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 Ile System ICS v10.0 / Sequel II Chemistry 2.0 / SMRT Link v10.0
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

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 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*

* 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.

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*
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 Ile 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.

**APPLICATIONS**

**RNA SEQUENCING**

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

[Link to documentation](https://www.pacb.com/support/documentation/)
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

**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

**SMRTbell Library Construction (4 hrs)**
- *Procedure & Checklist – Iso-Seq Express Template Preparation for Sequel and Sequel II Systems* (PN 101-763-800)
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.

A RIN \( \geq 7.0 \) (ideally \( \geq 8.0 \)) 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 – 2.0 (or higher) and a A260/A230 between 2.0 – 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, Sequel II and Sequel IIe Systems

<table>
<thead>
<tr>
<th>SEQUEL SYSTEM</th>
<th>SEQUEL II AND IIe 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 8-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 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 Ile 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
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
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**

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

*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

### List of Temperature-Sensitive Reagents Included in PacBio Iso-Seq Express Oligo Kit and Smrtbell Express TPK 2.0

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

<table>
<thead>
<tr>
<th>Tube Image #</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
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**

**Total RNA Starting Input (~300 ng)** (Contains poly(A)-tailed mRNA)

- **Revers transcription (1st strand synthesis)**
  - **RT Primer (NEB)**
  - **Oligo-dT<sub>30</sub>VN**

- **Template switching (from mRNA to TS oligo)**
  - **rG<sub>4</sub>rG<sub>3</sub>C<sub>3</sub>C<sub>3</sub>**
  - **TS oligo (PB)**

- **Second strand synthesis and PCR amplification**

**Forward PCR Primer (PB)**

- **G G G**
- **C C C**

**Reverse PCR Primer (NEB)**

- **G G G**
- **C C C**

* 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)

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Scale</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>bc1001-F</td>
<td>CACATATCAAGTGCCGGCAATGAAGTGCCAGGGTTG</td>
<td>25nm</td>
<td>STD</td>
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<tr>
<td>bc1001-R</td>
<td>ACACACAGACTGGAGAGCAGATGTTGTAACAGCAGAGT</td>
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<td>STD</td>
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<tr>
<td>bc1002-F</td>
<td>ACACACAGACTGGAGAGCAGATGTTGTAACAGCAGAGT</td>
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<td>STD</td>
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<td>bc1002-R</td>
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<td>STD</td>
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<tr>
<td>bc1003-F</td>
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<td>STD</td>
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<tr>
<td>bc1003-R</td>
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<td>STD</td>
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<td>bc1006-R</td>
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<td>bc1012-R</td>
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<td>bc1018-F</td>
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<td>bc1023-R</td>
<td>CAGAGAGATATACCTGGGCAATGAAGTGCCAGGGTTG</td>
<td>25nm</td>
<td>STD</td>
</tr>
</tbody>
</table>

Appendix 2: Recommended Barcoded NEBNext Single Cell cDNA PCR Primer and Iso-Seq Express cDNA PCR Primer Sequences

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:

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

<table>
<thead>
<tr>
<th>PacBio Instrument</th>
<th>Min. cDNA Amount for 1 sample</th>
<th>Min. cDNA Amount for Multiplexed Sample</th>
<th>Recommendation for Samples with Low Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequel System</td>
<td>80-500 ng</td>
<td>80-500* ng</td>
<td>If total mass is &lt;80 ng (&lt;1.75 ng/μL) → Go to Appendix 1</td>
</tr>
<tr>
<td>Sequel II/Ile System</td>
<td>160-500 ng</td>
<td>160-500* ng</td>
<td>If total mass is &lt;160 ng (&lt;3.5 ng/μL) → Go to Appendix 1</td>
</tr>
</tbody>
</table>

* 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/Ile Systems require different amounts (ng) of cDNA for SMRTbell library construction. The Sequel System requires >80 ng of DNA, while the Sequel II/Ile 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:

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

*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/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
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.

* PacBio recommends Sequel Binding Kit 3.0 for all Iso-Seq Express workflows (Short, Standard, and Long)
SAMPLE SETUP RECOMMENDATIONS FOR ISO-SEQ EXPRESS LIBRARIES – SEQUEL II AND Ile 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/Ile 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.

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.
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)

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Sample Description¹</th>
<th>Protocol²</th>
<th>Raw Polymerase Read Bases (Gb)</th>
<th># CCS Reads</th>
<th>% FLNC³ Reads</th>
<th>FLNC Mean Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UHR Standard</td>
<td>Standard</td>
<td>35.06</td>
<td>598,345</td>
<td>85%</td>
<td>2895</td>
</tr>
<tr>
<td>2</td>
<td>UHR Standard</td>
<td>Standard</td>
<td>35.17</td>
<td>553,037</td>
<td>87%</td>
<td>2879</td>
</tr>
<tr>
<td>3</td>
<td>UHR Standard</td>
<td>Standard</td>
<td>37.19</td>
<td>581,159</td>
<td>84%</td>
<td>2811</td>
</tr>
<tr>
<td>4</td>
<td>UHR Standard</td>
<td>Standard</td>
<td>39.91</td>
<td>530,629</td>
<td>86%</td>
<td>2850</td>
</tr>
<tr>
<td>5</td>
<td>Normal Brain</td>
<td>Standard</td>
<td>32.55</td>
<td>595,550</td>
<td>83%</td>
<td>2751</td>
</tr>
<tr>
<td>6</td>
<td>Alzheimer’s</td>
<td>Standard</td>
<td>36.96</td>
<td>509,404</td>
<td>87%</td>
<td>2565</td>
</tr>
<tr>
<td>7</td>
<td>UHR Long</td>
<td>Long</td>
<td>33.96</td>
<td>550,365</td>
<td>85%</td>
<td>3744</td>
</tr>
<tr>
<td>8</td>
<td>UHR Short</td>
<td>Short</td>
<td>41.02</td>
<td>612,392</td>
<td>84%</td>
<td>2366</td>
</tr>
<tr>
<td>9</td>
<td>UHR Barcoded (2-plex)</td>
<td>Barcoded</td>
<td>40.57</td>
<td>595,290</td>
<td>76%⁴</td>
<td>2839</td>
</tr>
</tbody>
</table>

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)

<table>
<thead>
<tr>
<th>Replicate #</th>
<th>Sample Description</th>
<th>Raw Polymerase Read Bases (Gb)</th>
<th># CCS Reads</th>
<th># FLNC(^2) Reads</th>
<th>% FLNC Reads</th>
<th>FLNC Mean Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UHR</td>
<td>314.02</td>
<td>4,770,143</td>
<td>4,009,124</td>
<td>84%</td>
<td>3411</td>
</tr>
<tr>
<td>2</td>
<td>UHR</td>
<td>270.06</td>
<td>4,389,007</td>
<td>3,505,161</td>
<td>80%</td>
<td>3396</td>
</tr>
<tr>
<td>3</td>
<td>UHR</td>
<td>361.13</td>
<td>5,055,974</td>
<td>4,300,398</td>
<td>85%</td>
<td>3913</td>
</tr>
<tr>
<td>4</td>
<td>UHR</td>
<td>298.80</td>
<td>4,377,647</td>
<td>3,663,342</td>
<td>84%</td>
<td>3470</td>
</tr>
<tr>
<td>5</td>
<td>UHR</td>
<td>223.42</td>
<td>3,263,340</td>
<td>2,725,148</td>
<td>84%</td>
<td>3303</td>
</tr>
<tr>
<td>6</td>
<td>UHR</td>
<td>290.86</td>
<td>4,326,935</td>
<td>3,639,004</td>
<td>84%</td>
<td>3495</td>
</tr>
<tr>
<td>7</td>
<td>UHR</td>
<td>287.24</td>
<td>3,815,759</td>
<td>3,285,200</td>
<td>86%</td>
<td>3826</td>
</tr>
<tr>
<td>8</td>
<td>UHR</td>
<td>258.47</td>
<td>3,902,038</td>
<td>3,179,275</td>
<td>81%</td>
<td>3470</td>
</tr>
</tbody>
</table>

**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)

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Sample Description¹</th>
<th>Protocol²</th>
<th># FLNC³ Reads</th>
<th>% FLNC Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UHR</td>
<td>Standard</td>
<td>3,466,513</td>
<td>85%</td>
</tr>
<tr>
<td>2</td>
<td>Mouse Liver</td>
<td>Standard</td>
<td>3,431,638</td>
<td>87%</td>
</tr>
<tr>
<td>3</td>
<td>MCF7</td>
<td>Standard</td>
<td>3,531,419</td>
<td>84%</td>
</tr>
<tr>
<td>4</td>
<td>Brain</td>
<td>Standard</td>
<td>2,943,148</td>
<td>86%</td>
</tr>
<tr>
<td>5</td>
<td>Alz Brain Tissue</td>
<td>Standard</td>
<td>3,142,634</td>
<td>83%</td>
</tr>
<tr>
<td>6</td>
<td>Heart</td>
<td>Standard</td>
<td>2,753,509</td>
<td>87%</td>
</tr>
<tr>
<td>7</td>
<td>Liver</td>
<td>Long</td>
<td>3,542,983</td>
<td>85%</td>
</tr>
<tr>
<td>8</td>
<td>ColT Cell Line</td>
<td>Short</td>
<td>2,852,434</td>
<td>84%</td>
</tr>
</tbody>
</table>

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)

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

HiFi Reads = CCS Reads with ≥Q20
ISO-SEQ ANALYSIS WORKFLOW DETAILS (CONT.)

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

**CCS Reads**

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

**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

1. CCS Reads
2. Classify Full-Length Reads
3. Cluster Isoforms
ISO-SEQ ANALYSIS WORKFLOW DETAILS (CONT.)

4. Cluster Isoforms

5. Map to Genome

Isoform Clusters:
- Isoform 1
- Isoform 2
- Isoform 3

Gene Clusters:
- Gene A
- Gene B
- Gene C
ISO-SEQ ANALYSIS WORKFLOW DETAILS (CONT.)

Map to Genome

QC & Annotate

Gene A

Gene B

Gene C

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 Ile 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/Ile 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 Ile 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/Ile 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

<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</em> – Brain with Alzheimer’s Disease</td>
<td>HiFi Reads</td>
<td>Sequel II System</td>
</tr>
<tr>
<td>Whole Transcriptome</td>
<td><em>Homo sapiens</em> – Universal Human Reference RNA (UHRR)</td>
<td>HiFi Reads</td>
<td>Sequel II System</td>
</tr>
</tbody>
</table>
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/](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

Publications
- 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

<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.