULTIPLEXED ISO-SEQ ANALYSES

cDNA 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 ProNex beads, pooled together and then constructed into a SMRTbell library as a “single” sample.
- There are 24 pairs of barcoded primers supported by PacBio (see **Appendix 2** of the protocol)
- 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 2: Recommended Barcoded NEBNext Single Cell cDNA PCR Primer and Iso-Seq Express cDNA PCR Primer Sequences

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 Resources website:

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

PURIFICATION OF AMPLIFIED CDNA PRODUCTS

The specific method chosen to purify the amplified cDNA depends on the goal of the experiment and the expected size distribution of transcripts.

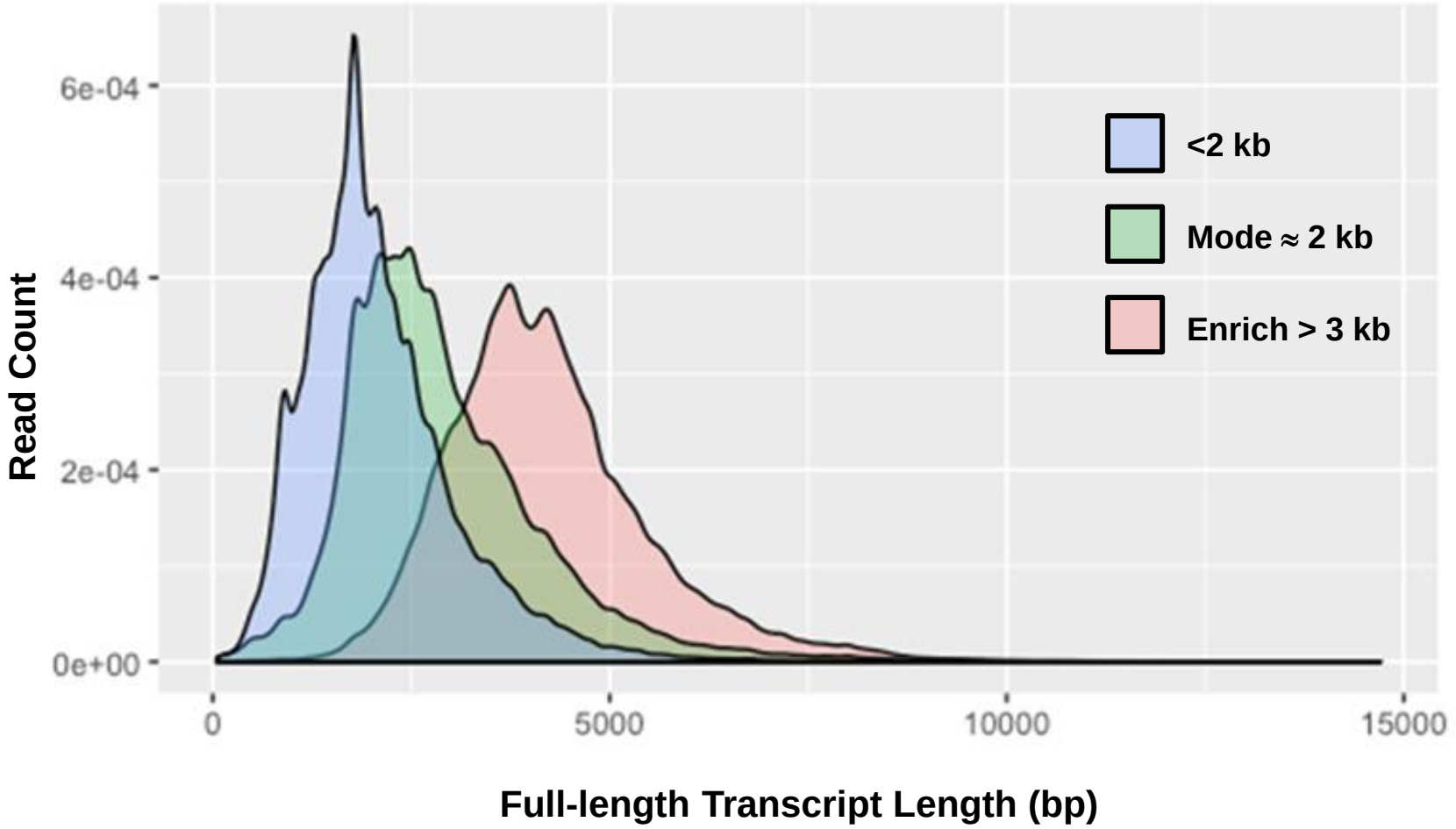
- Use **Pronex beads** for purification of amplified cDNA products according to the table below:

WORKFLOW	GOAL OF EXPERIMENT	PRONEX BEAD VOLUME
Standard	▪ Sample is composed primarily of transcripts centered at ~2 kb	86 µL
Short Transcripts	▪ Sample is composed primarily of transcripts <2 kb; or ▪ Transcripts of research interest are primarily <2 kb; or ▪ Sample is degraded and shows a low RIN number	95 µL
Long Transcripts	▪ To obtain material enriched for longer 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.

PURIFICATION OF AMPLIFIED CDNA PRODUCTS (CONT.)

ProNex 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

PacBio Instrument	Min. cDNA Amount for 1 sample	Min. cDNA Amount for Multiplexed Sample	Recommendation for Samples with Low Yield
Sequel System	80-500 ng	80-500* ng	If total mass is <80 ng (<1.75 ng/µL) → Go to **Appendix 1**
Sequel II/IIe System	160-500 ng	160-500* ng	If total mass is <160 ng (<3.5 ng/µL) → Go to **Appendix 1**

* This refers to the required **total mass** of the **pooled** cDNA samples (not individual samples of the pool). See "Sample Pooling" section for additional information.

- **Appendix 1:** Recommendations for Additional cDNA Amplification by PCR for Samples with a Lower Yield or to Enrich for Longer Transcripts*
  - The Sequel and Sequel II/IIe Systems require different amounts (ng) of cDNA for SMRTbell library construction. The Sequel System requires **>80 ng** of DNA, while the Sequel II/IIe System requires **>160 ng** DNA.
  - 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.

* If you want to **enrich for longer transcripts (>3 kb),** additional cDNA amplification (as described in **Appendix 1**) is **required**

SAMPLE POOLING FOR MULTIPLEXED ISO-SEQ ANALYSES

Equal molar pooling of barcoded cDNA samples is necessary to generate good representation of samples that are being multiplexed.

1. Use the 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 be >80 ng for Sequel and >160 ng for Sequel II/IIe to proceed to DNA Damage Repair.
   - If the volume required to achieve the minimum mass of the pooled cDNA exceeds 47.4 µL, concentrate the pooled cDNA by performing a 1X volume of ProNex beads and elute it in 48 µL. To account for potential losses during concentration at this step, start with ≥100 ng for the Sequel System and ≥200 ng for the Sequel II/IIe Systems.

3. The pooled cDNA can now be constructed into a SMRTbell library as a single sample. Proceed to the DNA Damage Repair step.

Iso-Seq Express Library Sequencing Workflow Details

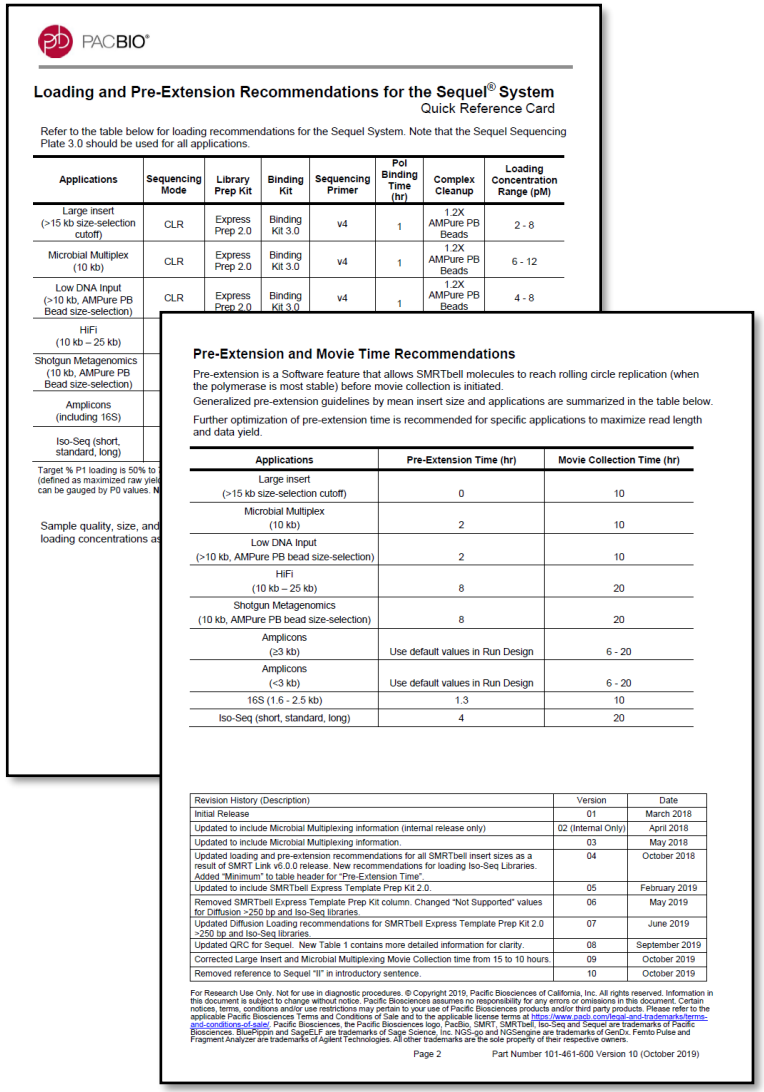
SAMPLE SETUP RECOMMENDATIONS FOR ISO-SEQ EXPRESS LIBRARIES – SEQUEL SYSTEM (CHEMISTRY 3.0)

- Follow **SMRT Link Sample Setup** instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel System for sequencing Iso-Seq samples.

Applications	Sequencing Mode	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
Iso-Seq (short, standard, long)	CCS	Express Prep 2.0	Binding Kit 3.0	v4	1	1.2X ProNex	2 - 8

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
Iso-Seq (short, standard, long)	4	20

* PacBio recommends **Sequel Binding Kit 3.0** for all Iso-Seq Express workflows (Short, Standard, and Long)



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Loading and Pre-Extension Recommendations for the Sequel® System
Quick Reference Card

Refer to the table below for loading recommendations for the Sequel System. Note that the Sequel Sequencing Plate 3.0 should be used for all applications.

Applications	Sequencing Mode	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
Large insert (>15 kb size-selection cutoff)	CLR	Express Prep 2.0	Binding Kit 3.0	v4	1	1.2X AMPure PB Beads	2 - 8
Microbial Multiplex (10 kb)	CLR	Express Prep 2.0	Binding Kit 3.0	v4	1	1.2X AMPure PB Beads	6 - 12
Low DNA Input (>10 kb, AMPure PB Bead size-selection)	CLR	Express Prep 2.0	Binding Kit 3.0	v4	1	1.2X AMPure PB Beads	4 - 8
HiFi (10 kb – 25 kb)							
Shotgun Metagenomics (10 kb, AMPure PB Bead size-selection)							
Amplicons (including 16S)							
Iso-Seq (short, standard, long)							

Target % P1 loading is 50% to
(defined as maximized raw yiel
can be gauged by P0 values. N

Sample quality, size, and
loading concentrations as

Pre-Extension and Movie Time Recommendations

Pre-extension is a Software feature that allows SMRTbell molecules to reach rolling circle replication (when the polymerase is most stable) before movie collection is initiated.
Generalized pre-extension guidelines by mean insert size and applications are summarized in the table below.

Further optimization of pre-extension time is recommended for specific applications to maximize read length and data yield.

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
Large insert (>15 kb size-selection cutoff)	0	10
Microbial Multiplex (10 kb)	2	10
Low DNA Input (>10 kb, AMPure PB bead size-selection)	2	10
HiFi (10 kb – 25 kb)	8	20
Shotgun Metagenomics (10 kb, AMPure PB bead size-selection)	8	20
Amplicons (≥3 kb)	Use default values in Run Design	6 - 20
Amplicons (<3 kb)	Use default values in Run Design	6 - 20
16S (1.6 - 2.5 kb)	1.3	10
Iso-Seq (short, standard, long)	4	20

Revision History (Description)	Version	Date
Initial Release	01	March 2018
Updated to include Microbial Multiplexing information (internal release only)	02 (Internal Only)	April 2018
Updated to include Microbial Multiplexing information.	03	May 2018
Updated loading and pre-extension recommendations for all SMRTbell insert sizes as a result of SMRT Link v6.0.0 release. New recommendations for loading Iso-Seq Libraries. Added "Minimum" to table header for "Pre-Extension Time".	04	October 2018
Updated to include SMRTbell Express Template Prep Kit 2.0.	05	February 2019
Removed SMRTbell Express Template Prep Kit column. Changed "Not Supported" values for Diffusion >250 bp and Iso-Seq libraries.	06	May 2019
Updated Diffusion Loading recommendations for SMRTbell Express Template Prep Kit 2.0 >250 bp and Iso-Seq libraries.	07	June 2019
Updated QRC for Sequel. New Table 1 contains more detailed information for clarity.	08	September 2019
Corrected Large Insert and Microbial Multiplexing Movie Collection time from 15 to 10 hours.	09	October 2019
Removed reference to Sequel "II" in introductory sentence.	10	October 2019

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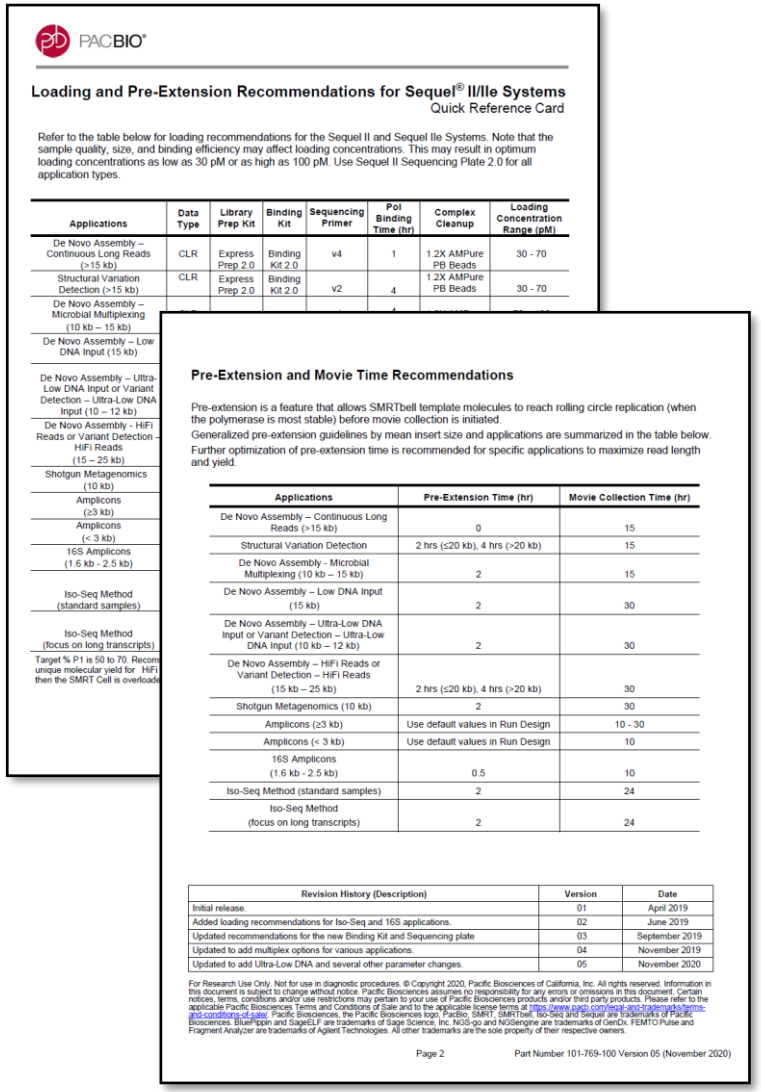
SAMPLE SETUP RECOMMENDATIONS FOR ISO-SEQ EXPRESS LIBRARIES – SEQUEL II AND IIe SYSTEMS (CHEMISTRY 2.0)

- Follow **SMRT Link Sample Setup** instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II/IIe Systems for sequencing Iso-Seq samples.
  - For **SMRT Link v10.0** (or higher): Select **'Iso-Seq Method'** from the **Application** field drop-down menu in the SMRT Link Sample Setup and SMRT Link Run Design user interface

Applications	Data Type	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
Iso-Seq Method (standard samples)	CCS	Express Prep 2.0	Binding Kit 2.1	v4	1	1.2X ProNex Beads	40 - 80
Iso-Seq Method (focus on long transcripts)	CCS	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X ProNex Beads	50 - 100

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
Iso-Seq Method (standard samples)	2	24
Iso-Seq Method (focus on long transcripts)	2	24

* PacBio recommends **Sequel II Binding Kit 2.1 for standard** Iso-Seq Express samples. For Iso-Seq samples with a focus on **long transcripts**, PacBio recommends **Sequel II Binding Kit 2.0**



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Loading and Pre-Extension Recommendations for Sequel® II/IIe Systems
Quick Reference Card

Refer to the table below for loading recommendations for the Sequel II and Sequel IIe Systems. Note that the sample quality, size, and binding efficiency may affect loading concentrations. This may result in optimum loading concentrations as low as 30 pM or as high as 100 pM. Use Sequel II Sequencing Plate 2.0 for all application types.

Applications	Data Type	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
De Novo Assembly – Continuous Long Reads (>15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 - 70
Structural Variation Detection (>15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v2	4	1.2X AMPure PB Beads	30 - 70
De Novo Assembly – Microbial Multiplexing (10 kb – 15 kb)							
De Novo Assembly – Low DNA Input (15 kb)							
De Novo Assembly – Ultra-Low DNA Input or Variant Detection – Ultra-Low DNA Input (10 – 12 kb)							
De Novo Assembly - HiFi Reads or Variant Detection – HiFi Reads (15 – 25 kb)							
Shotgun Metagenomics (10 kb)							
Amplicons (≥3 kb)							
Amplicons (< 3 kb)							
16S Amplicons (1.6 kb - 2.5 kb)							
Iso-Seq Method (standard samples)							
Iso-Seq Method (focus on long transcripts)							

Target % P1 is 50 to 70. Recom
unique molecular yield for HiFi
then the SMRT Cell is overload

Pre-Extension and Movie Time Recommendations

Pre-extension is a feature that allows SMRTbell template molecules to reach rolling circle replication (when the polymerase is most stable) before movie collection is initiated.
Generalized pre-extension guidelines by mean insert size and applications are summarized in the table below.
Further optimization of pre-extension time is recommended for specific applications to maximize read length and yield.

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
De Novo Assembly – Continuous Long Reads (>15 kb)	0	15
Structural Variation Detection	2 hrs (≤20 kb), 4 hrs (>20 kb)	15
De Novo Assembly - Microbial Multiplexing (10 kb – 15 kb)	2	15
De Novo Assembly – Low DNA Input (15 kb)	2	30
De Novo Assembly – Ultra-Low DNA Input or Variant Detection – Ultra-Low DNA Input (10 kb – 12 kb)	2	30
De Novo Assembly – HiFi Reads or Variant Detection – HiFi Reads (15 kb – 25 kb)	2 hrs (≤20 kb), 4 hrs (>20 kb)	30
Shotgun Metagenomics (10 kb)	2	30
Amplicons (≥3 kb)	Use default values in Run Design	10 - 30
Amplicons (< 3 kb)	Use default values in Run Design	10
16S Amplicons (1.6 kb - 2.5 kb)	0.5	10
Iso-Seq Method (standard samples)	2	24
Iso-Seq Method (focus on long transcripts)	2	24

Revision History (Description)	Version	Date
Initial release.	01	April 2019
Added loading recommendations for Iso-Seq and 16S applications.	02	June 2019
Updated recommendations for the new Binding Kit and Sequencing plate	03	September 2019
Updated to add multiplex options for various applications.	04	November 2019
Updated to add Ultra-Low DNA and several other parameter changes.	05	November 2020

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Part Number 101-769-100 Version 05 (November 2020)

Iso-Seq Express Library Example Sequencing Performance Data

EXAMPLE ISO-SEQ LIBRARY SEQUENCING PERFORMANCE RESULTS FOR UHR CONTROL AND BRAIN TISSUE SAMPLES (SEQUEL SYSTEM)

Sequel System Chemistry 3.0 (20-h Movie Collection; 4-h Pre-Extension Time)

Sample #	Sample Description[1]	Protocol[2]	Raw Polymerase Read Bases (Gb)	# CCS Reads	% FLNC[3] Reads	FLNC Mean Length (bp)
1	UHR	Standard	35.06	598,345	85%	2895
2	UHR	Standard	35.17	553,037	87%	2879
3	UHR	Standard	37.19	581,159	84%	2811
4	UHR	Standard	39.91	530,629	86%	2850
5	Normal Brain	Standard	32.55	595,550	83%	2751
6	Alzheimer's	Standard	36.96	509,404	87%	2565
7	UHR	Long	33.96	550,365	85%	3744
8	UHR	Short	41.02	612,392	84%	2366
Multiplexed Transcriptome Samples						
9	UHR	Barcoded (2-plex)	40.57	595,290	76%[4]	2839

[1] UHR = Universal Human Reference RNA

[2] Protocol: Standard = ProNex Bead Purification to target transcripts ~2 kb; Long = ProNex Bead Purification to target transcripts >2 kb; Short = ProNex Bead Purification to target transcripts <2 kb

[3] % FLNC: % of CCS Reads that are Full-length non-concatemer reads.

[4] For Sample #9: % **Barcoded** FLNC reads observed for each different barcoded sample in a 2-plex run within a multiplexed Iso-Seq library pool was 36 – 40%, consistent with approximately 80% total recovery of FLNC reads distributed evenly across the multiplexed samples.

EXAMPLE ISO-SEQ LIBRARY SEQUENCING PERFORMANCE RESULTS FOR UHR CONTROL SAMPLE (SEQUEL II SYSTEM)

Sequel II System Chemistry 1.0 (24-h Movie Collection; 2-h Pre-Extension Time)

Replicate #	Sample Description[1]	Raw Polymerase Read Bases (Gb)	# CCS Reads	# FLNC[2] Reads	% FLNC Reads	FLNC Mean Length (bp)
1	UHR	314.02	4,770,143	4,009,124	84%	3411
2	UHR	270.06	4,389,007	3,505,161	80%	3396
3	UHR	361.13	5,055,974	4,300,398	85%	3913
4	UHR	298.80	4,377,647	3,663,342	84%	3470
5	UHR	223.42	3,263,340	2,725,148	84%	3303
6	UHR	290.86	4,326,935	3,639,004	84%	3495
7	UHR	287.24	3,815,759	3,285,200	86%	3826
8	UHR	258.47	3,902,038	3,179,275	81%	3470
AVERAGE		**288**	**4,237,605**	**3,538,331**	**83.5%**	**3535**

[1] All UHR (Universal Human Reference RNA) samples were processed using the standard ProNex Bead Purification workflow to target transcripts ~2 kb

[2] FLNC: Full-length non-concatemer reads

EXAMPLE ISO-SEQ LIBRARY SEQUENCING PERFORMANCE RESULTS FOR VARIOUS TISSUE SAMPLE TYPES (SEQUEL II SYSTEM)

Sequel II System Chemistry 1.0 (24-h Movie Collection; 2-h Pre-Extension Time)

Sample #	Sample Description[1]	Protocol[2]	# FLNC[3] Reads	% FLNC Reads
1	UHR	Standard	3,466,513	85%
2	Mouse Liver	Standard	3,431,638	87%
3	MCF7	Standard	3,531,419	84%
4	Brain	Standard	2,943,148	86%
5	Alz Brain Tissue	Standard	3,142,634	83%
6	Heart	Standard	2,753,509	87%
7	Liver	Long	3,542,983	85%
8	ColT Cell Line	Short	2,852,434	84%

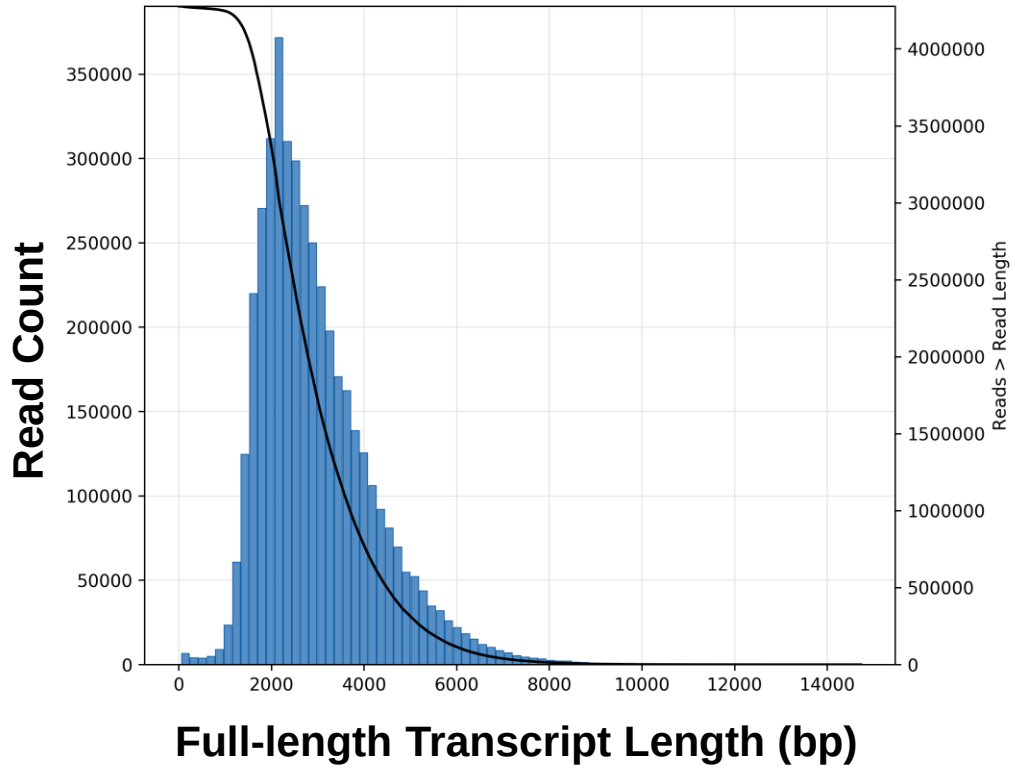
[1] UHR (Universal Human Reference RNA)

[2] Protocol: Standard = ProNex Bead Purification to target transcripts ~2 kb; Long = ProNex Bead Purification to target transcripts >2 kb; ProNex Bead Purification to target transcripts <2 kb

[3] FLNC: Full-length non-concatemer reads

EXAMPLE ISO-SEQ LIBRARY SEQUENCING PERFORMANCE RESULTS FOR AN ALZHEIMER'S BRAIN TISSUE SAMPLE ON SEQUEL II SYSTEM

Sequel II System Chemistry 1.0 (24-h Movie Collection; 2-h Pre-Extension Time)



CCS Read Classification

Value	Analysis Metric
5,052,827	Reads
4,327,705	Reads with 5' and 3' Primers
4,292,971	Non-Concatamer Reads with 5' and 3' Primers
4,277,293	Non-Concatamer Reads with 5' and 3' Primers and Poly-A Tail
2,973	Mean Length of Full-Length Non-Concatamer Reads
1	Unique Primers
4,327,705	Mean Reads per Primer
4,327,705	Max. Reads per Primer
4,327,705	Min. Reads per Primer
725,122	Reads without Primers

4 million full-length non-concatemer reads generated on a **single** Sequel II System SMRT Cell 8M (85% yield from total reads generated)*

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

Iso-Seq Data Analysis Workflow Overview (SMRT Link GUI)

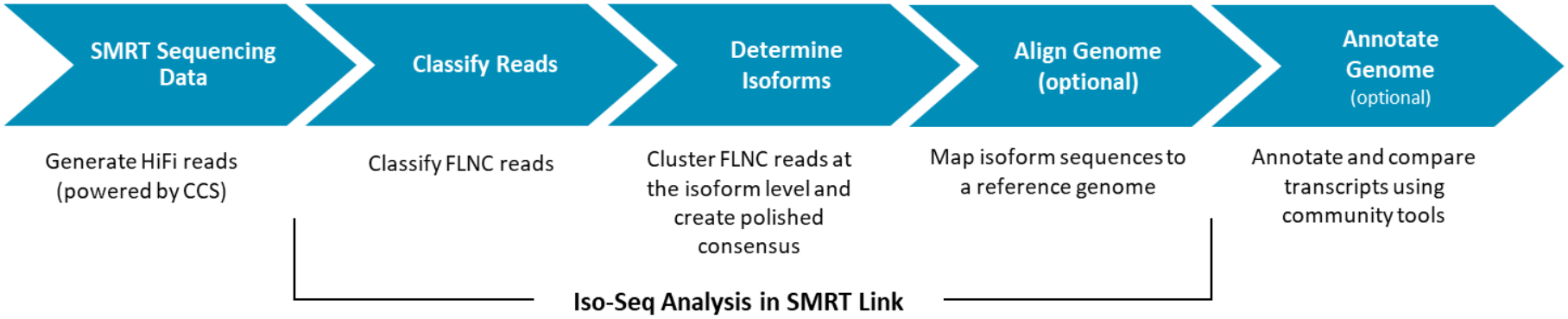
SMRT LINK ISO-SEQ ANALYSIS APPLICATION

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

- 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

PacBio highly recommends using **SMRT Link v9.0** or higher to perform de-multiplexing and analysis of your Iso-seq data sets

Iso-Seq Analysis Workflow Summary Overview*

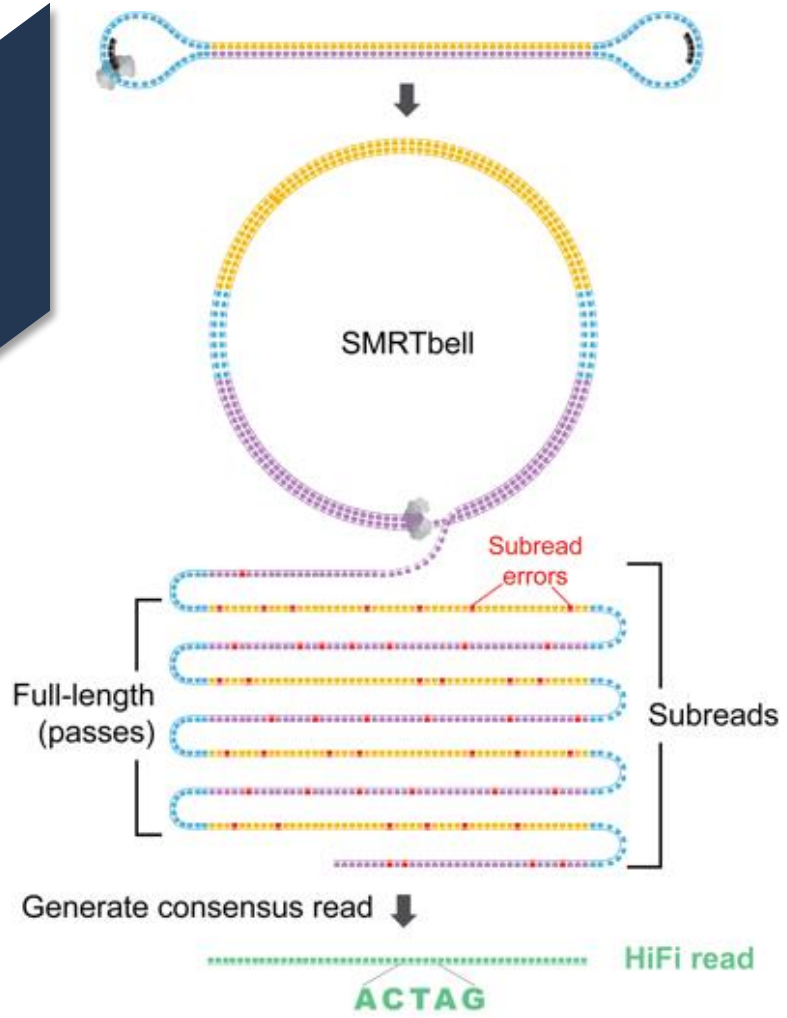


* Advanced users may optionally install the Linux developer's version of IsoSeq and its related downstream analysis under the Anaconda environment

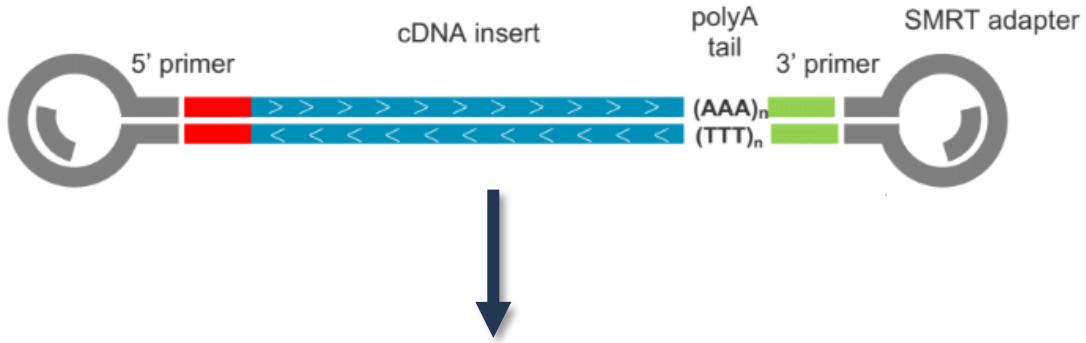
ISO-SEQ ANALYSIS WORKFLOW DETAILS

1

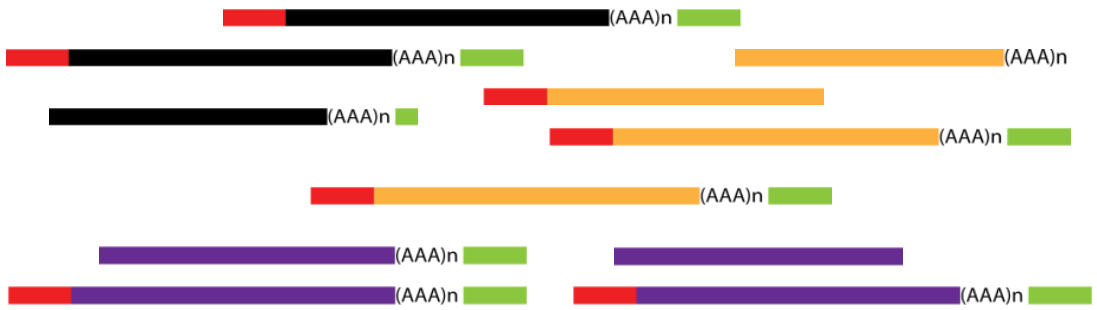
CCS Reads



Iso-Seq Library



CCS Reads

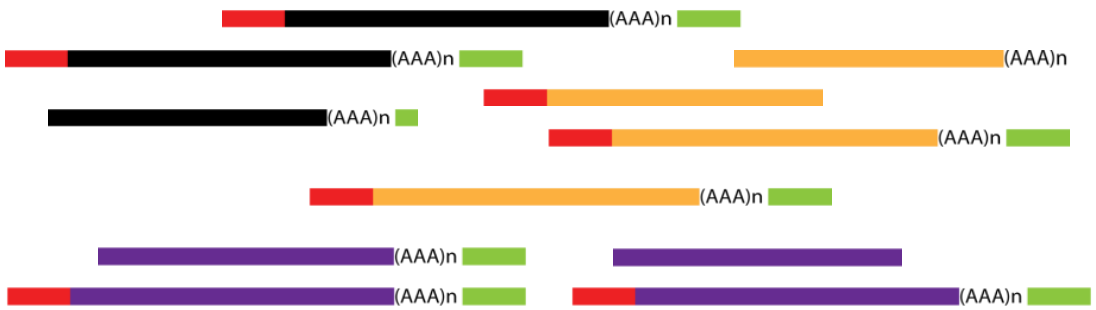


HiFi Reads = CCS Reads with ≥Q20

ISO-SEQ ANALYSIS WORKFLOW DETAILS (CONT.)

1. CCS Reads
2. Classify Full-Length Reads

CCS Reads



With multiplexed Iso-Seq samples, cDNA primers + barcodes together are treated as *custom primers*

Full-Length Transcript Reads

Full-length transcript reads:

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

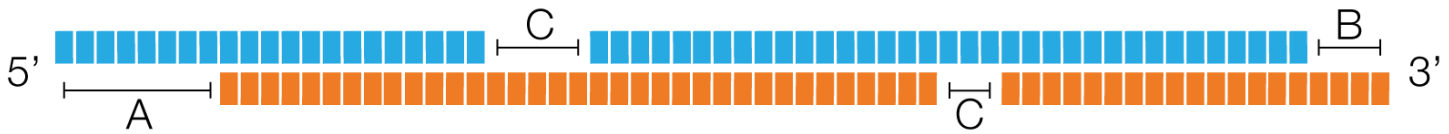
ISO-SEQ ANALYSIS WORKFLOW DETAILS (CONT.)

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

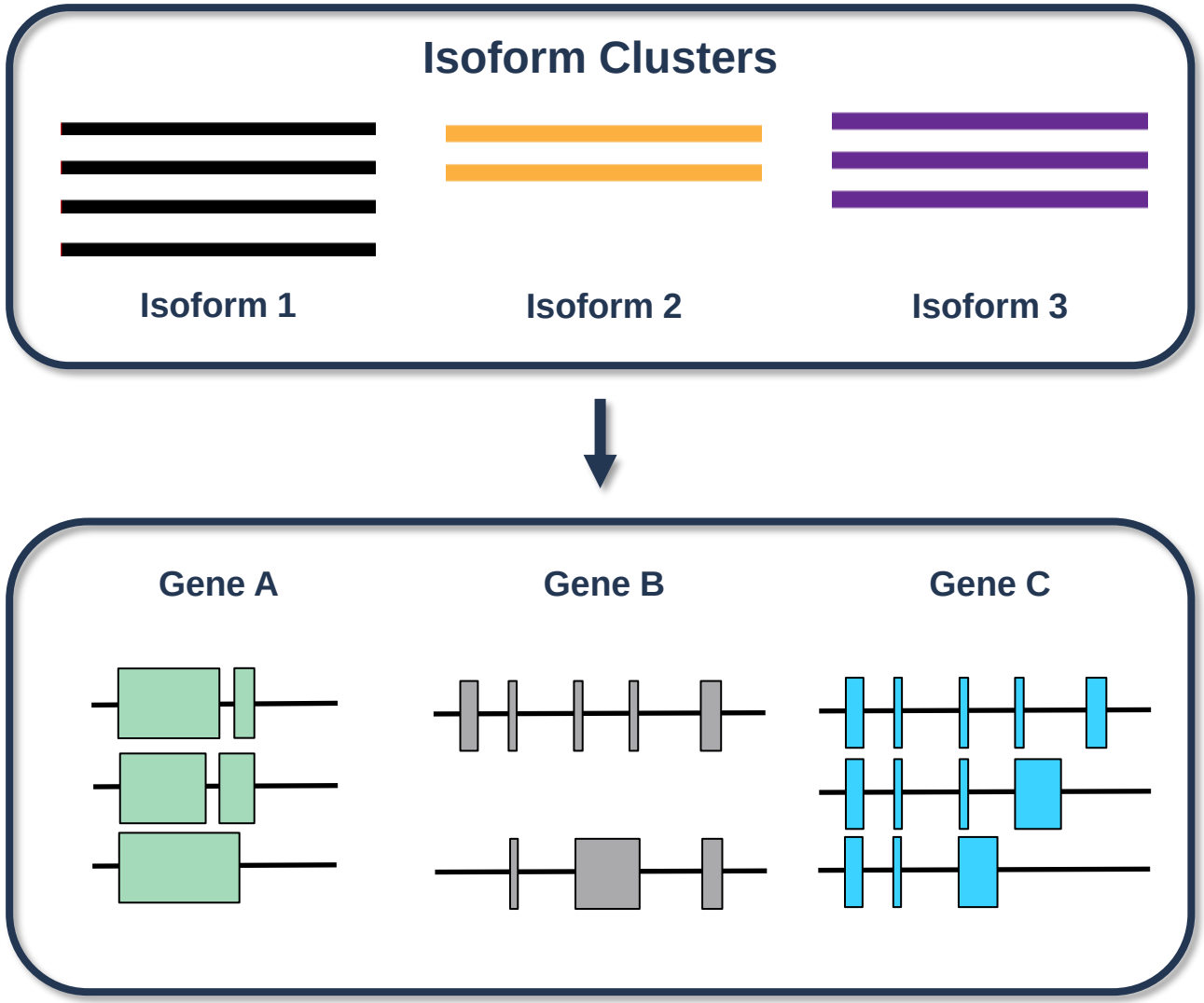
C. <10 bp in internal gap (exon), no limit on the number of gaps



ISO-SEQ ANALYSIS WORKFLOW DETAILS (CONT.)

4 Cluster Isoforms

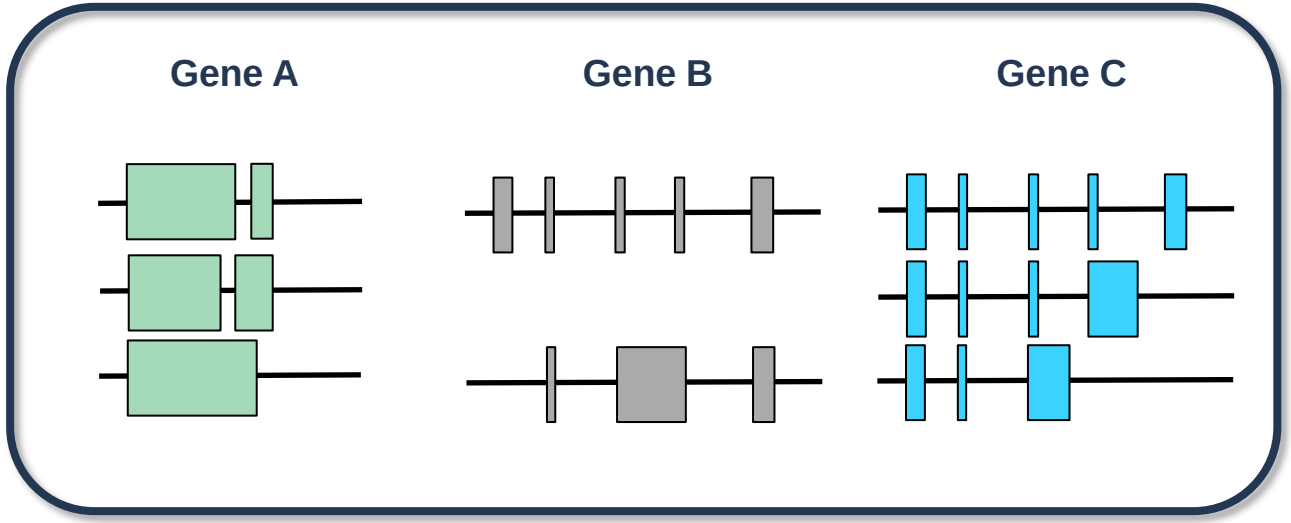
5 Map to Genome



ISO-SEQ ANALYSIS WORKFLOW DETAILS (CONT.)

5 Map to Genome

6 QC & Annotate



SQANTI

TAPPAS

CAT

TAMA

Technical Documentation & Applications Support Resources

BEST PRACTICES: LONG-READ RNA SEQUENCING (ISO-SEQ ANALYSIS)

Template Preparation with SMRTbell Express Template Prep Kit 2.0

- Prepare full-length cDNA from 300 ng of total RNA using the NEBNext Single Cell/Low Input cDNA Synthesis & Amplification Module kit
- Use the SMRTbell Express Template Prep Kit 2.0 to prepare libraries in one day
- Multiplex up to 12 samples with barcoding

Sequence on the Sequel, Sequel II or Sequel IIe System

- Maximize output and turn-around-time with adjustable sequencing parameters
  - Sequel System: 20-hour movies with 4 hours pre-extension is recommended
  - Sequel II System: 24-hour movies with 2 hours pre-extension is recommended
- Use the Sequel System to generate up to 500,000* full-length, non-concatemer (FLNC) reads per SMRT Cell 1M
- Use the Sequel II/IIe System to generate up to 4 million* FLNC reads per SMRT Cell 8M
- Scale throughput based on project needs – With a single Sequel II System SMRT Cell 8M you can:
  - Characterize a whole transcriptome
  - Multiplex multiple tissues for genome annotation

Data Analysis Solutions with the PacBio Analytical Portfolio

- Utilize SMRT Link to generate highly accurate and long single-molecule reads (HiFi reads) using the Circular Consensus Sequencing (CCS) analysis application or perform CCS analysis on-instrument using the Sequel IIe System
- Use the SMRT Link Iso-Seq analysis application 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

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

TECHNICAL DOCUMENTATION AND APPLICATIONS SUPPORT RESOURCES FOR ISO-SEQ LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS

Sample Preparation Literature

- Application Brief: Long-read RNA sequencing - Best Practices (PN BP103-062619)
- Procedure & Checklist – Iso-Seq Express Template Preparation for Sequel and Sequel II Systems (PN 101-763-800)
- Quick Reference Card – Loading and Pre-extension Recommendations for the Sequel System (PN 101-461-600)
- Quick Reference Card – Loading and Pre-extension Recommendations for the Sequel II/IIe Systems PN 101-769-100)
- Overview – Sequel Systems Application Options and Sequencing Recommendations (PN 101-851-300)
- Application Consumable Bundles Purchasing Guide (PN PG100-051320)
- Technical Overview: Iso-Seq Express Library Preparation Using SMRTbell Express Template Prep Kit 2.0 (PN 101-814-400)

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

TECHNICAL DOCUMENTATION AND APPLICATIONS SUPPORT RESOURCES FOR ISO-SEQ LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS (CONT.)

Data Analysis Resources

- SMRT Analysis Barcoding Overview (v9.0) (PN 101-923-200)
  - Contains detailed information on barcoding experimental design options and describes QC metrics for evaluation of barcoding performance using SMRT Link
- PacBio Multiplexing Resources Website: https://www.pacb.com/smrt-science/smrt-sequencing/multiplexing/
  - Barcoding Overview documents for different SMRT Link software versions
  - PacBio barcode sequence files (compressed FASTA) for use with Sequel, Sequel II and Sequel IIe Systems
  - Barcoded oligo ordering sheets

Videos & Webinars

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

TECHNICAL DOCUMENTATION AND APPLICATIONS SUPPORT RESOURCES FOR ISO-SEQ LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS (CONT.)

Posters

- PacBio AGBT 2020 Poster: A complete solution for full-length transcript sequencing using the PacBio Sequel II System
- PacBio PAG 2020 Poster: A complete solution for high-quality genome annotation using the PacBio Iso-Seq method
- PacBio ASHG 2019 Poster: Full-Length RNA-seq of Alzheimer brain on the PacBio Sequel II System

Publications

- Wang, B. et al. (2020) Variant Phasing and Haplotypic Expression from Single-molecule Long-read Sequencing in Maize. Commun Biol. 3:78.
- 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.
- 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.
- Zhang, Xiujuan et al. (2019) Full-length transcriptome analysis of Litopenaeus vannamei reveals transcript variants involved in the innate immune system. Fish Shellfish Immunol. 87:346-359.
- Minio, Andrea et al. (2019) Iso-Seq Allows Genome-Independent Transcriptome Profiling of Grape Berry Development. G3. 9(3):755-767.
- See the PacBio RNA Sequencing Applications website for a list of other publications.

Appendix: RNA Isolation Kit Options for Iso-Seq Express 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 R&D 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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