



PACIFIC
BIOSCIENCES®



Iso-Seq Library Prep



PACBIO®

What is Iso-Seq?

ISO-SEQ: FULL-LENGTH RNA SEQUENCING

Iso-Seq is...

- Full-Length cDNA sequencing – no assembly required
- Targeted or whole transcriptome

Iso-Seq can...

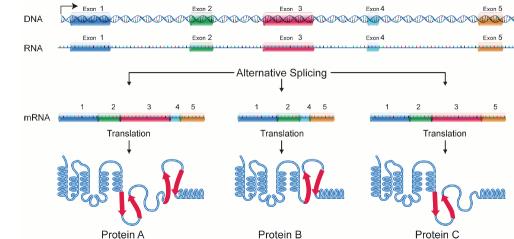
- Discover novel genes and isoforms
- Improve genome annotation, with or without reference genome
- Increase the accuracy of RNA-seq quantification at isoform-level resolution

You can do Iso-Seq with...

- 1-Day Library Prep
- 1 SMRT Cell 8M
- Full bioinformatics solution



RNA SEQUENCING



ANNOTATION



LIBRARY PREP

1 DAY



SMRT SEQUENCING

1 DAY



DATA ANALYSIS

1 DAY



ISO-SEQ EXPRESS WORKFLOW KEY FEATURES & BENEFITS

New streamlined and accelerated workflow for Iso-Seq transcriptome SMRTbell libraries in one day

Iso-Seq Express Workflow Using SMRTbell Express Template Prep Kit 2.0



- **Improved formulation** for reduced Total RNA input requirement at 300 ng
- **Significantly faster workflow** from RNA to SMRTbell library in one day
- **No size-selection required**
- **Minimal handling-induced DNA damage**
- **Capture full-length transcriptomes** in a single SMRT Cell
- **Supports multiplexing up to 12 Iso-Seq library samples** per SMRT Cell

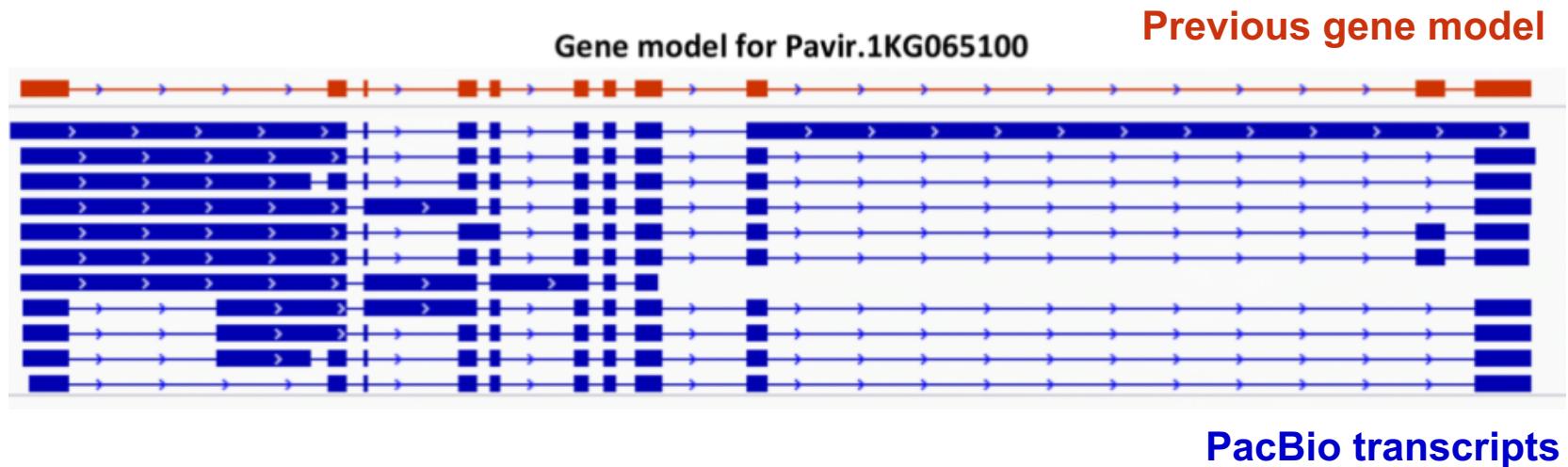
One SMRTbell Express Template Prep Kit 2.0 supports preparation of up to 18 large-insert gDNA and Iso-Seq transcriptome libraries, 48 microbial gDNA libraries or 96 amplicon template preparations



PACBIO®

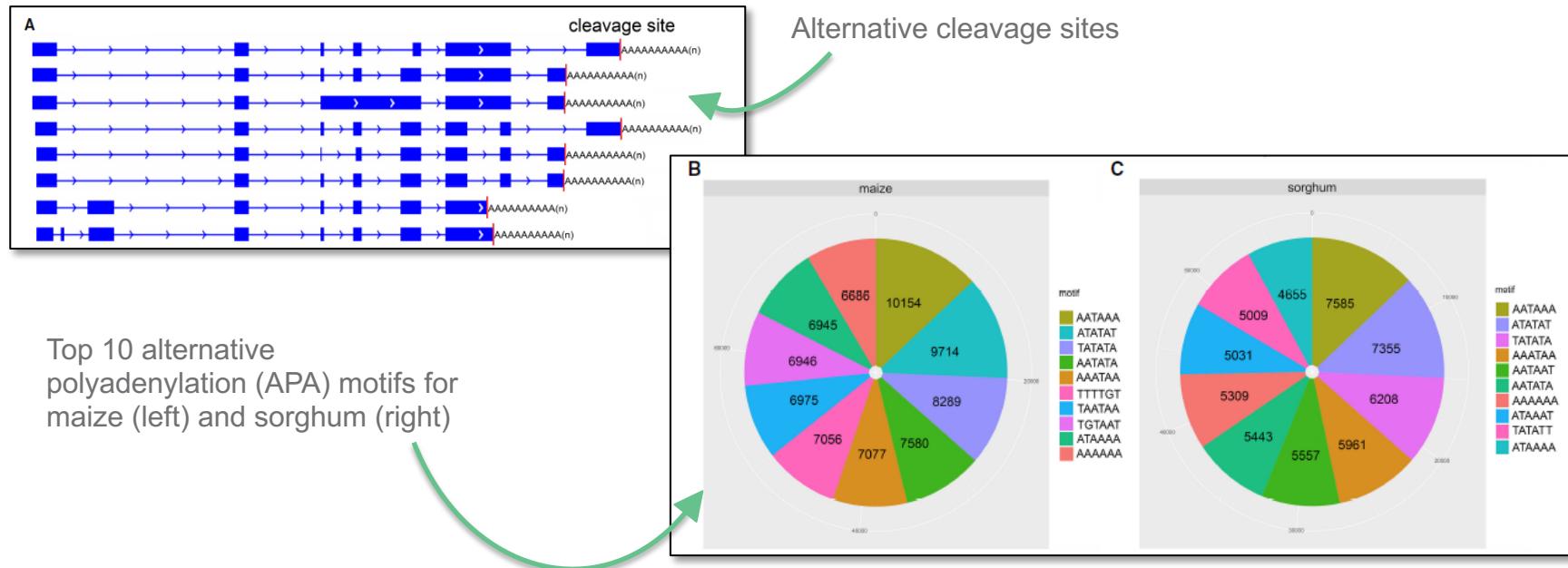
Who is using Iso-Seq?

ACCURATELY CHARACTERIZE TRANSCRIPT DIVERSITY



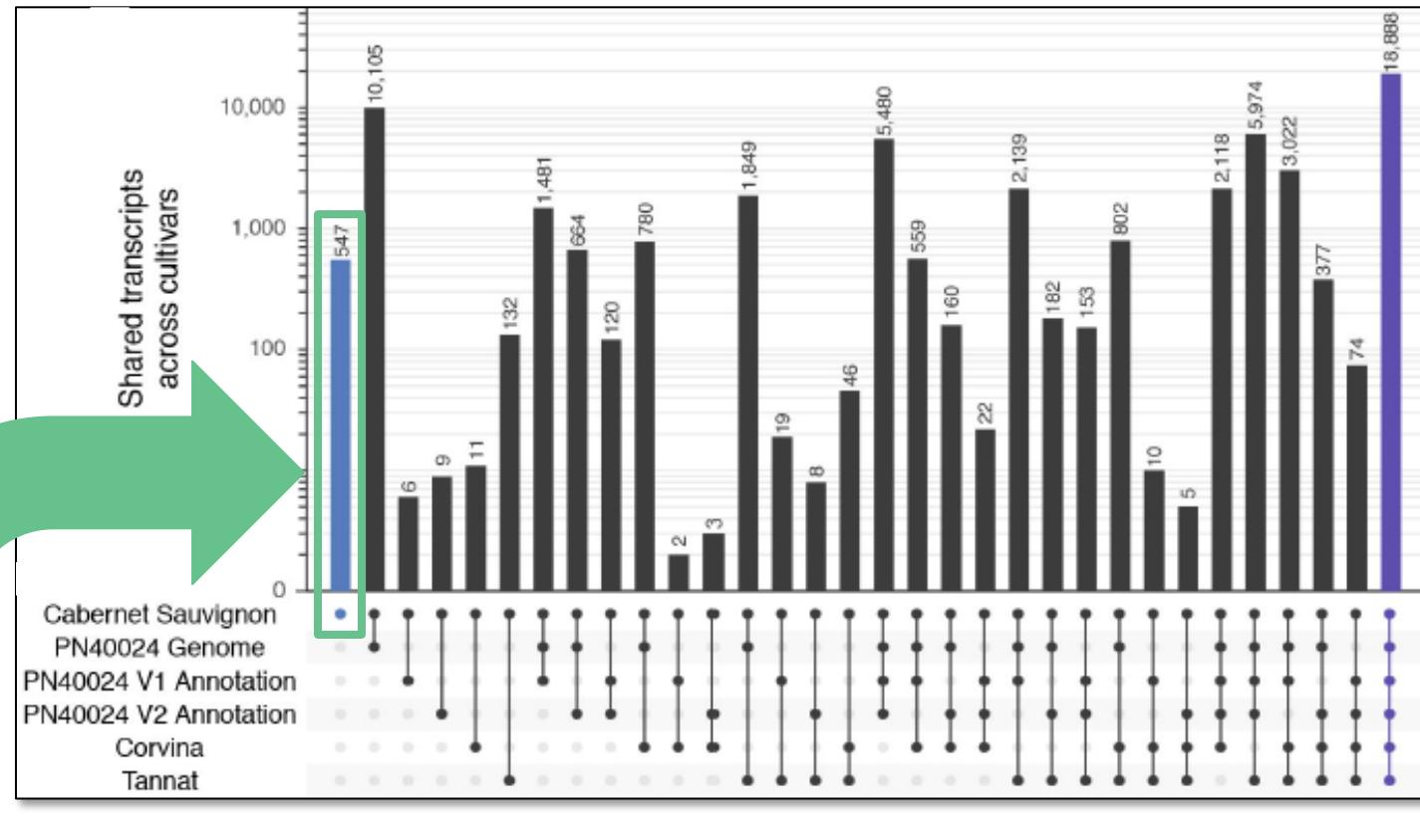
“Reliable identification of full-length transcripts is clearly an **advantage of the PacBio data**, which has enabled us to infer **considerably more alternatively spliced** transcripts than before.”

GAIN POLY-ADENYLATION SITE INFORMATION



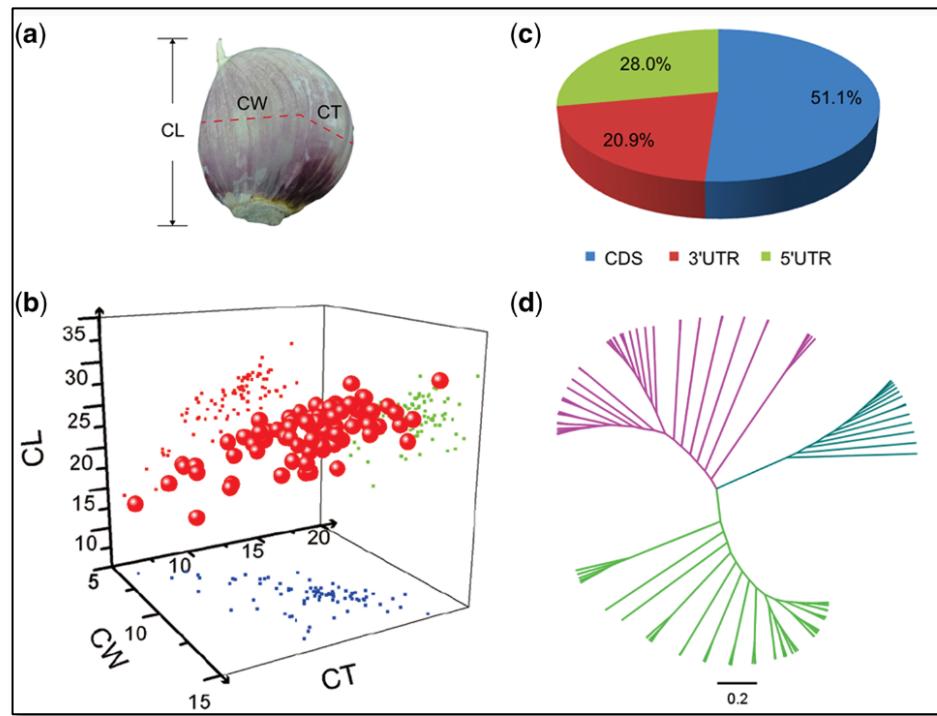
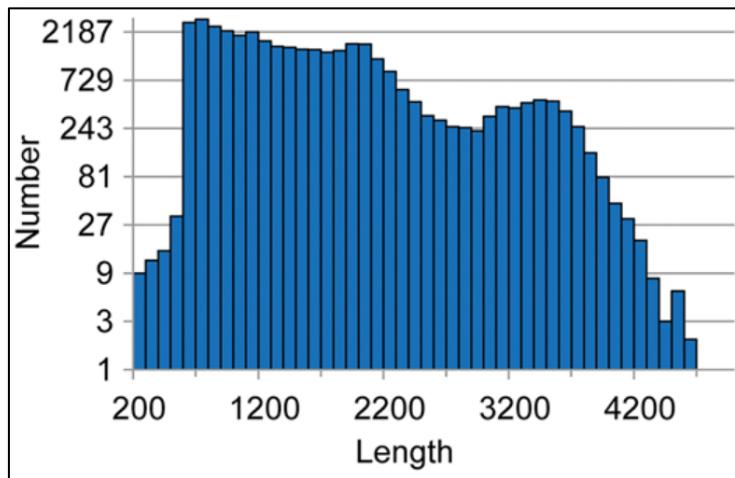
“We generated **comprehensive** and **high-resolution** maps of genome-wide poly(A) sites, allowing **systematic characterization** of the role of APA in... agronomically important species.”

IMPROVE SHORT-READ RNA-SEQ ISOFORM QUANTIFICATION



>500 Cabernet Sauvignon-specific transcripts were found when the transcriptome was compared other grape cultivars

INVESTIGATE TRANSCRIPTOMES WITHOUT REFERENCE GENOMES



"A large number of transcripts in *[previous] transcriptomes were incomplete*...therefore, we used single-molecule long-read sequencing technology for RNA sequencing, which *significantly improved the transcriptome quality*."

ISO-SEQ METHOD: FULL-LENGTH RNA SEQUENCING

Sequence full-length cDNA sequences – from 5' end to the poly-A tail – without the need for transcript reconstruction.

The PacBio Iso-Seq method allows you to:

- Profile whole transcriptomes exhaustively at the isoform level
- Discover novel genes and isoforms
- Identify exon skipping events and alternative 5' / 3' sites
- Characterize function without reference genomes
- Combine with and complement RNA-seq to quantify at isoform-level





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Iso-Seq Express 2.0

Reagent kit configuration and workflow overview

LONG-READ RNA SEQUENCING

—With Iso-Seq Express 2.0 and the Sequel Systems you can:

- Easily and affordably sequence complete transcript isoforms in genes of interest or across the entire transcriptome.
- Generate full-length cDNA sequences up to 15 kb in length — with no assembly required — to confidently characterize full-length transcript isoforms.

—Scaled throughput on the Sequel Systems:

- Use the Sequel II System to generate up to 4 million full-length, non-concatemer (FLNC) reads per SMRT Cell 8M
- Use the Sequel System to generate up to 500,000 FLNC reads per SMRT Cell 1M

With a single SMRT Cell 8M you can:

- Characterize a whole transcriptome
- Multiplex multiple tissues for genome annotation



NEW STREAMLINED AND ACCELERATED WORKFLOW FOR ISO-SEQ TRANSCRIPTOME LIBRARIES IN ONE DAY



Iso-Seq Express Workflow

- **Improved formulation** for reduced input requirement at 300 ng
- **Significantly faster workflow** from RNA to SMRTbell library in one day.
- **Minimal handling-induced cDNA damage**
- **Capture full-length transcriptomes** in a single SMRT Cell
- **Supports multiplexing up to 12 samples**
- **No size-selection required**

ISO-SEQ EXPRESS OLIGO KIT CONFIGURATION



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

Accessory kit contains **Iso-Seq Express Template Switching Oligo** and **cDNA PCR Primer** to be used in conjunction with:

NEBNext Single Cell/Low Input RNA Library Prep Kit (E6420)

ISO-SEQ EXPRESS WORKFLOW SUMMARY OVERVIEW



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 for sequencing on a single SMRT Cell
- Purify amplified samples with ProNex Beads followed by equimolar pooling

~ 3.5 h



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 $\geq 50\%$

~ 4 h



4. Sample A/B/C & Sequence

- Anneal v4 Primer, Bind Polymerase, and perform ProNex Bead Complex Cleanup
- # of SMRT Cells per library prep: >3 Sequel SMRT Cell 1M; >1 Sequel II SMRT Cell 8M



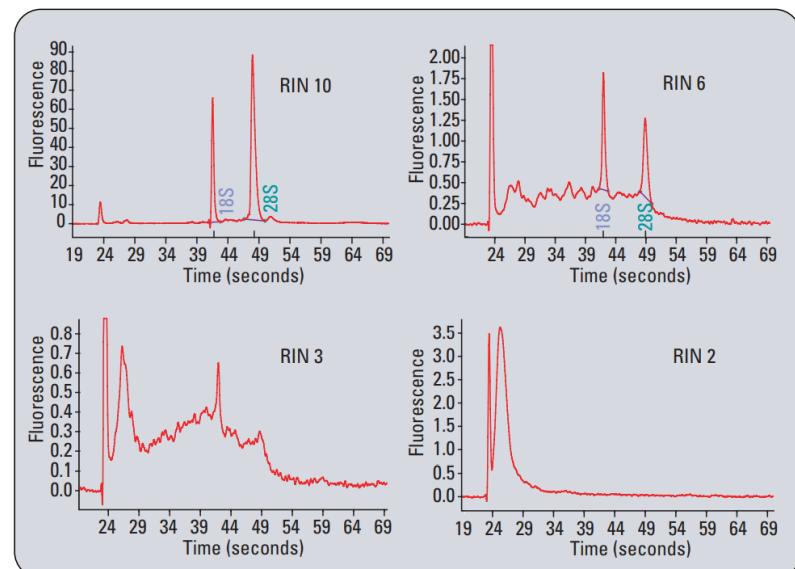
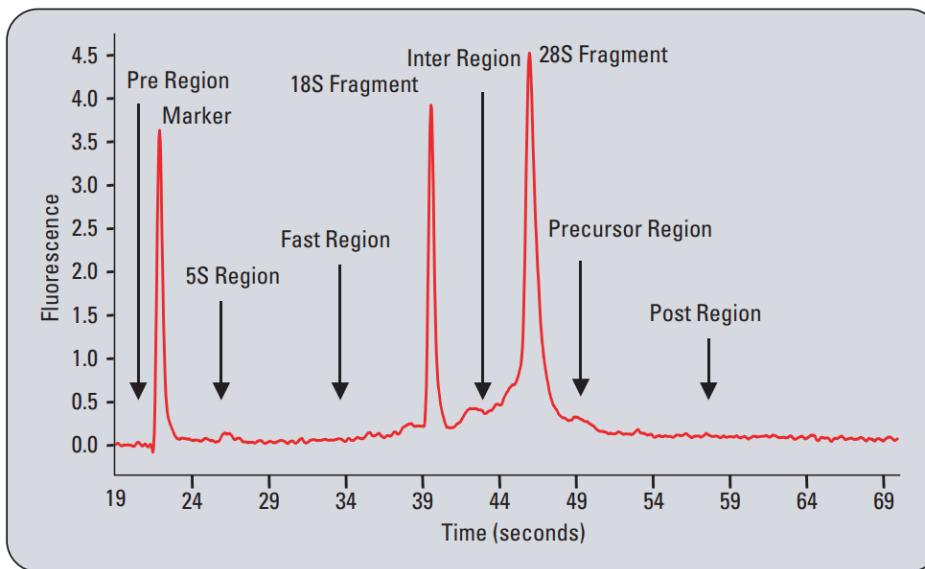
5. Analyze

- Use the Iso-Seq analysis application in SMRT Link v7.0 GUI to output high-quality, full-length transcript FASTA sequences, with no assembly required, to characterize transcripts and splice variants

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.

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 and 2.0 (or higher) and a A260/A230 between 2.0 and 2.5.



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

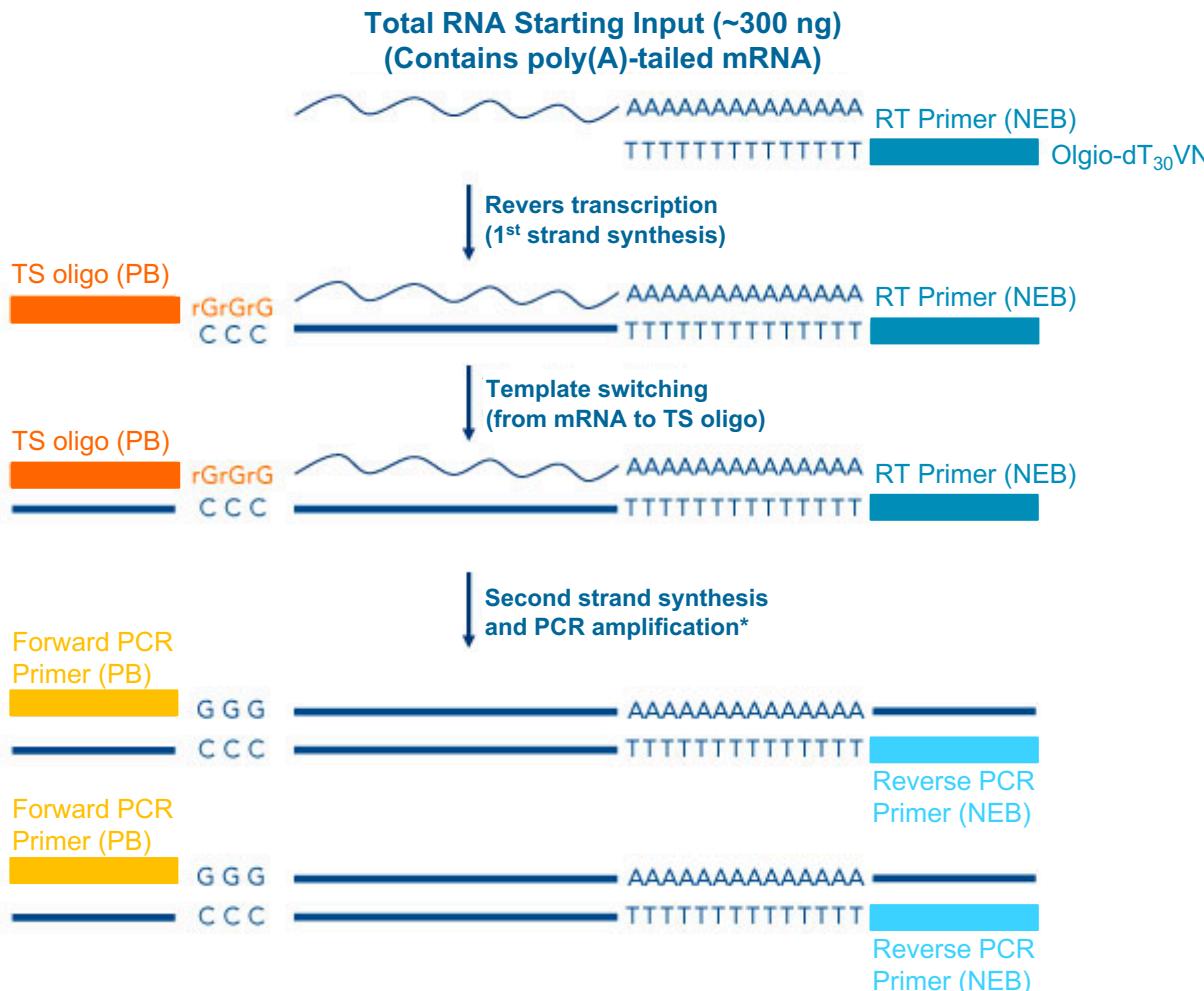
Iso-Seq Express Use Case Recommendations for Sequel and Sequel II Systems

SEQUEL SYSTEM	SEQUEL II SYSTEM
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

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



*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.

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 12 pairs of barcoded primers supported by PacBio and they are listed in **Appendix 2** of the protocol
- Barcoded forward and reverse primers may be ordered from any oligo synthesis company
- 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	CACATATCAGAGTGGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1001-R	CACATATCAGAGTGGCAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1002-F	ACACACAGACTGTGAGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1002-R	ACACACAGACTGTGAGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1003-F	ACACATCTGTGAGAGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1003-R	ACACATCTGTGAGAGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1004-F	CACGCCACACCGCGGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1004-R	CACGCCACACCGCGGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1005-F	CACTCGACTCTCGCGTGAATGAAGTCGCAGGGTTG	25nm	STD
bc1005-R	CACTCGACTCTCGCGTAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1006-F	CATATATATCAGCTGTGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1006-R	CATATATATCAGCTGTGAAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1008-F	ACAGTCGAGCGCTGGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1008-R	ACAGTCGAGCGCTGGGAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1012-F	ACACTAGATCGCGTGTGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1012-R	ACACTAGATCGCGTGTGAAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1018-F	TCACGTGCTCACTGTGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1018-R	TCACGTGCTCACTGTGAAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1019-F	ACACACTTATCAGATGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1019-R	ACACACTTATCAGATAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1020-F	CACGACACGACGATGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1020-R	CACGACACGACGATGCAAGCAGTGGTATCAACGCAGAGT	25nm	STD
bc1023-F	CAGAGAGATATCTGGCAATGAAGTCGCAGGGTTG	25nm	STD
bc1023-R	CAGAGAGATATCTGAAGCAGTGGTATCAACGCAGAGT	25nm	STD

Oligo Order Sheet for 12 Barcoded Iso-Seq primers:

https://www.pacb.com/wp-content/uploads/IsoSeq_Primers_12_Barcodes_v1_Ordering_Sheet.xlsx

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.

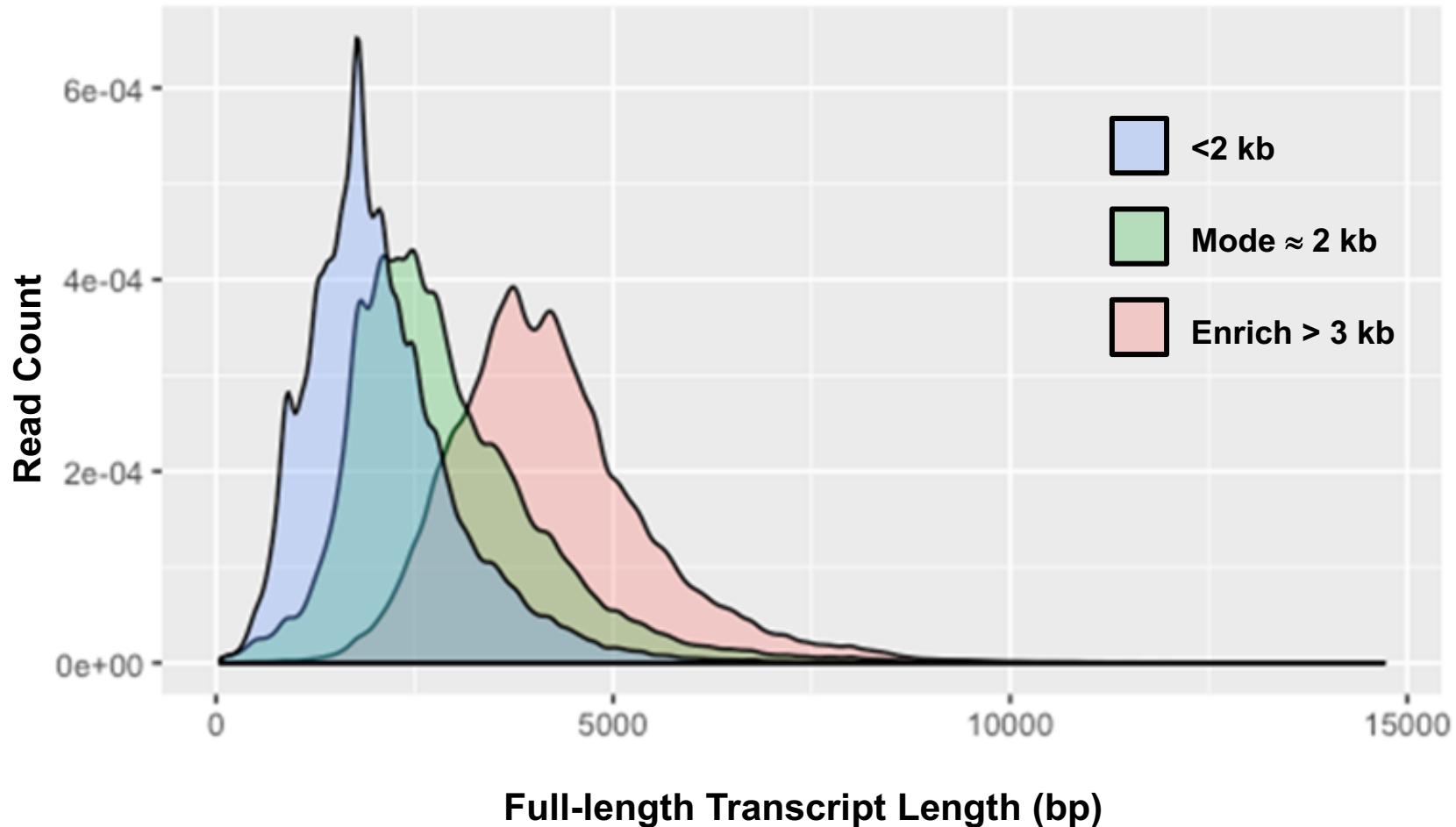
- Use **Pronex Beads** for purification of amplified cDNA products according to the table below:

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



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:

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

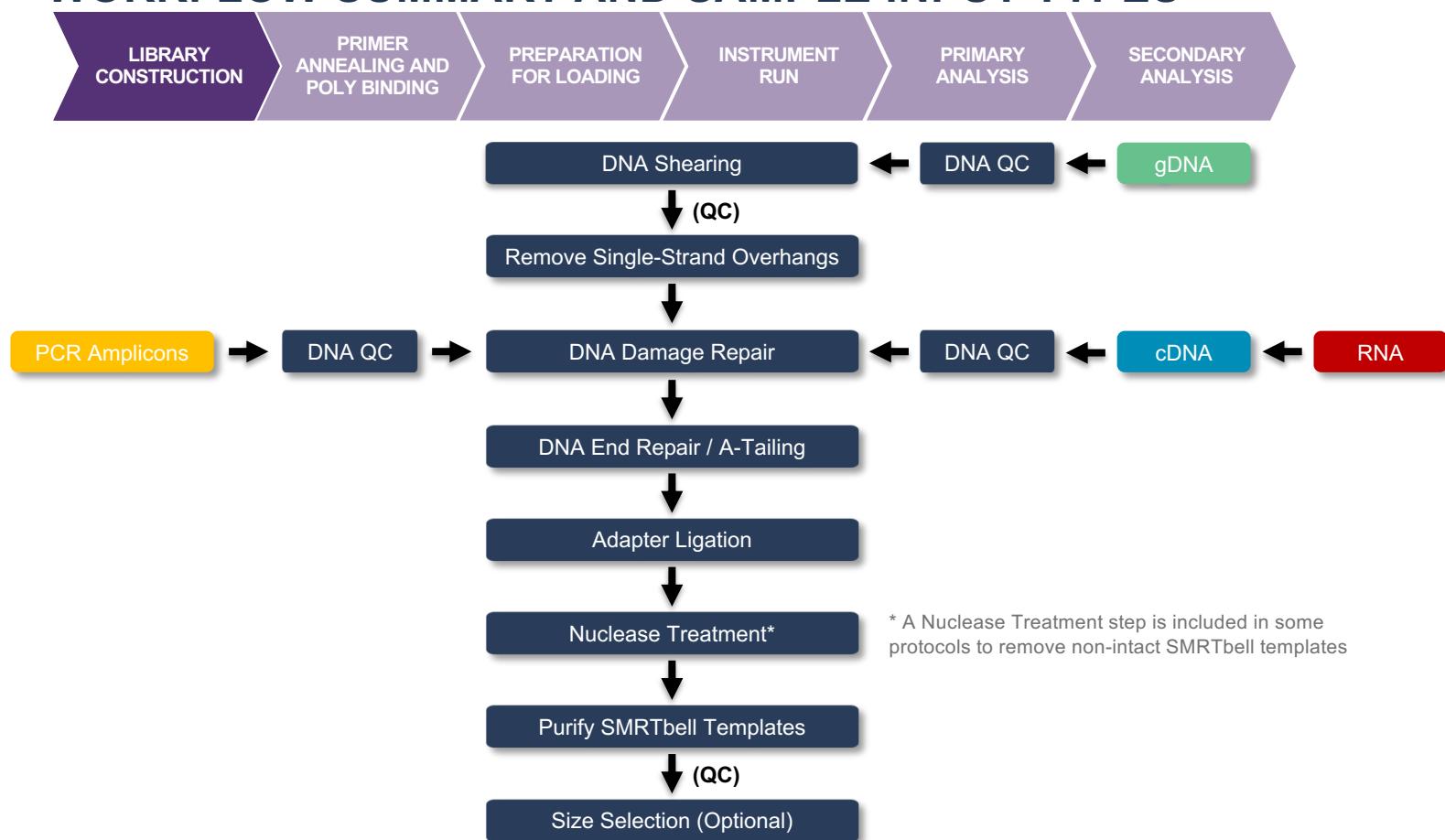
*To determine the average library size using a Bioanalyzer System, select the region of interest by defining the start of the smear at 200 bp and the end point at 9500 bp (when using a High Sensitivity DNA assay kit).

2. Pool equal molar quantities of the barcoded cDNA.

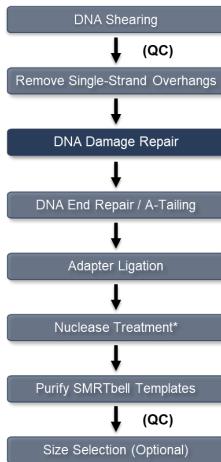
- Use the maximum total combined mass possible without exceeding 500 ng in 47.4 μL .
- The total combined mass must be >80 ng for Sequel and >160 ng for Sequel II to proceed to DNA Damage Repair.
- If the volume required to achieve the minimum mass of the pooled cDNA exceeds 47.4 μL , concentrate the 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 Sequel and ≥ 200 ng for Sequel II.

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

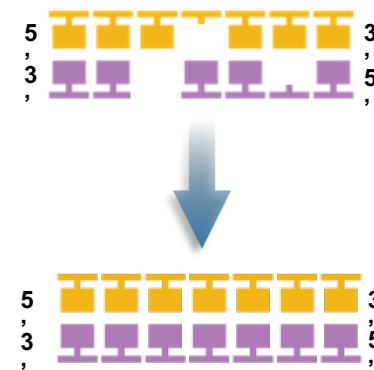
SMRTBELL EXPRESS LIBRARY CONSTRUCTION WORKFLOW SUMMARY AND SAMPLE INPUT TYPES



SMRTBELL LIBRARY CONSTRUCTION: DNA DAMAGE REPAIR



- Recommended for all library insert sizes
- DNA Damage Repair enzymes and buffer are included in the SMRTbell Express Template Prep Kit 2.0
- Repairs abasic sites, nicks, thymine dimers, blocked 3'-ends, oxidized guanines / pyrimidines, deaminated cytosine

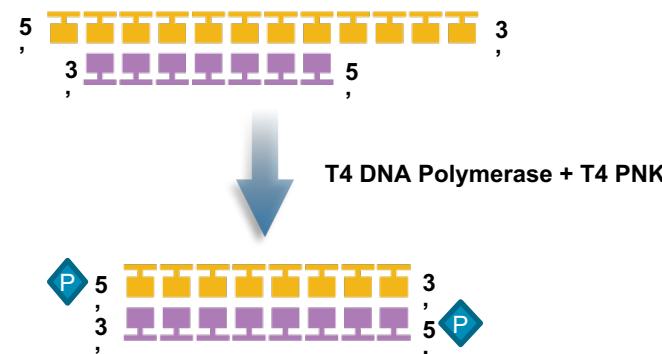


SMRTBELL LIBRARY CONSTRUCTION: DNA END REPAIR / A-TAILING



1. DNA End Repair reaction polishes ends of fragments prior to the A-Tailing reaction:

- 5' overhangs are filled-in by T4 DNA Polymerase
- 3' overhangs are removed by T4 DNA Polymerase
- T4 PNK phosphorylates the 5' hydroxyl group



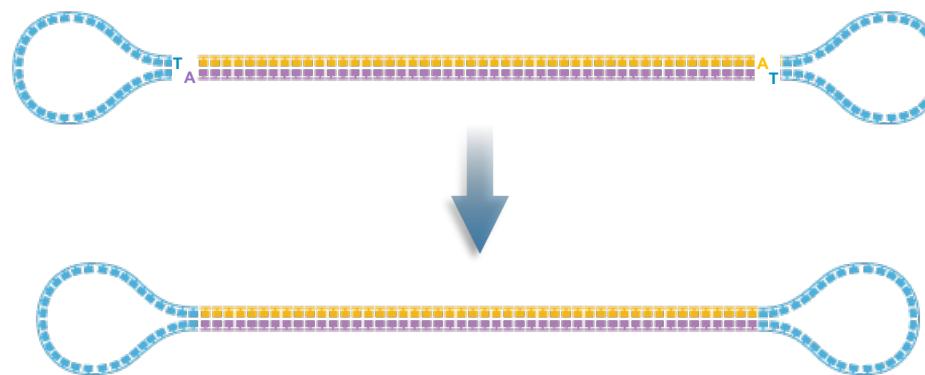
2. After DNA End Repair, an A-Tailing reaction is performed to generate single-nucleotide overhangs (A-tail):



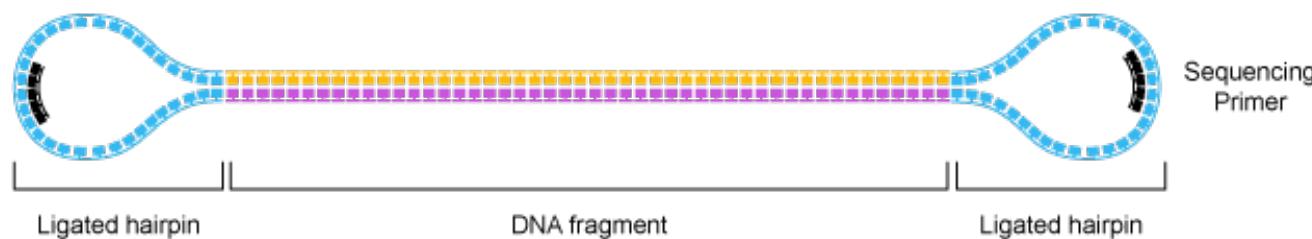
SMRTBELL LIBRARY CONSTRUCTION: ADAPTER LIGATION



- SMRTbell hairpin overhang adapters are ligated to repaired and A-tailed dsDNA ends
- Pre-annealed hairpin overhang adapters are included in the SMRTbell Express Template Prep Kit 2.0
- Overhang adapter ligation reaction incubation time is typically 1 hour



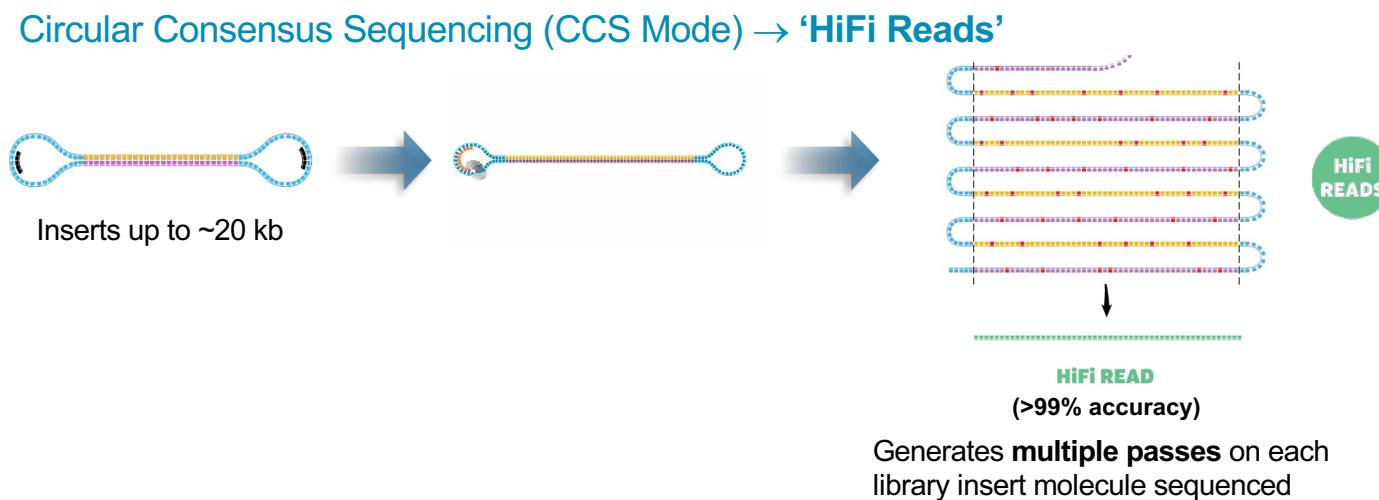
DNA SMRTBELL LIBRARY TEMPLATE STRUCTURE



SMRTbell Template:

- Structurally linear
- Topologically circular
- Structural homogeneity of templates
- Provides sequences of both forward and reverse strands in the same sequence

DNA SMRTBELL LIBRARY INSERT SIZE DETERMINES THE NUMBER OF TEMPLATE PASSES GENERATED DURING SEQUENCING



<https://www.pacb.com/wp-content/uploads/2015/09/Pacific-Biosciences-Glossary-of-Terms.pdf>

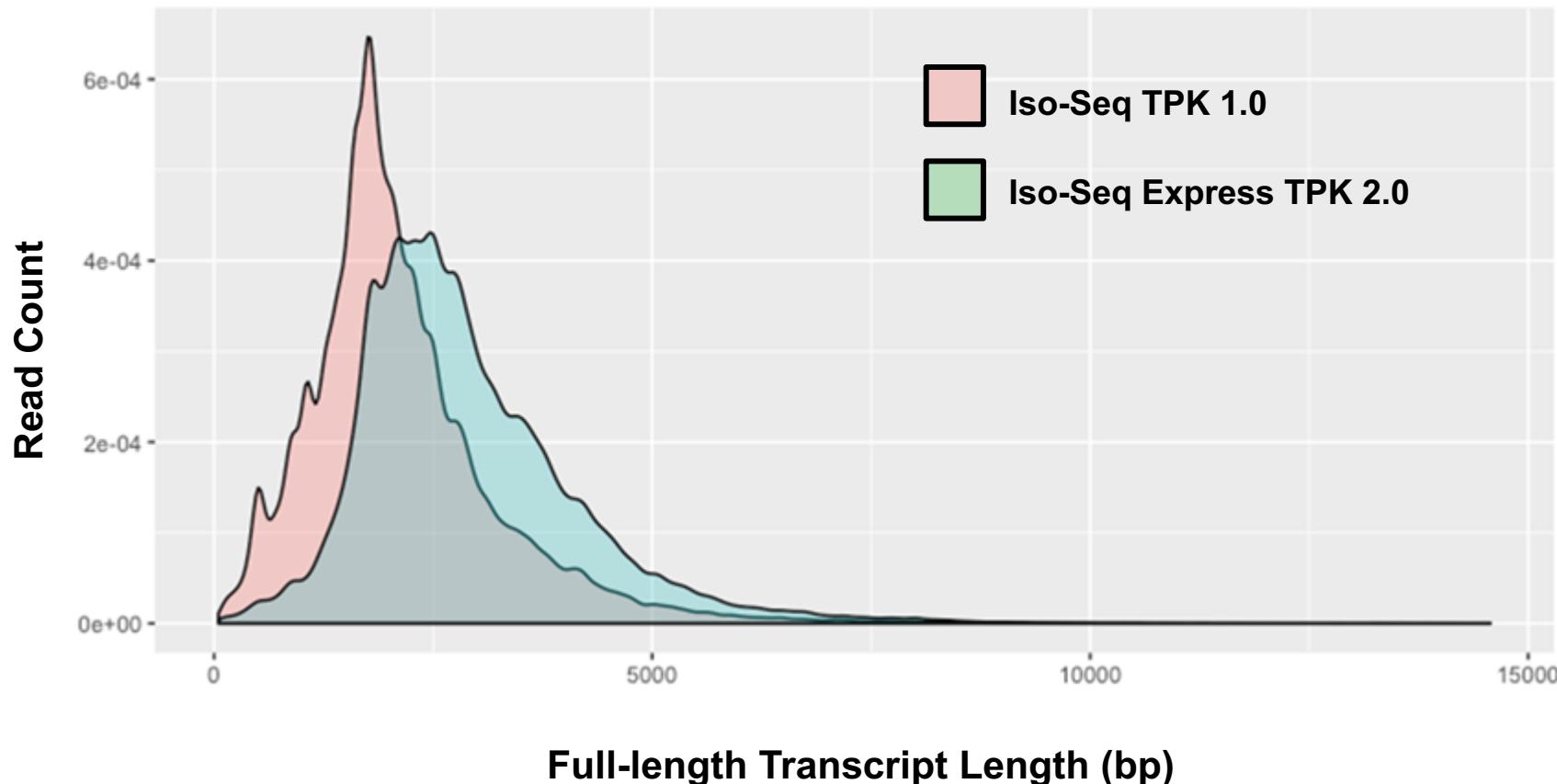


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Iso-Seq Express Library Example Sequencing Performance Data

FULL-LENGTH TRANSCRIPT SIZE DISTRIBUTION COMPARISON FOR ISO-SEQ TPK 1.0 LIBRARY VERSUS ISO-SEQ EXPRESS TPK 2.0 LIBRARY (SEQUEL SYSTEM CHEMISTRY 3.0)

Standard (2 kb) Iso-Seq Express TPK 2.0 workflow using ProNex Bead purification generated a higher proportion of longer full-length transcripts compared to Iso-Seq TPK 1.0 libraries using AMPure PB Bead purification



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%

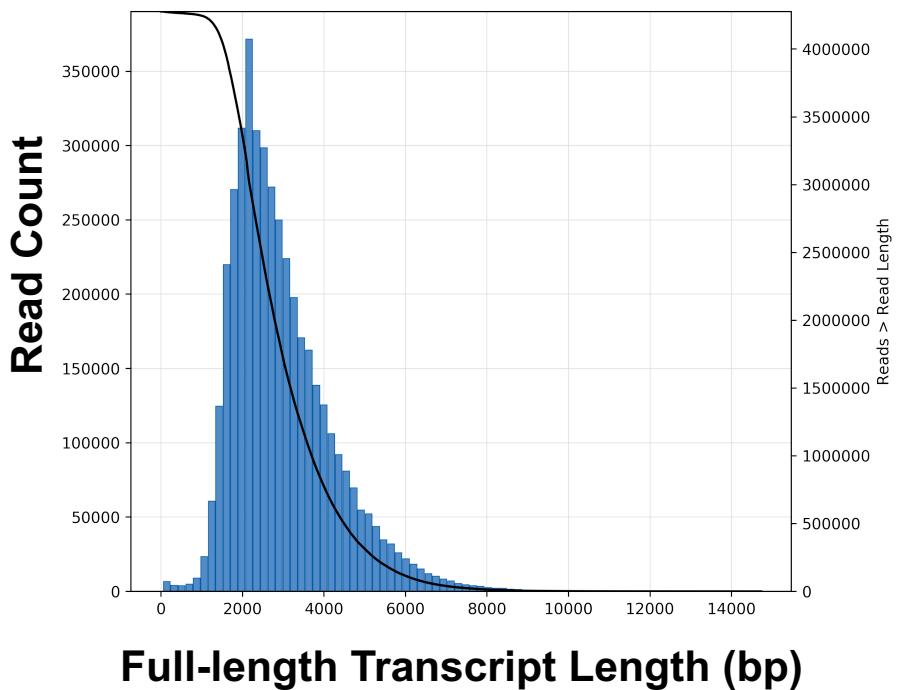
¹ UHR (Universal Human Reference RNA)

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-concatamer reads generated on a **single** Sequel II System SMRT Cell 8M (85% yield from total reads generated)



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What can you do with one SMRT Cell?

What Can You Do with One SMRT Cell?



RNA
SEQUENCING

Whole Transcriptome: Characterize alternative splicing with full-length transcripts

Genome Annotation: Sequence full-length transcripts and multiplex up to 8 tissues

Efficient Workflows



LIBRARY
PREP

1 DAY



SMRT
SEQUENCING

1 DAY

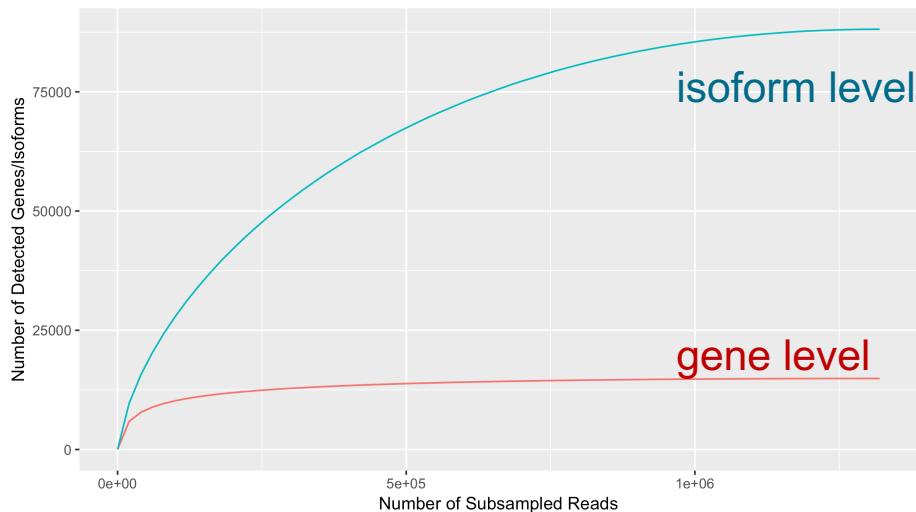


DATA
ANALYSIS

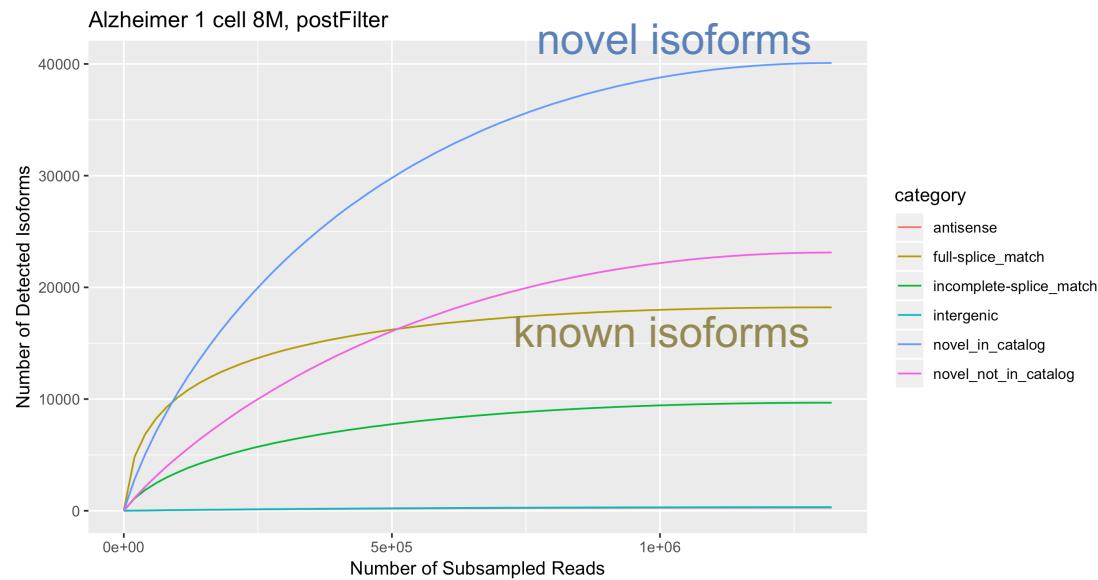
1 DAY

1 SMRT CELL 8M SATURATES KNOWN GENES & ISOFORMS

Alzheimer 1 cell 8M, postFilter

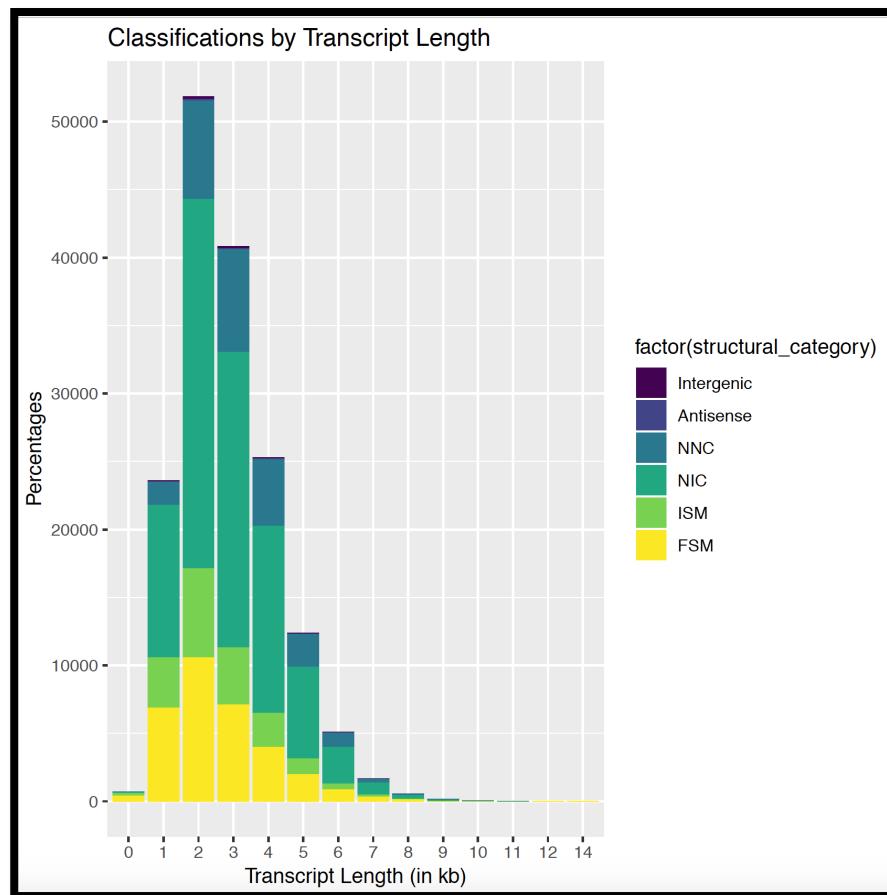


Alzheimer 1 cell 8M, postFilter



1 SMRT CELL 8M DETECTS SHORT & LONG TRANSCRIPTS

Alzheimer brain, 1 SMRT Cell 8M using
Iso-Seq Express kit



163,815 transcripts

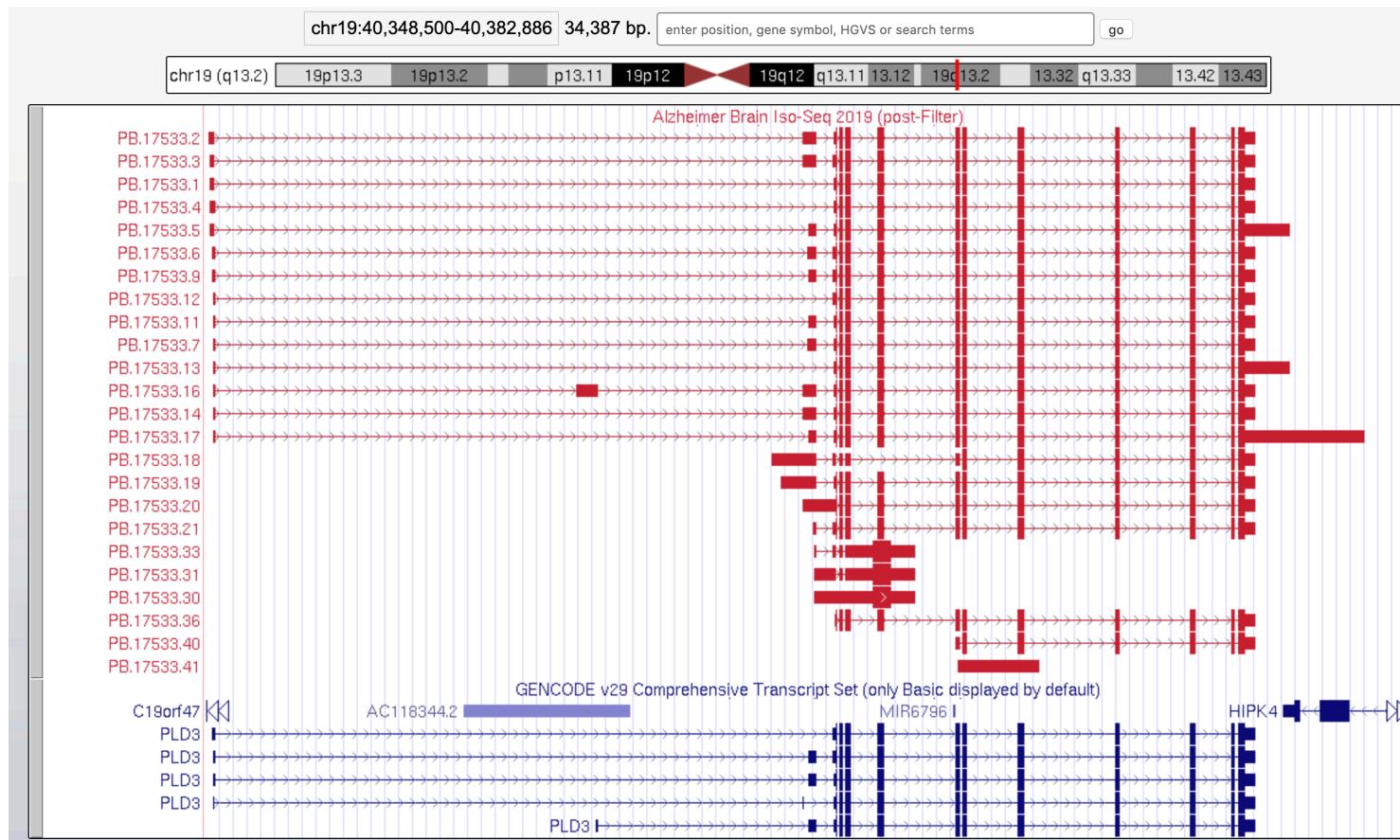
Min: 80 bp

Max: 14,288 bp

Mean: 3,351 bp

1 SMRT CELL 8M DETECTS KNOWN & NOVEL TRANSCRIPTS

Alzheimer brain, 1 SMRT cell 8M
Post-processed using [SQANTI2](#)



PLANNING YOUR HUMAN TRANSCRIPTOME PROJECT

WORKFLOW STEP	DETAILS	COST
 SAMPLE PREP	Prepare full-length transcripts from 300 ng of total RNA	NEBNext® kit – \$27
 LIBRARY PREP	Use the SMRTbell Express Template Prep Kit 2.0 to prepare libraries in 1 day	Express Template Prep Kit 2.0 – \$85.64
 SMRT SEQUENCING	Sequence 1 SMRT Cell 8M per sample on Sequel II System. 24-hour movie with 2-hour pre-extension	1 SMRT Cell 8M – \$1,203.98 SMRT Cell run reagents/cons. – \$22.99
 DATA ANALYSIS	Generate up to 4 million full-length, non-concatemer reads per SMRT Cell 8M Fully-supported Iso-Seq Analysis in SMRT Link	Software license cost - \$0

PacBio consumables: \$1312.61 for 1 SMRT Cell 8M per human transcriptome



PACBIO®

Where can I learn more?

APPLICATIONS

RNA SEQUENCING



<https://www.pacb.com/applications/rna-sequencing/>

RNA SEQUENCING

Human RNA Sequencing

RNA Sequencing for Plant
and Animal Sciences

BRING CERTAINTY TO ISOFORM DETECTION

Changes in isoform variants and relative abundances are an important clue in deciphering how genomic variants drive the phenotypic differences between health and disease. Only long-read sequencing can provide unbiased, direct detection of all the isoforms present in your sample.

OBTAI^N A MORE COMPREHENSIVE SET OF TRANSCRIPT ISOFORMS

The PacBio Isoform Sequence (Iso-Seq) method generates full-length cDNA sequences – from 5' to the poly-A tail – to confidently characterize the full transcriptome.

LONG-READ RNA SEQUENCING: BEST PRACTICE GUIDE



- Use the SMRTbell® Express Template Prep Kit 2.0 to prepare libraries in one day

Procedure & Checklist - Iso-Seq Express Template Preparation for Sequel and Sequel II Systems.

Application Brief: Learn more about these best practices for long-read RNA sequencing

BEST PRACTICES: LONG READ RNA SEQUENCING (ISO-SEQ ANALYSIS) (SEQUEL AND SEQUEL II SYSTEMS)



LIBRARY PREP



**SMRT
SEQUENCING**



DATA 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 or Sequel II System (CCS Sequencing Mode)

- Maximize output and turn-around-time with adjustable sequencing parameters
 - Sequel System: Diffusion loading, 20 hour movies with 4 hours pre-extension is recommended
 - Sequel II System: Diffusion loading, 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 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

- Generate highly accurate long reads (HiFi reads), with single-molecule resolution using circular consensus sequencing (CCS) mode
- Use the Iso-Seq analysis 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 [SQANTI2](#), [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.

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

- Qiagen RNeasy Plus Kits
 - <https://www.qiagen.com/ca/shop/sample-technologies/rna/total-rna/rneasy-plus-micro-and-mini-kits/#productdetails>
- Ambion Poly(A) PuristTM MAG Kit
 - <https://www.thermofisher.com/order/catalog/product/AM1922>
- Sigma Spectrum Plant Total RNA Kit
 - <https://www.sigmaaldrich.com/life-science/molecular-biology/plant-biotechnology/plant-molecular-biology/product-highlights/spectrum-plant-total-rna-kit.html>
- iNtRON Easy Spin Total RNA
 - https://intronbio.com:6001/intronbioen/product/product_view.php?PRDT_ID=28
- TRIzol™ Reagent can be used to isolate total RNA from tissues or cells, including lipid-rich and difficult samples
 - <https://www.thermofisher.com/ca/en/home/brands/product-brand/trizol.html>
- RNALater is an aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA
 - <https://www.thermofisher.com/ca/en/home/brands/product-brand/rnalater.html>

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.

ACCESS ISO-SEQ SEQUEL II DATA

Universal Human Reference (UHRR) 2 SMRT Cells 8M

SAMPLE

Universal Human Reference RNA (Agilent) + SIRV Isoform Mix E0 (Lexogen)

METHODS

- Library prep Iso-Seq Template Preparation for Sequel Systems (PN 101-070-200)
- Sequencing Sequel System II with "Early Access" binding kit (101-490-800) and chemistry (101-490-900)
- Run time:
 - 4 hrs. pre-extension
 - 15 hrs. run time per SMRT Cell
- Reference hs37d5 (GRCh37 with decoy)
- Analysis SMRTLink 7.0 "IsoSeq With Mapping protocol" with hg38+SIRV combined reference genome

FILES

Users are encouraged to work with the mapped, unique transcripts in GFF and FASTQ format provided in the "PolishedMappedTranscripts" subfolder. The two raw movie files are in "subreads" subfolder. The intermediate data, full-length reads, are available in "FullLengthReads" folder.

```

PolishedMappedTranscripts/
  - out.abundance.txt
  - out.fasta
  - out.gff

FullLengthReads/
  - flnc.bam

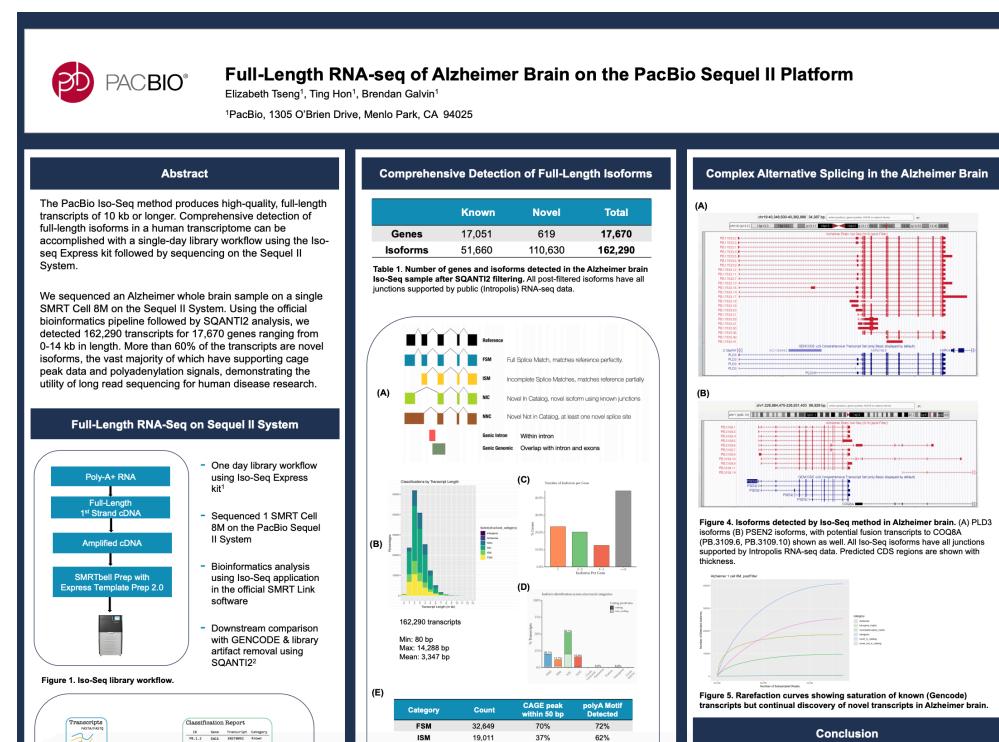
Subreads/
  - m64012_181221_231243.subreads.bam
  - m64012_181221_231243.subreads.bam.pbi
  - m64012_181222_192540.subreads.bam
  - m64012_181222_192540.subreads.bam.pbi

```

Download

Download from China: https://downloads-ap.pacbcloud.com/public/dataset/UHR_IsoSeq/

Alzheimer Whole Brain 1 SMRT Cell 8M (data release pending)



[AGBT-PH2019 poster](#)
[Link to data](#)

[Link to data](#)



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