SOLiD™ Total RNA-Seq Kit
Protocol
For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

Information in this document is subject to change without notice.

APPLIED BIOSYSTEMS DISCLAIMS ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. TO THE FULLEST EXTENT ALLOWED BY LAW, IN NO EVENT SHALL APPLIED BIOSYSTEMS BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF, WHETHER OR NOT FORESEEABLE AND WHETHER OR NOT APPLIED BIOSYSTEMS IS ADVISED OF THE POSSIBILITY OF SUCH DAMAGES.

LITERATURE CITATION:
When describing a procedure for publication using this product, please refer to it as the SOLiD™ Total RNA-Seq Kit.

WARRANTY AND LIABILITY:
Applied Biosystems is committed to delivering superior product quality and performance, supported by industry-leading global service and technical support teams. Warranty information for the accompanying consumable product is available at www.ambion.com/info/warranty in “Limited Warranty for Consumables,” which is subject to the exclusions, conditions, exceptions, and limitations set forth under the caption “EXCLUSIONS, CONDITIONS, EXCEPTIONS, AND LIMITATIONS” in the full warranty statement. Please contact Applied Biosystems if you have any questions about our warranties or would like information about post-warranty support.

PATENTS AND LICENSING NOTIFICATIONS:
The SOLiD™ Total RNA-Seq Kit is covered by US and foreign patents pending.

TRADEMARKS
Trademarks of Life Technologies Corporation and its affiliates include: AB Logo™, Ambion®, Applied Biosystems®, ArrayScript™, DNAZap™, ElectroZap™, FirstChoice®, flashPAGE™, GeneAmp®, KinaseMax™, LEGend™, MicroPoly(A)Purist™, mirVana™, Novex®, NucAway™, PARIS®, PureLink™, Quant-iT™, Qubit®, RiboMinus™, SOLiD™, SureLock™, SYBR®, Veriti™.

AmpliTaq® is a registered trademark of Roche Molecular Systems, Inc.

MinElute® is a registered trademark of Qiagen.

All other trademarks are the sole property of their respective owners.

© 2010 Life Technologies Corp. All rights reserved.

Part Number 4452437 Rev. A
01/2010
## Contents

**About This Guide** ................................. 7
- Safety information ........................................ 7
- Safety alert words ....................................... 7
- SDSs .................................................. 7

**CHAPTER 1**

**SOLiD™ Total RNA-Seq Kit** ..................... 9
- Product information ...................................... 9
- Purpose of the product .................................. 9
- Kit contents and storage ............................ 9
- Materials and equipment required but not included ...................... 10
  - Equipment ........................................ 10
  - Supplies ....................................... 10
  - Reagents ....................................... 11

**CHAPTER 2**

**Prepare Whole Transcriptome Libraries** ........... 13
- Fragment the whole transcriptome RNA ................ 14
  - Guidelines for RNA sample type and amount .......... 14
- Fragment the RNA ................................... 14
- Clean up the RNA ................................... 15
- Assess the yield and size distribution of the fragmented RNA .......... 16
- Typical results of fragmentation of whole transcriptome RNA ........ 17
- Construct the amplified whole transcriptome library ........ 18
  - Hybridize and ligate the RNA ...................... 18
  - Perform reverse transcription .................... 19
  - Purify the cDNA ................................ 20
  - Size select the cDNA ............................. 21
  - Example of size selection ...................... 24
  - Expected lengths of the insert and PCR product according to excised cDNA length .... 25
  - Amplify the cDNA ................................. 25
  - Purify the amplified DNA ...................... 27
  - Assess the yield and size distribution of the amplified DNA .......... 28
  - Proceed with SOLiD™ System templated bead preparation .......... 29
  - Typical size profiles of amplified libraries ........ 29
  - Expected yields ................................ 30
- Troubleshooting ..................................... 31
  - Using a positive control .......................... 32
Using 2100 expert software to assess whole transcriptome libraries .................. 62
  Perform a smear analysis ................................................................. 62
  Determine the median size .............................................................. 63
  Analyze multiple peaks as one peak ............................................... 63
Small RNA enrichment ........................................................................ 66
Using 2100 expert software to assess small RNA libraries ......................... 68
  Review the median size ................................................................. 68
  Perform a smear analysis .............................................................. 68
  Determine the % miRNA library ...................................................... 70

APPENDIX C  PCR Good Laboratory Practices ............................ 71

APPENDIX D  Safety ................................................................. 73
  Chemical safety ................................................................. 73
    General chemical safety .......................................................... 73
    SDSs ................................................................. 73
    Biological hazard safety ....................................................... 74

Documentation and Support ......................................................... 75
  Kit documentation ................................................................. 75
  Related documentation .......................................................... 75
  Obtaining support ............................................................... 76

Bibliography .................................................................................. 77
About This Guide

Safety information

Note: For general safety information, see this section and Appendix D, “Safety” on page 73. When a hazard symbol and hazard type appear by an instrument hazard, see the “Safety” Appendix for the complete alert on the instrument.

Safety alert words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—IMPORTANT, CAUTION, WARNING, DANGER—implies a particular level of observation or action, as defined below:

IMPORTANT! – Indicates information that is necessary for proper instrument operation, or accurate chemistry kit use.

CAUTION! – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING! – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER! – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

SDSs

The Safety Data Sheets (SDSs) for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining SDSs, see “SDSs” on page 73.

IMPORTANT! For the SDSs of chemicals not distributed by Applied Biosystems or Ambion contact the chemical manufacturer.
SOLiD™ Total RNA-Seq Kit

Product information

Purpose of the product

Use the SOLiD™ Total RNA-Seq Kit (PN 4445374) to convert RNA transcripts expressed in a cell or tissue into a cDNA library for analysis on the Applied Biosystems SOLiD™ Sequencing System:

- For whole transcriptome libraries, follow the procedures in Chapter 2 on page 13.
- For small RNA libraries, follow the procedures in Chapter 3 on page 33.

When you use the SOLiD Total RNA-Seq Kit with one of the SOLiD™ RNA Barcoding Kits, you can prepare barcoded libraries to enable sequencing of multiple samples in a single, multiplexed, SOLiD System sequencing run. Sequencing of multiplexed libraries is fully supported by the SOLiD 4 System. Instructions for using the SOLiD™ RNA Barcoding Kits are included in this protocol.

Kit contents and storage

Sufficient reagents are supplied in the SOLiD Total RNA-Seq Kit to prepare cDNA libraries from 12 samples for high-throughput sequencing with the SOLiD System.

Upon receipt of the SOLiD Total RNA-Seq Kit, immediately store the components at –20 °C. You may store the Nuclease-free Water at room temperature, 4 °C, or –20 °C.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Cap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free Water</td>
<td>1.75 mL</td>
<td>clear</td>
</tr>
<tr>
<td>10X RNase III Buffer</td>
<td>20 µL</td>
<td>red</td>
</tr>
<tr>
<td>RNase III</td>
<td>20 µL</td>
<td>red</td>
</tr>
<tr>
<td>SOLiD™ Adaptor Mix</td>
<td>30 µL</td>
<td>green</td>
</tr>
<tr>
<td>Hybridization Solution</td>
<td>40 µL</td>
<td>green</td>
</tr>
<tr>
<td>2X Ligation Buffer</td>
<td>150 µL</td>
<td>green</td>
</tr>
<tr>
<td>Ligation Enzyme Mix</td>
<td>30 µL</td>
<td>green</td>
</tr>
<tr>
<td>10X RT Buffer</td>
<td>50 µL</td>
<td>yellow</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>500 µL</td>
<td>white</td>
</tr>
<tr>
<td>SOLiD™ RT Primer</td>
<td>30 µL</td>
<td>yellow</td>
</tr>
<tr>
<td>ArrayScript™ Reverse Transcriptase</td>
<td>20 µL</td>
<td>yellow</td>
</tr>
</tbody>
</table>

Note: The SOLiD™ Adaptor Mix is not equivalent to Adaptor Mix A or Adaptor Mix B in other kits.
### Materials and equipment required but not included

For optional materials and equipment and more ordering information, see Appendix A, “Ordering Information” on page 53.

#### Equipment

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal cycler with heated lid, capable of holding 0.2-mL tubes:</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>• Veriti® 96-Well Thermal Cycler</td>
<td></td>
</tr>
<tr>
<td>• GeneAmp® PCR System 9700</td>
<td></td>
</tr>
<tr>
<td>XCell SureLock™ Mini-Cell</td>
<td>Invitrogen PN EI0001</td>
</tr>
<tr>
<td>Agilent 2100 Bioanalyzer</td>
<td>Agilent PN G2938A</td>
</tr>
<tr>
<td>NanoDrop™ Spectrophotometer</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Centrifugal vacuum concentrator (for example, SpeedVac)</td>
<td>MLS</td>
</tr>
<tr>
<td>Microcentrifuge</td>
<td>MLS</td>
</tr>
<tr>
<td>Pipettors, positive displacement or air-displacement</td>
<td>MLS</td>
</tr>
<tr>
<td>Transilluminator</td>
<td>MLS</td>
</tr>
</tbody>
</table>

#### Supplies

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-strip PCR Tubes &amp; Caps, RNase-free, 0.2-mL</td>
<td>Applied Biosystems PN AM12230</td>
</tr>
<tr>
<td>Non-Stick RNase-free Microfuge Tubes (0.5 mL), 500</td>
<td>Applied Biosystems PN AM12350</td>
</tr>
<tr>
<td>Non-Stick RNase-free Microfuge Tubes (1.5 mL), 250</td>
<td>Applied Biosystems PN AM12450</td>
</tr>
<tr>
<td>Pipette tips, RNase-free</td>
<td>MLS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Cap</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR Buffer</td>
<td>660 µL</td>
<td>white</td>
</tr>
<tr>
<td>AmpliTaq® DNA Polymerase</td>
<td>110 µL</td>
<td>white</td>
</tr>
<tr>
<td>SOLiD™ 5’ PCR Primer</td>
<td>100 µL</td>
<td>white</td>
</tr>
<tr>
<td>SOLiD™ 3’ PCR Primer</td>
<td>100 µL</td>
<td>blue</td>
</tr>
<tr>
<td>WT Control RNA (1 µg/µL HeLa total RNA)</td>
<td>50 µL</td>
<td>clear</td>
</tr>
<tr>
<td>Small RNA Control (1 µg/µL human placenta total RNA)</td>
<td>10 µL</td>
<td>purple</td>
</tr>
</tbody>
</table>
## Reagents

For the SDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

### Reagents for both libraries

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free Water [not DEPC-treated], 100 mL</td>
<td>Applied Biosystems PN AM9938</td>
</tr>
<tr>
<td>Novex® TBE-Urea Sample Buffer [2X], 10 mL</td>
<td>Invitrogen PN LC6876</td>
</tr>
<tr>
<td>Novex® TBE Running Buffer [5X], 1 L</td>
<td>Invitrogen PN LC6675</td>
</tr>
<tr>
<td>PureLink™ PCR Micro Kit, 50 preps</td>
<td>Invitrogen PN K310050</td>
</tr>
<tr>
<td>SYBR® Gold nucleic acid gel stain, 10,000X concentrate in DMSO, 500 µL</td>
<td>Invitrogen PN S-11494</td>
</tr>
<tr>
<td>Agilent DNA 1000 Kit</td>
<td>Agilent PN 5067-1504</td>
</tr>
<tr>
<td>Ethanol, 100%, ACS reagent grade or equivalent</td>
<td>MLS</td>
</tr>
<tr>
<td>MinElute® PCR Purification Kit [50]</td>
<td>Qiagen PN 28004</td>
</tr>
</tbody>
</table>

### Reagents for whole transcriptome libraries

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 bp DNA Ladder</td>
<td>Invitrogen PN 10416-014</td>
</tr>
<tr>
<td>Novex® 6% TBE-Urea Gels 1.0 mm, 10 well</td>
<td>Invitrogen PN EC6865BOX</td>
</tr>
<tr>
<td>Quanti-iT™ RNA Assay Kit, 100 assays</td>
<td>Invitrogen PN Q32852</td>
</tr>
<tr>
<td>RiboMinus™ Concentration Module, 6 preps</td>
<td>Invitrogen PN K1550-05</td>
</tr>
</tbody>
</table>

**Note:** The RiboMinus™ Concentration Module is not equivalent to the RiboMinus™ Eukaryote Kit for RNA-Seq or to the RiboMinus™ Plant Kit for RNA-Seq.

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA 6000 Pico Kit</td>
<td>Agilent PN 5067-1513</td>
</tr>
</tbody>
</table>
### Reagents for small RNA libraries

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 bp DNA Ladder</td>
<td>Invitrogen PN 10821-015</td>
</tr>
<tr>
<td>Novex® 10% TBE-Urea Gels 1.0 mm, 10 well</td>
<td>Invitrogen PN EC6875BOX</td>
</tr>
<tr>
<td>RNA 6000 Nano Kit</td>
<td>Agilent PN 5067-1511</td>
</tr>
<tr>
<td>Small RNA Chip Kit</td>
<td>Agilent PN 5067-1548</td>
</tr>
</tbody>
</table>
Prepare Whole Transcriptome Libraries

**Fragment the whole transcriptome RNA**

100–500 µg poly(A) RNA or 200–500 ng rRNA-depleted total RNA

Fragment the RNA [page 14]

Clean up the RNA [page 15]

Assess the yield and size distribution of the fragmented RNA [page 16]

**Construct the amplified whole transcriptome library**

Hybridize and ligate the RNA [page 18]

Perform reverse transcription [page 19]

Purify the cDNA [page 20]

Size select the cDNA [page 21]

Amplify the cDNA [page 25]

Purify the amplified DNA [page 27]

Assess the yield and size distribution of the amplified DNA [page 28]

**Proceed with SOLiD™ System templated bead preparation**

Refer to the *Applied Biosystems SOLiD™ 4 System Templated Bead Preparation Guide* [PN 4448378]
Fragment the whole transcriptome RNA

Fragmentation of the whole transcriptome RNA involves the following procedures:

1. **Fragment the RNA** (below)
2. **Clean up the RNA** (page 15)
3. **Assess the yield and size distribution of the fragmented RNA** (page 16)

Guidelines for RNA sample type and amount

Use 100–500 ng poly(A) RNA or 200–500 ng rRNA-depleted total RNA.

- For poly(A) RNA, Applied Biosystems recommends performing two rounds of oligo(dT) selection of the poly(A) RNA; for example, use the Applied Biosystems MicroPoly(A)Purist™ Kit. Also, confirm the absence of 18S and 28S rRNA; for example, check the profile of the poly(A) RNA on an Agilent 2100 Bioanalyzer.
- For rRNA-depleted total RNA, Applied Biosystems recommends that you remove rRNA from total RNA for transcriptome analysis using the Invitrogen RiboMinus™ Eukaryote Kit for RNA-Seq or the Invitrogen RiboMinus™ Plant Kit for RNA-Seq.

Use only high-quality RNA as your starting material. FirstChoice® Total RNA and Poly(A) RNA provide high-quality, intact RNA isolated from a variety of sources.

Fragment the RNA

Use components from the SOLiD™ Total RNA-Seq Kit:

- Nuclease-free Water
- 10X RNase III Buffer
- RNase III

1. For each RNA sample, assemble a reaction mixture on ice:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA sample and Nuclease-free Water:</td>
<td>8 µL</td>
</tr>
<tr>
<td>Poly(A) RNA: 100–500 ng</td>
<td></td>
</tr>
<tr>
<td>rRNA-depleted total RNA: 200–500 ng</td>
<td></td>
</tr>
<tr>
<td>WT Control RNA: 500 ng</td>
<td></td>
</tr>
<tr>
<td>10X RNase III Buffer</td>
<td>1 µL</td>
</tr>
<tr>
<td>RNase III</td>
<td>1 µL</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10 µL</strong></td>
</tr>
</tbody>
</table>

2. Flick the tube or pipet up and down a few times to mix, then spin briefly.

3. Incubate the reaction in a thermal cycler at 37 °C for 10 minutes.
4. *Immediately* after the incubation, add 90 µL of Nuclease-free Water, then place the fragmented RNA on ice. Go to the next step immediately, or leave the fragmented RNA on ice for less than 1 hour.

### Clean up the RNA

Use the RiboMinus™ Concentration Module (Invitrogen).

1. Prepare the Wash Buffer (W5) with ethanol, then store at room temperature:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% ethanol</td>
<td>6 mL</td>
</tr>
<tr>
<td>Wash Buffer [W5]</td>
<td>1.5 mL</td>
</tr>
</tbody>
</table>

2. Add to the fragmented RNA, then mix well:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding Buffer [L3]</td>
<td>100 µL</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>250 µL</td>
</tr>
</tbody>
</table>

3. Bind the RNA sample containing Binding Buffer (L3) and ethanol to the Spin Column:
   a. Place the Spin Column in a clean 1.5-mL Wash Tube.
   b. Load 450 µL of the RNA sample containing Binding Buffer (L3) and ethanol onto the Spin Column.
   c. Spin the column at 12,000 × g for 1 minute.
   d. Discard the flowthrough.

4. Wash the RNA:
   a. Return the Spin Column to the Wash Tube.
   b. Add 500 µL of Wash Buffer (W5) with ethanol to the Spin Column.
   c. Spin the column at 12,000 × g for 1 minute.
   d. Discard the flowthrough.
   e. Return the Spin Column in the Wash Tube.
   f. Spin the column at maximum speed for 2 minutes.

5. Elute the RNA in a clean Recovery Tube:
   a. Place the Spin Column in a clean Recovery Tube.
   b. Add 12 µL of RNase-Free Water to the center of the Spin Column.
   c. Wait 1 minute, then spin the column at maximum speed for 1 minute.

   **Note:** You should recover approximately 10 µL of fragmented RNA from the column.
Assess the yield and size distribution of the fragmented RNA

Use the Quant-iT™ RNA Assay Kit with the Qubit® Fluorometer (Invitrogen) and the RNA 6000 Pico Chip Kit with the Agilent® 2100 Bioanalyzer (Agilent).

**Note:** You can use a NanoDrop™ Spectrophotometer in place of the Quant-iT RNA Assay Kit and Qubit Fluorometer. However, RNA eluted from spin columns may contain extra salts or other components that affect readings on the NanoDrop Spectrophotometer. For increased accuracy, quantitate the RNA concentration using the Quant-iT RNA Assay Kit on the Qubit Fluorometer.

1. Quantitate the yield of the fragmented RNA using the Quant-iT RNA Assay Kit on the Qubit Fluorometer.
   Refer to the Quant-iT™ RNA Assay Kit Protocol or the Qubit® Fluorometer Instruction Manual by Invitrogen for instructions.

2. Assess the size distribution of the fragmented RNA:
   a. Dilute 1 µL of the sample 1:10 with Nuclease-Free Water.
   b. Run the diluted sample on an Agilent 2100 bioanalyzer with the RNA 6000 Pico Chip Kit. Follow the manufacturer’s instructions for performing the assay.
   c. Using the 2100 expert software, review the size distribution.

   The fragmentation procedure should produce a distribution of RNA fragment sizes from 35 nt to several hundred or a few thousand nt, depending on your sample type. The average size should be 100–200 nt. See Figures 1 and 2 on page 17.

   **Note:** For instructions on how to review the size distribution, refer to the Agilent 2100 Bioanalyzer 2100 Expert User’s Guide by Agilent.

3. Proceed according to the amount of fragmented RNA you have in 3 µL:

<table>
<thead>
<tr>
<th>Amount of fragmented RNA in 3 µL</th>
<th>Instructions</th>
</tr>
</thead>
<tbody>
<tr>
<td>• ≥50 ng poly(A) RNA</td>
<td>Proceed with “Construct the amplified whole transcriptome library” on page 18.</td>
</tr>
<tr>
<td>• ≥100 ng rRNA-depleted total RNA</td>
<td>Store the remaining RNA at −80 °C.</td>
</tr>
<tr>
<td>• ≥100 ng WT Control RNA</td>
<td>1. Dry 50–100 ng of the RNA completely in a centrifugal vacuum concentrator at low or medium heat (≤40 °C); this should take 10–20 minutes.</td>
</tr>
<tr>
<td>• &lt;50 ng poly(A) RNA</td>
<td>2. Resuspend in 3 µL Nuclease-free Water, then proceed with “Construct the amplified whole transcriptome library” on page 18.</td>
</tr>
</tbody>
</table>
| • <100 ng rRNA-depleted total RNA | ]
Typical results of fragmentation of whole transcriptome RNA

Figure 1 and Figure 2 show profiles from an Agilent 2100 Bioanalyzer after RNase III fragmentation and cleanup. Figure 1 shows results with HeLa poly(A) RNA. Figure 2 shows results with rRNA-depleted HeLa RNA.
Construct the amplified whole transcriptome library

Constructing the amplified whole transcriptome library involves the following procedures:

1. Hybridize and ligate the RNA (below)
2. Perform reverse transcription (page 19)
3. Purify the cDNA (page 20)
4. Size select the cDNA (page 21)
5. Amplify the cDNA (page 25)
6. Purify the amplified DNA (page 27)
7. Assess the yield and size distribution of the amplified DNA (page 28)
8. Proceed with SOLiD™ System templated bead preparation (page 29)

Hybridize and ligate the RNA

Use components from the SOLiD™ Total RNA-Seq Kit:
- SOLiD™ Adaptor Mix
- Hybridization Solution
- Nuclease-free Water
- 2× Ligation Buffer
- Ligation Enzyme Mix

1. On ice, prepare the hybridization master mix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for one reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOLiD™ Adaptor Mix</td>
<td>2 µL</td>
</tr>
<tr>
<td>Hybridization Solution</td>
<td>3 µL</td>
</tr>
<tr>
<td><strong>Total volume per reaction</strong></td>
<td><strong>5 µL</strong></td>
</tr>
</tbody>
</table>

† Include 5–10% excess volume to compensate for pipetting error.

2. Transfer 5 µL hybridization master mix to 3 µL fragmented RNA sample:
   - Fragmented poly(A) RNA: 50 ng
   - Fragmented rRNA-depleted total RNA: 100 ng
   - Fragmented WT Control RNA: 100 ng

3. Slowly pipet up and down a few times to mix well, then spin briefly.
4. Run the hybridization reaction in a thermal cycler:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>65 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>16 °C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

5. Add the RNA ligation reagents to each 8-µL hybridization reaction:

<table>
<thead>
<tr>
<th>Component (add in order shown)</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Ligation Buffer</td>
<td>10 µL</td>
</tr>
<tr>
<td>Ligation Enzyme Mix</td>
<td>2 µL</td>
</tr>
</tbody>
</table>

❗ **IMPORTANT!** You may observe a white precipitate in the 2X Ligation Buffer. If so, warm the tube at 37 °C for 2–5 minutes or until the precipitate is dissolved. 2X Ligation Buffer is very viscous; pipet slowly to dispense it accurately.

6. Flick the tube or slowly pipet up and down a few times to mix well, then spin briefly.

7. Incubate the 20-µL ligation reaction in a thermal cycler at 16 °C for 16 hours.

Note: If possible, set the temperature of the thermal cycler lid to match the block temperature. Otherwise, incubate the reaction with the heated lid turned off, or do not cover the reaction tubes with the heated lid.

### Perform reverse transcription

Use components from the SOLiD™ Total RNA-Seq Kit:

- Nuclease-free Water
- 10X RT Buffer
- dNTP Mix
- SOLiD™ RT Primer
- ArrayScript™ Reverse Transcriptase

1. Prepare RT master mix (without the ArrayScript™ Reverse Transcriptase):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for each reaction†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free Water</td>
<td>11 µL</td>
</tr>
<tr>
<td>10X RT Buffer</td>
<td>4 µL</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>2 µL</td>
</tr>
<tr>
<td>SOLiD™ RT Primer</td>
<td>2 µL</td>
</tr>
<tr>
<td><strong>Total volume per reaction</strong></td>
<td><strong>19 µL</strong></td>
</tr>
</tbody>
</table>

† Include 5–10% excess volume in the master mix to compensate for pipetting error.
2. Incubate the RT master mix with the ligated RNA sample:
   a. Add 19 µL of RT master mix to each 20-µL ligation reaction.
   b. Pipet up and down a few times to mix, then spin briefly.
   c. Incubate in a thermal cycler with a heated lid at 70 °C for 5 minutes, then snap-cool on ice.

3. Perform the reverse transcription reaction:
   a. Add 1 µL ArrayScript™ Reverse Transcriptase to each ligated RNA sample.
   b. *Gently* vortex to mix thoroughly, then spin briefly.
   c. Incubate in a thermal cycler with a heated lid at 42 °C for 30 minutes.

   **Note:** The cDNA can be stored at –20 °C for a few weeks, stored at –80 °C for long-term storage, or used immediately.

### Purify the cDNA

Use the MinElute® PCR Purification Kit (Qiagen).

   **Note:** The kit may be supplied with Buffer PB (without pH Indicator) or Buffer PBI (with pH Indicator). Either buffer can be used as is; it is not necessary to add pH Indicator to Buffer PB before use.

1. Add Nuclease-free Water and Buffer PB or Buffer PBI to the cDNA:
   a. Transfer all of the cDNA (40 µL) to a clean 1.5-mL microcentrifuge tube.
   b. Add 60 µL of Nuclease-free Water.
   c. Add 500 µL of Buffer PB or Buffer PBI, then mix well.

2. Load the cDNA onto the MinElute column:
   a. Load 600 µL of the sample containing Buffer PB or Buffer PBI onto the MinElute column.
   b. Spin the column at 13,000 × g for 1 minute.
   c. Discard the flowthrough.

3. Wash the cDNA:
   a. Return the MinElute column to the microcentrifuge tube.
   b. Add 750 µL of Buffer PE to the MinElute column.
   c. Spin the column at 13,000 × g for 1 minute.
   d. Discard the flowthrough.
   e. Return the MinElute column to the microcentrifuge tube.
   f. Spin the column at 13,000 × g for 1 minute.

4. Elute the cDNA in a clean microcentrifuge tube:
   a. Place the MinElute column in a clean microcentrifuge tube.
   b. Add 10 µL of Buffer EB to the center of the MinElute column.
   c. Wait 1 minute, then spin the column at 13,000 × g for 1 minute.
Size select the cDNA

Use Novex® pre-cast gel products (Invitrogen), 50 bp DNA Ladder (Invitrogen), and SYBR® Gold nucleic acid gel stain (Invitrogen):

- Novex® 6% TBE-Urea Gel 1.0 mM, 10 Well
- Novex® TBE Running Buffer (5×)
- Novex® TBE-Urea Sample Buffer (2×)
- XCell SureLock™ Mini-Cell
- 50 bp DNA Ladder
- SYBR® Gold nucleic acid gel stain

For more instructions on running Novex gels, refer to the Novex® Pre-Cast Gel Electrophoresis Guide by Invitrogen. For more instructions on staining the gel, refer to the SYBR® Gold Nucleic Acid Gel Stain manual by Invitrogen.

1. Prepare the gel as described in the Novex® Pre-Cast Gel Electrophoresis Guide by Invitrogen:
   a. Prepare 1000 mL of 1× TBE Running Buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novex® TBE Running Buffer (5×)</td>
<td>200 mL</td>
</tr>
<tr>
<td>Deionized water</td>
<td>800 mL</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Prepare 1000 mL of 1× TBE Running Buffer using Novex® TBE Running Buffer (5×).

   b. Place the Novex® 6% TBE-Urea Gel in the XCell SureLock™ Mini-Cell.
   c. Use a marker to draw a line that is 1 cm below the middle of the gel, as shown in the figure in step 6 on page 22.
   d. Add 1× TBE Running Buffer to the Upper Buffer Chamber and the Lower Buffer Chamber.

2. Dilute the 50 bp DNA Ladder:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 bp DNA Ladder</td>
<td>1 µL</td>
<td>1 µg/µL</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>24 µL</td>
<td>–</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>25 µL</td>
<td>40 ng/µL</td>
</tr>
</tbody>
</table>

3. Prepare the cDNA and the DNA ladder:
   a. Mix 5 µL of the cDNA with 5 µL of 2× Novex TBE-Urea Sample Buffer.
   b. Mix 5 µL of the 40 ng/µL 50 bp DNA Ladder with 5 µL of 2× Novex TBE-Urea Sample buffer.
c. Heat the cDNA and the DNA Ladder at 95 °C for 3 minutes.

d. Snap-cool the tubes on ice. Leave the tubes on ice for less than 30 minutes.

**Note:** Do not leave denatured samples on ice for longer than 30 minutes. If the denatured samples are left on ice for longer than 30 minutes, repeat step 3c before loading the samples.

4. Immediately before you load each sample, flush the well of the gel several times with 1X TBE Running Buffer to remove urea from the wells.

**Note:** Flushing the wells is important to obtain sharp bands.

5. Load the cDNA samples and the DNA Ladder.

**Note:** Follow these guidelines when loading the gel:
   - Do not use the lanes next to the edges of the gel (lanes #1 and #10).
   - Load the DNA Ladder on both sides of each cDNA sample to help you make accurate cuts.
   - While loading, place the pipette tip as close to the bottom of the well as possible, and load the sample slowly. It is important to keep the sample compact in the gel.

6. Run the gel at 180 V until the leading dye front is 1 cm below the middle of the gel (~25 minutes).

![](image)

**IMPORTANT!** Do not run the gel too long.

7. Add 5 µL of the SYBR Gold nucleic acid gel stain to 50 mL of 1X TBE Running Buffer, then stain the gel for 5–10 minutes.
8. Illuminate the stained gel, then excise the gel containing 150–250 nt cDNA:

   **Note:** Be careful not to include extra gel that does not contain any cDNA.

   a. Using a clean razor blade, make horizontal cuts to excise the gel containing 150–250 nt cDNA.

   ![Image of gel with horizontal cuts](image)

   **Note:** If you are using a UV transilluminator to visualize the reaction products, work quickly to limit their exposure to UV radiation.

   **Note:** To obtain the desired insert length, you can adjust the cuts. However, too much gel will severely inhibit the PCR reactions. See Table 1 on page 25 for the expected lengths of the insert and PCR product according to the length of the cDNA that is excised from the gel.

   b. Reduce the width of the gel piece by making vertical cuts on both edges of the smear.

   ![Image of gel with vertical cuts](image)

   **Note:** The width of the smear is normally more narrow than the width of the well in the gel. Make the cuts carefully to minimize the amount of extra gel in the gel piece.
9. Transfer the gel piece to a clean working area, maintaining the orientation of the gel, then cut the gel vertically into 4 pieces using a clean razor blade. Each gel slice should be about 1 mm × 6 mm.

![Cut the gel vertically into 4 pieces](image)

10. Place the two gel slices from the middle of the lane individually into clean 0.2-mL PCR tubes, and place the outside gel slices into a clean 1.5-mL microcentrifuge tube for storage.

To generate sufficient cDNA for accurate quantitation, you need to run 2 amplification reactions using 2 gel pieces, 1 gel piece in each reaction. You may store the other 2 pieces for 2 weeks at –20 °C.

**Note:** To maximize the yield for SOLiD System sequencing, use the 2 gel pieces from the middle of the lane.

**Example of size selection**

*Figure 3* shows 5 µL of purified cDNA from HeLa poly(A) RNA run on a Novex 6% TBE-Urea Gel with the Invitrogen 50 bp DNA Ladder. The white rectangle indicates the area of the gel to excise. Each vertical slice can be used for one 100-µL PCR.

*Figure 3* Example of size selection of cDNA from HeLa poly(A) RNA

![Region of excision](image)
Expected lengths of the insert and PCR product according to excised cDNA length

<table>
<thead>
<tr>
<th>Excised cDNA length (nt)</th>
<th>Insert length (bp)</th>
<th>PCR product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>~0</td>
<td>~100</td>
</tr>
<tr>
<td>100</td>
<td>~50</td>
<td>~150</td>
</tr>
<tr>
<td>150</td>
<td>~100</td>
<td>~200</td>
</tr>
<tr>
<td>200</td>
<td>~150</td>
<td>~250</td>
</tr>
<tr>
<td>250</td>
<td>~200</td>
<td>~300</td>
</tr>
</tbody>
</table>

Amplify the cDNA

Use components from the SOLiD™ Total RNA-Seq Kit:

- Nuclease-free Water
- 10X PCR Buffer
- dNTP Mix
- SOLiD™ 5’ PCR Primer
- AmpliTaq® DNA Polymerase
- SOLiD™ 3’ PCR Primer

(Optional) To prepare cDNA libraries for multiplex SOLiD System sequencing, substitute with the barcoded SOLiD™ 3’ Primers from one of the SOLiD™ RNA Barcoding Kits.

Note: Plan your experiments to include multiples of four different barcoded libraries in every multiplex sequencing pool, to preserve color balance on the SOLiD System sequencing run. Use the SOLiD 3’ Primers from the SOLiD RNA Barcoding Kit in the appropriate color-balanced groups. For more information, refer to the product insert for your SOLiD RNA Barcoding Kit and the Applied Biosystems SOLiD™ 4 System SETS Software User Guide (PN 4448411).

1. For each cDNA sample, prepare duplicate in-gel amplification reactions to generate sufficient cDNA for emulsion PCR:
   a. Ensure that each gel slice from step 10 on page 24 is placed in a 0.2-mL PCR tube. If necessary, transfer the gel slice to the PCR tube using a clean pipette tip.
b. For each cDNA sample, prepare 98 µL PCR mix for each gel slice:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One 100-µL reaction</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>76.8</td>
</tr>
<tr>
<td>10X PCR Buffer</td>
<td>10.0</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>8.0</td>
</tr>
<tr>
<td>SOLiD™ 5′ PCR Primer</td>
<td>2.0</td>
</tr>
<tr>
<td>AmpliTaq® DNA Polymerase</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>98.0</strong></td>
</tr>
</tbody>
</table>

† Includes 10% excess volume to compensate for pipetting error.

c. Transfer 98 µL PCR mix into each 0.2-mL PCR tube.
d. Add 2 µL SOLiD 3′ PCR Primer to each tube.

**Note:** To prepare cDNA libraries for multiplex SOLiD System sequencing, substitute with the barcoded SOLiD™ 3′ PCR Primers from your SOLiD™ RNA Barcoding Kit.

2. Run the PCR reactions in a thermal cycler:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>95 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>Cycle (15 cycles)</td>
<td>95 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>62 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>72 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Hold</td>
<td>72 °C</td>
<td>7 min</td>
</tr>
</tbody>
</table>

**Note:** Run 15 cycles if you started with 50–100 ng of fragmented RNA. If necessary, adjust the number of cycles according to the amount of input fragmented RNA, but for optimal results run between 12 and 18 cycles. Too many cycles results in overamplification and changes the expression profile.
Purify the amplified DNA

Use the PureLink™ PCR Micro Kit (Invitrogen):
- PureLink™ Micro Kit Column
- Collection Tube
- Binding Buffer (B2)
- Wash Buffer (W1)
- PureLink™ Elution Tube

⚠️ IMPORTANT! Do not use other PCR purification kits. Other purification kits are not as effective in the removal of unincorporated primers. Unincorporated primers can affect the final quantitation and emulsion PCR.

1. Prepare the sample:
   a. Combine the two 100-µL PCR reactions in a new 1.5-mL tube.
      ⚠️ IMPORTANT! If you used two different barcoded SOLiD 3′ Primers for your sample, do not combine the PCRs at this step.
   b. Add 800 µL of Binding Buffer (B2) to the tube, then mix well.

2. Load the sample onto the PureLink™ Micro Kit Column:
   a. Place the PureLink™ Micro Kit Column in a clean Collection Tube.
   b. Load 500 µL of the sample containing Binding Buffer (B2) onto the column.
   c. Spin the column at 10,000 × g for 1 minute.
   d. Discard the flowthrough.
   e. Load the remaining 500 µL of the sample containing Binding Buffer (B2) onto the column.
   f. Spin the column at 10,000 × g for 1 minute.
   g. Discard the flowthrough.

3. Wash the DNA:
   a. Return the column to the Collection Tube.
   b. Add 600 µL of Wash Buffer (W1) to the column.
   c. Spin the column at 10,000 × g for 1 minute.
   d. Discard the flowthrough.
   e. Return the column to the Collection Tube.
   f. Spin the column at 14,000 × g for 1 minute.

4. Elute the DNA in a clean PureLink™ Elution Tube:
   a. Place the column in a clean PureLink™ Elution Tube.
   b. Add 10 µL of Elution Buffer to the center of the membrane.
   c. Wait 1 minute, then spin the column at 14,000 × g for 1 minute.
   d. Repeat step 4b through step 4c for a total elution volume of 20 µL.
Assess the yield and size distribution of the amplified DNA

Use a NanoDrop spectrophotometer, and the Agilent 2100 Bioanalyzer with the DNA 1000 Kit (Agilent).

1. Measure the concentration of the purified DNA with a NanoDrop spectrophotometer, and if necessary, dilute the DNA to <50 ng/µL for accurate quantitation with the DNA 1000 Kit.

2. Run 1 µL of the purified DNA on an Agilent 2100 Bioanalyzer with the DNA 1000 Kit. Follow the manufacturer’s instructions for performing the assay.

3. Using the 2100 expert software, perform a smear analysis to quantify the percentage of DNA that is 25–200 bp.

<table>
<thead>
<tr>
<th>Percent of DNA in the 25–200 bp range</th>
<th>Next steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 20%</td>
<td>Proceed with SOLiD™ System templated bead preparation [see page 29].</td>
</tr>
<tr>
<td>Greater than 20%</td>
<td>Follow the troubleshooting instructions for “Normal yield and bad size distribution in the amplified library” on page 31.</td>
</tr>
</tbody>
</table>

Note: For instructions on how to perform the smear analysis, see “Perform a smear analysis” on page 62, and refer to the Agilent 2100 Bioanalyzer 2100 Expert User’s Guide by Agilent.

4. Determine the median peak size (bp) and molar concentration (nM) of the cDNA library using the Agilent software. For more information, see “Determine the median size” on page 63.

Note: The mass concentration of the cDNA must be <50 ng/µL for accurate quantitation with the DNA 1000 Kit.

Alternatively, obtain the mass concentration by another method, and convert the mass concentration to molar concentration. A concentration conversion calculator is available at:

www4.appliedbiosystems.com/techlib/append/concentration_calculator.html
Proceed with SOLiD™ System templated bead preparation

When less than 20% of the amplified DNA is in the 25–200 bp range, you can proceed with the SOLiD™ System templated bead preparation stage, in which each library template is clonally amplified on SOLiD™ P1 DNA Beads by emulsion PCR. Refer to the Applied Biosystems SOLiD™ 4 System Templated Bead Preparation Guide (PN 4448378).

Barcoded libraries are pooled prior to templated bead preparation. For more information refer to the product insert for your SOLiD™ RNA Barcoding Kit.

Note: When optimizing SOLiD Total RNA-Seq Kit library concentrations (singleplex or multiplex sequencing pools) by workflow analysis (WFA), library concentrations of 0.4 pM and 0.8 pM for ePCR are recommended.

Typical size profiles of amplified libraries

Typical size distributions (Agilent 2100 Bioanalyzer profiles) of amplified libraries prepared from HeLa poly(A) RNA (Figure 4) and rRNA-depleted HeLa RNA (Figure 5) using the SOLiD™ Total RNA-Seq Kit are shown. Figure 6 is an example of a sub-optimal size distribution with >20% DNA 25–200 bp in length, prepared from rRNA-depleted FirstChoice® Human Brain Reference RNA.

Figure 4  Size distribution of amplified library prepared from HeLa poly(A) RNA

Figure 5  Size distribution of amplified library prepared from rRNA-depleted HeLa total RNA
Chapter 2  Prepare Whole Transcriptome Libraries

Construct the amplified whole transcriptome library

Expected yields

The recovery of your experimental RNA will depend on its source and quality. The following results are typically seen with Human Brain Reference and HeLa RNAs.

<table>
<thead>
<tr>
<th>Workflow</th>
<th>Input amount</th>
<th>Typical recovery amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment the whole transcriptome RNA (page 14)</td>
<td>500 ng poly(A) RNA, total RNA, or rRNA-depleted total RNA</td>
<td>300–400 ng RNA</td>
</tr>
<tr>
<td>Construct the amplified whole transcriptome library (page 18)</td>
<td>50–100 ng fragmented RNA</td>
<td>&gt;200 ng cDNA</td>
</tr>
</tbody>
</table>

Figure 6  Sub-optimal size distribution of amplified library
## Troubleshooting

<table>
<thead>
<tr>
<th>Observation</th>
<th>Possible cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent software doesn’t calculate one concentration and peak size</td>
<td>The software detects multiple peaks in the amplified cDNA profile</td>
<td>Refer to “Analyze multiple peaks as one peak” on page 63.</td>
</tr>
<tr>
<td>Low yield and bad size distribution in the amplified library</td>
<td>You recovered &lt;20% of the input RNA after you fragmented and cleaned up the RNA</td>
<td>Decrease the RNase III digestion from 10 minutes to 5 minutes (step 3 on page 14).</td>
</tr>
<tr>
<td>Low yield in the amplified library and very few differences in the 2100 bioanalyzer traces before and after you fragment the RNA</td>
<td>RNA fragmentation failed</td>
<td>Purify the RNA sample again to remove the extra salts that may affect the RNase III activity. If RNA fragmentation still fails, increase the RNase III digestion from 10 minutes to 20 minutes (step 3 on page 14).</td>
</tr>
<tr>
<td>Low yield and no PCR products</td>
<td>The gel ran too long or too much gel was added to the PCR</td>
<td>Reduce the running time (step 6 on page 22) and add less gel to the PCR (step 1 on page 25).</td>
</tr>
<tr>
<td></td>
<td>An enzymatic reaction or column purification performed after RNase III treatment failed</td>
<td>1. Dilute the cDNA 1:10, then use 1 µL in a 100-µL PCR.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Check the yield before and after purification using the PureLink™ PCR Micro Kit.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. If you get the same results, repeat the ligation with more fragmented RNA, and run a parallel ligation reaction with fragmented Control RNA.</td>
</tr>
<tr>
<td>Normal or high yield but the purified amplified cDNA shows one or more sharp peaks between 100 and 150 bp in the Agilent 2100 Bioanalyzer trace</td>
<td>Nonspecific amplification</td>
<td>Increase the PCR annealing temperature to 68–72 °C (step 2 on page 26).</td>
</tr>
<tr>
<td>Normal or high yield but PCR products larger than 300 bp</td>
<td>Too many PCR cycles resulted in overamplification</td>
<td>Decrease the number of PCR cycles (step 2 on page 26).</td>
</tr>
<tr>
<td>Normal yield and bad size distribution in the amplified library</td>
<td>Too much sample was loaded on the Novex® TBE-Urea Gel</td>
<td>Decrease the volume of sample loaded to less than 10 µL (step 5 on page 22).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Before you load the samples, flush the wells of the gel several times with 1X TBE Running Buffer to remove urea from the wells and to obtain sharp bands (step 4 on page 22).</td>
</tr>
<tr>
<td>You decreased the volume of sample loaded on the Novex® TBE-Urea Gel, but smear analysis of the purified amplified cDNA shows that &gt;20% of the cDNA is in the 25–200 bp range</td>
<td>Fragmented RNA sample contains too many small fragments</td>
<td>Perform “Second-round size selection of amplified cDNA” on page 59.</td>
</tr>
<tr>
<td></td>
<td>Size selection was not successful</td>
<td></td>
</tr>
</tbody>
</table>
Using a positive control

A general troubleshooting strategy is to perform the SOLiD™ Total RNA-Seq Kit procedure using the WT Control RNA (HeLa total RNA) provided with the kit.

- Use 500 ng WT Control RNA for the fragmentation procedure starting on page 14.
- Use 100 ng fragmented WT Control RNA in the amplified library construction procedure starting on page 18.

See the expected yields for the WT Control RNA on page 30.
CHAPTER 3

Prepare Small RNA Libraries

**Prepare the starting material**

Assess the amount and quality of small RNA in your total RNA samples [page 34]

Enrich the sample for small RNA [page 36]

Assess the quality and quantity of the small RNA-enriched sample [page 36]

Determine the input amount [page 37]

**Construct the amplified small RNA library**

Hybridize and ligate the RNA [page 38]

Perform reverse transcription [page 39]

Purify the cDNA [page 40]

Size select the cDNA [page 41]

Amplify the cDNA [page 44]

Purify the amplified DNA [page 46]

Assess the yield and size distribution of the amplified DNA [page 47]

**Proceed with SOLiD™ System templated bead preparation**

Refer to the Applied Biosystems SOLiD™ 4 System Templated Bead Preparation Guide [PN 4448378]
Chapter 3  Prepare Small RNA Libraries

Prepare the starting material

Preparing the starting material involves the following procedures:

1. Assess the amount and quality of small RNA in your total RNA samples (below)
2. Enrich the sample for small RNA (page 36)
3. Assess the quality and quantity of the small RNA-enriched sample (page 36)
4. Determine the input amount (page 37)

Guidelines for obtaining small RNA

For this protocol, the total RNA must contain the small RNA fraction (microRNA or miRNA, 10–40 nt). For optimal results, use RNA that has been size selected for miRNA.

Applied Biosystems recommends using the following products:

- **Recommended RNA source:** Use Ambion FirstChoice® Total RNA, which is certified to contain miRNA and other small RNAs.
- **Recommended RNA isolation kits:** Use the Ambion mirVana™ miRNA Isolation Kit or the Ambion mirVana PARIS™ Kit to isolate total RNA that includes the small RNA fraction. Use the PureLink™ miRNA Isolation Kit (Invitrogen) to isolate small RNA from tissues or cells.

Assess the amount and quality of small RNA in your total RNA samples

Before you prepare the library, determine the quality of the total RNA sample. Use the NanoDrop® Spectrophotometer and the Agilent 2100 Bioanalyzer with the RNA 6000 Nano Kit and the Small RNA Chip Kit (Agilent).

1. Quantitate the amount of RNA in the sample using the NanoDrop® Spectrophotometer.

   **Note:** If you used the PureLink™ miRNA Isolation Kit to isolate small RNA from samples, you can skip to step 1 on page 36.

2. Determine the quality of the small RNA in your sample:

   a. Dilute the sample to ~50 to 100 ng/µL.
   b. Run 1 µL of diluted sample using the RNA 6000 Nano Kit with the Agilent® 2100 Bioanalyzer (Agilent) to determine the concentration of total RNA. Follow the manufacturer’s instructions for performing the assay.
   c. Using the 2100 expert software, determine the mass of total RNA in the sample, and save for step 3c.
d. Using the 2100 expert software, review the RNA Integrity Number (RIN). You can proceed if the RIN is ≥ 6.

**Note:** If the RIN is < 6, the sample is not suitable for small RNA library construction because the RNA degradation products may affect the quantitation of small RNA in the sample and interfere with small RNA ligation. Repeat RNA isolation; to obtain high-quality RNA, avoid RNase contamination (see Appendix 3, “PCR Good Laboratory Practices” on page 33).

3. Determine the percentage of small RNA in your sample:
   a. Run 1 μL of diluted RNA on the Agilent® 2100 Bioanalyzer with the Small RNA Chip. Follow the manufacturer’s instructions for performing the assay.
   b. Using the 2100 expert software, determine the mass of small RNA (miRNA, 10–40 nts) from the Small RNA Chip.
   c. Calculate the miRNA content in your RNA sample using the following formula:

   \[
   \% \text{ miRNA} = \left( \frac{\text{mass of miRNA (10–40 nts) from the Small RNA Chip}}{\text{mass of total RNA from the RNA 6000 Nano Chip}} \right) \times 100
   \]

4. Determine whether small RNA enrichment is needed and the type of enrichment to perform:

<table>
<thead>
<tr>
<th>How much miRNA (10–40 nt) is in your RNA sample?</th>
<th>Recommendations for small RNA enrichment and next steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥0.5% miRNA</td>
<td>You can use the total RNA in the ligation reaction, and small RNA enrichment is not needed. However, for optimal results, Applied Biosystems recommends enrichment of all total RNA samples. Proceed with “Enrich the sample for small RNA” on page 36 or skip to “Determine the input amount” on page 37.</td>
</tr>
<tr>
<td>0.1–0.5% miRNA</td>
<td>Small RNA enrichment is strongly recommended. Applied Biosystems recommends using the Invitrogen PureLink™ miRNA Isolation Kit. Proceed with “Enrich the sample for small RNA” on page 36.</td>
</tr>
<tr>
<td>&lt;0.1% miRNA</td>
<td>Small RNA purification is strongly recommended. Applied Biosystems recommends using Ambion flashPAGE™ purification products. Proceed with “Enrich the sample for small RNA” on page 36.</td>
</tr>
</tbody>
</table>

**Note:** When total RNA is used in the SOLiD Total RNA-Seq Kit procedure, the resulting reaction products comprise a larger size range than those produced from small RNA-enriched samples.
Chapter 3  Prepare Small RNA Libraries

Prepare the starting material

Guidelines for enriching for small RNA

The amount of small RNA in samples varies widely according to the tissue source and the RNA isolation method. A survey by Agilent provides a guide for the relative proportion of miRNA of 40 different tissues (Tissot, 2008). If the tissues or cell lines you are using contain a small fraction of small RNA, Applied Biosystems recommends that you enrich the RNA samples for small RNA.

Enrich the sample for small RNA

If needed, enrich the sample for small RNA using one of the methods below:

• If your RNA sample contains <0.1% miRNA – Use the Ambion flashPAGE™ Fractionator System and flashPAGE™ Clean-up Kit. Follow the instructions provided with the kit. Up to 100 µg total RNA can be loaded on the flashPAGE Fractionator.

  Expected recovery after flashPAGE fractionation and cleanup: From 5 µg total placenta RNA input, approximately 200 ng small RNA-enriched RNA can be recovered. For <5 µg total RNA input, use glycogen as a coprecipitant during the flashPAGE Clean-Up Kit procedure.

• If your RNA sample contains 0.1–0.5% miRNA – Use the Invitrogen PureLink™ miRNA Isolation Kit. Follow the instructions in “Small RNA enrichment” on page 66.

Assess the quality and quantity of the small RNA-enriched sample

Assess the quality and quantity of samples that are enriched for small RNA. Use the Agilent 2100 Bioanalyzer with the Small RNA Chip Kit (Agilent).

1. Run 1 µL of purified and enriched small RNA sample on the Agilent 2100 Bioanalyzer with the Small RNA Chip. Follow the manufacturer’s instructions for performing the assay.

2. Using the 2100 expert software, determine the quality and quantity of recovered small RNA.

\[
\text{% miRNA} = \left( \frac{\text{mass of miRNA (10–40 nts) from the Small RNA Chip}}{\text{mass of enriched small RNA from the Small RNA Chip}} \right) \times 100
\]

3. Compare the bioanalyzer traces to those of the sample before enrichment (step 2 in “Assess the amount and quality of small RNA in your total RNA samples” on page 34), and determine whether the RNA is degraded.

Expected results:

• For small RNA purified using flashPAGE, peaks from 10 to 40 nt are expected and very low peaks or no peaks should be observed after 40 nt.
• For enriched small RNA samples, peaks should be from 10 to 200 nt.
**Determine the input amount**

Using the results from the Agilent 2100 Bioanalyzer and the Small RNA Chip Kit, determine the amount of total RNA to use according to the type of RNA you ran and the amount of miRNA in 1 µL:

<table>
<thead>
<tr>
<th>Input sample type</th>
<th>Amount of miRNA (10 to 40 nt) in 1 µL on the Small RNA Chip</th>
<th>Total RNA input†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA</td>
<td>5 to 100 ng</td>
<td>≤1 µg</td>
</tr>
<tr>
<td>Enriched small RNA</td>
<td>1 to 100 ng</td>
<td>≤1 µg</td>
</tr>
<tr>
<td>Purified small RNA</td>
<td>1 to 100 ng</td>
<td>100 ng</td>
</tr>
</tbody>
</table>

† The yield drops if you use more than 1 µg of RNA for ligation.
Chapter 3  Prepare Small RNA Libraries

Construct the amplified small RNA library

Constructing the amplified small RNA library involves the following procedures:

1. **Hybridize and ligate the RNA** (below)
2. **Perform reverse transcription** (page 39)
3. **Purify the cDNA** (page 40)
4. **Size select the cDNA** (page 41)
5. **Amplify the cDNA** (page 44)
6. **Purify the amplified DNA** (page 46)
7. **Assess the yield and size distribution of the amplified DNA** (page 47)
8. **Proceed with SOLiD™ System templated bead preparation** (page 47)

Hybridize and ligate the RNA

Use components from the SOLiD™ Total RNA-Seq Kit:

- Hybridization Solution
- Nuclease-free Water
- SOLiD™ Adaptor Mix
- 2X Ligation Buffer
- Ligation Enzyme Mix

1. On ice, prepare the hybridization mix in 0.5-mL PCR tubes:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small RNA sample:</td>
<td></td>
</tr>
<tr>
<td>• 5 to 100 ng of miRNA in ≤ 1 µg of total RNA</td>
<td>3</td>
</tr>
<tr>
<td>• 1 to 100 ng of miRNA in ≤ 1 µg of enriched small RNA</td>
<td></td>
</tr>
<tr>
<td>• 1 to 100 ng of purified small RNA</td>
<td></td>
</tr>
<tr>
<td>Hybridization Solution</td>
<td>3</td>
</tr>
<tr>
<td>SOLiD™ Adaptor Mix</td>
<td>2</td>
</tr>
<tr>
<td>Total volume per reaction</td>
<td>8</td>
</tr>
</tbody>
</table>

2. Slowly pipet up and down a few times to mix well, then spin briefly.
3. Run the hybridization reaction in a thermal cycler:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>65 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>16 °C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

4. Add the RNA ligation reagents to the 8-µL hybridization reactions:

<table>
<thead>
<tr>
<th>Component (add in order shown)</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Ligation Buffer</td>
<td>10</td>
</tr>
<tr>
<td>Ligation Enzyme Mix</td>
<td>2</td>
</tr>
</tbody>
</table>

**IMPORTANT!** You may observe a white precipitate in the 2X Ligation Buffer. If so, warm the tube at 37 °C for 2–5 minutes or until the precipitate is dissolved. 2X Ligation Buffer is very viscous; pipet slowly to dispense it accurately.

5. Flick the tube or slowly pipet up and down a few times to mix well, then spin briefly.

6. Incubate the 20-µL ligation reaction in a thermal cycler at 16 °C for 16 hours.

**IMPORTANT!** Turn off the heated lid or leave the thermal cycler open during the incubation.

Perform reverse transcription

Use components from the SOLiD™ Total RNA-Seq Kit:

- Nuclease-free Water
- 10X RT Buffer
- dNTP Mix
- SOLiD™ RT Primer
- ArrayScript™ Reverse Transcriptase

1. On ice, prepare RT master mix (*without* the ArrayScript™ Reverse Transcriptase):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for each reaction (µL)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free Water</td>
<td>11</td>
</tr>
<tr>
<td>10X RT Buffer</td>
<td>4</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>2</td>
</tr>
<tr>
<td>SOLiD™ RT Primer</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total volume per reaction</strong></td>
<td><strong>19</strong></td>
</tr>
</tbody>
</table>

† Include 5–10% excess volume in the master mix to compensate for pipetting error.
2. Incubate the RT master mix with the ligated RNA sample:
   a. Add 19 µL of RT master mix to each 20-µL ligation reaction.
   b. Pipet up and down a few times to mix, then spin briefly.
   c. Incubate in a thermal cycler with a heated lid at 70 °C for 5 minutes, then
   snap-cool on ice.

3. Perform the reverse transcription reaction:
   a. Add 1 µL ArrayScript™ Reverse Transcriptase to each ligated RNA sample.
   b. Gently vortex to mix thoroughly, then spin briefly.
   c. Incubate in a thermal cycler with a heated lid at 42 °C for 30 minutes.

   **Note:** The cDNA can be stored at –20 °C for a few weeks, stored at –80 °C for
   long-term storage, or used immediately.

**Purify the cDNA**

Use the MinElute® PCR Purification Kit (Qiagen).

**Note:** The kit may be supplied with Buffer PB (without pH Indicator) or Buffer
PBI (with pH Indicator). Either buffer can be used as is; it is not necessary to add
pH Indicator to Buffer PB before use.

1. Add Nuclease-free Water and Buffer PB or Buffer PBI to the cDNA:
   a. Transfer all of the cDNA (40 µL) to a clean 1.5-mL microcentrifuge tube.
   b. Add 60 µL of Nuclease-free Water.
   c. Add 500 µL of Buffer PB or Buffer PBI, then mix well.

2. Load the cDNA onto the MinElute column:
   a. Load 600 µL of the sample containing Buffer PB or Buffer PBI onto the
MinElute column.
   b. Spin the column at 13,000 × g for 1 minute.
   c. Discard the flowthrough.

3. Wash the cDNA:
   a. Return the MinElute column to the microcentrifuge tube.
   b. Add 750 µL of Buffer PE to the MinElute column.
   c. Spin the column at 13,000 × g for 1 minute.
   d. Discard the flowthrough.
   e. Return the MinElute column to the microcentrifuge tube.
   f. Spin the column at 13,000 × g for 1 minute.

4. Elute the cDNA in a clean microcentrifuge tube:
   a. Place the MinElute column in a clean microcentrifuge tube.
   b. Add 10 µL of Buffer EB to the center of the MinElute column.
   c. Wait 1 minute, then spin the column at 13,000 × g for 1 minute.
Size select the cDNA

Use Novex® pre-cast gel products (Invitrogen), a 10 bp DNA Ladder (Invitrogen), and SYBR® Gold nucleic acid gel stain (Invitrogen):

- Novex® 10% TBE-Urea Gel 1.0 mM, 10 Well
- Novex® TBE Running Buffer (5X)
- Novex® TBE-Urea Sample Buffer (2X)
- XCell SureLock™ Mini-Cell
- 10 bp DNA Ladder
- SYBR® Gold nucleic acid gel stain

For more instructions on running Novex gels, refer to the Novex® Pre-Cast Gel Electrophoresis Guide by Invitrogen. For more instructions on staining the gel, refer to the SYBR® Gold Nucleic Acid Gel Stain manual by Invitrogen.

1. Prepare the gel as described in the Novex® Pre-Cast Gel Electrophoresis Guide by Invitrogen:
   a. Prepare 1000 mL of 1× TBE Running Buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novex® TBE Running Buffer (5X)</td>
<td>200</td>
</tr>
<tr>
<td>Deionized water</td>
<td>800</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>1000</td>
</tr>
</tbody>
</table>

   b. Place the Novex® 10% TBE-Urea Gel in the XCell SureLock™ Mini-Cell.
   c. Add 1× TBE Running Buffer to the Upper Buffer Chamber and the Lower Buffer Chamber.

2. Dilute the 10-bp DNA Ladder:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-bp DNA Ladder</td>
<td>1</td>
<td>1 µg/µL</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>24</td>
<td>–</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>25</td>
<td>40 ng/µL</td>
</tr>
</tbody>
</table>

3. Prepare the cDNA and the DNA ladder:
   a. Mix 5 µL of the cDNA with 5 µL of 2X Novex TBE-Urea Sample Buffer.
   b. Mix 5 µL of the 40 ng/µL 50 bp DNA Ladder with 5 µL of 2X Novex TBE-Urea Sample buffer.
   c. Heat the cDNA and the DNA Ladder at 95 °C for 3 minutes.
   d. Snap-cool the tubes on ice. Leave the tubes on ice for less than 30 minutes.

   **Note:** Do not leave denatured samples on ice for longer than 30 minutes. If the denatured samples are left on ice for longer than 30 minutes, repeat step 3c before loading the samples.
4. Before you load the samples, flush the wells of the gel several times with 1X TBE Running Buffer to remove urea from the wells.

   Note: Flushing the wells is important to obtain sharp bands.

5. Load the cDNA samples and the DNA Ladder.

   Note: Follow these guidelines when loading the gel:
   • Do not use the lanes next to the edges of the gel (lanes #1 and #10).
   • Load the DNA Ladder on both sides of each cDNA sample to help you make accurate cuts.
   • While loading, place the pipette tip as close to the bottom of the well as possible, and load the sample slowly. It is important to keep the sample compact in the gel.

6. Run the gel at 180 V until the second dye front just passes the middle of the gel (~45 minutes).

   IMPORTANT! Do not run the gel too short or too long.

7. Add 5 µL of the SYBR Gold nucleic acid gel stain to 50 mL of 1X TBE Running Buffer, then stain the gel for 5–10 minutes.
8. Illuminate the stained gel, then excise the gel containing 60 to 80 nt of cDNA:

**Note:** Be careful not to include extra gel that does not contain any cDNA.

a. Using a clean razor blade, make horizontal cuts directly on the 60-nt and 80-nt bands to excise the gel between 60 and 80 nt of cDNA.

**Note:** If you are using a UV transilluminator to visualize the reaction products, work quickly to limit their exposure to UV radiation.

**Note:** To obtain the desired insert length, you can adjust the cuts. However, too much gel will severely inhibit the PCR reactions. See page 44 for the expected lengths of the insert and PCR product according to the length of the cDNA that is excised from the gel.

b. Reduce the width of the gel piece by making vertical cuts on both edges of the smear.

9. Transfer the gel piece to a clean working area, maintaining the orientation of the gel, then cut the gel vertically into 4 pieces using a clean razor blade. Each gel slice should be about 1 mm × 6 mm.
Chapter 3  Prepare Small RNA Libraries  
Construct the amplified small RNA library

10. Place the two gel slices from the middle of the lane individually into clean 0.2-mL PCR tubes, and place the outside gel slices into a clean 1.5-mL microcentrifuge tube for storage.

To generate sufficient cDNA for accurate quantitation, you need to run 2 amplification reactions using 2 gel pieces, 1 gel piece in each reaction. You may store the other 2 pieces for 2 weeks at –20 °C.

Note: To maximize the yield for SOLiD System sequencing, use the 2 gel pieces from the middle of the lane.

### Expected lengths of the insert and PCR product according to excised cDNA length

<table>
<thead>
<tr>
<th>Excised cDNA length (nt)</th>
<th>Insert length (bp)</th>
<th>PCR product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>~0</td>
<td>~93</td>
</tr>
<tr>
<td>60</td>
<td>~18</td>
<td>~110</td>
</tr>
<tr>
<td>80</td>
<td>~38</td>
<td>~130</td>
</tr>
</tbody>
</table>

**Amplify the cDNA**

Use components from the SOLiD™ Total RNA-Seq Kit:

- Nuclease-free Water
- 10X PCR Buffer
- 2.5 mM dNTP Mix
- SOLiD™ 5′ PCR Primer
- AmpliTaq® DNA Polymerase
- SOLiD™ 3′ PCR Primer
  (Optional) To prepare cDNA libraries for multiplex SOLiD System sequencing, substitute with the barcoded SOLiD™ 3′ Primers from one of the SOLiD™ RNA Barcoding Kits.

Note: Plan your experiments to include multiples of four different barcoded libraries in every multiplex sequencing pool, to preserve color balance on the SOLiD System sequencing run. Use the SOLiD 3′ Primers from the SOLiD RNA Barcoding Kit in the appropriate color-balanced groups. For more information, refer to the product insert for your SOLiD RNA Barcoding Kit and the *Applied Biosystems SOLiD™ 4 System SETS Software User Guide* (PN 4448411).

1. For each cDNA sample, prepare duplicate in-gel amplification reactions to generate sufficient cDNA for emulsion PCR:
   a. Ensure that each gel slice from step 10 on page 44 is placed in a 0.2-mL PCR tube. If necessary, transfer the gel slice to the PCR tube using a clean pipette tip.
b. For each cDNA sample, prepare 98 µL PCR mix for each gel slice:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
<th>One 100-µL reaction</th>
<th>Two 100-µL reactions†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free Water</td>
<td>76.8</td>
<td></td>
<td>169.0</td>
</tr>
<tr>
<td>10X PCR Buffer</td>
<td>10.0</td>
<td></td>
<td>22.0</td>
</tr>
<tr>
<td>2.5 mM dNTP Mix</td>
<td>8.0</td>
<td></td>
<td>17.6</td>
</tr>
<tr>
<td>SOLiD™ 5′ PCR Primer</td>
<td>2.0</td>
<td></td>
<td>4.4</td>
</tr>
<tr>
<td>AmpliTaq® DNA Polymerase</td>
<td>1.2</td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>Total volume</td>
<td>98.0</td>
<td></td>
<td>215.6</td>
</tr>
</tbody>
</table>

† Includes 10% excess volume to compensate for pipetting error.

c. Transfer 98 µL of PCR master mix into each 0.2-mL PCR tube.
d. Add 2 µL SOLiD 3′ PCR Primer to each tube.

**Note:** To prepare cDNA libraries for multiplex SOLiD System sequencing, substitute with the barcoded SOLiD™ 3′ PCR Primers from your SOLiD™ RNA Barcoding Kit.

2. Run the PCR reactions in a thermal cycler:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>95 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>Cycle (15 cycles)</td>
<td>95 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>62 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>72 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Hold</td>
<td>72 °C</td>
<td>7 min</td>
</tr>
</tbody>
</table>

**Note:** If you started with total RNA and input 1–25 ng, run 18 cycles. If you started with enriched/purified small RNA and input 1–10 ng, run 18 cycles.
Chapter 3  Prepare Small RNA Libraries

Construct the amplified small RNA library

Purify the amplified DNA

Use the PureLink™ PCR Micro Kit (Invitrogen):

- PureLink™ Micro Kit Column
- Collection Tube
- Binding Buffer (B2)
- Wash Buffer (W1)
- PureLink™ Elution Tube

⚠️ IMPORTANT! Do not use other PCR purification kits. Other purification kits are not as effective in the removal of unincorporated primers. Unincorporated primers can affect the final quantitation and emulsion PCR.

1. Prepare the sample:
   a. Combine the two 100-µL PCR reactions in a new 1.5-mL tube.
      ⚠️ IMPORTANT! If you used two different barcoded SOLiD 3′ Primers for your sample, do not combine the PCRs at this step.
   b. Add 800 µL of Binding Buffer (B2) to the tube, then mix well.

2. Load the sample onto the PureLink™ Micro Kit Column:
   a. Place the PureLink™ Micro Kit Column in a clean Collection Tube.
   b. Load 500 µL of the sample containing Binding Buffer (B2) onto the column.
   c. Spin the column at 10,000 × g for 1 minute.
   d. Discard the flowthrough.
   e. Load the remaining 500 µL of the sample containing Binding Buffer (B2) onto the column.
   f. Spin the column at 10,000 × g for 1 minute.
   g. Discard the flowthrough.

3. Wash the DNA:
   a. Return the column to the Collection Tube.
   b. Add 600 µL of Wash Buffer (W1) to the column.
   c. Spin the column at 10,000 × g for 1 minute.
   d. Discard the flowthrough.
   e. Return the column to the Collection Tube.
   f. Spin the column at 14,000 × g for 1 minute.

4. Elute the DNA in a clean PureLink™ Elution Tube:
   a. Place the column in a clean PureLink™ Elution Tube.
   b. Add 12 µL of Elution Buffer to the center of the membrane.
   c. Wait 1 minute, then spin the column at 14,000 × g for 1 minute.
Assess the yield and size distribution of the amplified DNA

Use the Agilent 2100 Bioanalyzer with the DNA 1000 Kit (Agilent).

1. Run 1 µL of the purified DNA on an Agilent 2100 Bioanalyzer with the DNA 1000 Kit. Follow the manufacturer’s instructions for performing the assay.

2. Using the 2100 expert software, perform a smear analysis to determine whether you can proceed with SOLiD™ System templated bead preparation:
   a. Measure the area for the DNA that is 25–150 bp (the size range for ligation products with no insert and ligation products with short inserts) and 120–130 bp (the size range for the desired miRNA ligation products).
   b. Calculate the ratio of 120–130-bp DNA: 25–150-bp DNA:
      \[
      \frac{\text{Area (120–130 bp)}}{\text{Area (25–150 bp)}}
      \]

   \[
   \text{Note: Samples that are run on a bioanalyzer typically show 5 to 8 bp larger than their actual size.}
   \]

**Ratio of 120–130-bp DNA:25–150-bp DNA**

<table>
<thead>
<tr>
<th>Ratio of 120–130-bp DNA:25–150-bp DNA</th>
<th>Next steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greater than 50%</td>
<td>“Proceed with SOLiD™ System templated bead preparation” on page 47</td>
</tr>
<tr>
<td>Less than 50%</td>
<td>“Second-round size selection of amplified cDNA” on page 59</td>
</tr>
</tbody>
</table>

**Proceed with SOLiD™ System templated bead preparation**

You can proceed with the SOLiD™ System templated bead preparation stage, in which each library template is clonally amplified on SOLiD™ P1 DNA Beads by emulsion PCR. Refer to the Applied Biosystems SOLiD™ 4 System Templated Bead Preparation Guide (PN 4448378).

Barcoded libraries are pooled prior to templated bead preparation. For more information refer to the product insert for your SOLiD™ RNA Barcoding Kit.

**Note:** When optimizing SOLiD Total RNA-Seq Kit library concentrations (singleplex or multiplex sequencing pools) by workflow analysis (WFA), library concentrations of 0.4 pM and 0.8 pM for ePCR are recommended.
Typical size profiles of amplified libraries

Figure 7  Size distribution of amplified library prepared from placenta total RNA

Figure 8  Size distribution of amplified library prepared from HeLa total RNA and required a second round of size selection
Figure 9  Sub-optimal size distribution of amplified library from partially degraded placenta total RNA
## Troubleshooting

<table>
<thead>
<tr>
<th>Observation</th>
<th>Possible cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent software does not calculate one concentration and peak size</td>
<td>The software detects multiple peaks in the amplified cDNA profile</td>
<td>Refer to “Analyze multiple peaks as one peak” on page 63.</td>
</tr>
<tr>
<td>Low yield in the desired size range and high background of small sizes</td>
<td>Your input amount is too low</td>
<td>• Use enriched or purified small RNA instead of total RNA for ligation.</td>
</tr>
<tr>
<td>(120-bp and 130-bp byproducts)</td>
<td></td>
<td>• Use more RNA (&lt;1 µg) for ligation.</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
<td></td>
<td>• Perform another gel purification of PCR products to select the desired range (110–140 bps)</td>
</tr>
<tr>
<td>Low yield in the desired size range and high background of large sizes</td>
<td>tRNA is partially degraded</td>
<td>Run the remaining 5 µL cDNA on a 10% TBU gel and select only 60–70-nt sizes (see Figure 8 on page 43).</td>
</tr>
<tr>
<td>(~135-bp byproducts) (see Figure 9 on page 49)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal yield at desired size range but background (100 bp, 115 bp) is too</td>
<td>The gel selection step does not exclude small ligation products</td>
<td>• Run the remaining cDNA on another gel; cut right on 60 nt so that you do not include anything smaller than the 18-nt insert.</td>
</tr>
<tr>
<td>high (see Figure 8 on page 48)</td>
<td></td>
<td>• Perform another gel purification of PCR products to select the desired size range (110–140 bps).</td>
</tr>
<tr>
<td>Low yield and no PCR products</td>
<td>The gel ran too long or too much gel was added to the PCR</td>
<td>Reduce the running time (step 6 on page 42) and add less gel to the PCR (step 1 on page 44).</td>
</tr>
<tr>
<td></td>
<td>An enzymatic reaction or column purification failed</td>
<td>1. Dilute the cDNA 1:10, then use 1 µL in a 50-µL PCR.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Check the yield before and after purification using the PureLink™ PCR Micro Kit.</td>
</tr>
<tr>
<td>Normal or high yield but PCR products larger than 150 bp</td>
<td>Too many PCR cycles resulted in overamplification</td>
<td>Decrease the number of PCR cycles (step 2 on page 45).</td>
</tr>
<tr>
<td>Normal yield and bad size distribution in the amplified library</td>
<td>Too much sample was loaded on the Novex® TBE-Urea Gel</td>
<td>Decrease the volume of sample loaded to less than 10 µL (step 5 on page 42).</td>
</tr>
<tr>
<td></td>
<td>The wells of the Novex TBE-Urea Gel contained urea</td>
<td>Before you load the samples, flush the wells of the gel several times with 1X TBE Running Buffer to remove urea from the wells and to obtain sharp bands (step 4 on page 42).</td>
</tr>
</tbody>
</table>
Using a positive control

A general troubleshooting strategy is to perform the SOLiD™ Total RNA-Seq Kit procedure using the Small RNA Control (human placenta total RNA) provided with the kit. Use 1 µg of Small RNA Control for the hybridization and ligation procedure starting on page 38.

Alternatively, you can use Ambion® flashPAGE™ Pre-cast Gels to purify small RNA or use the Invitrogen PureLink miRNA isolation Kit to enrich small RNA from control RNA and use that as input for ligation.

Note: If starting from total RNA, the yield will be very low, so the use of purified or enriched small RNA is highly recommended.

Another option is to use miRNA Reference Panel as a positive control. However, it needs to be kinased by the KinaseMax™ Kit before use:

1. Mix the components listed below in a 0.2-mL PCR tube, then spin.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNA Reference Panel</td>
<td>5.4</td>
</tr>
<tr>
<td>10X Kinase buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>9.6</td>
</tr>
<tr>
<td>10 mM ATP</td>
<td>1.0</td>
</tr>
<tr>
<td>T4 Polynucleotide Kinase</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>20.0</strong></td>
</tr>
</tbody>
</table>

2. Incubate at 37 °C for 30 minutes. The concentration of miRNA in the reaction is ~2 µM.

3. Make aliquots, transfer 2 µL to a new tube, then add 18 µL of Nuclease-free Water to make a working stock. The miRNA concentration in the working stock is ~0.2 µM (200 fmol/µL).

4. Make a 1:10 dilution and use 20 fmol of kinased miRNA reference panel for each ligation.

5. Store aliquots and working stock at -80 °C.
How to order

SOLiD™ Total RNA-Seq Kit

For information on the SOLiD™ Total RNA-Seq Kit, go to the Applied Biosystems website at www.appliedbiosystems.com and select:

Products > DNA Sequencing by Ligation > Library Preparation Reagents & Tubes > SOLiD™ Total RNA-Seq Kit

Barcoded primers for multiplex SOLiD™ System sequencing

The Applied Biosystems SOLiD™ RNA Barcoding Kits are sets of 16 SOLiD 3’ PCR Primers that are individually “barcoded” for multiplex SOLiD System sequencing.

For information on the available modules, go to the Applied Biosystems website at www.appliedbiosystems.com and select:

Products > DNA Sequencing by Ligation > Library Preparation Reagents & Tubes > SOLiD™ RNA Barcoding Kits
Optional materials and equipment not included

For the SDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Optional for both libraries

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 M Ammonium Acetate, 500 mL</td>
<td>Applied Biosystems PN AM9071</td>
</tr>
<tr>
<td>FirstChoice® Total RNA</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Gel Loading Solution (All-purpose, Native Agarose)</td>
<td>Applied Biosystems PN AM8556</td>
</tr>
<tr>
<td>Glycogen (5 mg/mL) (1 mL tube)</td>
<td>Applied Biosystems PN AM9510</td>
</tr>
<tr>
<td>Spin Columns and Tubes</td>
<td>Applied Biosystems PN AM10065</td>
</tr>
<tr>
<td>TE, pH 8.0, 500 mL</td>
<td>Applied Biosystems PN AM9849</td>
</tr>
<tr>
<td>Novex 6% TBE Gel, 1.0 mM, 10 well</td>
<td>Invitrogen PN EC6265BOX</td>
</tr>
<tr>
<td>RiboMinus™ Eukaryote Kit for RNA-Seq</td>
<td>Invitrogen PN A1083708</td>
</tr>
<tr>
<td>RiboMinus™ Plant Kit for RNA-Seq</td>
<td>Invitrogen PN A1083808</td>
</tr>
<tr>
<td>21-gauge needle</td>
<td>Major laboratory supplier (MLS)</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>MLS</td>
</tr>
</tbody>
</table>

Optional for whole transcriptome libraries

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>FirstChoice® Poly(A) RNA</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>MicroPoly(A)Purist™ Kit</td>
<td>Applied Biosystems PN AM1919</td>
</tr>
<tr>
<td>RiboMinus™ Eukaryote Kit for RNA-Seq</td>
<td>Invitrogen PN A1083708</td>
</tr>
<tr>
<td>RiboMinus™ Plant Kit for RNA-Seq</td>
<td>Invitrogen PN A1083808</td>
</tr>
</tbody>
</table>
Optional for small RNA libraries

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ElectroZap™ Electrode Decontamination Solution</td>
<td>Applied Biosystems PN AM9785</td>
</tr>
<tr>
<td>flashPAGE™ Buffer Kit (Type A)</td>
<td>Applied Biosystems PN AM9015</td>
</tr>
<tr>
<td>flashPAGE™ Fractionator Apparatus</td>
<td>Applied Biosystems PN AM13100</td>
</tr>
<tr>
<td>flashPAGE™ Pre-cast Gels (Type A)</td>
<td>Applied Biosystems PN AM10010</td>
</tr>
<tr>
<td>flashPAGE™ Reaction Clean-Up Kit</td>
<td>Applied Biosystems PN AM12200</td>
</tr>
<tr>
<td>KinaseMax™ 5’ End-Labeling Kit, 30 reactions</td>
<td>Applied Biosystems PN AM1520</td>
</tr>
<tr>
<td>mirVana™ miRNA Isolation Kit, 40 purifications</td>
<td>Applied Biosystems PN AM1560</td>
</tr>
<tr>
<td>mirVana™ miRNA Reference Panel v9.1</td>
<td>Applied Biosystems PN 4388891</td>
</tr>
<tr>
<td>mirVana™ PARIS™, 40 purifications</td>
<td>Applied Biosystems PN AM1556</td>
</tr>
<tr>
<td>NucAway™ Spin Columns Kit, 30 each</td>
<td>Applied Biosystems PN AM10070</td>
</tr>
<tr>
<td>PureLink™ miRNA Isolation Kit, 25 preps</td>
<td>Invitrogen PN K1570-01</td>
</tr>
</tbody>
</table>
Supplemental Information

This appendix contains:
- Amplified library construction concepts ............................................. 57
- Sequences of the SOLiD™ primers included in the kit .......................... 58
- Second-round size selection of amplified cDNA ................................. 59
- Using 2100 expert software to assess whole transcriptome libraries ....... 62
- Small RNA enrichment ........................................................................ 66
- Using 2100 expert software to assess small RNA libraries ................. 68

Amplified library construction concepts

The procedures in this protocol are based on Applied Biosystems Ligase-Enhanced Genome Detection (LEGenD™) technology (patent pending).

Hybridization and ligation to the Adaptor Mix

The RNA samples are hybridized and ligated with the SOLiD™ Adaptor Mix. The SOLiD™ Adaptor Mix is a set of oligonucleotides with a single-stranded degenerate sequence at one end and a defined sequence required for SOLiD™ System sequencing at the other end. The SOLiD™ Adaptor Mix constrains the orientation of the RNA in the ligation reaction such that hybridization with the SOLiD™ Adaptor Mix yields template for SOLiD System sequencing from the 5' end of the sense strand. The downstream emulsion PCR primer alignment and the resulting products of templated bead preparation for SOLiD System sequencing are illustrated in Figure 10.

Figure 10 Strand-specific RNA sequence information from SOLiD™ Total RNA-Seq Kit products

Reverse transcription and size selection

The RNA population with ligated adaptors is reverse transcribed to generate single-stranded cDNA copies of the fragmented RNA molecules. After a cleanup step using the MinElute® PCR Purification Kit, the sample is subjected to denaturing gel electrophoresis, and gel slices containing cDNA in the desired size range are excised.
In-gel cDNA library amplification (single- or multiplex) and final cleanup

The size-selected cDNA is amplified using 15–18 cycles of PCR that take place in the gel slices. This step appends required terminal sequences to each molecule and generates sufficient template for SOLiD System sequencing. Limiting the cycle number minimizes the synthesis of spurious PCR products and better preserves the RNA profile of the sample.

- To prepare template for singleplex SOLiD System sequencing, use the PCR primers included in the SOLiD Total RNA-Seq Kit.
- For multiplex SOLiD System sequencing, use the 3′ PCR primers supplied in a SOLiD™ RNA Barcoding Kit.

The 3′ (reverse) PCR primers in the SOLiD™ RNA Barcoding Kits contain the P2 sequence required for SOLiD emulsion PCR, a unique barcode sequence, and an internal adaptor sequence necessary for sequencing the barcode (Figure 10). Use a different 3′ PCR primer in the amplification reaction for each cDNA sample to generate a barcoded cDNA library that can be mixed with other barcoded libraries for multiplex SOLiD System sequencing. Plan the in-gel PCRs so that barcoded libraries are generated using multiples of the color-balanced groups of four 3′ primers, to preserve color balance during multiplexed sequencing on the SOLiD System. For more information, refer to the product insert for your SOLiD™ RNA Barcoding Kit and Applied Biosystems SOLiD™ 4 System SETS Software User Guide (PN 4448411).

The 5′ PCR primer is identical in each kit; its sequence corresponds to SOLiD emulsion PCR primer 1 (P1 in Figure 10).

After the PCR, the amplified cDNA is cleaned up using the PureLink™ PCR Micro Kit. The yield and size distribution of each cDNA library is assessed; it is important to have sufficient cDNA for accurate quantitation prior to SOLiD System templated bead preparation, and to use only libraries with sufficiently long inserts for SOLiD System sequencing.

Sequences of the SOLiD™ primers included in the kit

SOLiD™ 5′ PCR primer

The 5′ PCR primer is the SOLiD emulsion PCR primer P1.

5′ - CCA CTA CGC CTC CGC TTT CCT TAT GGG CAG TCG GTG AT -3′

SOLiD™ 3′ PCR primer

5′ - CTG CCC CGG GTT CCT CAT TCT CGT TAT TGC GTG AT -3′
Second-round size selection of amplified cDNA

Perform a second round of size selection of the amplified, double-stranded cDNA using non-denaturing polyacrylamide gel electrophoresis if:

- For whole transcriptome libraries: The percent of DNA that is 25–200 bp is greater than 20% (from step 3 on page 28).
- For small RNA libraries: The ratio of 120–130-bp DNA: 25–150-bp DNA is less than 50% (from step 2 on page 47)

Size-select the amplified cDNA

Use Novex® pre-cast gel products (Invitrogen), 10 bp or 50 bp DNA Ladder (Invitrogen), and SYBR® Gold nucleic acid gel stain (Invitrogen):

- Novex® 6% TBE Gel 1.0 mM, 10 Well
- Novex® TBE Running Buffer (5X)
- Novex® TBE Sample Buffer (2X)
- XCell SureLock™ Mini-Cell
- 50 bp DNA Ladder for whole transcriptome libraries or the 10 bp DNA Ladder for small RNA libraries
- SYBR® Gold nucleic acid gel stain

For more instructions on running Novex gels, refer to the Novex® Pre-Cast Gel Electrophoresis Guide by Invitrogen.

For more instructions on staining the gel, refer to the SYBR® Gold Nucleic Acid Gel Stain manual by Invitrogen.

1. Add Gel Loading Solution (AM8556) to the eluted cDNA:
   - For whole transcriptome libraries: Add 4 µL Gel Loading Solution to the eluted cDNA (~20 µL) from step 4 on page 27.
   - For small RNA libraries: Add 2 µL Gel Loading Solution to the eluted cDNA (~10 µL) from step 4 on page 46.

   **IMPORTANT!** Do not heat the samples before loading.

2. Prepare the gel as described in the Novex® Pre-Cast Gel Electrophoresis Guide by Invitrogen:
   a. Prepare 1000 mL of 1X TBE Running Buffer using Novex® TBE Running Buffer (5X).

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novex® TBE Running Buffer (5X)</td>
<td>200 mL</td>
</tr>
<tr>
<td>Deionized water</td>
<td>800 mL</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

   b. Place the Novex® 6% TBE Gel in the XCell SureLock™ Mini-Cell.
   c. Add 1X TBE Running Buffer to the Upper Buffer Chamber and the Lower Buffer Chamber.
3. Load the sample on the Novex 6% TBE Gel:
   • For whole transcriptome libraries: Load the sample into 3 adjacent wells (8 µL per well) and include a separate well for 5 µL 50 bp DNA Ladder (40 ng/µL)
   • For small RNA libraries: Load the sample into 2 adjacent wells (6 µL per well) and include a separate well for 5 µL 10 bp DNA Ladder (40 ng/µL)
   **Note:** Include a separate well with 5 µL 50 bp Ladder (40 ng/µL).

4. Run the gel at ~140 V for ~45 minutes, or until the front dye reaches the bottom of the gel.
   **Note:** Nondenaturing gels must be run slowly to avoid heat denaturation of the samples.

5. Stain the gel with SYBR® Gold dye, following the manufacturer’s instructions.

6. Illuminate the stained gel, then excise the gel in the appropriate size range for your library:
   • For whole transcriptome libraries: 200–300 bp
   • For small RNA libraries: 110–130 bp
   **Note:** If you are using a UV transilluminator to visualize the nucleic acid, work quickly to limit its exposure to UV radiation.

**Purify the amplified cDNA from the gel**

Use PAGE Elution Buffer (recipe below) and Spin Columns and Tubes (Applied Biosystems PN AM10065).

1. Prepare ~600 µL PAGE Elution Buffer for each sample.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE Buffer, pH 8 (10 mM Tris-HCl, ph 8, 1 mM EDTA)</td>
<td>5</td>
</tr>
<tr>
<td>5 M ammonium acetate (2.5 M final concentration)</td>
<td>5</td>
</tr>
<tr>
<td><strong>Final volume</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>

2. Shred the gel piece:
   a. Use a 21-gauge needle to puncture through the bottom-center of a 0.5-mL microcentrifuge tube.
   b. Place the gel piece in the punctured 0.5-mL tube, then place the 0.5-mL tube into a larger, 1.5-mL, nuclease-free microcentrifuge tube.
   c. Spin for 3 minutes at 13,000 x g to shred the gel.
   d. Place the 1.5-mL tube containing the shredded gel piece on ice.
   e. Inspect the 0.5-mL tube, and if any gel pieces remain, repeat the centrifugation step into a fresh 1.5-mL tube. Pool the gel pieces into one collection tube using a pipette tip.
3. Elute the DNA in PAGE elution buffer:
   a. Add 300 µL of PAGE Elution Buffer to the shredded gel pieces.
   b. Incubate the mixture overnight at room temperature, with gentle agitation.
   c. Transfer the buffer, which contains eluted DNA, to a fresh tube, leaving the gel fragments behind.
      Store the DNA on ice during the second elution (step 3e).
   d. Add another 300 µL of PAGE Elution Buffer to the shredded gel pieces.
   e. Incubate the buffer and gel pieces for 1 to 2 hours at 37°C, with gentle agitation.

4. Remove the gel pieces from the sample using a filter spin column:
   a. Combine the PAGE elution buffer from step 3c with the buffer plus gel slurry from step 3e.
   b. Cut a pipette tip to make a larger opening and use it to transfer the combined PAGE elution buffer and gel slurry from each sample to a Spin Column.
   c. Spin the Spin Column at top speed for 5 minutes to remove gel pieces. The DNA is now in the flowthrough.
      Alternatively, you can use a 0.45 µm-filter spin column from another manufacturer for this step, following the manufacturer’s instructions for the maximum centrifugation speed.

5. Precipitate the DNA, then resuspend in 20 µL of Nuclease-free Water:
   a. Add 1/100 volume of glycogen and 0.7 volume of isopropanol to each sample.
   b. Mix thoroughly, then incubate at room temperature for 5 minutes.
   c. Spin the sample at 13,000 x g for 20 minutes at room temperature.
   d. Carefully remove and discard the supernatant, then air dry the pellet.
   e. Resuspend the DNA pellet in 20 µL of Nuclease-free Water.

   **Note:** Accurate quantitation of the DNA is important for the downstream SOLiD™ System emulsion PCR titration step. The resuspension volume should yield DNA sufficiently concentrated for accurate measurements (~10 ng/µL).
Using 2100 expert software to assess whole transcriptome libraries

Perform a smear analysis

Perform a smear analysis to quantify the percentage of DNA in the 25–200 bp size range.

1. In the 2100 expert software, select View ▶ Setpoints.

2. On the Global tab, select Advanced settings.

3. In the Sample Setpoints section of the Advanced settings, select the Perform Smear Analysis checkbox, then double-click Table.

4. Set the smear regions in the Smear Regions dialog box:
   a. Click Add, then enter 25 bp and 200 bp for the lower and upper limits, respectively.
      These settings are used to determine the percentage of total product that is 25–200 bp in length.
   b. Click Add, enter 25 bp and 225 bp, then click OK.
      This is an arbitrary upper limit which is used to determine the median size.
5. Select the **Region Table** tab.

6. In the Region Table, review the percentage of the total product in the size ranges you set.

<table>
<thead>
<tr>
<th>Peak [bp]</th>
<th>To [bp]</th>
<th>Area%</th>
<th>% of Total</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>230</td>
<td>99.0</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>230</td>
<td>411.0</td>
<td>50</td>
</tr>
</tbody>
</table>

Determine the median size

On the Region Table tab, drag the upper limit line that you set in step b on page 62 to the left or right until the Region Table indicates 50% of Total.

Analyze multiple peaks as one peak

On the Peak Table tab, you may observe that the bioanalyzer software identified multiple peaks in a region that you want to consider as one peak. To obtain one concentration and automatically determine median size for a peak region, manually set the size range of the desired peak region.

1. In the bottom left corner of the software window, select the **Peak Table** tab.
2. Right-click anywhere on the electropherogram, then select **Manual Integration**.

3. To remove multiple peaks:
   a. Place the cursor on the peak to remove, right-click, then select **Remove Peak**.

   b. Repeat until one peak remains within the region of interest.
c. Drag the lower and upper region limits of the region until the entire library is included.

4. The software recalculates the median size (bp), concentration (ng/µL), and molarity (nM) of the peak region and displays the values in the Peak Table.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>4.30</td>
<td>604.2 Ladder Markers</td>
<td></td>
</tr>
<tr>
<td>221</td>
<td>20.18</td>
<td>153.3</td>
<td></td>
</tr>
<tr>
<td>1,000</td>
<td>2.10</td>
<td>21 Upper Marker</td>
<td></td>
</tr>
</tbody>
</table>
Small RNA enrichment

If your RNA sample contains 0.1–0.5% miRNA, perform small RNA enrichment using the Invitrogen PureLink™ miRNA Isolation Kit using this purification procedure. If you are using the PureLink™ miRNA Isolation Kit to isolate small RNA from cells or tissue, refer to the PureLink™ miRNA Isolation Kit Instruction Manual by Invitrogen.

1. Prepare the sample:
   a. Resuspend 5–50 µg total RNA in 90 µL Nuclease-free Water.
   b. Add 300 µL Binding Buffer (L3) and 210 µL 100% ethanol, then vortex to mix well.

2. Load the total RNA onto a Spin Cartridge:
   a. Load 600 µL of the sample containing Binding Buffer and ethanol onto the Spin Cartridge in a Collection Tube.
   b. Spin the cartridge at 12,000 × g for 1 minute.
   c. Discard the cartridge.
   **IMPORTANT!** Total RNA is bound to the cartridge and small RNA is in the flowthrough. Keep the flowthrough.

3. Add 700 µL 100% ethanol to the flowthrough, then vortex to mix well.

4. Load the small RNA onto a second Spin Cartridge:
   a. Load 700 µL of the sample containing 100% ethanol onto a second Spin Cartridge in a Collection Tube.
   b. Spin the cartridge at 12,000 × g for 1 minute.
   c. Discard the flowthrough.
   d. Return the cartridge to the collection tube.
   e. Load the remaining 700 µL of the sample containing 100% ethanol onto the cartridge.
   f. Spin the cartridge at 12,000 × g for 1 minute.
   g. Discard the flowthrough.

5. Wash the small RNA:
   a. Return the cartridge to the collection tube.
   b. Add 500 µL of Wash Buffer (W5) with ethanol to the Spin Cartridge.
   c. Spin the cartridge at 12,000 × g for 1 minute.
   d. Discard the flowthrough.

6. Wash the small RNA a second time:
   a. Return the cartridge to the collection tube.
   b. Add 500 µL of Wash Buffer (W5) with ethanol to the Spin Cartridge.
   c. Spin the cartridge at 12,000 × g for 1 minute.
   d. Discard the flowthrough and the collection tube.
7. Remove any residual Wash Buffer:
   a. Place the Spin Cartridge in a Wash Tube supplied with the kit.
   b. Spin the cartridge at 16,000 × g for 3 minutes.
   c. Discard the flowthrough and the Wash Tube.

8. Elute the small RNA:
   a. Place the Spin Cartridge in a clean Recovery Tube supplied with the kit.
   b. Add 50 µL of Sterile, RNase-free Water to the center of the Spin Cartridge.
   c. Incubate the Spin Cartridge at room temperature for 1 minute.
   d. Spin the cartridge at 16,000 × g for 1 minute.

Store the small RNA at –80 °C or assess the quality and quantity of the small RNA-enriched sample (page 36).
Using 2100 expert software to assess small RNA libraries

Review the median size

The 2100 expert software automatically calculates the median size (bp) of miRNA ligation products.

Select the Peak Table tab, then review the median size in the Peak Table and at the top of the peak in the electropherogram. The median size should be ~122–125 bp.

![Electropherogram chart showing the median size at the top of the peak.]

Perform a smear analysis

Perform a smear analysis to quantify the percentage of DNA in the 25–150 bp and 120–130 bp size range. The desired size range for miRNA ligation products is 120–130 bp.

1. In the 2100 expert software, select View ▶ Setpoints.

2. On the Global tab, select Advanced settings.
3. In the Sample Setpoints section of the Advanced settings, select the **Perform Smear Analysis** checkbox, then double-click **Table**.

```
  Sample Setpoints
    Alignment
    Align to Upper Marker  X
    Align to Lower Marker  X
    Quantitation
    Concentration of Upper... 2.1
    Concentration of Lower... 4.2
    Sizing
    Standard Curve  Point to Point
    Smear Analysis
    Perform Smear Analysis  X
    Regions  Table ...
```

4. Set the smear regions in the Smear Regions dialog box:
   a. Click **Add**, then enter 25 bp and 150 bp for the lower and upper limits, respectively.
   b. Click **Add**, enter 120 bp and 130 bp, then click **OK**.

```
Smear Regions (Global Setpoints)

<table>
<thead>
<tr>
<th>From [bp]</th>
<th>To [bp]</th>
<th>Name</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>150</td>
<td>150-150 bp</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>130</td>
<td>130-130 bp</td>
<td></td>
</tr>
</tbody>
</table>
```

5. Select the **Region Table** tab.

6. In the Region Table, review the area values for each of the size ranges you set.

```
<table>
<thead>
<tr>
<th>From [bp]</th>
<th>To [bp]</th>
<th>Area</th>
<th>% of Total</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>150</td>
<td>58</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>130</td>
<td>70</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>
```
Determine the % miRNA library

Using the area values from the Region Table, calculate the % miRNA library in the 120–130 bp region as a fraction of the 25–150 bp region using the formula:

\[
\text{% miRNA library} = \left( \frac{\text{Area from 120–130 bp}}{\text{Area from 25–150 bp}} \right) \times 100
\]

If the % miRNA library is less than 50%, Applied Biosystems strongly recommends that you perform a second-round size selection using a nondenaturing polyacrylamide gel, such as the Novex 6% TBE Gel (page 59).

Example % miRNA library calculation

In the example below, the % miRNA library is 73%.

<table>
<thead>
<tr>
<th>From [bp]</th>
<th>To [bp]</th>
<th>Area</th>
<th>% of Total</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>150</td>
<td>92.0</td>
<td>96</td>
</tr>
<tr>
<td>1</td>
<td>120</td>
<td>130</td>
<td>67.3</td>
<td>70</td>
</tr>
</tbody>
</table>

\[
\text{% miRNA library} = \left( \frac{67.3}{92.0} \right) \times 100 = 73\%
\]
When preparing samples for PCR amplification:

- Use a positive-displacement pipette or aerosol-resistant pipette tips.
- Follow proper pipette-dispensing techniques to prevent aerosols.
- Wear clean gloves and a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation).
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Centrifuge tubes before opening. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Clean lab benches and equipment periodically with 10% bleach solution. Use DNAZap™ Solution (PN AM9890).
This appendix covers:

- Chemical safety ................................................................. 73
  General chemical safety .................................................... 73
  SDSs .................................................................................. 73
  Biological hazard safety ....................................................... 74

**Chemical safety**

**General chemical safety**

**Chemical safety guidelines**

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See “About SDSs” on page 73.)

- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.

- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.

- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer’s cleanup procedures as recommended in the SDS.

- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

**SDSs**

**About SDSs**

Chemical manufacturers supply current Safety Data Sheets (SDSs) with shipments of hazardous chemicals to new customers. They also provide SDSs with the first shipment of a hazardous chemical to a customer after an SDS has been updated. SDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new SDS packaged with a hazardous chemical, be sure to replace the appropriate SDS in your files.
Appendix D  Safety

Chemical safety

Obtaining SDSs

To obtain Safety Data Sheets (SDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At www.appliedbiosystems.com, select Support → SDS. Search by chemical name, product name, product part number, or SDS part number. Right-click to print or download the SDS of interest.
- At www.ambion.com, go to the web catalog page for the product of interest. Select SDS, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com), telephone (650-554-2756; USA), or fax (650-554-2252; USA) your request, specifying the catalog or part number(s) and the name of the product(s). The associated SDSs will be e-mailed unless you request fax or postal delivery. Requests for postal delivery require 1 to 2 weeks for processing.

Note: For the SDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.

Biological hazard safety

General biohazard

**WARNING! BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories (www.cdc.gov/od/ohs/biosfty/bmb15/bmb15toc.htm).
- Your company’s/institution’s Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at: www.cdc.gov
Documentation and Support

Kit documentation

The following documents are available for the SOLiD™ Total RNA-Seq Kit:

<table>
<thead>
<tr>
<th>Document</th>
<th>Part number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOLiD™ Total RNA-Seq Kit Protocol</td>
<td>4452437</td>
<td>Provides product information, step-by-step instructions for using the SOLiD Total RNA-Seq Kit, troubleshooting information, ordering information, and supplemental information.</td>
</tr>
<tr>
<td>SOLiD™ Total RNA-Seq Kit for Whole Transcriptome Libraries Quick Reference Card</td>
<td>4452438</td>
<td>Provides abbreviated instructions for using the SOLiD Total RNA-Seq Kit to create an amplified cDNA library of the whole transcriptome.</td>
</tr>
<tr>
<td>SOLiD™ Total RNA-Seq Kit for Small RNA Libraries Quick Reference Card</td>
<td>4452439</td>
<td>Provides abbreviated instructions for using the SOLiD Total RNA-Seq Kit to create an amplified cDNA library of the small RNAs.</td>
</tr>
</tbody>
</table>

Related documentation

When using this protocol, you may find the Applied Biosystems documents listed below useful. To obtain this and additional documentation, see “Obtaining support” on page 76.

<table>
<thead>
<tr>
<th>Document</th>
<th>Part number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied Biosystems SOLiD™ 4 System SETS Software User Guide</td>
<td>4448411</td>
</tr>
<tr>
<td>Applied Biosystems SOLiD™ 4 System Templated Bead Preparation Guide</td>
<td>4448378</td>
</tr>
</tbody>
</table>
Obtaining support

For the latest services and support information for all locations, go to:

www.appliedbiosystems.com

At the Applied Biosystems web site, you can:

• Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
• Search through frequently asked questions (FAQs).
• Submit a question directly to Technical Support.
• Order Applied Biosystems user documents, SDSs, certificates of analysis, and other related documents.
• Download PDF documents.
• Obtain information about customer training.
• Download software updates and patches.
Technical Resources and Support
For the latest technical resources and support information for all locations, please refer to our Web site at www.appliedbiosystems.com/support