ambion®
by life technologies™

Ambion® RNA-Seq Library Construction Kit

Protocol

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Safety information

Note: For general safety information, see this section and Appendix D, "Safety" on page 75. When a hazard symbol and hazard type appear by an instrument hazard, see the "Safety" Appendix for the complete alert on the instrument.

Safety alert words

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

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

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

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

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

SDSs

The Safety Data Sheets (SDSs) for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining SDSs, see "SDSs" on page 75.

IMPORTANT! For the SDSs of chemicals not distributed by Applied Biosystems or Ambion contact the chemical manufacturer.

Ambion® RNA-Seq Library Construction Kit

Product information

Purpose of the product

Use the Ambion® RNA-Seq Library Construction Kit (PN 4454073) to convert RNA transcripts expressed in a cell or tissue into a cDNA library for sequencing analysis:

- For whole transcriptome libraries, follow the procedures in Chapter 2 on page 11.
- For small RNA libraries, follow the procedures in Chapter 3 on page 33.

Kit contents and storage

Sufficient reagents are supplied in the Ambion RNA-Seq Library Construction Kit to prepare cDNA libraries from 12 samples for high-throughput sequencing.

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

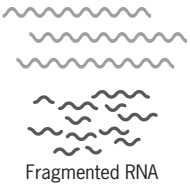
Component	Amount	Cap
Nuclease-free Water	1.75 mL	clear
10× RNase III Buffer	20 µL	red
RNase III	20 µL	red
Ambion Adaptor Mix	30 µL	green
Hybridization Solution	40 µL	green
2× Ligation Buffer	150 µL	green
Ligation Enzyme Mix	30 µL	green
10× RT Buffer	50 µL	yellow
dNTP Mix	500 µL	white
Ambion RT Primer	30 µL	yellow
ArrayScript™ Reverse Transcriptase	20 µL	yellow
10× PCR Buffer	660 µL	white
AmpliTaq® DNA Polymerase	110 µL	white
Ambion 5′ PCR Primer	100 µL	white
Ambion 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

For more ordering information, required materials and equipment that are not included, and optional materials and equipment, see Appendix A, "Ordering Information" on page 51.

Prepare Whole Transcriptome Libraries

Fragment the whole transcriptome RNA

100–500 ng poly(A) RNA or 200–500 ng rRNA-depleted total RNA (and ERCC spike-in control mixes [available soon])



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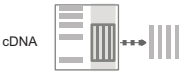

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Amplify the cDNA (page 24)

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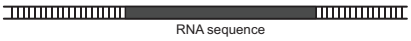
Purify the amplified DNA (page 25)



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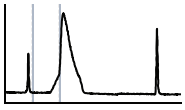
Assess the yield and size distribution of the amplified DNA (page 26)



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Proceed with cluster generation

Refer to the Illumina *Paired-End Sequencing User Guide* (PN1003880). See the chapter "Using the Cluster Station."

Fragment the whole transcriptome RNA

Fragmentation of the whole transcriptome RNA involves the following procedures:

1. Fragment the RNA (below)
2. Clean up the RNA (page 13)
3. Assess the yield and size distribution of the fragmented RNA (page 14)

Guidelines for RNA sample type and amount

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

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

Use only high-quality RNA as your starting material. FirstChoice® Total RNA provides high-quality, intact RNA isolated from a variety of sources.

Fragment the RNA

Use components from the Ambion® RNA-Seq Library Construction Kit:

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

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

Component	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 Buffer	1 µL
RNase III	1 µL
Total volume	**10 µL**

IMPORTANT! To reduce fragmentation variability, accurately pipet 1 µL 10✕ RNase III Buffer to each reaction. **Do not** make master mix using only 10✕ RNase III 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. Go to the next step immediately, or leave the fragmented RNA on ice for less than 1 hour.

Clean up the RNA

Use the RiboMinus™ Concentration Module (Invitrogen).

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

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

2. Add binding buffer and ethanol to the fragmented RNA, then mix well:

Component	Volume
Binding Buffer (L3)	100 µL
100% ethanol	250 µL

3. Bind the RNA sample containing Binding Buffer (L3) and ethanol to the Spin Column:
   a. Place the Spin Column in a clean 1.5-mL Wash Tube.
   b. Load 450 µL of the RNA sample containing Binding Buffer (L3) and ethanol onto the Spin Column.
   c. Spin the column at 12,000 × g for 1 minute.
   d. Discard the flow-through.
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 flow-through.
   e. Return the Spin Column in the Wash Tube.
   f. Spin the column at maximum speed for 2 minutes.
5. Elute the RNA in a clean Recovery Tube:
   a. Place the Spin Column in a clean Recovery Tube.
   b. Add 12 µL of RNase-Free Water to the center of the Spin Column.
   c. Wait 1 minute, then spin the column at maximum speed for 1 minute.

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

Assess the yield and size distribution of the fragmented RNA

Use the Quant-iT™ RNA Assay Kit with the Qubit® Fluorometer (Invitrogen) and the RNA 6000 Pico Chip Kit with the Agilent 2100 Bioanalyzer 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 Invitrogen *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 average size should be 100–200 nt. See Figures 1 on page 15 and 2 on page 16.

      Note: If the fragmented RNA profile does not meet the specifications, RNAseIII digestion optimization is recommended. See "Troubleshooting" on page 31 for guidance.

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

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

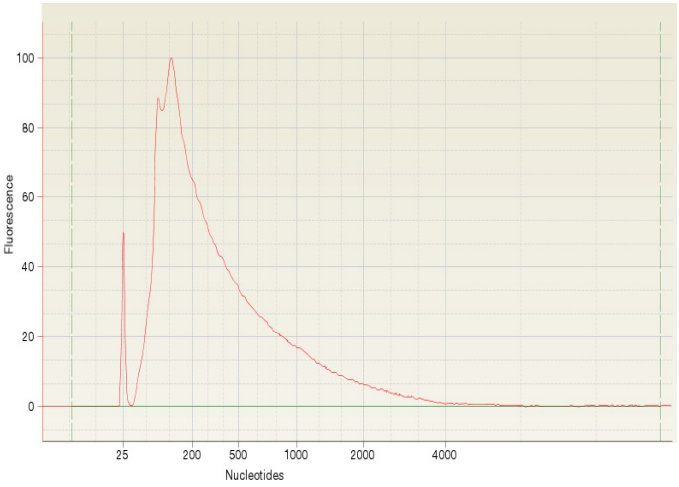
Amount of fragmented RNA in 3 µL	Instructions
• ≥50 ng poly(A) RNA • ≥100 ng rRNA-depleted total RNA • ≥100 ng WT Control RNA	Proceed with "Construct the amplified whole transcriptome library" on page 17. Store the remaining RNA at –80 °C.

Amount of fragmented RNA in 3 µL	Instructions
• <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 17.

Typical results of fragmentation of whole transcriptome RNA

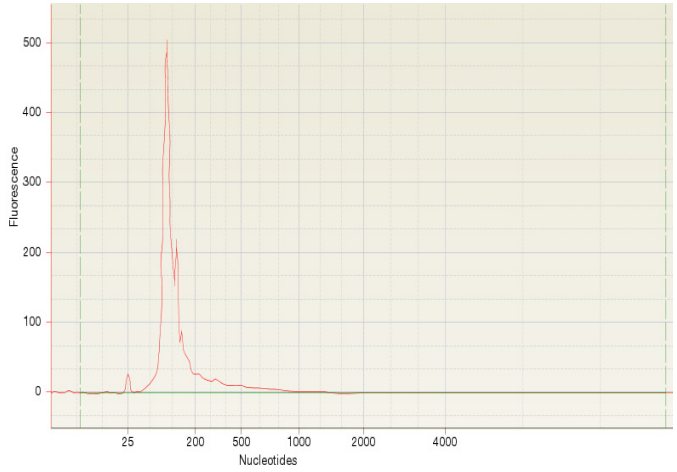
Figure 1 and Figure 2 show profiles from an Agilent 2100 Bioanalyzer Instrument after RNase III fragmentation and cleanup. Figure 1 shows results with HeLa poly(A) RNA. Figure 2 shows results with rRNA-depleted HeLa RNA. Figure 3 shows results with WT control RNA (HeLa total RNA).

Figure 1 Size distribution of fragmented HeLa poly(A) RNA



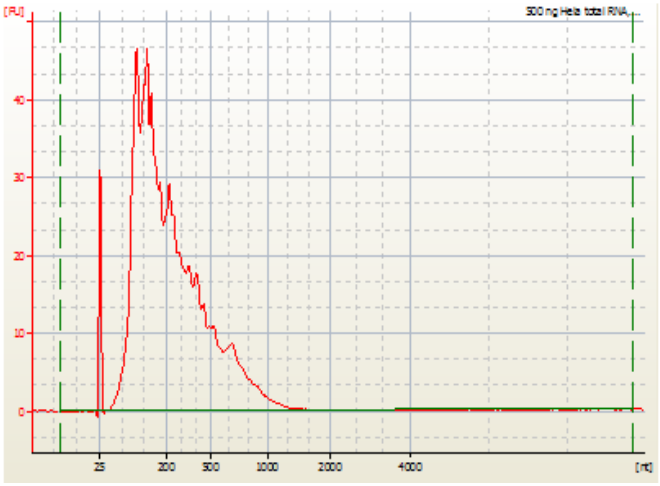
Median size equals 165 nts

Figure 2 Size distribution of fragmented rRNA-depleted HeLa RNA



Median size equals 140 nts

Figure 3 Size distribution of fragmented WT control RNA.



Median size equals 195 nts

Construct the amplified whole transcriptome library

Constructing the amplified whole transcriptome library involves the following procedures:

1. Hybridize and ligate the RNA (below)
2. Perform reverse transcription (page 18)
3. Purify the cDNA (page 19)
4. Size select the cDNA (page 20)
5. Amplify the cDNA (page 24)
6. Purify the amplified DNA (page 25)
7. Assess the yield and size distribution of the amplified DNA (page 26)

If less than 25% of the amplified DNA is in the 25—270 bp range, you can proceed with cluster generation (page 27).

Hybridize and ligate the RNA

Use components from the Ambion® RNA-Seq Library Construction Kit:

- Ambion 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†
Ambion 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
2× Ligation Buffer	10 µL
Ligation Enzyme Mix	2 µL

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.

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 Ambion® RNA-Seq Library Construction Kit:

- Nuclease-free Water
- 10× RT Buffer
- dNTP Mix
- Ambion RT Primer
- ArrayScript™ Reverse Transcriptase

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

Component	Volume for each reaction†
Nuclease-free Water	11 µL
10× RT Buffer	4 µL
dNTP Mix	2 µL
Ambion 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 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 flow-through.
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 flow-through.
   e. Return the MinElute column to the microcentrifuge tube.
   f. Spin the column at 13,000 × g for 1 minute.
4. Elute the cDNA in a clean microcentrifuge tube:
   a. Place the MinElute column in a clean microcentrifuge tube.
   b. Add 10 µL of Buffer EB to the center of the MinElute column.
   c. Wait 1 minute, then spin the column at 13,000 × g for 1 minute.

Size select the cDNA

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

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

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

1. Prepare the gel as described in the Invitrogen *Novex® Pre-Cast Gel Electrophoresis Guide*:
   a. Using Novex TBE Running Buffer (5✕), prepare 1000 mL of 1✕ TBE Running Buffer:

Component	Volume
Novex® TBE Running Buffer (5✕)	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 21.
   d. Add 1✕ 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 1✕ TBE Running Buffer to remove urea from the wells.

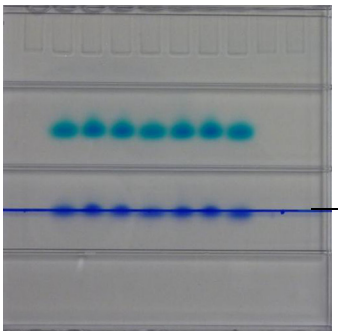
Note: Flushing the wells is important to obtain sharp bands.

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).



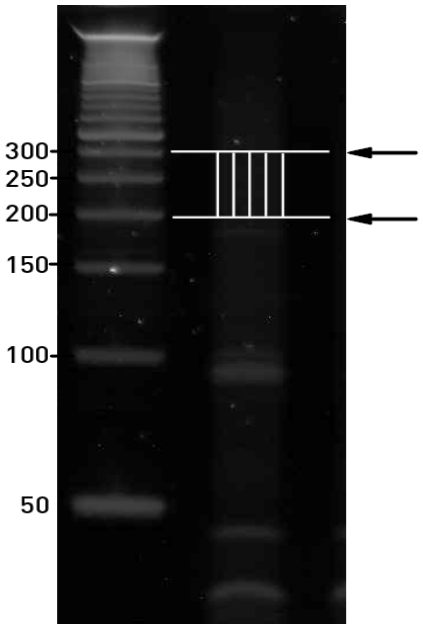
IMPORTANT! Shortening or elongating the running time results in inferior resolution.

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 200–300 nt cDNA:

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

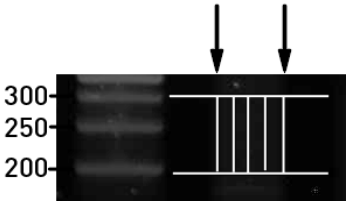
   a. Using a clean razor blade, make horizontal cuts to excise the gel containing 200–300 nt cDNA.



   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 the table on page 24 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 × 4 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.

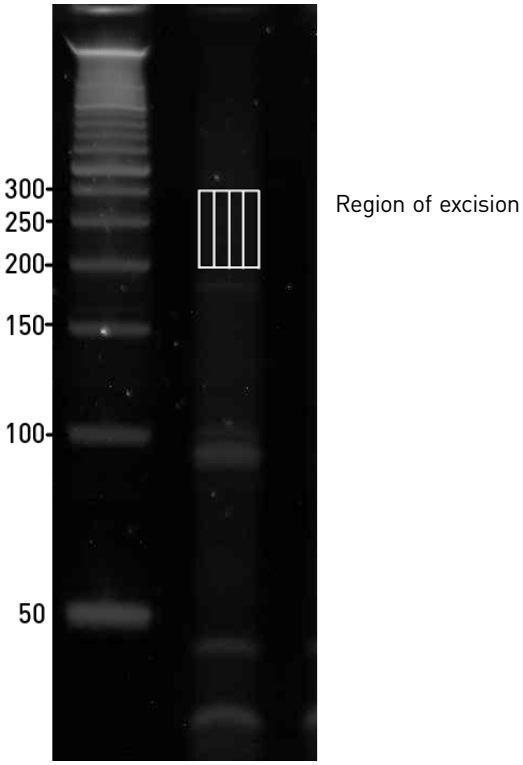
    To generate sufficient cDNA for accurate quantitation, we recommend running 2 amplification reactions using 2 gel pieces, 1 gel piece in each reaction. You may store the other 2 pieces for 2 weeks at –20 °C.

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

Example of size selection

Figure 4 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 4 Example of size selection of cDNA from HeLa poly(A) RNA



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

The following table shows 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	~120
100	~50	~170
200	~150	~270
300	~250	~320

Amplify the cDNA

Use components from the Ambion® RNA-Seq Library Construction Kit:

- Nuclease-free Water
- 10× PCR Buffer
- dNTP Mix
- Ambion 5′ PCR Primer
- Ambion 3′ PCR Primer
- AmpliTaq® DNA Polymerase

1. For each cDNA sample, prepare duplicate in-gel amplification reactions to generate sufficient cDNA for accurate evaluation of library quality and quantitation:
   a. Ensure that each gel slice from step 10 on page 23 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 100 µL PCR mix for each gel slice:

Component	Volume	
	One 100-µL reaction	Two 100-µL reactions†
Nuclease-free Water	76.8 µL	169.0 µL
10× PCR Buffer	10.0 µL	22.0 µL
dNTP Mix	8.0 µL	17.6 µL
Ambion 5′ PCR Primer	2.0 µL	4.4 µL
Ambion 3′ PCR Primer	2.0 µL	4.4 µL
AmpliTaq® DNA Polymerase	1.2 µL	2.6 µL
Total volume	**100.0 µL**	**220.0 µL**

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

   c. Transfer 100 µL PCR mix into each 0.2-mL PCR tube.

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

Purify the amplified DNA

Use the PureLink™ PCR Micro Kit (Invitrogen):

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

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

1. Before using the PureLink™ Micro Kit Column, centrifuge the column with a collection tube 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.
   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. Place the PureLink™ Micro Kit Column in a clean Collection Tube.
   b. Load 500 µL of the sample containing Binding Buffer (B2) onto the column.
   c. Spin the column at 10,000 × g for 1 minute.
   d. Discard the flow-through.
   e. Load the remaining 500 µL of the sample containing Binding Buffer (B2) onto the column.
   f. Spin the column at 10,000 × g for 1 minute.
   g. Discard the flow-through.

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 flow-through.
   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 and 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.

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—270 bp.

Percent of DNA in the 25—270 bp range	Next steps
Less than 25%	Proceed with cluster generation (page 27).
Greater than 25%	Follow the troubleshooting instructions for "Normal yield and bad size distribution in the amplified library" on page 31.

Note: For instructions on how to perform the smear analysis, see "Perform a smear analysis" on page 61, and refer to the *Agilent 2100 Bioanalyzer 2100 Expert User's Guide* by Agilent.

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

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

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

www4.appliedbiosystems.com/techlib/append/concentration_calculator.html

Generate clusters

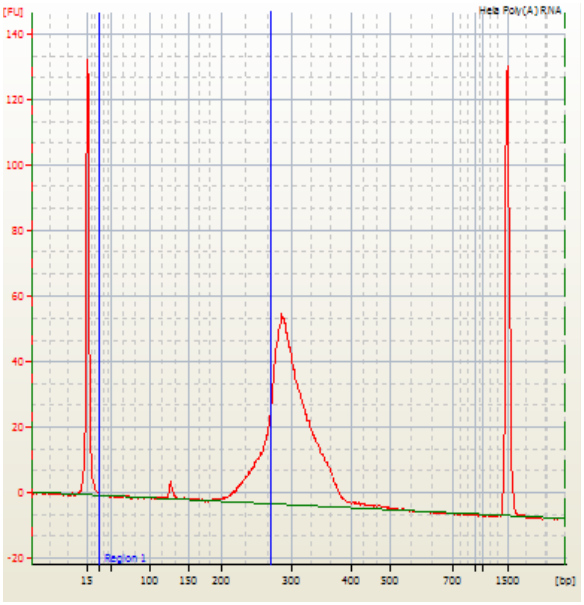
If less than 25% of the amplified DNA is in the 25—270 bp range, you can proceed with cluster generation in which each library template is attached to the flow cell and amplified to form clusters for sequencing on the Genome Analyzer. Refer to the Illumina *Paired-End Sequencing User Guide* (PN 1003880). See the chapter "Using the Cluster Station."

Typical size profiles of amplified libraries

Typical size distributions (Agilent 2100 Bioanalyzer Instrument profiles) of amplified libraries prepared from HeLa poly(A) RNA (Figure 5), Hela rRNA-RNA (Figure 6), and WT Control RNA (Figure 7) using the Ambion® RNA-Seq Library Construction Kit are shown. Figure 8 is an example of a sub-optimal size distribution with >25% DNA 25—270 bp in length.

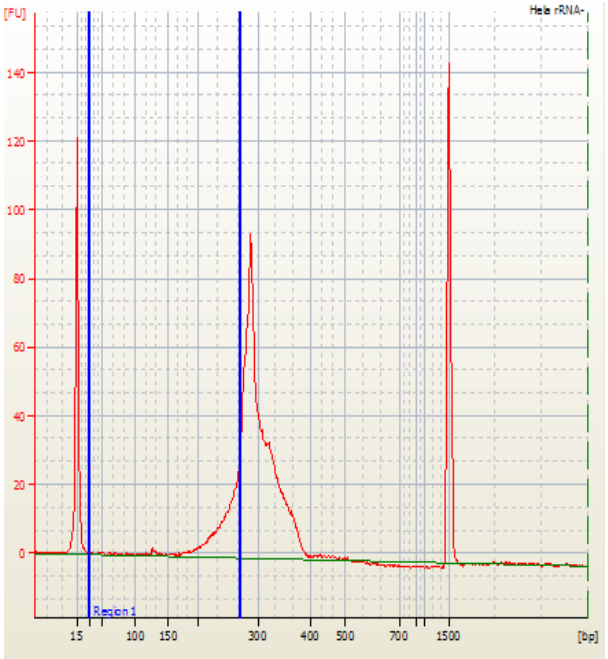
In general, we see a positive correlation between RNA-Seq mapping statistics and the percent of DNA library inserts that are above 270 bp in length. We recommend minimizing the percent of inserts that fall in the 25—270 bp range. This recommendation is based on our experience. Please use your judgement when deciding the best way to proceed with libraries from precious samples that fall close to these recommended quality values of having less than 25% DNA 25—270 bp in length.

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



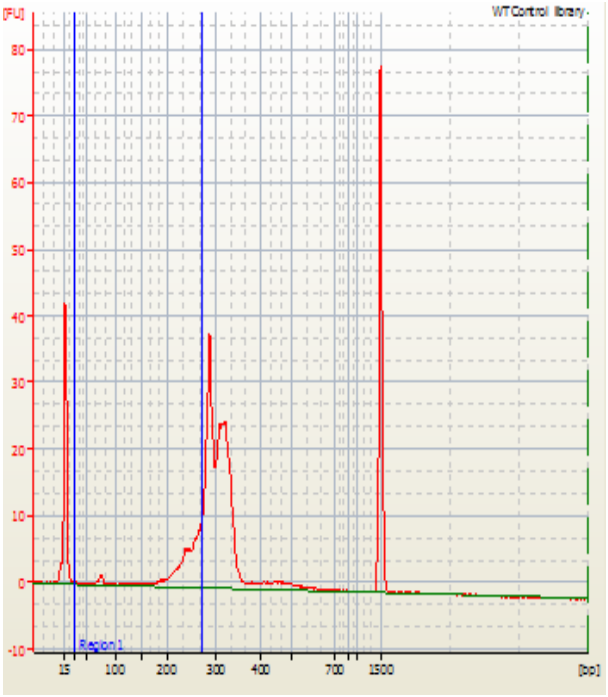
25—270 bp % = 18%
Median size= 296 bp

Figure 6 Size distribution of amplified library prepared from Hela rRNA- RNA



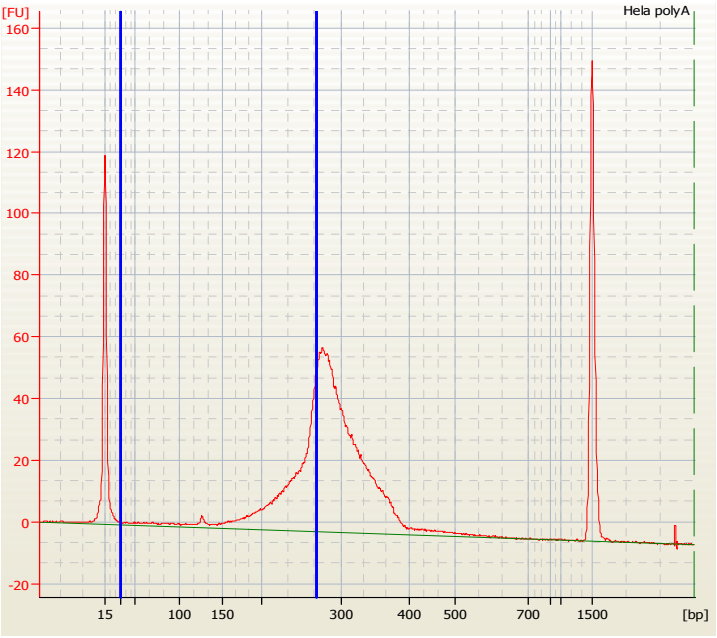
25—270 bp % = 20%
Median size= 291 bp

Figure 7 Size distribution of amplified library prepared from WT Control RNA



25—270 bp % = 20%
Median size= 297 bp

Figure 8 Sub-optimal size distribution of amplified library



25—270 bp % = 35%
Median size= 283 bp

Expected yields

The recovery of your experimental RNA depends 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 12)	500 ng poly(A) RNA, total RNA, or rRNA-depleted total RNA	300—400 ng RNA
Construct the amplified whole transcriptome library (page 17)	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 64.
Low yield and bad 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 13).
Low yield in the amplified library and very few differences in the Agilent 2100 Bioanalyzer Instrument traces before and after fragmenting 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 13).
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 21) and add less gel to the PCR (step 1 on page 24).
	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 the purified amplified cDNA shows one or more sharp peaks between 120 and 270 bp in the Agilent 2100 Bioanalyzer Instrument trace	Nonspecific amplification	Increase the PCR annealing temperature to 68–72 °C (step 2 on page 25).
Normal or high yield but PCR products larger than 400 bp	Too many PCR cycles resulted in overamplification	Decrease the number of PCR cycles (step 2 on page 25).
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 21).
	The wells of the Novex TBE-Urea Gel contained urea	Before you load the samples, flush the wells of the gel several times with 1× TBE Running Buffer to remove urea from the wells and to obtain sharp bands (step 4 on page 21).
You decreased the volume of sample loaded on the Novex® TBE-Urea Gel, but smear analysis of the purified amplified cDNA shows that >25% of the cDNA is in the 25—270 bp range	Fragmented RNA sample contains too many small fragments	Perform "Second-round size selection of amplified cDNA" on page 58.
	Size selection was not successful	

Using a positive control

A general troubleshooting strategy is to perform the Ambion® RNA-Seq Library Construction 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 12. Figure 3 on page 16 shows the Agilent 2100 Bioanalyzer Instrument profile for fragmented WT control RNA.
- Use 100 ng fragmented WT Control RNA in the amplified library construction procedure starting on page 17. Figure 6 on page 28 shows the Agilent 2100 Bioanalyzer Instrument profile for final library.

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

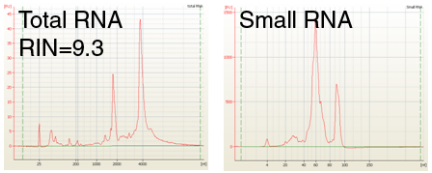
ERCC RNA spike-in control mixes

Ambion External RNA Controls Consortium (ERCC) RNA spike-in control mixes will be available soon. The control mixes provide a set of external RNA controls that enable you to assess the performance of a variety of technology platforms used for gene expression experiments including RNA sequencing. The control mixes are pre-formulated sets of 92 polyadenylated transcripts from the ERCC plasmid reference library. The transcript sequences are traceable through the manufacturing process to the National Institute of Standards and Technology (NIST) plasmid reference material and can be used to monitor library preparation, sequencing performance, and analysis parameters.

Prepare Small RNA Libraries

Prepare the starting material

Assess the amount and quality of small RNA in your total RNA samples (page 34)



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Enrich the sample for small RNA (page 36)

↓

Assess the quality and quantity of the small RNA-enriched sample (page 36)

↓

Determine the input amount (page 37)

↓

Construct the amplified small RNA library

Hybridize and ligate the RNA (page 37)

↓

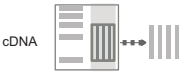
Perform reverse transcription (page 38)

↓

Purify the cDNA (page 39)

↓

Size select the cDNA (page 40)



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Amplify the cDNA (page 43)

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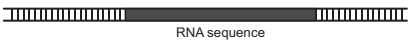
Purify the amplified DNA (page 44)



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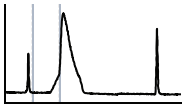
Assess the yield and size distribution of the amplified DNA (page 45)



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Proceed with cluster generation

Refer to the Illumina *Paired-End Sequencing User Guide* (PN1003880). See the chapter "Using the Cluster Station."

Prepare the starting material

Preparing the starting material involves the following procedures:

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

Guidelines for obtaining small RNA

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

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 total RNA that includes the small RNA fraction. Use the Invitrogen™ PureLink™ miRNA Isolation Kit to enrich small RNA from total RNA.

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 Chip 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 assessing sample quality and quantity (step 1 on page 36).

2. Determine the quality of the small RNA in your sample:
   a. Dilute the sample to ~50 to 100 ng/µL.
   b. Run 1 µL of diluted sample using the RNA 6000 Nano Kit with the Agilent 2100 Bioanalyzer 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 to be used in calculating miRNA content (step 3c).

d. Using the 2100 expert software, review the RNA Integrity Number (RIN). You can proceed if the RIN is ≥6.

Note: If the RIN is <6, the sample is not recommended for small RNA library construction because the RNA degradation products may affect the quantitation of small RNA in the sample and interfere with small RNA ligation. Repeat RNA isolation; to obtain high-quality RNA, avoid RNase contamination (see Appendix C, "Good Laboratory Practices for RNA Work" on page 71).

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 and the type of enrichment to perform:

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 36 or skip to "Determine the input amount" on page 37.
0.1–0.5% 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 36.
<0.1% miRNA	Small RNA purification is strongly recommended. We recommend using Ambion flashPAGE™ purification products. Proceed with "Enrich the sample for small RNA" on page 36.

Note: When total RNA is used in the Ambion RNA-Seq Library Construction Kit procedure, the resulting reaction products comprise a larger size range than those produced from small RNA-enriched samples.

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 one of the methods below:

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

  Expected recovery after flashPAGE fractionation and cleanup: From 5 µg total placenta RNA input, approximately 200 ng small RNA-enriched RNA can be recovered. For <5 µg total RNA input, use glycogen as a coprecipitant during the flashPAGE Clean-Up Kit procedure.
- If your RNA sample contains 0.1–0.5% miRNA – Use the Invitrogen PureLink™ miRNA Isolation Kit. Follow the instructions in "Small RNA enrichment" on page 66.

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

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

1. Run 1 µL of purified and 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 Agilent 2100 Bioanalyzer Instrument traces to those of the sample before enrichment (step 2 in "Assess the amount and quality of small RNA in your total RNA samples" on page 34), and determine whether the RNA is degraded.

The expected results are shown in the following table.

Sample	Results
Small RNA purified using flashPAGE	Peaks from 10 to 40 nt, and very low peaks or no peaks should be observed after 40 nt.
Enriched small RNA	Peaks should be from 10 to 200 nt.

Determine the input amount

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

Input sample type	Amount of miRNA (10 to 40 nt) in 1 µL on the Small RNA Chip	Total RNA input†
Total RNA	5 to 100 ng	≤1 µg
Enriched small RNA	1 to 100 ng	≤1 µg
Purified small RNA	1 to 100 ng	100 ng

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

Note: In very rare cases, too much input inhibits ligation reaction. In cases when very poor library yield is observed from more than 25 ng miRNA input, reduce the input amount to 5-10 ng.

Construct the amplified small RNA library

Constructing the amplified small RNA library involves the following procedures:

1. Hybridize and ligate the RNA (below)
2. Perform reverse transcription (page 38)
3. Purify the cDNA (page 39)
4. Size select the cDNA (page 40)
5. Amplify the cDNA (page 43)
6. Purify the amplified DNA (page 44)
7. Assess the yield and size distribution of the amplified DNA (page 45)

After you assess the yield and size distribution of the amplified DNA, you can proceed with cluster generation (page 46).

Hybridize and ligate the RNA

Use components from the Ambion® RNA-Seq Library Construction Kit:

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

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

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

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
2✕ Ligation Buffer	10 µL
Ligation Enzyme Mix	2 µL

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 Ambion® RNA-Seq Library Construction Kit:

- Nuclease-free Water
- 10✕ RT Buffer
- dNTP Mix
- Ambion RT Primer
- ArrayScript™ Reverse Transcriptase

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

Component	Volume for each reaction†
Nuclease-free Water	11 µL
10× RT Buffer	4 µL
dNTP Mix	2 µL
Ambion 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 the master mix 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 the samples 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 flow-through.

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

Size select the cDNA

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

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

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

1. Prepare the gel as described in the Invitrogen *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® 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	Concentration
10-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 10-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, heat the cDNA and DNA Ladder again (step 3c) before loading the samples.

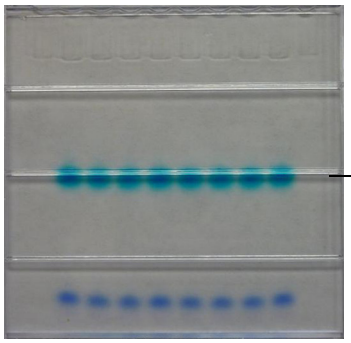
4. Flush the wells of the gel several times with 1× TBE Running Buffer to remove urea from the wells.

   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).



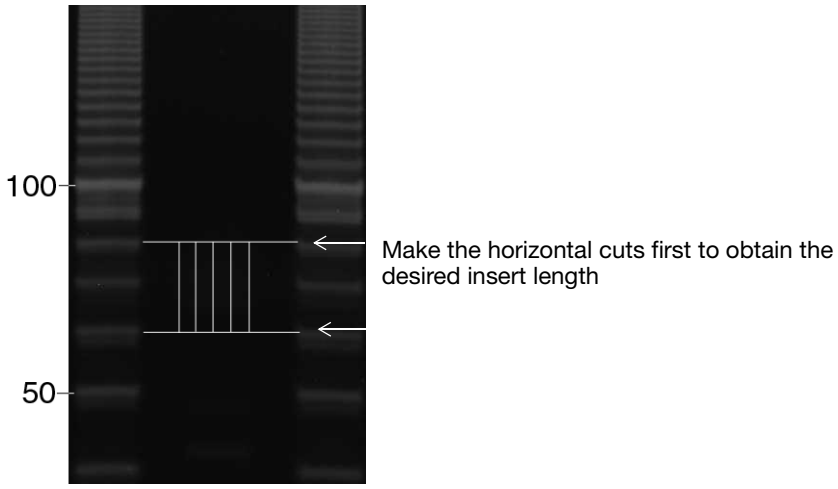
   IMPORTANT! Shortening or elongating the running time results in inferior resolution.

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 60 to 80 nt of cDNA:

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

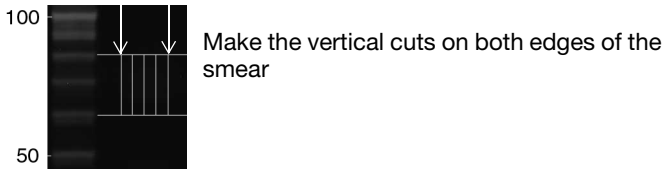
   a. Using a clean razor blade, make horizontal cuts directly on the 60-nt and 80-nt bands to excise the gel between 60 and 80 nt of cDNA.



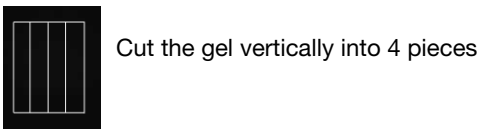
   Note: If you are using a UV transilluminator to visualize the reaction products, work quickly to limit their exposure to UV radiation.

   To obtain the desired insert length, you can adjust the cuts. However, too much gel will severely inhibit the PCR reactions. See page 43 for the expected lengths of the insert and PCR product according to the length of the cDNA that is excised from the gel.

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



9. Transfer the gel piece to a clean working area, maintaining the orientation of the gel, then cut the gel vertically into 4 pieces using a clean razor blade. Each gel slice should be about 1 mm × 6 mm.



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, use 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	~119
60	~18	~137
80	~38	~157

Amplify the cDNA

Use components from the Ambion® RNA-Seq Library Construction Kit:

- Nuclease-free Water
- 10× PCR Buffer
- 2.5 mM dNTP Mix
- Ambion 5′ PCR Primer
- Ambion 3′ PCR Primer
- AmpliTaq® DNA Polymerase

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 42) 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 100 µL PCR mix for each gel slice:

Component	Volume	
	One 100-µL reaction	Two 100-µL reactions†
Nuclease-free Water	76.8 µL	169.0 µL
10× PCR Buffer	10.0 µL	22.0 µL
2.5 mM dNTP Mix	8.0 µL	17.6 µL
Ambion 5′ PCR Primer	2.0 µL	4.4 µL
Ambion 3′ PCR Primer	2.0 µL	4.4 µL
AmpliTaq® DNA Polymerase	1.2 µL	2.6 µL
Total volume	**100.0 µL**	**220.0 µL**

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

c. Transfer 100 µL of PCR master mix into each 0.2-mL PCR tube.

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 PureLink™ PCR Micro Kit (Invitrogen):

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

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

Note: Before using the PureLink™ Micro Kit Column, centrifuge the column with a collection tube at 10,000 × g for 1 minute.

1. Before using the PureLink™ Micro Kit Column, centrifuge the column with a collection tube 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.
   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. Place the PureLink™ Micro Kit Column in a clean collection tube.
   b. Load 500 µL of the sample containing Binding Buffer (B2) onto the column.
   c. Spin the column at 10,000 × g for 1 minute.
   d. Discard the flow-through.
   e. Load the remaining 500 µL of the sample containing Binding Buffer (B2) onto the column.
   f. Spin the column at 10,000 × g for 1 minute.
   g. Discard the flow-through.

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 flow-through.
   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 1000 Kit to assess yield and size distribution of amplified DNA. For instructions on assessing libraries, see "Using 2100 expert software to assess small RNA libraries" on page 67, and refer to the *Agilent 2100 Bioanalyzer 2100 Expert User's Guide* by Agilent.

1. 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.

2. Using the 2100 expert software, perform a smear analysis to determine whether to proceed with cluster generation:
   a. Measure the area for the DNA that is 25—200 bp (the size range for ligation products with no insert and ligation products with short inserts) and 140-155 bp (the size range for the desired miRNA ligation products).
   b. Calculate the ratio of 140–155-bp DNA: 25—200-bp DNA:

      [Area (140–155 bp)] ÷ [Area (25–200 bp)]

      In general, we see a positive correlation between RNA-Seq mapping statistics and the percent of DNA library inserts that are above 270 bp in length. We recommend minimizing the percent of inserts that fall in the 25—270 bp range. This recommendation is based on our experience. 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 having less than 25% DNA 25—270 bp in length.

Ratio of 140—155 bp DNA: 25—200 bp DNA	Next steps
Greater than 50%	Proceed with cluster generation (page 46).
Less than 50%	Perform another size selection (see"Second-round size selection of amplified cDNA" on page 58).

Note: For more instructions on how to perform the smear analysis, see "Perform a smear analysis" on page 68, and refer to the *Agilent 2100 Bioanalyzer 2100 Expert User's Guide*.

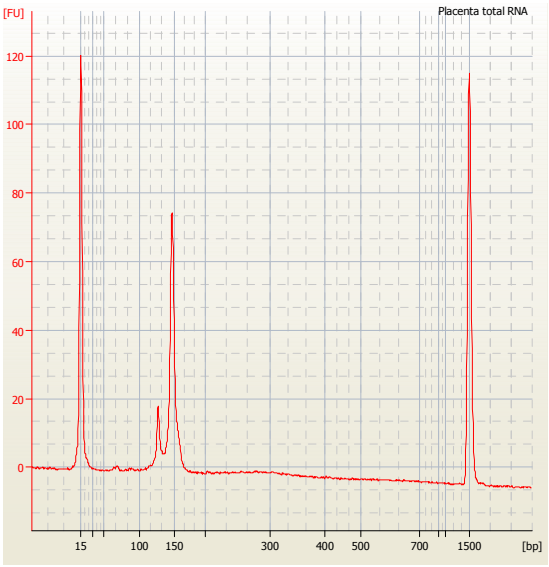
Note: Samples that are run on an Agilent 2100 Bioanalyzer Instrument typically show 5 to 8 bp larger than their actual size.

Generate clusters

You can proceed with cluster generation on the cluster station. Refer to the *Paired-End Sequencing User Guide* by Illumina (PN 1003880). See the chapter "Using the Cluster Station."

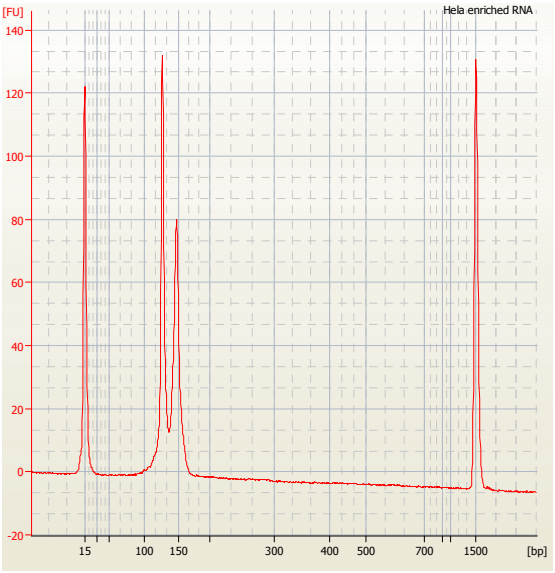
Typical size profiles of amplified libraries

Figure 9 Size distribution of amplified library prepared from placenta total RNA



140—155 bp% = 61.2%
Median peak size= 146 bp

Figure 10 Size distribution of amplified library prepared from HeLa total RNA and required a second round of size selection



140—155 bp% = 39.8%
Median peak size= 140 bp

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 64.
Low yield in the desired size range and high background of small sizes (120-bp and 130-bp byproducts)	Your input amount is too low	• Use enriched or purified 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 (130—160 bps)
Low yield in the desired size range and high background of large sizes (~160-bp byproducts)	tRNA is partially degraded	Run the remaining 5 µL cDNA on a 10% TBU gel and select only 60–70-nt sizes (see the horizontal cuts illustration on page 42).
Normal yield at desired size range but background (~127 bp on the Agilent 2100 Bioanalyzer Instrument) is too high (see Figure 10 on page 47)	The gel selection step does not exclude small ligation products	• Run the remaining cDNA on another gel; cut right on 60 nt so that anything smaller than the 18-nt insert is excluded. • Perform another gel purification of PCR products to select the desired size range (130—160 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 41) and add less gel to the PCR (step 1 on page 43).
	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.
Normal or high yield but PCR products larger than 180 bp	Too many PCR cycles resulted in overamplification	Decrease the number of PCR cycles (step 2 on page 44).
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 41).
	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 41).

Using a positive control

A general troubleshooting strategy is to perform the Ambion® RNA-Seq Library Construction Kit procedure using the Small RNA Control (human placenta total RNA) provided with the kit. Use 1 µg of Small RNA Control for the hybridization and ligation procedure starting on page 37.

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

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

Ordering Information

How to order

Ambion® RNA-Seq Library Construction Kit

For information on the Ambion® RNA-Seq Library Construction Kit (PN 4454073), go to the Invitrogen™ website at **www.invitrogen.com** and select:

Applications ▸ Nucleic Acid Amplification & Expression Profiling ▸ Sequencing ▸ Next Generation Sequencing Tools and Reagents ▸ Library Generation

Materials and equipment required but not included

Equipment

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

Supplies

Item	Source
8-strip PCR Tubes & Caps, RNase-free, 0.2-mL	Applied Biosystems PN AM12230
Non-Stick RNase-free Microfuge Tubes (0.5 mL), 500	Applied Biosystems PN AM12350
Non-Stick RNase-free Microfuge Tubes (1.5 mL), 250	Applied Biosystems PN AM12450
Pipette tips, RNase-free	MLS

Reagents

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

Reagents for both libraries

Item	Source
Nuclease-free Water (not DEPC-treated), 100 mL	Applied Biosystems PN AM9938
Novex® TBE-UREA Sample Buffer (2X), 10 mL	Invitrogen PN LC6876
Novex® TBE Running Buffer (5X), 1 L	Invitrogen PN LC6675
PureLink™ PCR Micro Kit, 50 preps	Invitrogen PN K310050
SYBR® Gold nucleic acid gel stain, 10,000X concentrate in DMSO, 500 µL	Invitrogen PN S-11494
Agilent® DNA 1000 Kit	Agilent PN 5067-1504
High Sensitivity DNA Kit (optional)	Agilent PN 5067-4626
Ethanol, 100%, ACS reagent grade or equivalent	MLS
MinElute® PCR Purification Kit (50)	Qiagen PN 28004

Reagents for whole transcriptome libraries

Item	Source
50 bp DNA Ladder	Invitrogen PN 10416-014
Novex® 6% TBE-Urea Gels 1.0 mm, 10 well	Invitrogen PN EC6865BOX
Quanti-iT™ RNA Assay Kit, 100 assays	Invitrogen PN Q32852
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.	Invitrogen PN K1550-05
RNA 6000 Pico Kit	Agilent PN 5067-1513
ERCC spike-in control mixes (available soon). For WT only.	External RNA Control Consortium

Reagents for small RNA libraries

Item	Source
10 bp DNA Ladder	Invitrogen PN 10821-015
Novex® 10% TBE-Urea Gels 1.0 mm, 10 well	Invitrogen PN EC6875BOX
RNA 6000 Nano Kit	Agilent PN 5067-1511
Small RNA Chip Kit	Agilent PN 5067-1548

Optional materials and equipment not included

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

Optional for both libraries

Item	Source
5 M Ammonium Acetate, 500 mL	Applied Biosystems PN AM9071
FirstChoice® Total RNA	Applied Biosystems
Gel Loading Solution (All-purpose, Native Agarose), 1.4 mL	Applied Biosystems PN AM8556
Glycogen (5 mg/mL) (1 mL tube)	Applied Biosystems PN AM9510

Item	Source
Spin Columns and Tubes	Applied Biosystems PN AM10065
TE, pH 8.0, 500 mL	Applied Biosystems PN AM9849
Novex 6% TBE Gel, 1.0 mM, 10 well	Invitrogen PN EC6265BOX
RiboMinus™ Eukaryote Kit for RNA-Seq	Invitrogen PN A1083708
RiboMinus™ Plant Kit for RNA-Seq	Invitrogen PN A1083808
21-gauge needle	Major laboratory supplier (MLS)
Isopropanol	MLS

Optional for whole transcriptome libraries

Item	Source
MicroPoly(A)Purist™ Kit	Applied Biosystems PN AM1919
RiboMinus™ Eukaryote Kit for RNA-Seq	Invitrogen PN A1083708
RiboMinus™ Plant Kit for RNA-Seq	Invitrogen PN A1083808
ERCC spike-in control mixes (available soon)	External RNA Control Consortium

Optional for small RNA libraries

Item	Source
Electro*Zap*™ Electrode Decontamination Solution	Applied Biosystems PN AM9785
flashPAGE™ Buffer Kit (Type A)	Applied Biosystems PN AM9015
flashPAGE™ Fractionator Apparatus	Applied Biosystems PN AM13100
flashPAGE™ Pre-cast Gels (Type A)	Applied Biosystems PN AM10010
flashPAGE™ Reaction Clean-Up Kit	Applied Biosystems PN AM12200

Item	Source
KinaseMax™ 5′ End-Labeling Kit, 30 reactions	Applied Biosystems PN AM1520
*mir*Vana™ miRNA Isolation Kit, 40 purifications	Applied Biosystems PN AM1560
*mir*Vana™ miRNA Reference Panel v9.1	Applied Biosystems PN 4388891
*mir*Vana™ PARIS™, 40 purifications	Applied Biosystems PN AM1556
NucAway™ Spin Columns Kit, 30 each	Applied Biosystems PN AM10070
PureLink™ miRNA Isolation Kit, 25 preps	Invitrogen PN K1570-01

Supplemental Information

This appendix contains:

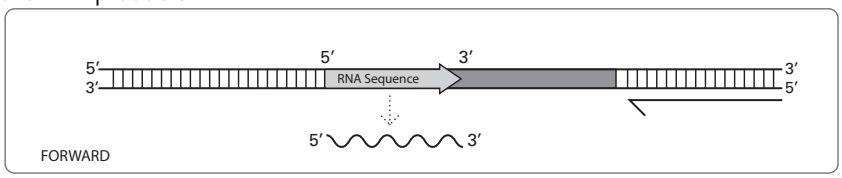
Amplified library construction concepts

The procedures in this protocol are based on Life Technologies' Ligase-Enhanced Genome Detection (LEGenD™) technology.

Hybridization and ligation to the RNA

The RNA samples are hybridized and ligated with the Ambion® Adaptor Mix, which is a set of oligonucleotides with a single-stranded degenerate sequence at one end and a defined sequence required for Genome Analyzer IIe sequencing at the other end. The Ambion Adaptor Mix constrains the orientation of the RNA in the ligation reaction such that hybridization yields template for sequencing from the 5′ end of the sense strand on the Genome Analyzer IIe.

Figure 11 Strand-specific RNA sequence information from Ambion RNA-Seq Library Construction Kit products



Reverse transcription and size selection

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

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

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

After 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 cluster generations, and to use only libraries with sufficiently long inserts for sequencing on the Genome Analyzer IIe.

Sequences of the primers included in the kit

Ambion 5′ PCR primer

5′ - AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T -3′

Ambion 3′ PCR primer

5′ - CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC TGA ACC GCT CTT CCG ATC T -3′

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—270 bp is greater than 25% (from step 3 on page 26).
- For small RNA libraries: The ratio of 140—155-bp DNA: 25—200-bp DNA is less than 50% (from step 2 on page 45)

Size-select the amplified cDNA

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

- Novex® 6% TBE Gel 1.0 mM, 10 Well
- Novex® TBE Running Buffer (5✕)
- Gel Loading Solution (AM8556)
- XCell *SureLock*™ Mini-Cell
- 50 bp DNA Ladder for whole transcriptome libraries or the 10 bp DNA Ladder for small RNA libraries
- SYBR® Gold nucleic acid gel stain

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

For more instructions on staining the gel, refer to the Invitrogen *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 26.
   - For small RNA libraries: Add 2 µL Gel Loading Solution to the eluted cDNA (~10 µL) from step 5 on page 45.

   IMPORTANT! Do not heat the samples before loading.

2. Prepare the gel as described in the Invitrogen *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: 270—370 bp
   - For small RNA libraries: 130—160 bp

   Note: If you are using a UV transilluminator to visualize the nucleic acid, work quickly to limit its exposure to UV radiation.

Purify the amplified cDNA from the gel

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

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

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 flow-through.

   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 the sample 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.

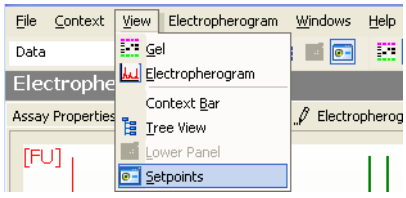
   Note: Accurate quantitation of the DNA is important for the downstream steps of the Genome Analyzer sequencing workflow. The resuspension volume should yield DNA sufficiently concentrated for accurate measurements (~10 ng/µL).

Using Agilent® 2100 Bioanalyzer™ Instrument expert software to assess whole transcriptome libraries

Perform a smear analysis

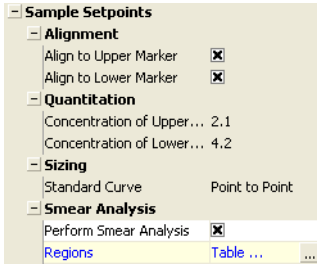
Perform a smear analysis to quantify the percentage of DNA in the 25—270 bp size range.

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**.

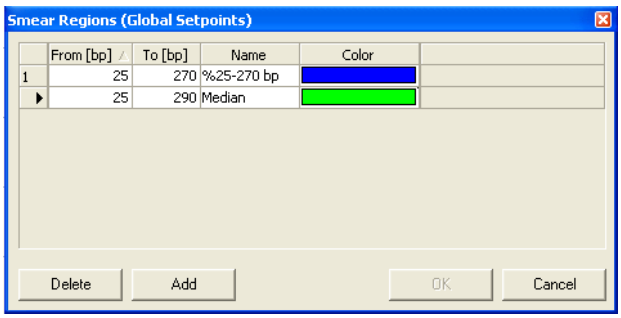


4. Set the smear regions in the Smear Regions dialog box:
   a. Click **Add**, then enter 25 bp and 270 bp for the lower and upper limits, respectively.

      These settings are used to determine the percentage of total product that is 25—270 bp in length.

   b. Click **Add**, enter 25 bp and 290 bp, then click **OK**.

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



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

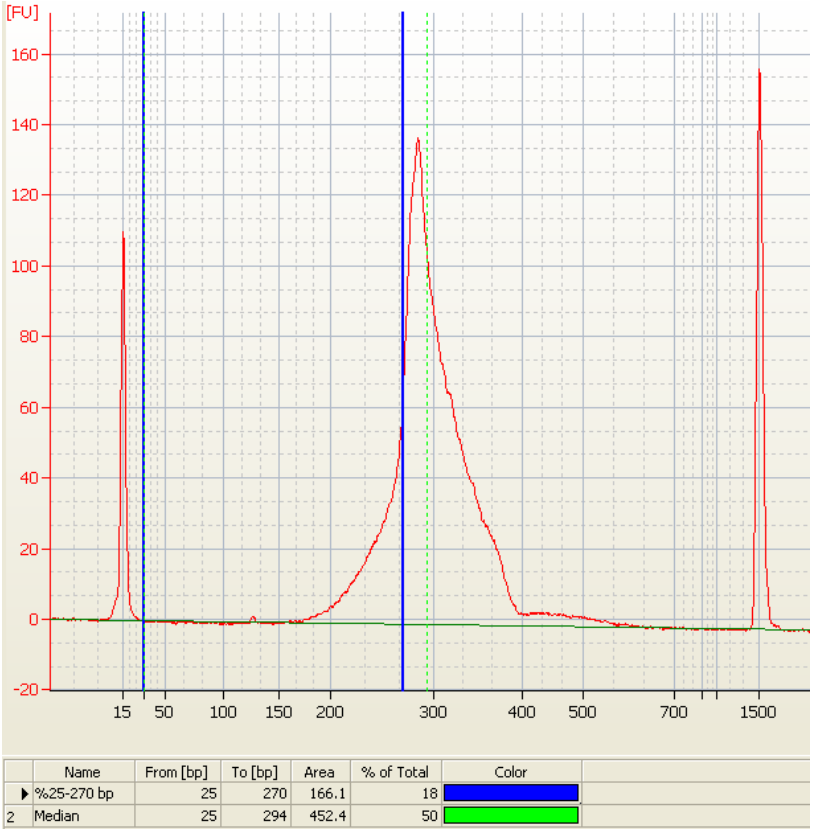
6. In the Region Table, review the percentage of the total product in the size ranges you set. Look at %25—270 bp.

	Name	From [bp]	To [bp]	Area	% of Total	Color
1	%25-270 bp	25	270	166.1	18	
2	Median	25	294	452.4	50	

Determine the median size

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

Figure 12 Determine median (whole transcriptome libraries)



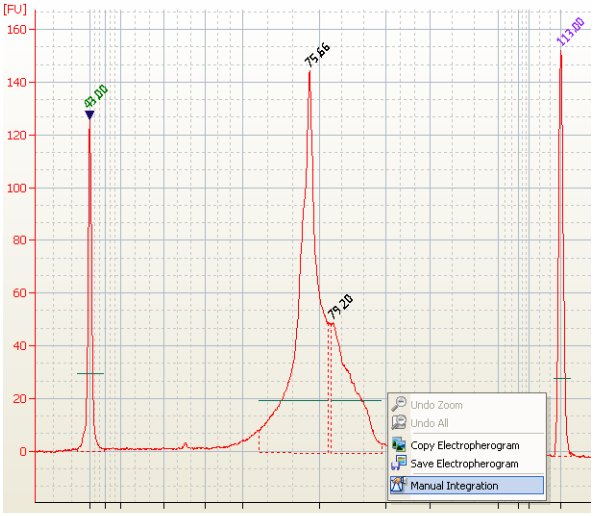
	Name	From [bp]	To [bp]	Area	% of Total	Color
▶	%25-270 bp	25	270	166.1	18	
2	Median	25	294	452.4	50	

Analyze multiple peaks as one peak

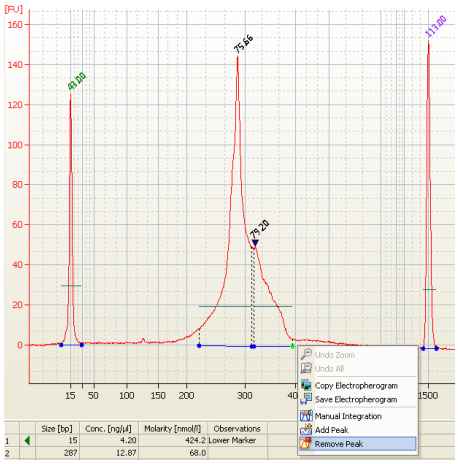
On the Peak Table tab, you may observe that the Agilent 2100 Bioanalyzer Instrument software identified multiple peaks in a region that you want to consider as one peak. To obtain one concentration and automatically determine median size for a peak region, manually set the size range of the desired peak 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**.

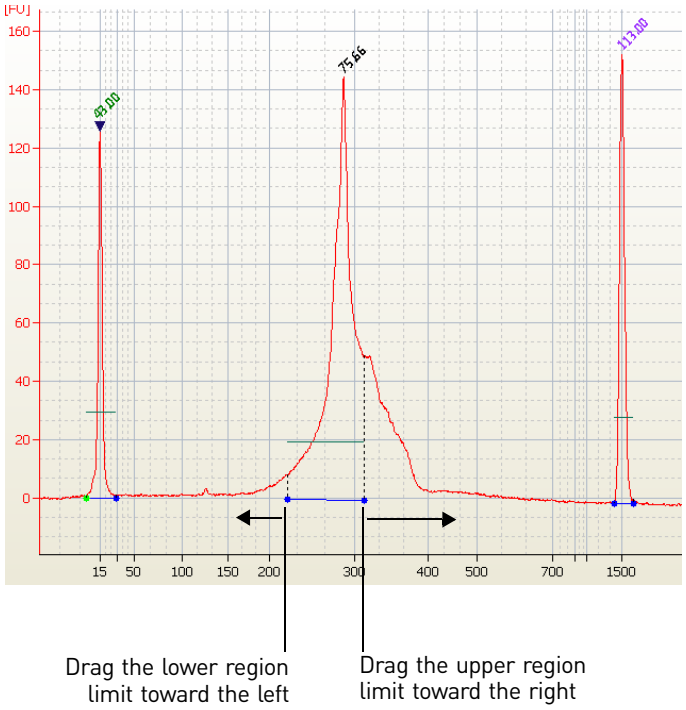


3. To remove multiple peaks:
   a. Place the cursor on the peak to remove, right-click, then select **Remove Peak**.



   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.



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		287	18.13	95.8	
3	▶	1,500	2.10	2.1	Upper Marker

Small RNA enrichment

If your RNA sample contains 0.1–0.5% miRNA, perform small RNA enrichment using the Invitrogen PureLink™ miRNA Isolation Kit using this purification procedure. If you are using the PureLink miRNA Isolation Kit to isolate small RNA from cells or tissue, refer to the Invitrogen *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 the tube 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 flow-through. **Keep the flow-through**. Transfer the flow-through to a new 1.5 mL tube with a lid for vortexing.

   c. Discard the cartridge.
3. Add 700 µL 100% ethanol to the flow-through, then vortex the tube to mix well.
4. Spin the small RNA:
   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 flow-through.
   d. Return the cartridge to the collection tube.
   e. Load the remaining 600 µL of the sample containing 100% ethanol onto the cartridge.
   f. Spin the cartridge at 12,000 × g for 1 minute.
   g. Discard the flow-through.
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 flow-through.
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 flow-through 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 flow-through and the Wash Tube.

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

Store the small RNA at –80 °C or assess the quality and quantity of the small RNA-enriched sample (page 36).

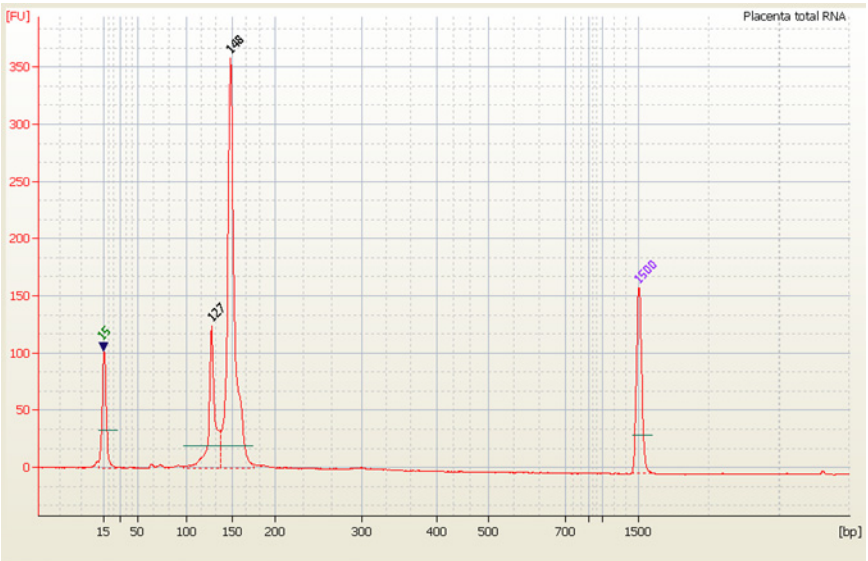
Using 2100 expert software to assess small RNA libraries

Review the median size

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

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

Figure 13 Determine median (small RNA libraries)

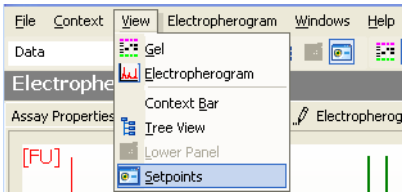


		Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
▶	◀	15	4.20	424.2	Lower Marker
2		127	4.20	50.1	
3		148	13.82	141.7	
4	▶	1,500	2.10	2.1	Upper Marker

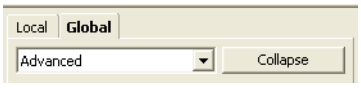
Perform a smear analysis

Perform a smear analysis to quantify the percentage of DNA in the 25—200 bp and 140—155 bp size range. The desired size range for miRNA ligation products is 140—155 bp.

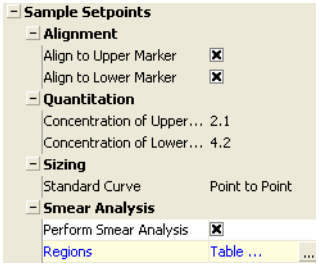
1. In the 2100 expert software, select **View** ▸ **Setpoints**.



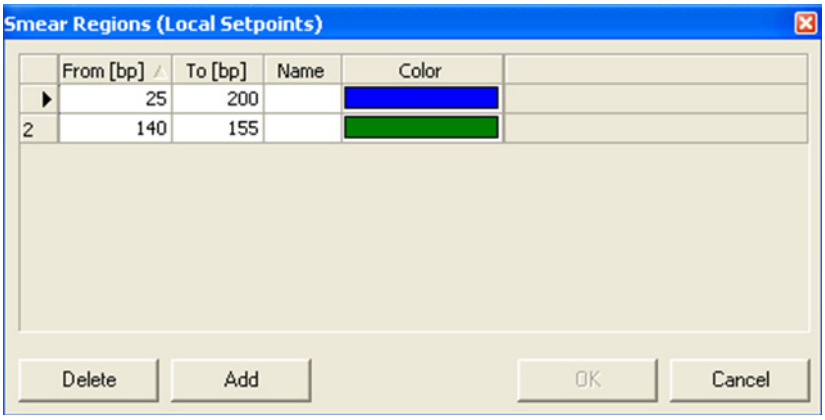
2. On the **Global** tab, select **Advanced** settings.



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 200 bp for the lower and upper limits, respectively.
   b. Click **Add**, enter 140 bp and 155 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.

	Name	From [bp]	To [bp]	Area	% of Total	Color
▶	Region 1	25	200	496.8	95	
2	Region 2	140	155	289.5	56	

Determine the % miRNA library

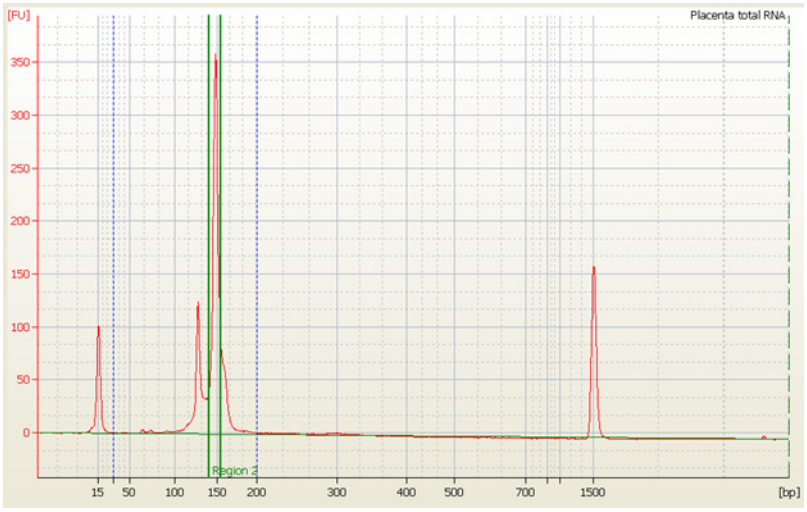
Using the area values from the Region Table, calculate the % miRNA library in the 140—155 bp region as a fraction of the 25—200 bp region using the formula:

$$\%\ \text{miRNA library} = \left(\frac{\text{Area from 140–155 bp}}{\text{Area from 25–200 bp}} \right) \times 100$$

If the % miRNA library is less than 50%, Applied Biosystems strongly recommends that you perform a second-round size selection using a nondenaturing polyacrylamide gel, such as the Novex 6% TBE Gel (page 58).

Example % miRNA library calculation

In the example below, the % miRNA library is 59%.



	Name	From [bp]	To [bp]	Area	% of Total	Color
1	Region 1	25	200	496.8	95	
▶	Region 2	140	155	289.5	56	

$$\%\ \text{miRNA} = (56/95) \times 100 = 59\%$$

Good Laboratory Practices for RNA Work

This appendix contains:

RNase precautions

Before working with RNA:

- Clean the lab bench and pipettors with an RNase decontamination solution (for example, Ambion RNase*Zap*® Solution, Cat No. AM9780).
- Wear laboratory gloves for this procedure.
- Use RNase-free pipette tips to handle the kit reagents, and avoid putting used tips into the reagent containers.

RNA purity and integrity

The quality of the RNA is a crucial factor for performance of the Ambion® RNA-Seq Library Construction Kit. RNA quality is a combination of the purity and integrity of the RNA preparation.

RNA purity

Impurities can lower the efficiencies of enzymatic reactions:

- RNA samples should be free of contaminating proteins, DNA, and other cellular material, as well as phenol, ethanol, and salts associated with RNA isolation procedures.
- A_{260} to A_{280}, an effective measure of RNA purity, should fall in the range of 1.7-2.1.

RNA integrity

The integrity of the RNA sample is the proportion that is full-length, is another important component of RNA quality. When the size distribution of the RNA is analyzed, primarily full-length RNA will exhibit discrete rRNA bands (that is, no significant smearing below each band), and a ratio of 28S to 18S rRNA bands that approaches 2:1. RNA integrity can be evaluated by:

- **Microfluidic analysis:** Use the Agilent® 2100 Bioanalyzer™ Instrument and Caliper RNA LabChip® Kits. With the Bioanalyzer Instrument, the RNA Integrity Number (RIN) can be calculated to further evaluate RNA integrity.
- **Denaturing agarose gel electrophoresis** and nucleic acid staining: this method requires microgram amounts of RNA.

Additional information

For more information about good library practices when working with RNA, refer to Invitrogen Technical Bulletin 159, *Working with RNA*, which is available at **www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Nucleic-Acid-Purification-and-Analysis/RNA-Purification/working-with-rna.html**.

RT-PCR Good Laboratory Practices

When preparing samples for RT-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 RT-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 RT-PCR products
- Never bring amplified PCR products into the RT-PCR setup area.
- Open and close all sample tubes carefully. Centrifuge tubes before opening. Try not to splash or spray RT-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).

Safety

This appendix covers:

General chemical safety

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About SDSs" on this page.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

SDSs

About SDSs

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

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

Obtaining SDSs

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

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

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

Biological hazard safety

General biohazard

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

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (**www.cdc.gov/biosafety/publications/index.htm**).
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **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

Documentation and Support

Kit documentation

The following documents are available for the Ambion® RNA-Seq Library Construction Kit:

Document	Part number	Description
Ambion® RNA-Seq Library Construction Kit *Protocol*	4452440	Provides product information, step-by-step instructions for using the Ambion RNA-Seq Library Construction Kit, troubleshooting information, ordering information, and supplemental information.
Ambion® RNA-Seq Library Construction Kit *for Whole Transcriptome Libraries Quick Reference Card*	4452441	Provides abbreviated instructions for using the Ambion RNA-Seq Library Construction Kit to create an amplified cDNA library of the whole transcriptome.
Ambion® RNA-Seq Library Construction Kit *for Small RNA Libraries Quick Reference Card*	4452442	Provides abbreviated instructions for using the Ambion RNA-Seq Library Construction Kit to create an amplified cDNA library of the small RNAs.

Related documents

Document	Part number	Description
Agilent® 2100 Bioanalyzer™ 2100 Expert User's Guide	Agilent G2946-90000	Provides instructions for running electrophoretic and flow cytometric assays with the Agilent 2100 Bioanalyzer Instrument.
Illumina Paired-End Sequencing User Guide	Illumina 1003880	Provides instructions for operating and maintaining the Illumina Cluster Station and Genome Analyzer.
Invitrogen SYBR Gold Nucleic Acid Gel Stain manual	Invitrogen MP 11494	Provides information about Molecular Probes SYBR Gold nucleic acid gel stain.
Novex® Pre-Cast Gel Electrophoresis Guide	Invitrogen IM-1002	Provides information about the Novex Pre-Cast gels and complete protocols for sample and buffer preparation, electrophoresis conditions, staining, and blotting.
Quant-iT™ RNA Assay Kit Protocol	Invitrogen MP 33140	Provides instructions for using the Quant-iT™ RNA Assay Kit.
Qubit® Fluorometer Instruction Manual	Invitrogen MP 32857	Provides instructions for inspecting, setting up, using, and troubleshooting the Qubit® Fluorometer.

Obtaining support

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

www.invitrogen.com

At the Applied Biosystems web site, you can:

- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, SDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

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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ambion®
by life technologies™

Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA
Phone 760.603.7200
www.lifetechnologies.com

Technical Resources and Support
For the latest technical resources and support information for all locations, please refer to our Web site at
www.invitrogen.com/support