PROTOCOL

applied biosystems®
by life technologies™

SOLiD® Total RNA-Seq Kit

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life technologies™

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About This Protocol

Purpose of this protocol

This protocol provides procedures for preparing whole transcriptome libraries or small RNA libraries using the SOLiD™ Total RNA-Seq Kit.

Revision history

Revision	Date	Description
B	04/2011	• Added procedures for preparing whole transcriptome libraries starting from low input amounts of poly(A) RNA. • Added chemical fragmentation procedures for the preparation of whole transcriptome libraries. • Added bead-based cDNA size selection for whole transcriptome libraries.

Safety information

IMPORTANT! Before using this product, read and understand the "Safety Information" appendix in this document.

Safety alert words

Four safety alert words appear in Life Technologies 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.

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:

- **Whole transcriptome libraries (standard input)**: To prepare whole transcriptome libraries starting from standard input amounts of poly(A) RNA (100–500 ng) or rRNA-depleted total RNA (200–500 ng), see page 13.
- **Whole transcriptome libraries (low input)**: To prepare whole transcriptome libraries starting from low input amounts of poly(A) RNA (5–25 ng), see page 33.
- **Small RNA libraries**: To prepare small RNA libraries, see page 51.

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 and 5500 Systems. Instructions for using the SOLiD™ RNA Barcoding Kits are included in product inserts that come with the kits and 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.

Component	Amount	Cap
Nuclease-free Water	1.75 mL	clear
10× RNase III Reaction Buffer	20 µL	red
RNase III	20 µL	red
SOLiD™ Adaptor Mix **Note:** The SOLiD™ Adaptor Mix is not equivalent to Adaptor Mix A or Adaptor Mix B in other kits.	30 µL	green
Hybridization Solution	40 µL	green
2× Ligation Buffer	150 µL	green
Ligation Enzyme Mix	30 µL	green
10× RT Buffer	50 µL	yellow
dNTP Mix	500 µL	white
SOLiD™ RT Primer	30 µL	yellow

Component	Amount	Cap
ArrayScript™ Reverse Transcriptase	20 µL	yellow
10× PCR Buffer	660 µL	white
AmpliTaq® DNA Polymerase	110 µL	white
SOLiD™ 5′ PCR Primer	100 µL	white
SOLiD™ 3′ PCR Primer	100 µL	blue
WT Control RNA (1 µg/µL HeLa total RNA)	50 µL	clear
Small RNA Control (1 µg/µL human placenta total RNA)	10 µL	purple

Materials and equipment required but not included

To order Applied Biosystems products, go to **www.appliedbiosystems.com**. For Ambion or Invitrogen products, go to **www.invitrogen.com**. For widely available products, go to major laboratory suppliers (MLS). For optional materials and equipment and more ordering information, see page 67.

Item	Catalog no.
Applied Biosystems thermal cycler with heated lid, capable of holding 0.2-mL tubes: • Veriti® 96-Well Thermal Cycler • GeneAmp® PCR System 9700	See the Applied Biosystems website
Agilent® 2100 Bioanalyzer™ Instrument	Agilent G2938A
Microcentrifuge	MLS
NanoDrop® Spectrophotometer	Thermo Scientific
Pipettors, positive displacement or air-displacement	MLS
Ambion® 8-strip PCR Tubes & Caps, RNase-free, 0.2-mL	AM12230
Ambion® Non-Stick RNase-free Microfuge Tubes (0.5 mL), 500	AM12350
Ambion® Non-Stick RNase-free Microfuge Tubes (1.5 mL), 250	AM12450
Ambion® Nuclease-free Water (not DEPC-treated), 500 mL	AM9930
Ethanol, 100%, ACS reagent grade or equivalent	MLS
Pipette tips, RNase-free	MLS

For whole transcriptome libraries: standard input

Item	Catalog no.
Ambion® ATP Soln (optional)	AM8110G
Ambion® T4 Polynucleotide Kinase (Cloned) 10 U/µL (optional)	AM2310
Invitrogen™ Qubit® 2.0 Fluorometer	Q32866
Centrifugal vacuum concentrator (for example, SpeedVac)	MLS
Invitrogen™ PureLink® PCR Micro Kit, 50 preps	K310050
Invitrogen™ Quant-iT™ RNA Assay Kit, 100 assays	Q32852

For whole transcriptome libraries: standard input

Item	Catalog no.
Invitrogen™ RiboMinus™ Concentration Module, 6 preps **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.	K1550-05
Agilent® DNA 1000 Kit	Agilent 5067-1504
Agilent® RNA 6000 Pico Kit	Agilent 5067-1513
Agencourt® AMPure® XP, 5mL	Beckman Coulter Genomics A63880
Ambion® Magnetic Stand-96	AM10027 OR AM10050
U-bottom microplates, 96-well	MLS
Ambion MicroPoly(A)Purist™ Kit (optional)	AM1919
Invitrogen RiboMinus™ Eukaryote Kit for RNA-Seq (optional)	A10837
Invitrogen RiboMinus™ Plant Kit for RNA-Seq (optional)	A10838

For whole transcriptome libraries: low input

Item	Catalog no.
Ambion® Magnetic Stand-96	AM10027
Invitrogen™ PureLink® RNA Micro Kit	12183-016
Agencourt® AMPure® XP, 5mL	Beckman Coulter Genomics A63880
Agilent® DNA 1000 Kit	Agilent 5067-1504
Agilent® High Sensitivity DNA Kit (optional)	Agilent 5067-4626
Agilent® RNA 6000 Pico Kit	Agilent 5067-1513
U-bottom microplates, 96-well	MLS
mRNA Catcher™*PLUS* Kit (optional)	Invitrogen K157002
Ambion® MicroPoly(A)Purist™ Kit (optional)	AM1919

For small RNA libraries

Item	Catalog no.
Invitrogen™ XCell *SureLock™* Mini-Cell	EI0001
Transilluminator	MLS
Invitrogen™ 10 bp DNA Ladder	10821-015
Invitrogen™ Novex® 10% TBE-Urea Gels 1.0 mm, 10 well	EC6875BOX
Invitrogen™ Novex® TBE-Urea Sample Buffer (2X), 10 mL	LC6876
Invitrogen™ Novex® TBE Running Buffer (5X), 1 L	LC6675
Invitrogen™ PureLink® PCR Micro Kit, 50 preps	K310050
Invitrogen™ SYBR® Gold nucleic acid gel stain, 10,000X concentrate in DMSO, 500 µL	S-11494
Agilent® High-Sensitivity DNA Kit (optional)	Agilent 5067-4626
Agilent® DNA 1000 Kit	Agilent 5067-1504
Agilent® RNA 6000 Nano Kit	Agilent 5067-1511
Agilent® Small RNA Kit	Agilent 5067-1548
MinElute® PCR Purification Kit (50)	Qiagen 28004

2 Whole Transcriptome Library Preparation: Standard Input

Fragment the whole transcriptome RNA (standard input)

Start with 100–500 ng poly(A) RNA or 200–500 ng rRNA-depleted total RNA
(We strongly recommend that you add ERCC RNA Spike-In Control Mixes to the input total RNA see page 14)

↓

Fragment the RNA:

Fragment the RNA using RNase III (page 15) **OR**
Fragment the RNA by chemical hydrolysis (page 16)

↓

Clean up the fragmented RNA (page 17)

↓

Assess the yield and size distribution of the fragmented RNA (page 18)

↓

Construct the amplified whole transcriptome library (standard input)

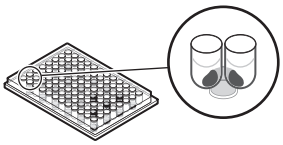
Hybridize and ligate the RNA (page 21)

↓

Perform reverse transcription (page 22)

↓

Purify and size-select the cDNA: round 1 (page 23)



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Purify and size-select the cDNA: round 2 (page 24)

↓

Amplify the cDNA (page 25)

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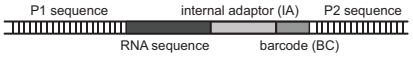
Purify the amplified DNA (page 27)



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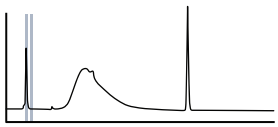
Assess the yield and size distribution of the amplified DNA (page 28)



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Proceed with SOLiD™ System templated bead preparation

Refer to the *Applied Biosystems SOLiD™ 4 System Templated Bead Preparation Guide* (PN 4448378) or to

SOLiD™ EZ Bead Emulsifier Getting Started Guide (Part no. 4441486)

Guidelines for RNA sample type and amount

We highly recommend the addition of Ambion® ERCC Spike-In Control Mixes (Part nos. 4456739 and 4456739) to the input RNA at the earliest step possible, preferably at the total RNA stage following the guidelines below, prior to whole transcriptome library preparation.

- The Ambion® ERCC RNA Spike-In Control Mixes provide a set of external RNA controls that enable performance assessment of a variety of technology platforms used for gene expression experiments. Add one Spike-In Mix to each RNA sample, and run the Spike-In Mix-containing samples on your platform. Then compare the Spike-In Mix data to known Spike-In Mix concentrations and ratios to assess the dynamic range, lower limit of detection, and fold-change response of your platform.
- The Ambion® ERCC RNA Spike-In Control Mixes are pre-formulated sets of 92 polyadenylated transcripts from the ERCC plasmid reference library. The transcripts are traceable through the manufacturing process to the NIST plasmid reference material. For detailed information, please refer to the ERCC Spike-In Control protocol. For your convenience, we have included the following table as reference during experimental design.

Guidelines for adding Spike-In Mixes to sample RNA

Amount of sample RNA	Volume of Spike-In Mix 1 or Mix 2 (dilution)†	
	Total RNA	Poly(A) RNA
20 ng	4 µL (1:10000)	2 µL (1:100)
50 ng	1 µL (1:1000)	5 µL (1:100)
100 ng	2 µL (1:1000)	1 µL (1:10)
500 ng	1 µL (1:100)	5 µL (1:10)
1000 ng	2 µL (1:100)	
5000 ng	1 µL (1:10)	

† ERCC RNA Spike-In Mix 1, ExFold Spike-In Mix 1, or ExFold Spike-In Mix 2.

Use highest-quality RNA available as your starting material. Ambion® FirstChoice® Total RNA provides high-quality, intact RNA isolated from a variety of sources. Best results are obtained when using RNA with an RNA integrity number (RIN) greater than 7.

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

- For poly(A) RNA, we recommend performing two rounds of oligo(dT) selection of the poly(A) RNA; for example, use the Ambion 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™ Instrument.
- For rRNA-depleted total RNA, we recommend 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. Also, confirm the absence of 18S and 28S rRNA; for example, check the profile of the rRNA-depleted RNA on an Agilent® 2100 Bioanalyzer™ Instrument.

Fragment the whole transcriptome RNA

In this guide, we provide procedures for two methods of RNA fragmentation, enzymatic and chemically induced. Each fragmentation method has pros and cons. Both fragmentation methods have been verified as part of the library preparation procedure outlined in this protocol. For more information and to help you select the best RNA fragmentation method for your experiment, see , "About the RNA fragmentation methods" on page 73. Use information about your experimental goals along with this information to choose the optimal fragmentation method for your study.

Fragmentation of the whole transcriptome RNA involves the following procedures:

1. Fragment the RNA:
   - Fragment the RNA using RNase III (page 15)
     OR
   - Fragment the RNA by chemical hydrolysis (page 16)
2. Clean up the fragmented RNA (page 17)
3. Assess the yield and size distribution of the fragmented RNA (page 18)

Fragment the RNA using RNase III

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

- Nuclease-free Water
- 10× RNase III Reaction Buffer
- RNase III

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

Component (add in order shown)	Volume
RNA sample and Nuclease-free Water: • Poly(A) RNA: 100–500 ng • rRNA-depleted total RNA: 200–500 ng • WT Control RNA: 500 ng	8 µL
10× RNase III Reaction Buffer	1 µL
RNase III	1 µL
Total volume	**10 µL**

IMPORTANT! To reduce fragmentation variability, accurately pipet 1 µL of 10× RNase III Reaction Buffer and 1 µL of RNase III to each sample. **Do not make a master mix that contains only 10× RNase III Reaction Buffer and RNase III**.

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. Proceed with cleaning up the fragmented RNA immediately (page 17), or leave the fragmented RNA on ice for less than 1 hour.

Fragment the RNA by chemical hydrolysis

Note: If you have fragmented the RNA using the RNase III procedure, skip this section and proceed to, "Clean up the fragmented RNA" on page 17.

For the RNA fragmentation, use components from the SOLiD™ Total RNA-Seq Kit:

- Nuclease-free Water
- 10× RNase III Reaction Buffer

For the kinase reaction, use the following components not included in the SOLiD Total RNA-Seq Kit:

- Ambion® T4 Polynucleotide Kinase (Cloned) 10 U/µL
- Ambion® ATP Soln.

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

Component (add in order shown)	Volume
RNA sample and Nuclease-free Water: • Poly(A) RNA: 100–500 ng • rRNA-depleted total RNA: 200–500 ng • WT Control RNA: 500 ng	9 µL
10× RNase III Reaction Buffer	1 µL
Total volume	**10 µL**

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 95°C for 10 minutes.

IMPORTANT! Incubate exactly 10 minutes. Shortening or lengthening the incubation time can result in suboptimal size distribution of the fragmented RNA.

4. *Immediately* after the incubation, snap-cool the tube on ice.

IMPORTANT! Proceed with the next step immediately.

5. To each 10 µL fragmented RNA sample, add the kinase reaction components:

Component (add in order shown)	Volume
Ambion® T4 Polynucleotide Kinase (Cloned) 10 U/µL	1 µL
Ambion® ATP Soln	1 µL

6. Flick the tube or pipet up and down a few times to mix, then spin briefly.
7. Incubate the reaction in a thermal cycler at 37°C for 30 minutes.
8. *Immediately* after the incubation, add 88 µL of Nuclease-free Water, then place the fragmented RNA on ice.

Proceed with cleaning up the fragmented RNA immediately (page 17), or leave the fragmented RNA on ice for less than 1 hour.

Clean up the fragmented RNA

Use the Invitrogen RiboMinus™ Concentration Module.

Note: Alternatively, use the Purelink® RNA Micro Kit, following the instructions on page 37. If you follow the alternative clean-up procedure, make sure that you return to the procedures in this chapter for constructing the amplified whole transcriptome library.

1. Add Binding Buffer (L3) and ethanol to the fragmented RNA, then mix well:
   - 100 µL of Binding Buffer (L3)
   - 250 µL of 100% ethanol
2. 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.
3. Prepare the Wash Buffer (W5) with ethanol, then store at room temperature:

Component	Volume
100% ethanol	6 mL
Wash Buffer (W5)	1.5 mL

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 Nuclease-Free water to the center of the Spin Column.

   IMPORTANT! Use the RNase-Free water from your SOLiD Total RNA-Seq Kit. Other nuclease-free water may contain DEPC which will affect downstream enzymatic reactions.

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

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* 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™ Instrument 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 median size is normally 125–200 nt. See Figures 1-5 starting on page 19.

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

      If the profile for the fragmented RNA does not meet the typical results, see "Troubleshooting" on page 31 for guidance.

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

Amount of fragmented RNA in 3 µL	Instructions
• ≥50 ng poly(A) RNA • ≥100 ng rRNA-depleted total RNA • ≥100 ng WT Control RNA	Proceed with "Construct the amplified whole transcriptome library" on page 20. Store the remaining RNA at –80°C.
• <50 ng poly(A) RNA • <100 ng rRNA-depleted total RNA	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. 2. Resuspend in 3 µL Nuclease-free Water, then proceed with "Construct the amplified whole transcriptome library" on page 20.

Typical results of fragmentation of whole transcriptome RNA

Figures 1-3 show profiles from an Agilent® 2100 Bioanalyzer™ Instrument after RNase III fragmentation and cleanup. Figure 1 shows results with HeLa poly(A) RNA. Figure 2 shows results with rRNA-depleted HeLa RNA. Figure 3 shows results with WT control total RNA. Figures 4-5 show profiles from an Agilent 2100 Bioanalyzer Instrument after chemical hydrolysis and cleanup. Figure 4 shows results with Hela poly(A) RNA. Figure 5 shows results with rRNA-depleted Hela RNA.

Figure 1 Size distribution of RNAse III fragmented HeLa poly(A) RNA (median size is 165 nt)

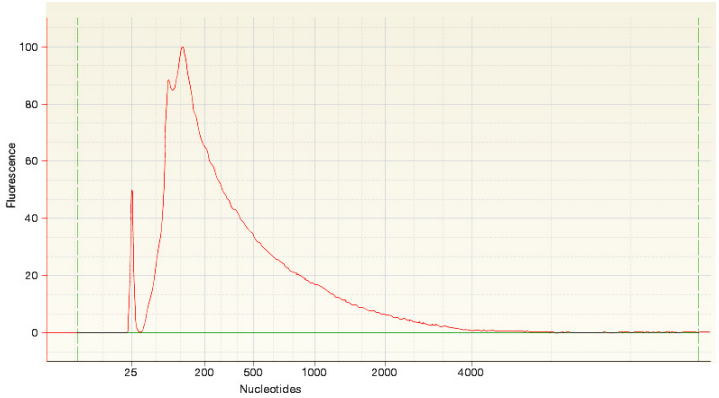


Figure 2 Size distribution of RNAse III fragmented rRNA-depleted HeLa RNA (median size is 140 nt)

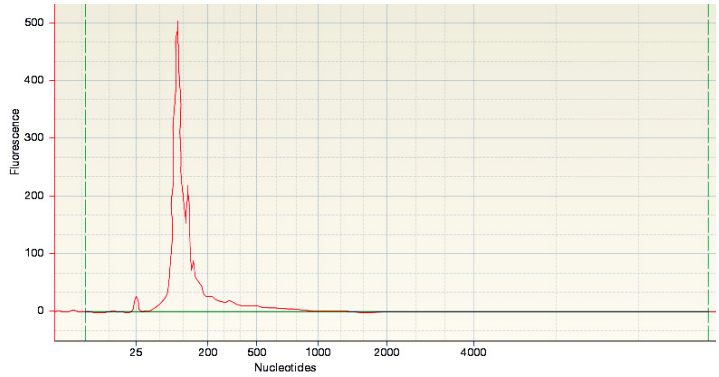


Figure 3 Size distribution of RNAse III fragmented WT Control total RNA (median size is 195 nt)

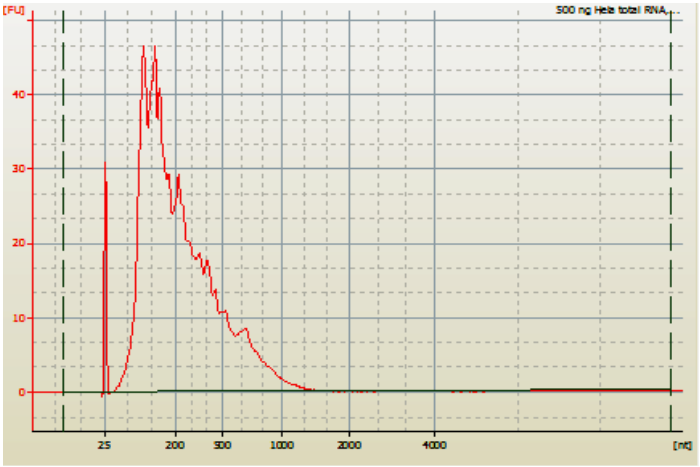


Figure 4 Size distribution of chemical fragmented Hela poly(A) RNA (median size is 165 nt)

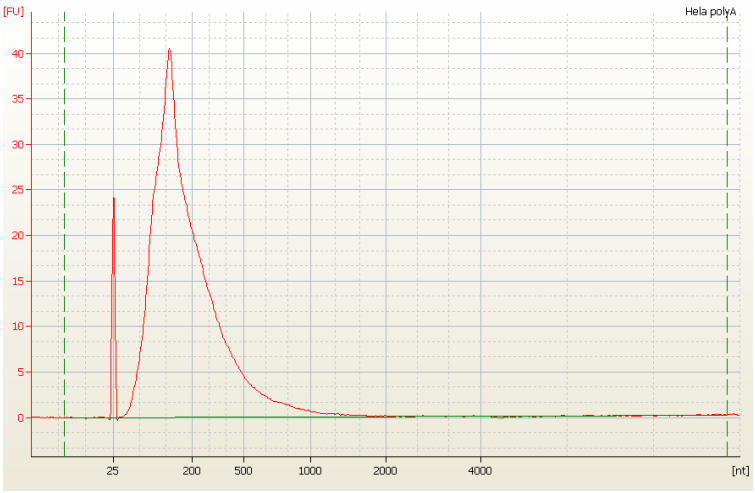
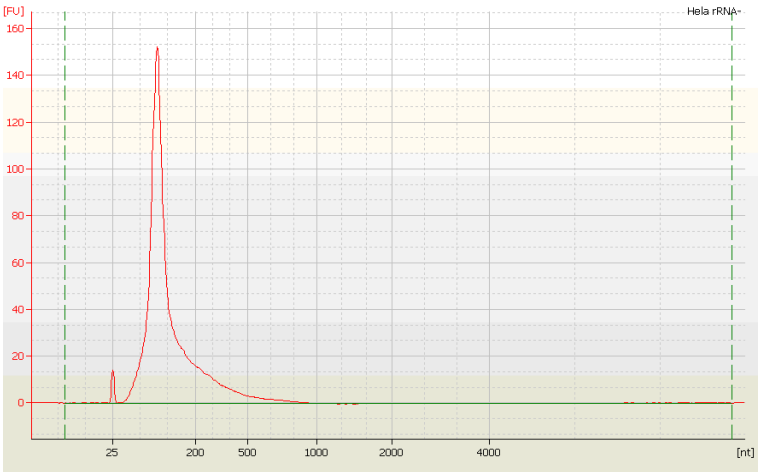


Figure 5 Size dist. of chemical fragmented rRNA-depleted Hela RNA (median size is 138 nt)



Construct the amplified whole transcriptome library

Constructing the amplified whole transcriptome library involves the following procedures:

1. Hybridize and ligate the RNA (page 21)
2. Perform reverse transcription (page 22)
3. Purify and size-select the cDNA: round 1 (page 23)
4. Purify and size-select the cDNA: round 2 (page 24)
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
- 2X Ligation Buffer
- Ligation Enzyme Mix

1. On ice, prepare the hybridization master mix:

Component	Volume for one reaction†
SOLiD™ Adaptor Mix	2 µL
Hybridization Solution	3 µL
Total volume per reaction	5 µL

† 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:

Temperature	Time
65°C	10 min
16°C	5 min

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

Component (add in order shown)	Volume
2X Ligation Buffer	10 µL
Ligation Enzyme Mix	2 µL

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
- 10× RT Buffer
- dNTP Mix
- SOLiD™ RT Primer
- ArrayScript™ Reverse Transcriptase

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

Component	Volume for one reaction†
Nuclease-free Water	11 µL
10X RT Buffer	4 µL
dNTP Mix	2 µL
SOLiD™ RT Primer	2 µL
Total volume per reaction	**19 µL**

† 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 and size-select the cDNA: round 1

Using Agencourt® AMPure® XP Reagent, perform two rounds of bead capture, wash, and elution to ensure complete capture and size-selection of the desired cDNA.

Note: If you require cDNA libraries with more accurate, defined insert sizes, you may wish to use the gel-based size selection protocol on page 74.

The first round of bead capture removes the salt and enzyme and binds cDNA products greater than 100 bp with a 1.8× addition of bead volume to the sample mixture. The subsequent washes remove fragments that are less than 100 bp. This process increases the percentage of library inserts that are in the desired size range.

The protocol described below uses an U-bottom 96 well plate and single or multi-channel pipets for mixing steps. Alternatively, a 1.2ml 96-Well plate and a plate shaker could be used assuming the beads are homogeneously resuspended at each mixing step.

Required materials:

- Agencourt® AMPure® XP Beads (place at room temperature 30 minutes before using)
- U-bottom microplate
- Magnetic Stand-96
- 70% ethanol, freshly prepared from 100% ethanol, ACS reagent grade or equivalent and nuclease-free water
- Nuclease-free Water

1. Bind the cDNA to the beads:
   a. Gently shake the Agencourt® AMPure® XP bottle to resuspend any magnetic particles that may have settled.
   b. Add 72 µL Agencourt® AMPure® XP Reagent to each 40 µL sample. Pipet up and down a few times to mix.
   c. Transfer the 112-µL bead and sample mixture to a U-bottom microplate.
   d. Incubate samples for 5 minutes at room temperature.
2. Remove the buffer from the beads:
   a. Place a U-bottom plate onto a Magnetic Stand-96 for 2-5 minutes to separate beads from the solution. Wait for the solution to clear before proceeding to the next step.
   b. While the plate is in the magnetic stand, aspirate the supernatant from the plate and discard.

   IMPORTANT! This step must be performed while the reaction plate is on the magnetic stand. Do not disturb the beads. If necessary, leave a few microliters of supernatant rather than disturb the beads.

3. Wash the beads with 70% ethanol:
   a. While the plate is on the magnetic stand, dispense 200 µL of 70% ethanol to each well of the U-bottom plate and incubate for 30 seconds at room temperature. Then aspirate the ethanol and discard.

      IMPORTANT! This step must be performed while the reaction plate is on the magnetic stand. Do not disturb the beads.

   b. Remove the plate from the Magnetic Stand-96.
4. Elute the cDNA from the beads:
   a. Add 40 µL of Nuclease-free water to each well of the plate.

      Note: Elution occurs quickly. It is not necessary for the beads to go back into solution for it to occur.

   b. Place U-bottom plate onto a Magnetic Stand-96 for 2-5 minutes to separate beads from the solution. Wait for the solution to clear before proceeding to the next step.
   c. Transfer 40 µL eluant to an empty well of the U-bottom plate for the second round of purification and size-selection. Then remove plate from Magnetic Stand-96.

      Note: If processing is equal to or less than 40 samples at one time, you may use the same U-bottom plate for both rounds of purification and size-selection. To avoid contamination, leave an empty row of wells between the two rounds.

Purify and size-select the cDNA: round 2

The second round of bead capture using Agencourt® AMPure® XP binds cDNA products greater than 150 bp with a 1.6× addition of bead volume to the sample mixture. Two rounds of size selection are required to increase the percentage of library inserts that are in the desired size range.

1. Bind the cDNA from the first bead capture to the beads:
   a. Gently shake the Agencourt® AMPure® XP Reagent bottle to resuspend any magnetic particles that may have settled.
   b. Add 64 µL Agencourt® AMPure® XP Reagent to each 40 µL of sample from the first bead capture.
   c. Mix thoroughly by pipette mixing 10 times. The color of the mixture should appear homogenous after mixing.
   d. Incubate samples for 5 minutes at room temperature to bind cDNA products to the magnetic beads.

2. Remove the buffer from the beads:
   a. Place U-bottom plate on a Magnetic Stand-96 for 2-5 minutes to separate beads from the solution. Wait for the solution to clear before proceeding to the next step.
   b. While the plate is in the magnetic stand, aspirate the supernatant from the plate and discard.

   IMPORTANT! This step must be performed while the reaction plate is on the magnetic stand. Do not disturb the beads. If necessary, leave a few microliters of supernatant rather than disturb the beads.

3. Wash the beads with 70% ethanol:
   a. While the plate is in the magnetic stand, dispense 200 µL of 70% ethanol to each well of the U-bottom plate and incubate for 30 seconds at room temperature.
   b. Aspirate the cleared solution from the reaction plate and discard.

   IMPORTANT! Perform these steps with U-bottom plate situated on the Magnetic Stand-96. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it is a known PCR inhibitor.

   c. Off the Magnetic Stand-96, allow the beads to dry for 5 minutes at room temperature.

   Note: Overdrying beads significantly decreases elution efficiency, however, leftover ethanol may inhibit PCR.

4. Elute the cDNA:
   a. While the plate is off the magnetic stand, add 40 µL of Nuclease-free water to each well of the U-bottom plate and pipette mix 10 times.

   Note: Elution occurs quickly. It is not necessary for the beads to go completely back into solution for elution to occur. Some beads may appear clumpy.

   b. Place U-bottom plate on a Magnetic Stand-96 for 2-5 minutes to separate beads from the solution. Wait for the solution to clear before proceeding to the next step.
   c. Transfer the eluant (40 µL) to a new 0.2mL tube or plate.

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 45 µL PCR mix.

Component	Volume (µL)
	50 µL PCR reaction
Nuclease-free water	33.4
10x PCR Buffer	5.0
dNTP Buffer	4.0
SOLiD 5′ PCR Primer	1.0
AmpliTaq® DNA Polymerase	0.6
Total Volume	**44.0**

2. Transfer 5 µL of each cDNA sample into a new 0.2mL PCR tube.
3. Add 44 µL PCR mix to each cDNA sample.
4. Add 1 µL 3' PCR Primer to each sample.

   Note: To prepare cDNA libraries for multiplex SOLiD™ System sequencing, substitute with the barcoded SOLiD™ 3' PCR Primers from one of the SOLiD™ RNA Barcoding Kits.

5. Run the PCR reactions in a thermal cycler:

Stage	Temperature	Time
Hold	95°C	5 minutes
Cycle (15 cycles)	95°C	30 seconds
	62°C	30 seconds
	72°C	30 seconds
Hold	72°C	7 minutes

Note: Run 15 cycles if you started with 50-100ng of fragmented RNA. If necessary, increase the volume of cDNA used for each PCR reaction, or adjust the numbers of cycles according to the amount of fragmented RNA used in the hybridization and ligation steps, 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 Invitrogen PureLink® PCR Micro Kit.

- 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. Before using the PureLink® Micro Kit Column, place it in a clean Collection Tube, then spin the column at 10,000 × g for 1 minute.
2. Prepare the sample:
   a. Transfer the 50-µL PCR product into 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 200 µL of Binding Buffer (B2) to the tube, then mix well.
3. Load the sample onto the PureLink® Micro Kit Column:
   a. Load 250 µL of the sample containing Binding Buffer (B2) onto the PureLink® Micro Kit Column.
   b. Spin the column at 10,000 × g for 1 minute.
   c. Discard the flowthrough.
4. 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.
5. 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 5b through step 5c 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™ Instrument with the DNA 1000 Kit (Agilent®). For instructions on how to perform a smear analysis, see page 88.

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™ Instrument 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.

   Note: In general, we see a positive correlation between RNA-Seq mapping statistics and the percent of DNA library inserts that are above 200 bp in length. We recommend minimizing the percent of inserts that fall in the 25-200 bp range. Please use your judgement when deciding the best way to proceed with libraries from precious samples that fall close to these recommended quality values of more than 50% DNA in 25-200 bp range.

Percent of DNA in the 25–200 bp range	Next steps
Less than 50%	Proceed with SOLiD™ System templated bead preparation.
50-60%	You may perform another round of purification and size-selection on the amplified DNA using Agencourt® AMPure® XP: 1. Bring the sample volume to 40 µL with Nuclease-free Water. 2. Follow the instructions in "Purify and size-select the cDNA: round 2" on page 24, using AMPureXP bead purification to decrease the % of amplified DNA in the 25-200bp range. However, in order to maintain a high concentration, elute with 20 µL of water, instead of with the 40 µL used in step 4 of the procedure. OR Alternatively, you may proceed with SOLiD System templated bead preparation and expect to see slightly higher % of filtered reads in your sequencing data when compared to libraries with less than 50% in the 25-200bp range.
More than 60%	It is recommended that you perform another round of purification and size-selection using Agencourt AMPureXP. See step 1 and step 2 above.

Note: For instructions on how to perform the smear analysis, see "Perform a smear analysis" on page 88. You may also 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 89.

   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.

Proceed with SOLiD™ System templated bead preparation

When the amplified DNA in the 25–200 bp range is minimal, 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) or to *SOLiD™ EZ Bead Emulsifier Getting Started Guide* (Part no. 4441486).

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™ Instrument profiles) of amplified libraries prepared from HeLa poly(A) RNA (Figure 6), HeLa WT Control total RNA (Figure 7), and rRNA-depleted HeLa RNA (Figure 8) using the SOLiD™ Total RNA-Seq Kit are shown.

Figure 6 Size distribution of amplified library prepared from HeLa poly(A) RNA

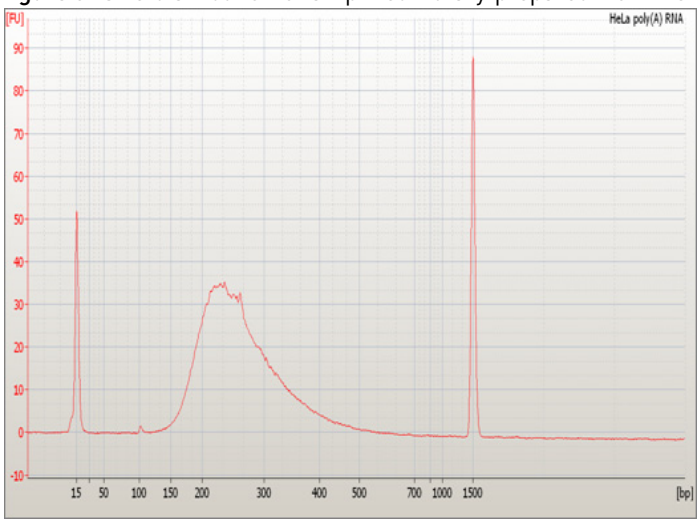


Figure 7 Size distribution of amplified library prepared from WT control total RNA

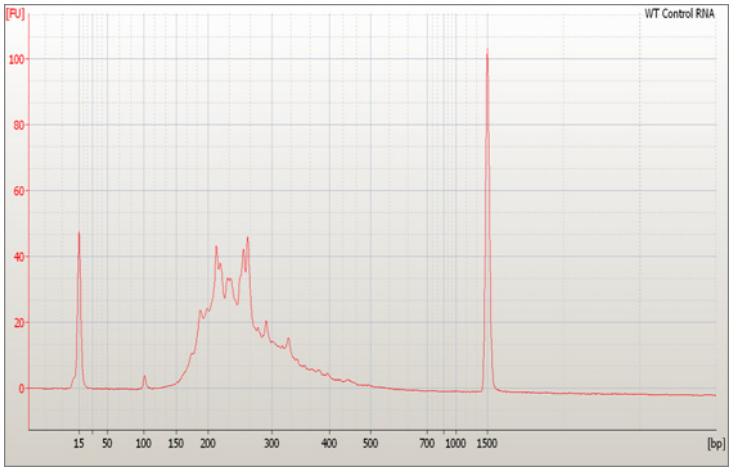
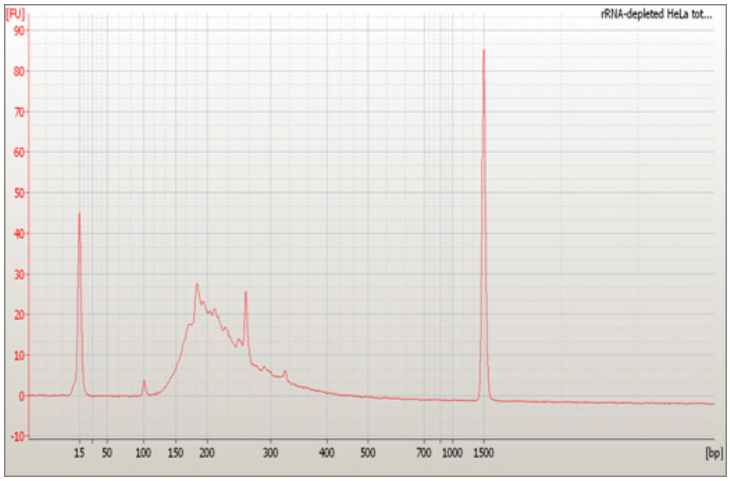


Figure 8 Size distribution of amplified library prepared from rRNA-depleted HeLa total RNA



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.

Workflow	Input amount	Typical recovery amount
Fragment the whole transcriptome RNA (page 15)	500 ng poly(A) RNA, total RNA, or rRNA-depleted total RNA	300–400 ng RNA
Construct the amplified whole transcriptome library (page 20)	50–100 ng fragmented RNA	>100 ng cDNA

Troubleshooting

Observation	Possible cause	Solution
Agilent® software doesn't calculate one concentration and peak size	The software detects multiple peaks in the amplified cDNA profile	Refer to "Analyze multiple peaks as one peak" on page 90.
Low yield and poor size distribution in the amplified library	You recovered <20% of the input RNA after you fragmented and cleaned up the RNA	Decrease the RNase III digestion from 10 minutes to 5 minutes (step 3 on page 15).
Low yield in the amplified library and very few differences in the Agilent® 2100 Bioanalyzer™ Instrument traces before and after you fragment the RNA	RNA fragmentation failed	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 15). For chemical fragmentation procedure, slowly increase the fragmentation time to 12-15 minutes.
Low yield and no PCR products	Too much ethanol introduced into PCR reaction.	Use optional drying step in bead clean up protocol.
	An enzymatic reaction or column purification performed after RNase III treatment failed	1. Dilute the cDNA 1:10, then use 1 µL in a 100-µL PCR. 2. Check the yield before and after purification using the PureLink® PCR Micro Kit. 3. If you get the same results, repeat the ligation with more fragmented RNA, and run a parallel ligation reaction with fragmented Control RNA.
Normal or high yield but PCR products larger than 300 bp	Too many PCR cycles resulted in overamplification	Decrease the number of PCR cycles. (step 5 on page 26).

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 15.
- Use 100 ng fragmented WT Control RNA in the amplified library construction procedure starting on page 20.

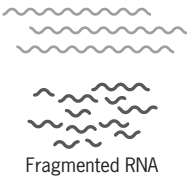
See the expected yields for the WT Control RNA on page 30.

3 Whole Transcriptome Library Preparation: Low Input

Fragment the low input whole transcriptome RNA (low input)

Start with 5-25 ng high quality poly(A) RNA

(We strongly recommend that you add ERCC RNA Spike-In Control Mixes to the input total RNA see page 34)



Fragment the RNA using RNase III (page 36)

Clean up the fragmented RNA (page 37)

Assess the yield and size distribution of the fragmented RNA (page 38)

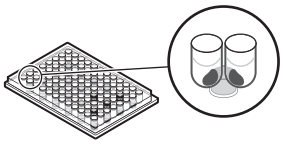
Construct the amplified low-input whole transcriptome library (low input)

Hybridize and ligate the low input poly(A) RNA (page 39)

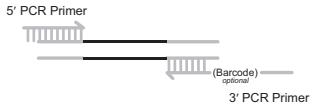
Perform reverse transcription (page 40)

Purify and size-select the cDNA: round 1 (page 41)

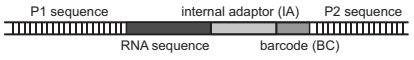
Purify and size-select the cDNA: round 2 (page 42)



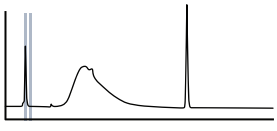
Amplify the cDNA (page 43)



Purify the amplified DNA (page 45)



Assess the yield and size distribution of the amplified DNA (page 46)



Proceed with SOLiD™ System templated bead preparation

Refer to the *Applied Biosystems SOLiD™ 4 System Templated Bead Preparation Guide* (PN 4448378) or to

SOLiD™ EZ Bead Emulsifier Getting Started Guide (Part no. 4441486)

About the low input procedures

IMPORTANT! Although the low input procedures are similar to the standard procedures, there are significant differences. For best results, follow these procedures as written.

We have developed and validated a procedure for preparing whole transcriptome libraries starting with 5–25 ng of poly(A) RNA. Modifications to the standard input procedures include:

- Reduced incubation time for RNA fragmentation to produce greater numbers of RNA fragments within the desired size range
- Modified purification methods after RNA fragmentation to maximize sample recovery
- Modified PCR conditions to maximize cDNA library yields without introducing a detectable bias in the expression profile

Note: We do not yet support preparation of whole transcriptome libraries starting with low inputs of rRNA-depleted total RNA. A protocol for low input rRNA depleted total RNA is under development. Although this protocol has been optimized for 2-25 ng inputs, we would recommend the use of the low input recommendations for input amounts from 25-100 ng as well.

We highly recommend the addition of ERCC Spike-In Control Mixes (Part nos. 4456739 and 4456739) to the input RNA at the earliest step possible, preferably at the total RNA stage following the guidelines below, prior to whole transcriptome library preparation.

- The Ambion®ERCC RNA Spike-In Control Mixes provide a set of external RNA controls that enable performance assessment of a variety of technology platforms used for gene expression experiments. Add one Spike-In Mix to each RNA sample, and run the Spike-In Mix-containing samples on your platform. Then compare the Spike-In Mix data to known Spike-In Mix concentrations and ratios to assess the dynamic range, lower limit of detection, and fold-change response of your platform.
- The ERCC RNA Spike-In Control Mixes are pre-formulated sets of 92 polyadenylated transcripts from the ERCC plasmid reference library. The transcripts are traceable through the manufacturing process to the NIST plasmid reference material. For detailed information, please refer to the ERCC Spike-In Control protocol. For your convenience, we have included the following table as reference during experimental design.

Guidelines for adding Spike-In Mixes to sample RNA

Amount of sample RNA	Volume of Spike-In Mix 1 or Mix 2 (dilution)†	
	Total RNA	Poly(A) RNA
20 ng	4 µL (1:10000)	2 µL (1:100)
50 ng	1 µL (1:1000)	5 µL (1:100)
100 ng	2 µL (1:1000)	1 µL (1:10)
500 ng	1 µL (1:100)	5 µL (1:10)
1000 ng	2 µL (1:100)	
5000 ng	1 µL (1:10)	

† ERCC RNA Spike-In Mix 1, ExFold Spike-In Mix 1, or ExFold Spike-In Mix 2.

Use the highest-quality RNA available as your starting material. Ambion® FirstChoice® Total RNA provides high-quality, intact RNA isolated from a variety of sources. Best results are obtained when using RNA with an RNA integrity number (RIN) greater than 7.

Fragment the low input whole transcriptome RNA

Fragmentation of the low input whole transcriptome RNA involves the following procedures:

1. Fragment the RNA using RNase III (page 36)
2. Clean up the fragmented RNA (page 37)
3. Assess the yield and size distribution of the fragmented RNA (page 38)

Guidelines for RNA sample type and amount

Use 5–25 ng of highest-quality poly(A) RNA available as your starting material. For example, for best results, use RNA with an RNA integrity number (RIN) greater than 7.

We recommend the following:

- Use the Invitrogen mRNA Catcher™ *PLUS* Kit to isolate mRNA from cells, tissues, blood, and total RNA.
- Use the Invitrogen mRNA Catcher™ PLUS 96-well plate and reagents for isolation of polyA-tailed mRNA from small sample sizes of 100ng of Total RNA up to 100µg of Total RNA.
  mRNA Catcher™ PLUS 96-well plate also accommodates polyA-tailed mRNA selection from 100 cells up to 10^6 cells per well.
- Alternatively, perform two rounds of oligo(dT) selection of the poly(A) RNA. For example, use the Ambion® MicroPoly(A)Purist™ Kit which accommodates 2–400 µg total RNA.
- Confirm the absence of 18S and 28S rRNA. For example, check the profile of the poly(A) RNA on an Agilent® 2100 Bioanalyzer™ Instrument.

Fragment the RNA using RNase III

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

- Nuclease-free Water
- 10× RNase III Reaction Buffer
- RNase III

1. For each poly(A) RNA sample, assemble the reaction on ice:

Component (add in order shown)	Volume
5–25 ng Poly(A) RNA sample in Nuclease-free Water	8 µL
10× RNase III Reaction Buffer	1 µL
RNase III	1 µL
Total volume	**10 µL**

IMPORTANT! To reduce fragmentation variability, accurately pipet 1 µL of 10× RNase III Reaction Buffer and 1 µL of RNase III to each sample. **Do not make a master mix that contains only 10× RNase III Reaction Buffer and RNase III**.

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 3 minutes.

IMPORTANT! The low input protocol only requires 3 minutes of incubation, as opposed to the 10 minutes required for the standard protocol. Do not over-incubate.

4. *Immediately* after the incubation, add 90 µL of Nuclease-free Water, then place the fragmented low-input RNA on ice.

Proceed with cleaning up the fragmented RNA immediately (page 37), or leave the fragmented RNA on ice for less than 1 hour.

Clean up the fragmented RNA

Use the Purelink® RNA Micro Kit (Invitrogen Cat no. 12183-016)

IMPORTANT! Use of the PureLink® RNA Micro Kit Columns instead of the RiboMinus™ Concentration Module is strongly recommended for optimum recovery of low input amounts of RNA.

Note: It is not necessary to add 2-mercaptoethanol or DTT to the Lysis Buffer.

1. Before using Wash Buffer II for the first time:
   a. Add 60 mL of 96-100% ethanol directly to the bottle.
   b. Check the box on the Wash Buffer II label to indicate that ethanol was added.
   c. Store Wash Buffer II with ethanol at room temperature.
2. Transfer the 100 µL fragmented RNA digestion mix to a 1.5mL tube. Add 100 µL of Lysis Buffer and 250 µL of 100% ethanol, then mix well.
3. Obtain a PureLink® RNA Micro Kit Spin Column with a collection tube, then verify that the column has a red o-ring.

   Note: The red o-ring differentiates the PureLink® RNA Micro Kit Spin Column from the unmarked PureLink® PCR Micro Kit Spin Column.
4. Bind the RNA to the spin column:
   a. Load 450 µL of the RNA sample containing Lysis Buffer and ethanol onto the Spin Column.
   b. Spin the column at 12,000 × g for 15 seconds.
   c. Discard the flowthrough.
5. Wash the RNA:
   a. Return the Spin Column to the Collection Tube.
   b. Add 500 µL of Wash Buffer II (W5) with ethanol to the Spin Column.
   c. Spin the column at 12,000 × g for 15 seconds.
   d. Discard the flowthrough.
6. Wash the RNA again:
   a. Return the Spin Column to the Collection Tube.
   b. Add another 500 µL of Wash Buffer II (W5) with ethanol to the Spin Column.
   c. Spin the column at 12,000 × g for 15 seconds.
   d. Discard the flowthrough.
   e. Return the Spin Column to the Collection tube.
   f. Using a pipette, carefully remove any buffer adhering to the top of the o-ring inside of the column.
   g. Spin the column at 12,000 × g for 1 minute.

7. Elute the RNA in a clean Recovery Tube:
   a. Place the Spin Column in a clean Recovery Tube.
   b. Add 12 µL of Nuclease-free Water to the center of the Spin Column.
   c. Wait 1 minute.
   d. Spin the column at 12,000 × g for 1 minute.

   Note: You should recover approximately 10 µL of fragmented low input RNA from the column.

Assess the yield and size distribution of the fragmented RNA

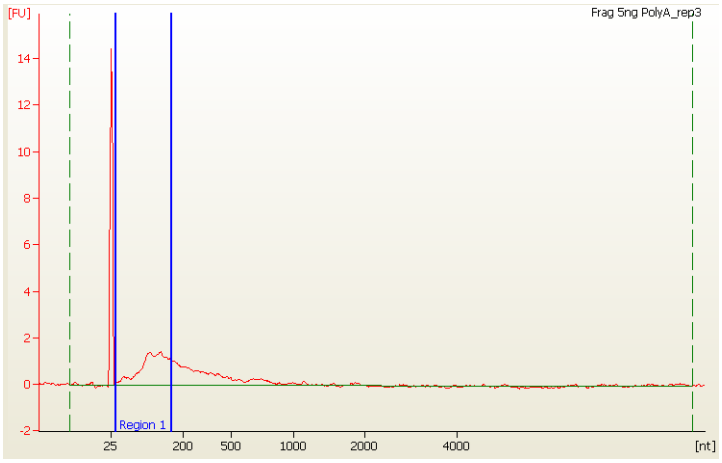
Use the RNA 6000 Pico Kit with the Agilent® 2100 Bioanalyzer™ Instrument.

1. Run 1 µL of undiluted sample on an Agilent® 2100 Bioanalyzer™ Instrument with the RNA 6000 Pico Kit. Follow the manufacturer's instructions for performing the assay.
2. 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. The average size should be 100–200 nt.

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

   Size distribution of RNAseIII fragmented 5ng input HeLa poly(A) RNA



3. Using a centrifugal vacuum concentrator at low or medium heat (≤40°C) for approximately 5 - 10 minutes, reduce the sample to 3 µL.

Construct the amplified low-input whole transcriptome library

Constructing the amplified, low-input, whole transcriptome library involves the following procedures:

1. Hybridize and ligate the low input poly(A) RNA (below)
2. Perform reverse transcription (page 40)
3. Purify and size-select the cDNA: round 1 (page 41)
4. Purify and size-select the cDNA: round 2 (page 42)
5. Amplify the cDNA (page 43)
6. Purify the amplified DNA (page 45)
7. Assess the yield and size distribution of the amplified DNA (page 46)
8. Proceed with SOLiD™ System templated bead preparation (page 47)

Hybridize and ligate the low input poly(A) 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:

Component	Volume for one reaction†
SOLiD™ Adaptor Mix	2 µL
Hybridization Solution	3 µL
Total volume per reaction	5 µL

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

2. Transfer 5 µL hybridization master mix to 3 µL fragmented low input RNA sample.
3. Slowly pipet up and down a few times to mix well, then spin briefly.
4. Run the hybridization reaction in a thermal cycler:

Temperature	Time
65°C	10 min
16°C	5 min

5. On ice, prepare the RNA ligation master mix:

Component (add in order shown)	Volume
2X Ligation Buffer	10 µL
Ligation Enzyme Mix	2 µL
Total volume	12 µL

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. Add 12 µL RNA ligation master mix to each 8-µL hybridization reaction.
7. Flick the tube or slowly pipet up and down a few times to mix well, then spin briefly.
8. 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):

Component	Volume for one reaction†
Nuclease-free Water	11 µL
10X RT Buffer	4 µL
dNTP Mix	2 µL
SOLiD™ RT Primer	2 µL
Total volume per reaction	**19 µL**

† 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 and size-select the cDNA: round 1

Using Agencourt® AMPure® XP, perform two rounds of bead capture, wash, and elution to ensure complete capture and size-selection of the desired cDNA.

The first round of bead capture binds cDNA products greater than 100 bp with a 1.8× addition of bead volume to the sample mixture. The subsequent washes remove fragments that are less than 100 bp. This process increases the percentage of library inserts that are in the desired size range.

Required materials:

- Agencourt® AMPure® XP Reagent (place at room temperature 30 minutes before using)
- U-bottom microplate
- Magnetic Stand-96
- 70% ethanol, freshly prepared from 100% ethanol, ACS reagent grade or equivalent
- Nuclease-free Water

1. Bind the cDNA to the beads:
   a. Gently shake the Agencourt® AMPure® XP Reagent bottle to resuspend any magnetic particles that may have settled.
   b. Add 72 µL Agencourt® AMPure® XP Reagent to each 40 µL sample.
   c. Transfer the 112-µL bead and sample mixture to a U-bottom microplate.
   d. Mix thoroughly by pipette mixing 10 times. The color of the mixture should appear homogenous after mixing.
   e. Incubate samples for 5 minutes at room temperature.
2. Remove the buffer from the beads:
   a. Place the U-bottom microplate onto a Magnetic Stand-96 for 2-5 minutes to separate beads from the solution. Wait for the solution to clear before proceeding to the next step.
   b. While the plate is in the magnetic stand, aspirate the supernatant from the plate and discard.

   IMPORTANT! Do not disturb the magnetic beads. If beads are drawn out during aspiration, leave a few microliters of supernatant behind.

3. Wash the beads with 70% ethanol:
   a. While the plate is on the magnetic stand, dispense 200 µL of 70% ethanol to each well of the U-bottom microplate and incubate for 30 seconds at room temperature.
   b. Aspirate the ethanol and discard.
   c. While the plate is on the magnetic stand, dispense 200 µL of 70% ethanol to each well of the plate and incubate for 30 seconds at room temperature.
   d. Aspirate the ethanol and discard.
   e. Air dry the beads at room temperature for 5 minutes to remove all traces of ethanol. Do not overdry beads (overdried beads appear cracked).

      Note: Overdrying beads significantly decreases elution efficiency, however, leftover ethanol may inhibit PCR.

   f. Remove the plate from the Magnetic Stand-96.
4. Elute the cDNA from the beads:
   a. Add 40 µL of Nuclease-free water to each well of the plate and pipette mix 10 times.

      Note: Elution occurs quickly. It is not necessary for the beads to go back into solution for it to occur. Some beads may appear clumpy.

   b. Place the U-bottom plate on the Magnetic Stand-96 for 1 minute to separate beads from the solution.
   c. Transfer 40 µL eluant to an empty well of a U-bottom plate for the second round of purification and size-selection. Then remove plate from Magnetic Stand-96.

Note: You may use the same U-bottom plate for both rounds of purification and size-selection. To avoid contamination, leave an empty row of wells between the two rounds.

Purify and size-select the cDNA: round 2

The second round of bead capture using Agencourt® AMPure® XP binds cDNA products greater than 150 bp with a 1.6× addition of bead volume to the sample mixture. Two rounds of size selection are required to increase the percentage of library inserts that are in the desired size range.

1. Bind the cDNA from the first bead capture to the beads:
   a. Gently shake the Agencourt® AMPure® XP Reagent bottle to resuspend any magnetic particles that may have settled.
   b. Add 64 µL Agencourt® AMPure® XP Reagent to each 40 µL of sample from the first bead capture.
   c. Mix thoroughly by pipette mixing 10 times. The color of the mixture should appear homogenous after mixing.
   d. Incubate samples for 5 minutes at room temperature to bind cDNA products to the magnetic beads.

2. Remove the buffer from the beads:
   a. Place the U-bottom microplate onto a Magnetic Stand-96 for 2-5 minutes to separate beads from the solution. Wait for the solution to clear before proceeding to the next step.
   b. While the plate is in the magnetic stand, aspirate the supernatant from the plate and discard.

   IMPORTANT! This step must be performed while the reaction plate is on the magnetic stand. Do not disturb the beads. If necessary, leave a few microliters of supernatant rather than disturb the beads.

3. Wash the beads with 70% ethanol:
   a. While the plate is on the magnetic stand, dispense 200 µL of 70% ethanol to each well of the U-bottom microplate and incubate for 30 seconds at room temperature.
   b. Aspirate the ethanol and discard.
   c. While the plate is on the magnetic stand, dispense 200 µL of 70% ethanol to each well of the plate and incubate for 30 seconds at room temperature.
   d. Aspirate the ethanol and discard.
   e. Air dry the beads at room temperature for 5 minutes to remove all traces of ethanol. Do not overdry beads (overdried beads appear cracked).

      Note: Overdrying beads significantly decreases elution efficiency, however, leftover ethanol may inhibit PCR.

   f. Remove the plate from the Magnetic Stand-96.

4. Elute the cDNA:
   a. While the plate is off the magnetic stand, add 40 µL of Nuclease-free water to each well of the plate and pipette mix 10 times.

      Note: Elution occurs quickly. It is not necessary for the beads to go back into solution for elution to occur. Some beads may appear clumpy.

   b. Place the U-bottom plate on the Magnetic Stand-96 for 1 minute to separate beads from the solution. Wait for the solution to clear before proceeding to the next step.
   c. Transfer the eluant (40 µL) to a new 0.2-mL tube.

Amplify the cDNA

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

- Nuclease-free Water
- 10× 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. Transfer 20 µL of the eluted cDNA from the second round of purification and size-selection to a new tube.
2. For each low input cDNA sample, prepare 29 µL PCR master mix:

Component	Volume for one reaction (µL)†
Nuclease-free water	18.4 µL
10× PCR Buffer	5 µL
dNTP Mix	4 µL
SOLiD™ 5′ PCR Primer	1 µL
AmpliTaq® DNA Polymerase	0.6 µL
Total Volume	**29 µL**

† Include 5–10% excess volume in the master mix to compensate for pipetting error.

3. Transfer 29 µL PCR master mix into each 20 µL cDNA sample.
4. Add 1 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.

5. Run the PCR reactions in a thermal cycler:

Stage	Temp	Time
Hold	95°C	5 min
Cycle (18 cycles)	95°C	30 sec
	62°C	30 sec
	72°C	30 sec
Hold	72°C	7 min

Purify the amplified DNA

Use the Invitrogen PureLink® PCR Micro Kit:

- PureLink® PCR 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. Before using the PureLink® PCR Micro Kit Column, place it in a clean Collection Tube, then spin the column at 10,000 × g for 1 minute.
2. Prepare the sample:
   a. Transfer the 50-µL PCR reaction into 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 200 µL of Binding Buffer (B2) to the tube, then mix well.
3. Load the sample onto the PureLink® PCR Micro Kit Column:
   a. Load 250 µL of the sample containing Binding Buffer (B2) onto the PureLink® PCR Micro Kit Column.
   b. Spin the column at 10,000 × g for 1 minute.
   c. Discard the flowthrough.
4. 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.
5. 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 5b through step 5c for a total elution volume of 20 µL.

Assess the yield and size distribution of the amplified DNA

Assess the yield and size distribution of the low input amplified DNA using the Agilent® DNA 1000 Kit (Agilent®). For instructions on how to perform a smear analysis, see page 88.

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™ Instrument 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.

   Note: In general, we see a positive correlation between RNA-Seq mapping statistics and the percent of DNA library inserts that are above 200 bp in length. We recommend minimizing the percent of inserts that fall in the 25-200 bp range. Please use your judgement when deciding the best way to proceed with libraries from precious samples that fall close to these recommended quality values of more than 50% DNA in 25-200 bp range.

Percent of DNA in the 25–200 bp range	Next steps
Less than 50%	Proceed with SOLiD™ System templated bead preparation (see page 47).
50-60%	You may perform another round of purification and size-selection on the amplified DNA using Agencourt® AMPure® XP: 1. Bring the sample volume to 40 µL with Nuclease-free Water. 2. Follow the instructions in "Purify and size-select the cDNA: round 2" on page 42, using AMPureXP bead purification to decrease the % of amplified DNA in the 25-200bp range. However, in order to maintain a high concentration, elute with 20 µL of water, instead of with the 40 µL used in step 4 of the procedure. OR Alternatively, you may proceed with SOLiD System templated bead preparation and expect to see slightly higher % of filtered reads in your sequencing data when compared to libraries with less than 50% in the 25-200bp range.
More than 60%	It is recommended that you perform another round of purification and size-selection using Agencourt® AMPure® XP. See step 1 and step 2 above.

Note: For instructions on how to perform the smear analysis, see "Perform a smear analysis" on page 88. 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 89.

   Note: It is recommended that the amplified DNA be <50 ng/µL for accurate quantitation with the DNA 1000 Kit. (DNA 1000 Kit has a quantitative range of 0.1-50 ng/µL)

   Alternatively, obtain the mass concentration by another method, and convert the mass concentration to molar concentration.

Note: If the sample concentration is too low, reassess the yield and size distribution using the Agilent® High Sensitivity DNA Kit. (HS DNA Assay has a quantitative range of 5-500 pg/µL)

Proceed with SOLiD™ System templated bead preparation

When less than 50% (or in some cases, less than 60%)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) or *SOLiD™ EZ Bead Emulsifier Getting Started Guide* (Part no. 4441486

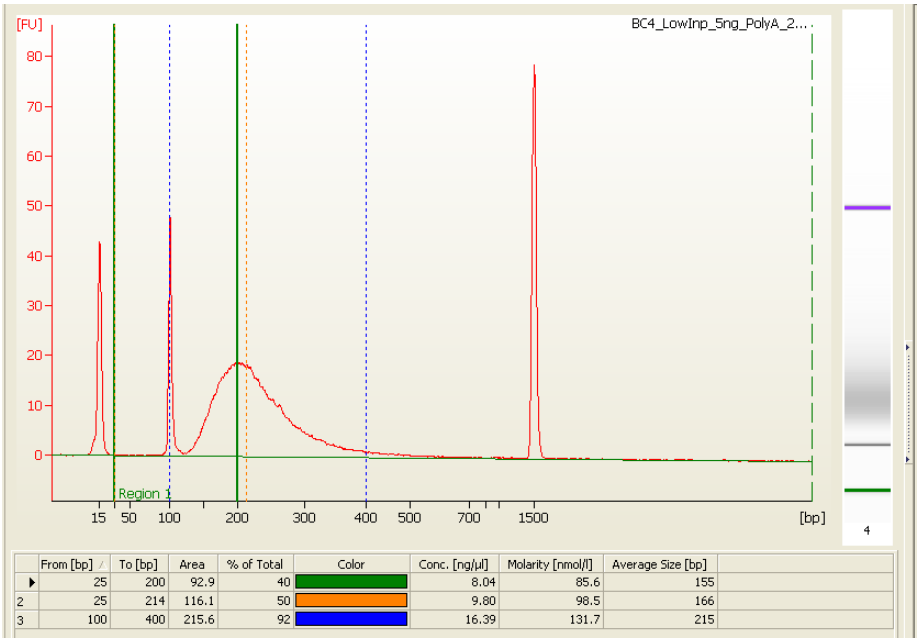
Barcoded libraries are pooled prior to templated bead preparation. For more information refer to the product insert for your SOLiD™ RNA Barcoding Kit.

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™ Instrument profiles) of amplified libraries prepared from HeLa poly(A) RNA (Figure 9) using the SOLiD™ Total RNA-Seq Kit are shown.

Figure 9 Size distribution of amplified library prepared from the 5 ng HeLa poly(A) RNA



	From [bp]	To [bp]	Area	% of Total	Color	Conc. [ng/µl]	Molarity [nmol/l]	Average Size [bp]
▶	25	200	92.9	40		8.04	85.6	155
2	25	214	116.1	50		9.80	98.5	166
3	100	400	215.6	92		16.39	131.7	215

Troubleshooting

See "Troubleshooting" on page 31.

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.

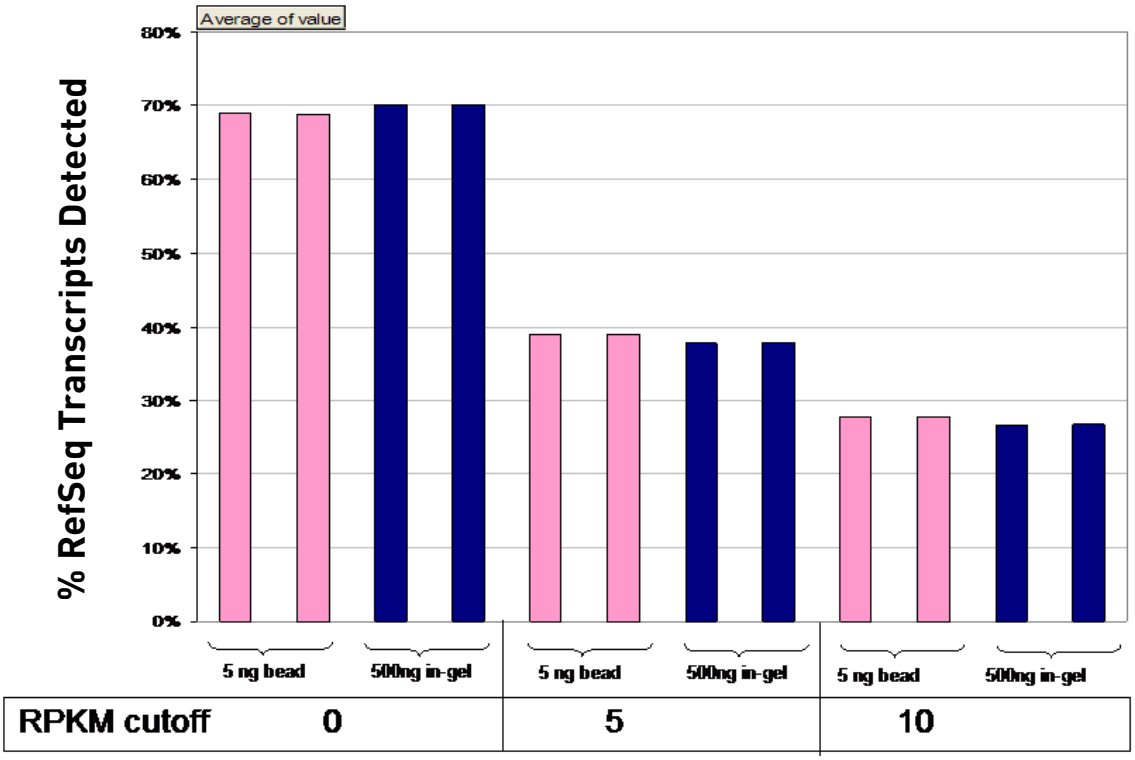
Use 5-25 ng WT Control RNA for the fragmentation procedure starting on page 35.

Typical results

Mapping statistics comparing the Low Input Workflow to the Standard input (Traditional In-Gel or AMPureXP bead size selection).

RNA Sample Input	cDNA Size Selection method	Total Number of Reads	%rRNA Reads	%Uniquely Mapped Reads	Number of Uniquely Mapped Reads
5ng HeLa PolyA_replicate 1	2 Rounds of AMPureXP Bead Purification	30,202,243	3.89%	31.66%	9,561,753.75
5ng HeLa PolyA_replicate 2		25,405,894	4.35%	38.58%	9,801,834.61
500ng HeLa PolyA_replicate 1	Traditional In-Gel (6% TBE-Urea Gel)	27,086,281	3.73%	56.62%	15,336,725.72
500ng HeLa PolyA_replicate 2		28,385,910	3.74%	56.03%	15,904,411.97
500ng HeLa PolyA_replicate 1	2 Rounds of AMPureXP Bead Purification	29,963,374	3.26%	54.58%	16,353,049.01
500ng HeLa PolyA_replicate 2		26,244,873	3.38%	57.51%	15,092,330.29

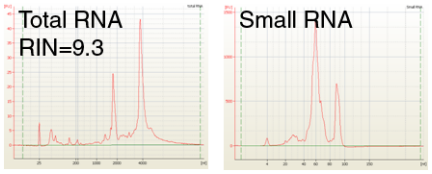
Uniquely mapped reads for low input with 5ng Poly(A) RNA are ~20% lower compared to the standard input at 500ng Poly(A). This is an attribute that is expected with the decrease in expression complexity of low input amounts of Poly(A) RNA when compared to the standard input. However, this decrease in % of uniquely mapped reads does not negatively impact % detection of RefSeq transcripts (graph below). In addition, low input Spearman correlations (by RefSeq RPKM) to the standard in-gel method are greater than 0.9625. (data not shown)


Average of value
% RefSeq Transcripts Detected
80%
70%
60%
50%
40%
30%
20%
10%
0%
5 ng bead
500ng in-gel
5 ng bead
500ng in-gel
5 ng bead
500ng in-gel
RPKM cutoff
0
5
10

4 Small RNA Library Preparation

Prepare the starting material

Assess the amount and quality of small RNA in your total RNA samples (page 52)



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Enrich the sample for small RNA (page 53)

↓

Assess the quality and quantity of the small RNA-enriched sample (page 54)

↓

Determine the input amount (page 54)

↓

Construct the amplified small RNA library

Hybridize and ligate the RNA (page 55)

↓

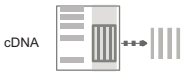
Perform reverse transcription (page 56)

↓

Purify the cDNA (page 56)

↓

Size select the cDNA (page 57)



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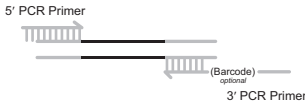
Amplify the cDNA (page 60)



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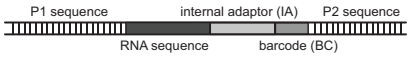
Purify the amplified DNA (page 61)



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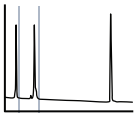
Assess the yield and size distribution of the amplified DNA (page 62)



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Proceed with SOLiD™ System templated bead preparation

Refer to the *Applied Biosystems SOLiD™ 4 System Templated Bead Preparation Guide* (PN 4448378) or to

SOLiD™ EZ Bead Emulsifier Getting Started Guide (Part no. 4441486)

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 53)
3. Assess the quality and quantity of the small RNA-enriched sample (page 54)
4. Determine the input amount (page 54)

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 enriched for miRNA.

We recommend 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 small RNA or total RNA that includes the small RNA fraction. Follow the procedure on page 91 to enrich small RNA from total RNA using the Invitrogen PureLink® miRNA Isolation Kit.
- ERCC spike in RNAs should not be used when constructing small RNA libraries. The ERCC RNAs will not be properly represented in the small RNA libraries because they are outside of the size range of RNAs selected for these libraries.

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™ Instrument with the RNA 6000 Nano Kit and the Small RNA Kit.

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 54.

2. Determine the quality and percentage of 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™ Instrument 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.

      Note: Best mapping results are seen when libraries are prepared from input RNA with RIN values ≥6. Use highest quality RNA available for library preparation. Libraries can be prepared from RNA with lower RIN values for precious samples. RNA degradation products may affect the quantitation of small RNA in the sample and compete with small RNA ligation affecting the number of sequencing reads uniquely mapping to miRBase (for instance).

3. Determine the percentage of small RNA in your sample:
   a. Run 1 µL of diluted RNA on the Agilent® 2100 Bioanalyzer™ Instrument 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:

How much miRNA (10–40 nt) is in your RNA sample?	Recommendations for small RNA enrichment and next steps
≥0.5% miRNA	You can use the total RNA in the ligation reaction, and small RNA enrichment is not needed. However, for optimal results, We recommend enrichment of all total RNA samples. Proceed with "Enrich the sample for small RNA" on page 53 or skip to "Determine the input amount" on page 54.
<0.5% miRNA	Small RNA enrichment is strongly recommended. We recommend using the Invitrogen PureLink® miRNA Isolation Kit. Proceed with "Enrich the sample for small RNA" on page 53.

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, we recommend that you enrich the RNA samples for small RNA.

Enrich the sample for small RNA

If needed, enrich the sample for small RNA using the method below:

- If your RNA sample contains <0.5% miRNA – Use the Invitrogen PureLink® miRNA Isolation Kit. Follow the instructions in "Small RNA enrichment" on page 91.

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™ Instrument with the Small RNA Chip Kit (Agilent®).

1. Run 1 µL of enriched small RNA sample on the Agilent® 2100 Bioanalyzer™ Instrument with the Small RNA Chip. Follow the manufacturer's instructions for performing the assay.
2. 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 52), and determine whether the RNA is degraded.
3. Using the 2100 expert software, determine the quality and quantity of recovered small RNA.

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

For enriched small RNA samples, peaks should be from 10 to 200 nt.

Determine the input amount

Using the following formula, determine the amount of total RNA to use according to the type of RNA you ran and the percentage of miRNA in the RNA sample.

$$\text{the amount of total RNA input} = \frac{\text{the amount of miRNA}}{\%\text{miRNA}}$$

Input sample type	Amount of miRNA (10 to 40 nt)	Total RNA input†
Total RNA	5 to 100 ng	≤ µg
Enriched small RNA	1 to 100 ng	≤ µg

† The yield drops if you use more than 1 µg of RNA for ligation.

Construct the amplified small RNA library

Constructing the amplified small RNA library involves the following procedures:

1. Hybridize and ligate the RNA (page 55)
2. Perform reverse transcription (page 56)
3. Purify the cDNA (page 56)
4. Size select the cDNA (page 57)
5. Amplify the cDNA (page 60)
6. Purify the amplified DNA (page 61)
7. Assess the yield and size distribution of the amplified DNA (page 62)
8. Proceed with SOLiD™ System templated bead preparation (page 63)

Hybridize and ligate the RNA

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

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

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

Component	Volume (µL)
Small RNA sample: • 5 to 100 ng of miRNA in ≤ µg of total RNA • 1 to 100 ng of miRNA in ≤ µg of enriched small RNA	3
Hybridization Solution	3
SOLiD™ Adaptor Mix	2
Total volume per reaction	**8**

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

Temperature	Time
65°C	10 min
16°C	5 min

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

Component (add in order shown)	Volume (µL)
2× Ligation Buffer	10
Ligation Enzyme Mix	2

IMPORTANT! You may observe a white precipitate in the 2× Ligation Buffer. If so, warm the tube at 37°C for 2–5 minutes or until the precipitate is dissolved. 2× 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
- 10× RT Buffer
- 2.5 mM dNTP Mix
- SOLiD™ RT Primer
- ArrayScript™ Reverse Transcriptase

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

Component	Volume for each reaction (µL)†
Nuclease-free Water	11
10X RT Buffer	4
dNTP Mix	2
SOLiD™ RT Primer	2
Total volume per reaction	**19**

† 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

Perform gel purification to separate library products from unused adaptors and primers. The gel purification method yields precise size-selection and the highest quality mapping.

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

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

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

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

Component	Volume (mL)
Novex® TBE Running Buffer (5X)	200
Deionized water	800
Total volume	1000

   b. Place the Novex® 10% TBE-Urea Gel in the XCell *SureLock*™ Mini-Cell.
   c. Add 1X TBE Running Buffer to the Upper Buffer Chamber and the Lower Buffer Chamber.
2. Dilute the 10-bp DNA Ladder:

Component	Volume (µL)	Concentration
10-bp DNA Ladder	1	1 µg/µL
RNase-free water	24	–
Total volume	**25**	**40 ng/µL**

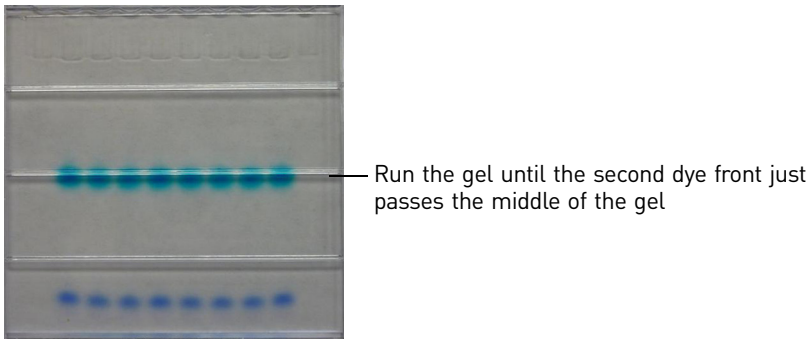
3. Prepare the cDNA and the DNA ladder:
   a. Mix 5 µL of the cDNA with 5 µL of 2X Novex TBE-Urea Sample Buffer.
   b. Mix 5 µL of the 40 ng/µL 10 bp DNA Ladder with 5 µL of 2X Novex TBE-Urea Sample buffer.
   c. Heat the cDNA and the DNA Ladder at 95°C for 3 minutes.
   d. Snap-cool the tubes on ice. 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).



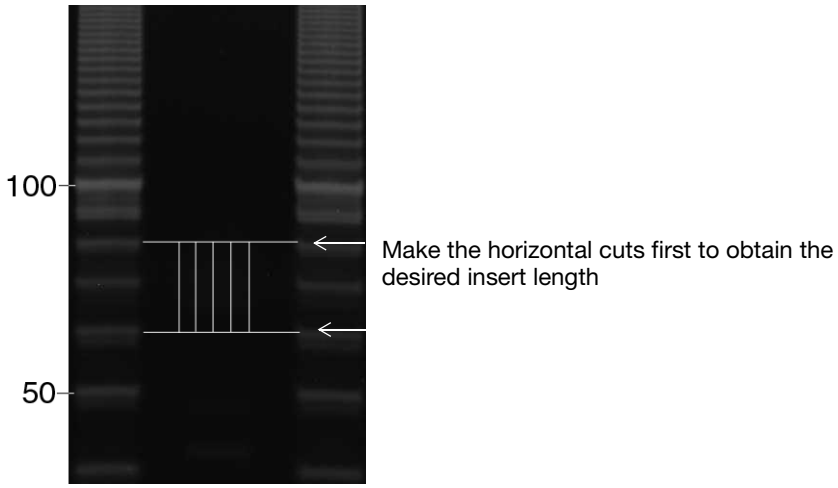
IMPORTANT! Shortening or lengthening the running time results in inferior resolution.

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.

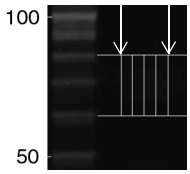
   a. Using a clean razor blade, make horizontal cuts directly on the 60-nt and 80-nt bands to excise the gel between 60 and 80 nt of cDNA.



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

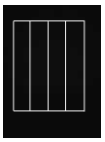
   Note: To obtain the desired insert length, you can adjust the cuts. However, too much gel will severely inhibit the PCR reactions. See page 60 for the expected lengths of the insert and PCR product according to the length of the cDNA that is excised from the gel.

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



Make the 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.



Cut the gel vertically into 4 pieces

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

Excised cDNA length (nt)	Insert length (bp)	PCR product length (bp)
42	~0	~93
60	~18	~110
80	~38	~130

Amplify the cDNA

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

- Nuclease-free Water
- 10× 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 60 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:

Component	Volume (µL)	
	One 100-µL reaction	Two 100-µL reactions†
Nuclease-free Water	76.8	169.0
10× PCR Buffer	10.0	22.0
2.5 mM dNTP Mix	8.0	17.6
SOLiD™ 5′ PCR Primer	2.0	4.4
AmpliTaq® DNA Polymerase	1.2	2.6
Total volume	**98.0**	**215.6**

† 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:

Stage	Temp	Time
Hold	95°C	5 min
Cycle (15 cycles)	95°C	30 sec
	62°C	30 sec
	72°C	30 sec
Hold	72°C	7 min

   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.

Purify the amplified DNA

Use the Invitrogen PureLink® PCR Micro Kit:

- 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. Before using the PureLink® Micro Kit Column, place it in a clean Collection Tube, then spin the column at 10,000 × g for 1 minute.
2. 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.
3. Load the sample onto the PureLink® Micro Kit Column:
   a. Load 500 µL of the sample containing Binding Buffer (B2) onto the PureLink® Micro Kit Column.
   b. Spin the column at 10,000 × g for 1 minute.
   c. Discard the flowthrough.
   d. Load the remaining 500 µL of the sample containing Binding Buffer (B2) onto the column.
   e. Spin the column at 10,000 × g for 1 minute.
   f. Discard the flowthrough.
4. 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.
5. 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™ Instrument with the DNA High Sensitivity Kit or DNA 1000 Kit (Agilent®). For instructions on how to perform a smear analysis, see page 88.

1. Run 1 µL of the purified DNA on an Agilent® 2100 Bioanalyzer™ Instrument with the DNA High Sensitivity Kit or 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:

      [Area (120–130 bp)]÷[Area (25–150 bp)]

      Note: In general, we see a positive correlation between the percentage of small RNA reads mapped to miRBase and the percentage of the DNA library that is between 120-130 bp. We recommend maximizing the percentage of inserts that fall into the 120-130 bp range. Please use your judgement when deciding the best way to proceed with libraries from precious samples that fall close to these recommended quality values.

Ratio of 120–130-bp DNA:25–150-bp DNA	Next steps
Greater than 50%	See "Proceed with SOLiD™ System templated bead preparation" on page 63
Less than 50%	Consider "Second-round size selection of amplified cDNA" on page 85. Alternatively, proceed with templated bead prep recommendations with potentially fewer sequencing reads uniquely mapping to miRBase.

Note: Samples that are run on a bioanalyzer typically show 5 to 8 bp larger than their actual size.

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* (Part. no. 4448378) or *SOLiD™ EZ Bead Emulsifier Getting Started Guide* (Part. no. 4441486).

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 10 Size distribution of amplified library prepared from placenta total RNA

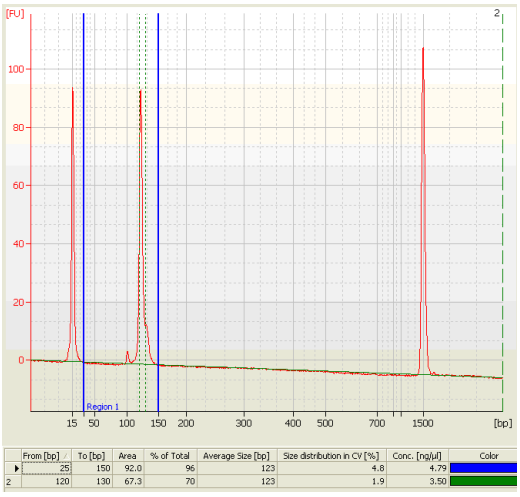


Figure 11 Size distribution of amplified library prepared from HeLa total RNA and required a second round of size selection

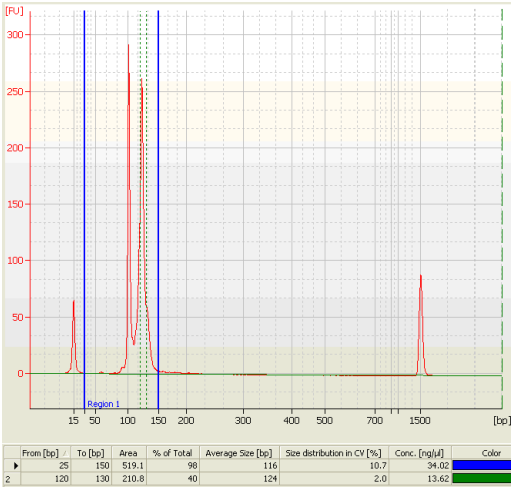
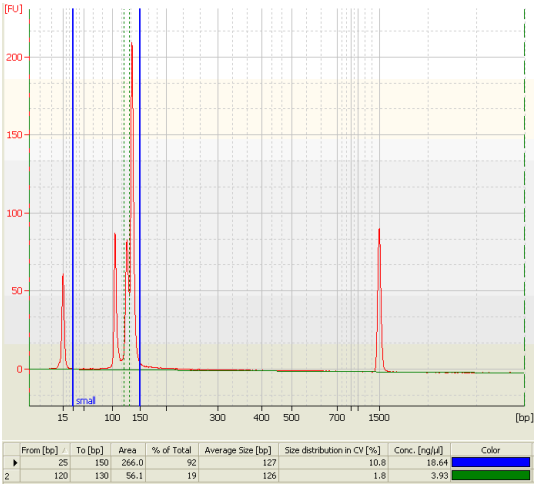


Figure 12 Sub-optimal size distribution of amplified library from partially degraded placenta total RNA



Troubleshooting

Observation	Possible cause	Solution
Agilent® software does not calculate one concentration and peak size	The software detects multiple peaks in the amplified cDNA profile	Refer to "Analyze multiple peaks as one peak" on page 90.
Low yield in the desired size range and high background of small sizes (~100-bp and 115-bp by-products)	Your input amount is too low	• Use enriched small RNA instead of total RNA for ligation. • Use more RNA (<1 µg) for ligation. • Perform another gel purification of PCR products to select the desired range (110–140 bps)
Low yield in the desired size range and high background of large sizes (~135-bp by-products) (see Figure 12 on page 64)	tRNA is partially degraded	Run the remaining 5 µL cDNA on a 10% TBU gel and select only 60–70-nt sizes (see page 59).
Normal yield at desired size range but background (100 bp, 115 bp) is too high (see Figure 11 on page 64)	The gel selection step does not exclude small ligation products	• 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. • Perform another gel purification of PCR products to select the desired size range (110–140 bps).
Low yield and no PCR products	The gel ran too long or too much gel was added to the PCR	Reduce the running time (step 6 on page 59) and add less gel to the PCR (step 1 on page 61).
	An enzymatic reaction or column purification failed	1. Dilute the cDNA 1:10, then use 1 µL in a 50-µL PCR. 2. Check the yield before and after purification using the PureLink® PCR Micro Kit.
Low yield with miRNA input >25 ng.	In rare cases, too much input inhibits the ligation reaction.	Reduce the input amount to 5–10 ng of miRNA (page 54).
Normal or high yield but PCR products larger than 150 bp	Too many PCR cycles resulted in overamplification	Decrease the number of PCR cycles (step 2 on page 61).
Normal yield and bad size distribution in the amplified library	Too much sample was loaded on the Novex® TBE-Urea Gel	Decrease the volume of sample loaded to less than 10 µL (step 5 on page 58).
	The wells of the Novex TBE-Urea Gel contained urea	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 58).

Using a positive control

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

- Use 500 ng small RNA for hybridization and ligation starting on page 55.
- Use 5 µg small RNA control for small RNA enrichment starting on page 91. Follow the instructions on page 56 to access the quality land quantity of small RNA-enriched control sample and use 25 ng of miRNA for hybridization and ligation.

A

Ordering Information

How to order

For more information on the SOLiD™ Total RNA-Seq Kit and SOLiD™ RNA Barcoding Kits, go to **www.appliedbiosystems.com**.

Item	Catalog/Part no.
SOLiD™ Total RNA-Seq Kit	4445374
SOLiD™ RNA Barcoding Kit, Modules 1-16	4427046
SOLiD™ RNA Barcoding Kit, Modules 17-32	4453189
SOLiD™ RNA Barcoding Kit, Modules 33-48	4453191
SOLiD™ RNA Barcoding Kit, Modules 49-64	4456501
SOLiD™ RNA Barcoding Kit, Modules 65-80	4456502
SOLiD™ RNA Barcoding Kit, Modules 81-96	4456503
SOLiD™ RNA Barcoding Kit, Modules 1-48	4461565
SOLiD™ RNA Barcoding Kit, Modules 49-96	4461566
SOLiD™ RNA Barcoding Kit, Modules 1-96	4461567

Optional materials and equipment not included

To order Ambion or Invitrogen products, go to **www.invitrogen.com**.

For whole transcriptome libraries (standard input)

Item	Catalog/Part no.
Ambion® 5 M Ammonium Acetate, 500 mL	AM9071
Ambion® FirstChoice® Total RNA	Go to **www.invitrogen.com**
Ambion® Gel Loading Solution (All-purpose, Native Agarose)	AM8556
Ambion® Glycogen (5 mg/mL) (1 mL tube)	AM9510
Ambion® MicroPoly(A)Purist™ Kit	AM1919
Ambion® Spin Columns and Tubes	AM10065
Ambion® TE, pH 8.0, 500 mL	AM9849
Invitrogen Novex 6% TBE Gel, 1.0 mM, 10 well	EC6265BOX
Invitrogen RiboMinus™ Eukaryote Kit for RNA-Seq	A1083708

Item	Catalog/Part no.
Invitrogen RiboMinus™ Plant Kit for RNA-Seq	A1083808
21-gauge needle	Major laboratory supplier (MLS)
Isopropanol	Major laboratory supplier (MLS)
ERCC RNA Spike-In Mix	4456740
ERCC ExFold RNA Spike-In Mixes	4456739

For whole transcriptome libraries (low input)

Item	Catalog/Part no.
Ambion® MicroPoly(A)Purist™ Kit	AM1919
Invitrogen mRNA Catcher™*PLUS* Kit	K1570-02
ERCC RNA Spike-In Mix	4456740
ERCC ExFold RNA Spike-In Mixes	4456739

For small RNA libraries

Item	Catalog/Part no.
Ambion® 5 M Ammonium Acetate, 500 mL	AM9071
Ambion® Electro*Zap*™ Electrode Decontamination Solution	AM9785
Ambion® FirstChoice® Total RNA	Go to **www.invitrogen.com**.
Ambion® Gel Loading Solution (All-purpose, Native Agarose)	AM8556
Ambion® Glycogen (5 mg/mL) (1 mL tube)	AM9510
Ambion® *mir*Vana™ miRNA Isolation Kit, 40 purifications	AM1560
Ambion® *mir*Vana™ PARIS™, 40 purifications	AM1556
Ambion® NucAway™ Spin Columns Kit, 30 each	AM10070
Ambion® Spin Columns and Tubes	AM10065
Ambion® TE, pH 8.0, 500 mL	AM9849
Invitrogen Novex 6% TBE Gel, 1.0 mM, 10 well	EC6265BOX
Invitrogen PureLink® miRNA Isolation Kit, 25 preps	K1570-01
21-gauge needle	Major laboratory supplier (MLS)
Isopropanol	Major laboratory supplier (MLS)

For whole transcriptome libraries (standard input): Gel-based size selection

Item	Catalog no.
Invitrogen™ XCell *SureLock*™ Mini-Cell	EI0001
Transilluminator	Major laboratory supplier (MLS)
Invitrogen™ 50 bp DNA Ladder	10416-014
Invitrogen™ Novex® 6% TBE-Urea Gels 1.0 mm, 10 well	EC6865BOX
Invitrogen™ Novex® TBE Running Buffer (5×), 1 L	LC6675
Invitrogen™ Novex® TBE-Urea Sample Buffer (2×), 10 mL	LC6876
Invitrogen™ SYBR® Gold nucleic acid gel stain, 10,000× concentrate in DMSO, 500 µL	S-11494
MinElute® PCR Purification Kit (50)	Qiagen 28004
(optional) ERCC RNA Spike-In Mix	4456740
(optional) ERCC ExFold RNA Spike-In Mixes	4456739

B Supplemental Information

This appendix contains:

- Amplified library construction concepts. 71
- Sequences of the SOLiD™ primers included in the kit. 72
- About the RNA fragmentation methods. 73
- Gel size selection for Whole Transcriptome workflow standard input 75
- Second-round size selection of amplified cDNA. 85
- Using 2100 expert software to assess whole transcriptome libraries 88
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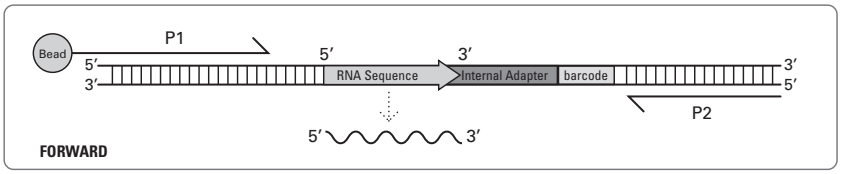
Amplified library construction concepts

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 13.

Figure 13 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. cDNA with insert sizes greater than 100 bp is selected by two rounds of Agencourt® AMPure® XP bead capture, wash, and elution.

cDNA library amplification (single- or multiplex) and final cleanup

The size-selected cDNA is amplified using 15–18 cycles of PCR. 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 13). 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) or *SOLiD™ EZ Bead™ Amplifier Getting Started Guide* (Part no. 4443494).

The 5′ PCR primer is identical in each kit; its sequence corresponds to SOLiD emulsion PCR primer 1 (P1 in Figure 13).

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 CTC TAT GGG CAG TCG GTG AT -3′

SOLiD™ 3′ PCR primer

5′ - CTG CCC CGG GTT CCT CAT TCT CTG TGT AAG AGG CTG CTG TAC GGC CAA GGC G -3′

About the RNA fragmentation methods

To generate whole transcriptome cDNA libraries with insert sizes suitable for SOLiD™ sequencing, you need to fragment the poly(A) RNA or rRNA-depleted total RNA to 100–200 nts before proceeding to ligation.

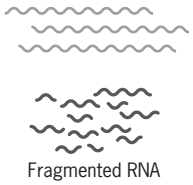
In this guide, we provide procedures for two methods of RNA fragmentation. Select the fragmentation method from the two options presented by using the guidance below:

- **RNase III fragmentation**: Provides convenience and robustness for comprehensive transcriptome analysis using the SOLiD™ system. RNase III digestion leaves 5′ phosphate and 3′ hydroxyl groups on the RNA fragments, and the resulting RNA fragments can be directly used in the hybridization and ligation reactions. The RNase III fragmentation method produces more consistent sequencing results because this method is effective across a wide range of enzyme activities, incubation times, and input amounts. We have observed RNaseIII fragmentation results in lower amounts of rRNA fragments being sequenced than when chemical fragmentation is used. This is assumed to be related to the role RNaseIII has in processing rRNAs. This method is useful for performing transcript-level quantification.
- **Chemical fragmentation**: The resulting RNA fragments need to be repaired using T4 polynucleotide kinase before proceeding to ligation. Because RNA fragmentation by chemical hydrolysis occurs rapidly, we recommend that you determine the optimal conditions for your experimental system. As noted above, we have observed a higher percentage of rRNA sequences are present in libraries constructed from chemically fragmented RNA than with RNaseIII fragmented RNA. Therefore, if minimizing rRNA sequences in the final library is very important, RNaseIII should be considered for fragmentation. The chemical fragmentation method produces a more uniform distribution of sequence tags across the transcripts. This method is useful for quantifying exon levels, identifying novel splicing, and discovering fusion genes.

Gel-based whole transcriptome library preparation: Standard Input

Fragment the whole transcriptome RNA (standard input)

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



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Fragment the RNA:

Fragment the RNA using RNase III (page 15) or
Fragment the RNA by chemical hydrolysis (page 16)

↓

Clean up the fragmented RNA (page 17)

↓

Assess the yield and size distribution of the fragmented RNA (page 18)

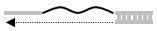
↓

Construct the amplified whole transcriptome library (standard input)

Hybridize and ligate the RNA (page 21)

↓

Perform reverse transcription (page 22)



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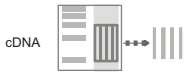
Purify the cDNA (page 75)

↓

Size select the cDNA (page 76)



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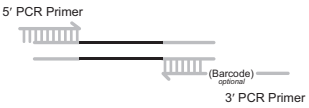
Amplify the cDNA (page 80)



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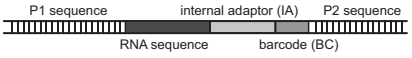
Purify the amplified DNA (page 81)



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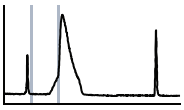
Assess the yield and size distribution of the amplified DNA (page 82)



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Proceed with SOLiD™ System templated bead preparation

Refer to the *Applied Biosystems SOLiD™ 4 System Templated Bead Preparation Guide* (PN 4448378) or to

SOLiD™ EZ Bead Emulsifier Getting Started Guide (Part no. 4441486)

Gel size selection for Whole Transcriptome workflow standard input

Although bead size selection is convenient for removing inserts that are too short from libraries, the size ranges of the final libraries are usually broad, ranging from 150 to 400 bps.

Gel size selection of cDNA generates libraries with more accurate, defined insert sizes. The gel procedure below generates whole transcriptome libraries with insert sizes from 100 to 200 bps. In addition, the insert size range can be adjusted by changing the cutting range on the gel.

Note: See the workflow on page 74 for more information. Follow the procedures from "Fragment the whole transcriptome RNA" on page 15 to "Hybridize and ligate the RNA" on page 21 and "Perform reverse transcription" on page 22. Then proceed with the following procedures for cDNA cleanup and gel size selection.

Purify the cDNA

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

Perform gel purification for precise size-selection and for the highest quality mapping.

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

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

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

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

Component	Volume
Novex® TBE Running Buffer (5X)	200 mL
Deionized water	800 mL
Total volume	1000 mL

   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 77.
   d. Add 1X TBE Running Buffer to the Upper Buffer Chamber and the Lower Buffer Chamber.
2. Dilute the 50 bp DNA Ladder:

Component	Volume	Concentration
50 bp DNA Ladder	1 µL	1 µg/µL
RNase-free water	24 µL	–
Total volume	**25 µL**	**40 ng/µL**

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.

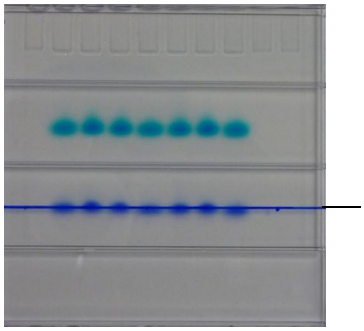
4. Immediately before you load each sample, flush the well of the gel several times with 1X TBE Running Buffer to remove urea from the wells.

   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).



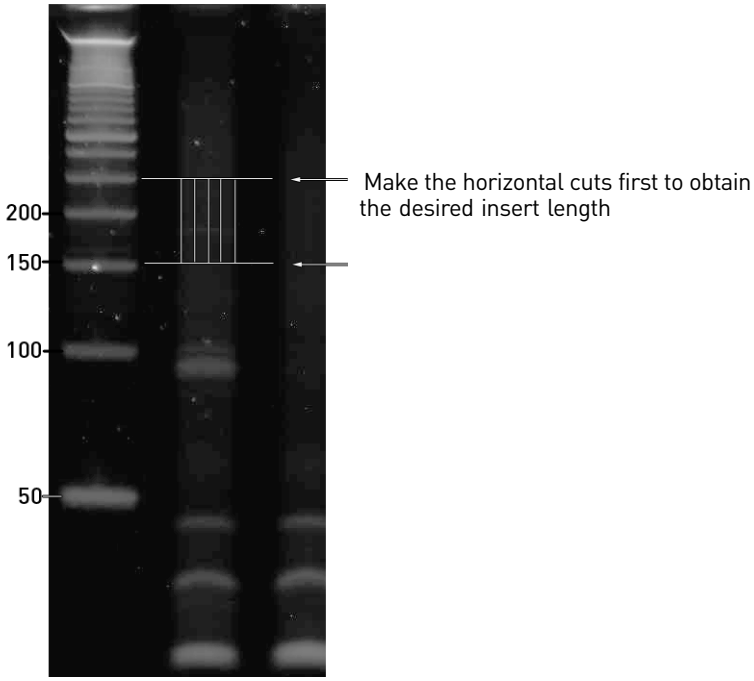
IMPORTANT! Shortening or lengthening the running time results in inferior resolution.

7. Add 5 µL of the SYBR Gold nucleic acid gel stain to 50 mL of 1✕ 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.

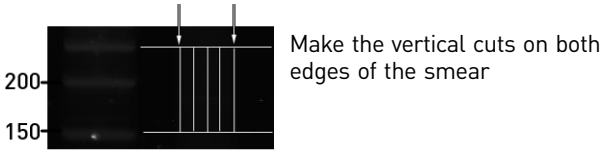
   a. Using a clean razor blade, make horizontal cuts to excise the gel containing 150–250 nt cDNA.



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

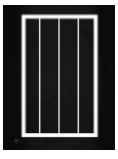
   Note: To obtain the desired insert length, you can adjust the cuts. However, too much gel will severely inhibit the PCR reactions. See Table 1 on page 79 for the expected lengths of the insert and PCR product according to the length of the cDNA that is excised from the gel.

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



   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.



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.

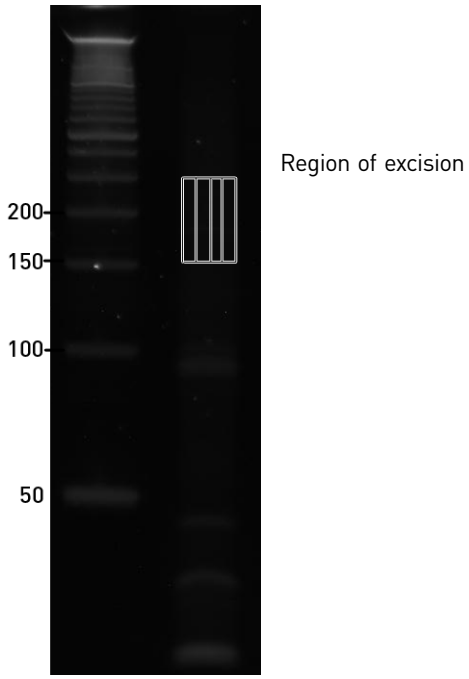
    To generate sufficient cDNA for accurate quantitation, you need to run 2 amplification reactions using 2 gel pieces, 1 gel piece in each reaction. You may store the other 2 pieces for 2 weeks at –20°C.

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

Example of 1st round size selection

Figure 14 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 14 Example of size selection of cDNA from HeLa poly(A) RNA



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

Table 1 Expected lengths of the insert and PCR according to excised cDNA length

Excised cDNA length (nt)	Insert length (bp)	PCR product length (bp)
50	~0	~100
100	~50	~150
150	~100	~200
200	~150	~250
250	~200	~300

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

1. For each cDNA sample, prepare 95 µL PCR mix.

Component	Volume (µL)	
	One100-µL reaction	Two 100-µL reactions
Nuclease-free water	71.8	143.6
10x PCR Buffer	10.0	20.0
dNPT Buffer	8.0	16.0
SOLiD 5′ PCR Primer	2.0	4.0
AmpliTaq® DNA Polymerase	1.2	2.4
Total Volume	**93.0**	**186.0**

2. Transfer 5 µL of each cDNA sample into a new 0.2mL PCR tube.
3. Add 93 µL PCR mix to each cDNA sample.
4. Add 2 µL 3' PCR Primer to each sample.

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

5. Run the PCR reactions in a thermal cycler:

Stage	Temperature	Time
Hold	95°C	5 minutes
Cycle (15 cycles)	95°C	30 seconds
	62°C	30 seconds
	72°C	30 seconds
Hold	72°C	7 minutes

Note: Run 15 cycles if you started with 50-100ng of fragmented RNA. If necessary, adjust the numbers of cycles accordingly 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 Invitrogen PureLink® PCR Micro Kit:

- 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. Before using the PureLink® Micro Kit Column, place it in a clean Collection Tube, then spin the column at 10,000 × g for 1 minute.
2. 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.
3. Load the sample onto the PureLink® Micro Kit Column:
   a. Load 500 µL of the sample containing Binding Buffer (B2) onto the PureLink® Micro Kit Column.
   b. Spin the column at 10,000 × g for 1 minute.
   c. Discard the flowthrough.
   d. Load the remaining 500 µL of the sample containing Binding Buffer (B2) onto the column.
   e. Spin the column at 10,000 × g for 1 minute.
   f. Discard the flowthrough.
4. 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.
5. 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 5b through step 5c 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.

Percent of DNA in the 25–200 bp range	Next steps
Less than 20%	Proceed with SOLiD™ System templated bead preparation. Refer to the *Applied Biosystems SOLiD™ 4 System Templated Bead Preparation Guide* (PN 4448378) or to *SOLiD™ EZ Bead Emulsifier Getting Started Guide* (Part no. 4441486)
Greater than 20%	Follow the troubleshooting instructions for "Normal yield and bad size distribution in the amplified library" on page 84.

Note: For instructions on how to perform the smear analysis, 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 89.

   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.

Typical size profiles of amplified libraries

Typical size distributions (Agilent 2100 Bioanalyzer profiles) of amplified libraries prepared from HeLa poly(A) RNA (Figure 15) and rRNA-depleted HeLa RNA (Figure 16) using the SOLiD™ Total RNA-Seq Kit are shown.

Figure 15 Size distribution of amplified library prepared from HeLa poly(A) RNA

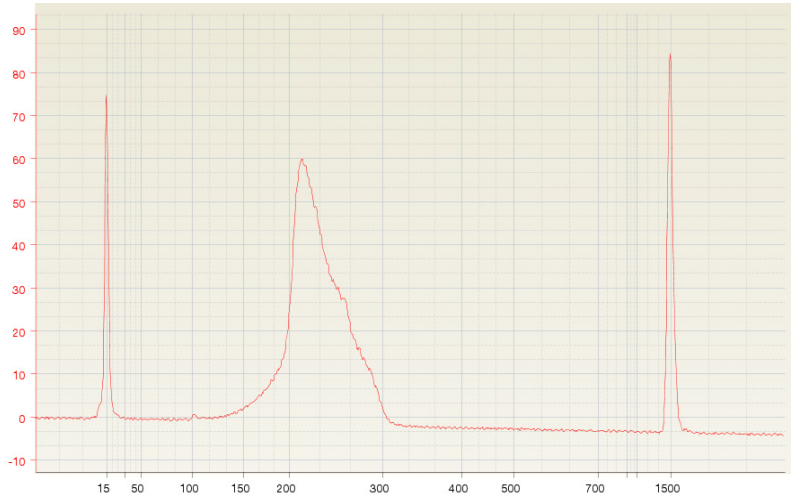


Figure 16 Size distribution of amplified library prepared from rRNA-depleted HeLa total RNA

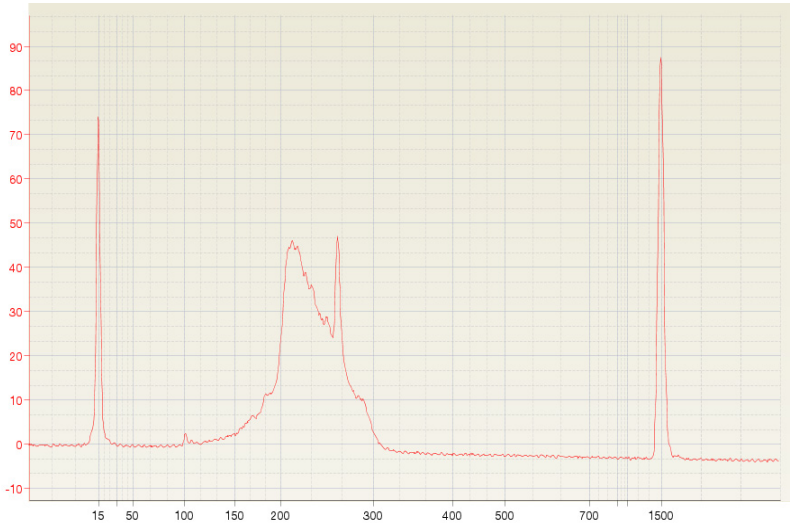
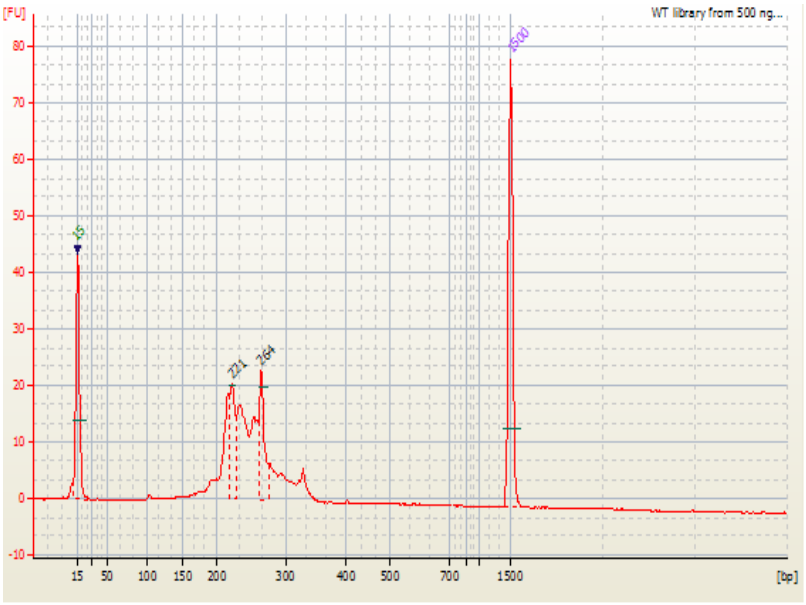


Figure 17 Size distribution of amplified library prepared from WT control total RNA



Troubleshooting

Observation	Possible cause	Solution
Low yield and no PCR products	The gel ran too long or too much gel was added to the PCR	Reduce the running time (step 6 on page 77) and add less gel to the PCR.
Normal yield and bad size distribution in the amplified library	Too much sample was loaded on the Novex® TBE-Urea Gel	Decrease the volume of sample loaded to less than 10 µL (step 5 on page 77).
	The wells of the Novex TBE-Urea Gel contained urea	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 77).
You decreased the volume of sample loaded on the Novex® TBE-Urea Gel, but smear analysis of the purified amplified cDNA shows that >20% of the cDNA is in the 25–200 bp range	Fragmented RNA sample contains too many small fragments	Perform "Second-round size selection of amplified cDNA" on page 85.
	Size selection was not successful	
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™ Instrument trace	Nonspecific amplification	Increase the PCR annealing temperature to 68–72 °C.

Second-round size selection of amplified cDNA

Perform a second round of size selection of the amplified, double-stranded cDNA using *nondenaturing* 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 18).
- For small RNA libraries: The ratio of 120–130-bp DNA: 25–150-bp DNA is less than 50% (from step 2 on page 63)

Size-select the amplified cDNA

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

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

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

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

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 5 on page 81.
   - For small RNA libraries: Add 2 µL Gel Loading Solution to the eluted cDNA (~10 µL) from step 4 on page 57.

   IMPORTANT! Do not heat the samples before loading.

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

Component	Volume
Novex® TBE Running Buffer (5X)	200 mL
Deionized water	800 mL
Total volume	1000 mL

   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 Ambion® Spin Columns and Tubes (AM10065).

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

Component	Volume (mL)
TE Buffer, pH 8 (10 mM Tris-HCl, ph 8, 1 mM EDTA)	5
5 M ammonium acetate (2.5 M final concentration)	5
Final volume	**10**

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.

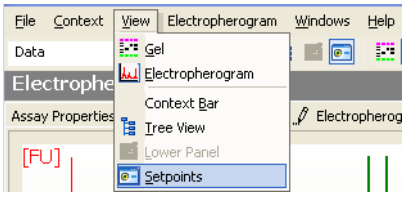
      Note: Accurate quantitation of the DNA is important for the downstream SOLiD™ System emulsion PCR titration step. The resuspension volume should yield DNA sufficiently concentrated for accurate measurements (~10 ng/µL).

Using 2100 expert software to assess whole transcriptome libraries

Perform a smear analysis

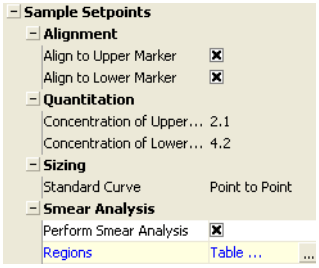
Perform a smear analysis to quantify the percentage of DNA in the 25–200 bp size range.

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**.

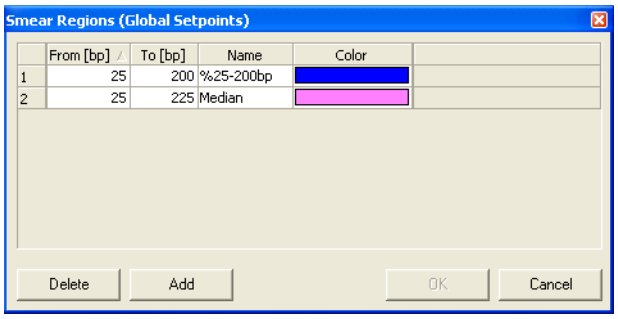


4. Set the smear regions in the Smear Regions dialog box:
   a. Click **Add**, then enter 25 bp and 200 bp for the lower and upper limits, respectively.

      These settings are used to determine the percentage of total product that is 25–200 bp in length.

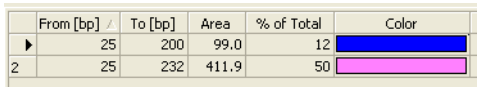
   b. Click **Add**, enter 25 bp and 225 bp, then click **OK**.

      This is an arbitrary upper limit which is used to determine the median size.



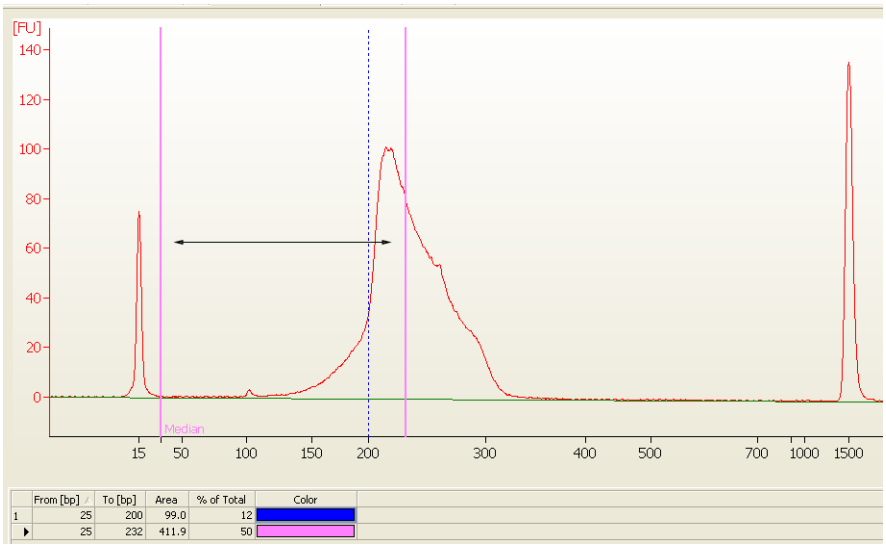
5. Select the **Region Table** tab.

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



Determine the median size

On the Region Table tab, drag the upper limit line that you set in step b on page 89 to the left or right until the Region Table indicates 50% of Total.

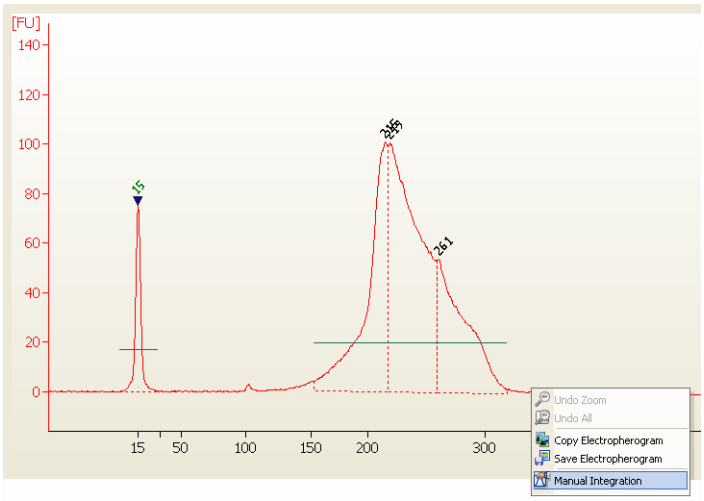


Analyze multiple peaks as one peak

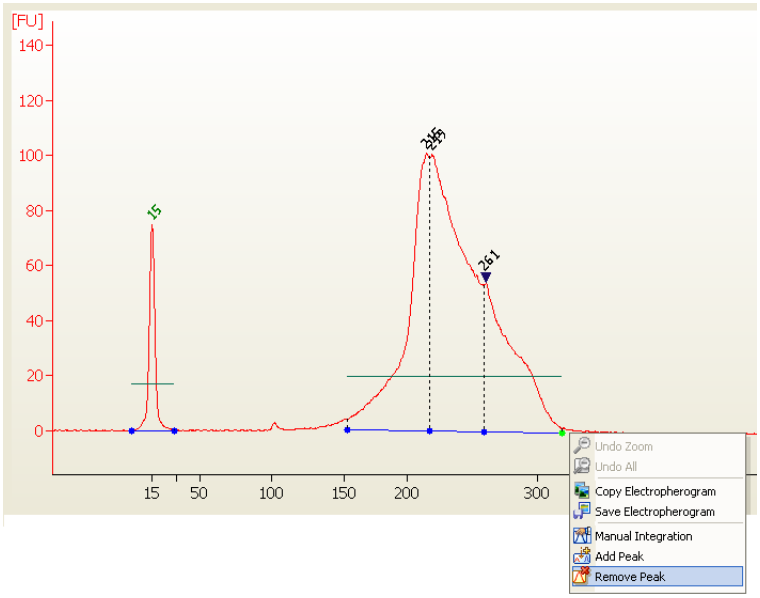
On the Peak Table tab, you may observe that the bioanalyzer software identified multiple peaks in a region that you want to consider as one peak. To obtain one concentration and automatically determine median size for a peak region, manually set the size range of the desired peak region.

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**.

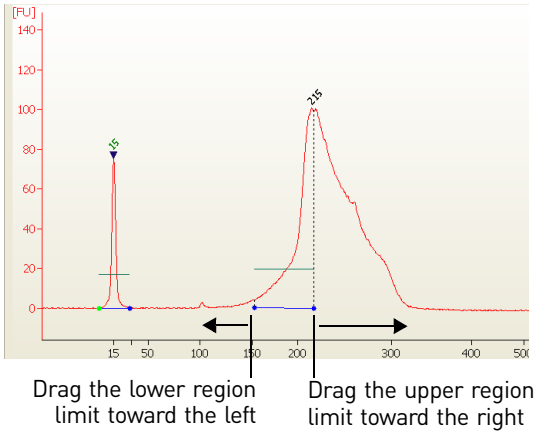


3. To remove multiple peaks:
   a. Place the cursor on the peak to remove, right-click, then select **Remove Peak**.

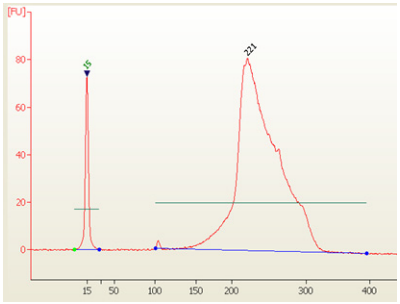


   b. Repeat until one peak remains within the region of interest

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.



		Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
▶	◀	15	4.20	424.2	Lower Marker
2		221	28.18	193.3	
3	▶	1,500	2.10	2.1	Upper Marker

Small RNA enrichment

If your RNA sample contains < 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*.

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.

IMPORTANT! Total RNA is bound to the cartridge and small RNA is in the flowthrough. **Keep the flowthrough**.

c. Transfer the flowthrough to a new 1.5-mL tube with a lid.

d. Discard the cartridge.

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.

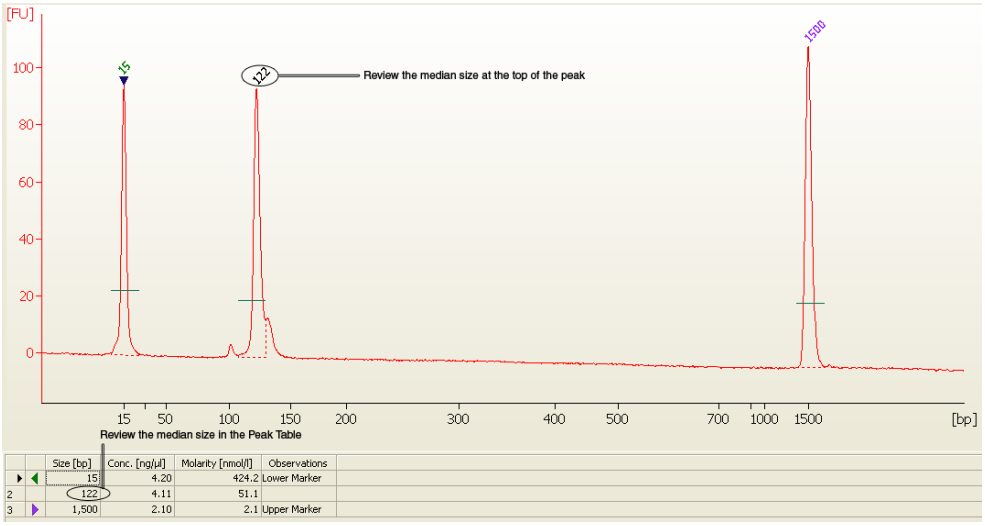
Store the small RNA at –80°C or assess the quality and quantity of the small RNA-enriched sample (page 54).

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.

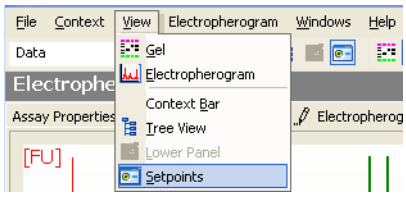
Select the **Peak Table** tab, then review the median size in the Peak Table and at the top of the peak in the electropherogram. The median size should be ~122–125 bp.



Perform a smear analysis

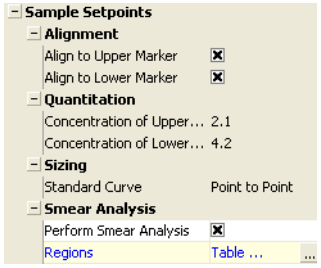
Perform a smear analysis to quantify the percentage of DNA in the 25–150 bp and 120–130 bp size range. The desired size range for miRNA ligation products is 120–130 bp.

1. In the 2100 expert software, select **View ▸ Setpoints**.

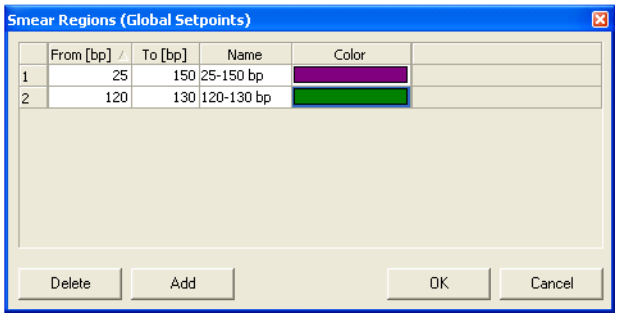


2. On the **Global** tab, select **Advanced** settings.

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

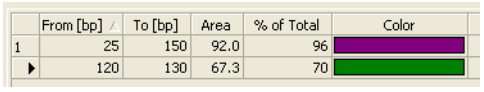


4. Set the smear regions in the Smear Regions dialog box:
   a. Click **Add**, then enter 25 bp and 150 bp for the lower and upper limits, respectively.
   b. Click **Add**, enter 120 bp and 130 bp, then click **OK**.



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

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



Determine the % miRNA library

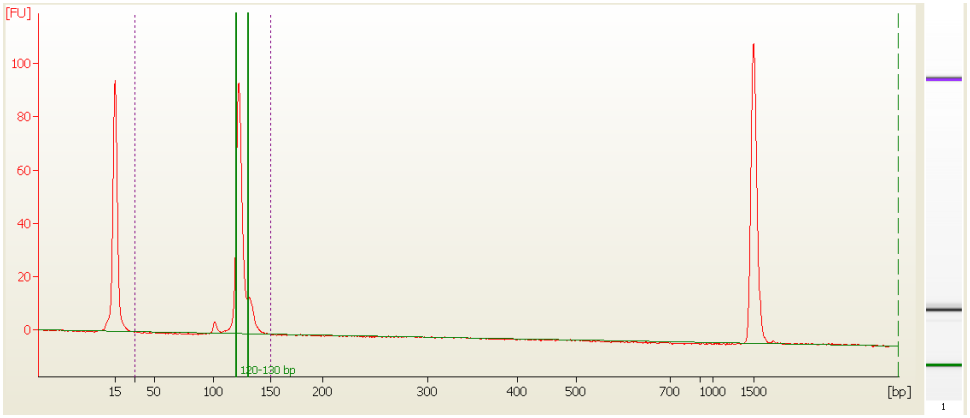
Using the area values from the Region Table, calculate the % miRNA library in the 120–130 bp region as a fraction of the 25–150 bp region using the formula:

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

If the % miRNA library is less than 50%, we strongly recommend that you perform a second-round size selection using a nondenaturing polyacrylamide gel, such as the Novex 6% TBE Gel (page 85).

Example % miRNA library calculation

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



	From [bp]	To [bp]	Area	% of Total	Color
1	25	150	92.0	96	
▶	120	130	67.3	70	

$$\%\ \text{miRNA library} = \left(\frac{67.3}{92.0} \right) \times 100 = 73\%$$

C PCR Good Laboratory Practices

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 DNA*Zap*™ Solution (PN AM9890).

D

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- 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. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

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:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**
- 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**

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available from:

- **www.invitrogen.com/sds**

  or

- **www.appliedbiosystems.com/sds**

Note: For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining support

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

- **www.invitrogen.com**

  or

- **www.appliedbiosystems.com**

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Kit documentation

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

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

Related documentation

When using this protocol, you may find the documents listed below useful. To obtain this and additional documentation, see "Obtaining support" on page 103.

Document	Part number
Applied Biosystems SOLiD™ 4 System SETS Software User Guide	4448411
Applied Biosystems SOLiD™ 4 System Templated Bead Preparation Guide	4448378
SOLiD™ EZ Bead Emulsifier Getting Started Guide	4441486
SOLiD™ EZ Bead™ Amplifier Getting Started Guide	4443494

Bibliography

Tissot, C. (Agilent® Technologies), 2008. Analysis of miRNA content in total RNA preparations using the Agilent® 2100 Bioanalyzer™. 8 pp. Publication Number 5989-7870EN.

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