iontorrent

Ion Total RNA-Seq Kit v2

USER GUIDE

for use with:
Ion PGM™ System
Ion Proton™ System
Ion S5™ System
Ion S5™ XL System

Catalog Numbers 4475936, 4479789, 4475485
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Revision C.0

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Revision history: Pub. No. MAN0010654

Revision	Date	Description of Change
C.0	11 January 2017	• Redundant steps deleted from procedure "Perform reverse transcription" in Chapter 2, "Prepare Whole Transcriptome Libraries". • Minor editing
B.0	30 June 2016	• Updated for Ion S5™ sequencing platforms. • General rebranding. • Minor editing.
A.0	21 July 2014	• Version numbering changed to alphanumeric format and reset to A.0 in conformance with internal document control procedures. • Updated "Determine library dilution for template preparation" section to yield 100 pM concentration.

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1 Product information

IMPORTANT! Before using this product, read and understand the information in Appendix C, "Safety" in this document.

Product description

Use the Ion Total RNA-Seq Kit v2.0 (Cat. Nos. 4475936 and 4479789) to convert RNA transcripts expressed in a cell or tissue into representative cDNA libraries for strand-specific RNA sequencing on the Ion Torrent™ Personal Genome Machine™ (PGM™), Ion Proton™, Ion S5™, and the Ion S5™ XL Systems. This user guide supports library preparation for up to 200-base-read sequencing:

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

Preparing barcoded libraries

The Ion Total RNA-Seq Kit v2.0 supports barcoded library preparation to enable sequencing of multiple samples in a single, multiplexed sequencing run. To prepare barcoded libraries, replace the adaptors in the kit with adaptors from the Ion Xpress™ RNA-Seq Barcode 01–16 Kit (Cat. No. 4475485).

Kit contents and storage

Ion Total RNA-Seq Kit v2, 12-Reaction Kit

Sufficient reagents are supplied in the Ion Total RNA-Seq Kit v2, 12-Reaction Kit (Cat. No. 4475936) to prepare cDNA libraries from 12 samples for sequencing analysis with the Ion PGM™, Ion Proton™, Ion S5™, or Ion S5™ XL Systems.

Components[1]	Amount	Storage
Ion RNA-Seq Core Kit v2, 12-Reaction Kit (Part No. 4474906)		
Nuclease-free Water (clear cap)	2 × 1.75 mL	15°C to 30°C (room temperature)
10X RNase III Reaction Buffer (red cap)	20 µL	–30°C to –10°C
RNase III (red cap)	20 µL	
Hybridization Solution (green cap)	40 µL	
2X Ligation Buffer (green cap)	150 µL	
Ligation Enzyme Mix (green cap)	30 µL	

Components[1]	Amount	Storage
10X RT Buffer (yellow cap)	56 µL	–30°C to –10°C
2.5 mM dNTP Mix (white cap)	30 µL	
10X SuperScript™ III Enzyme Mix (yellow cap)	56 µL	
Platinum™ PCR SuperMix High Fidelity (blue cap)	900 µL	
WT Control RNA (1 µg/µL HeLa total RNA; clear cap)	50 µL	
Small RNA Control (1 µg/µL human placenta total RNA; purple cap)	10 µL	
Ion RNA-Seq Primer Set v2, 12-Reaction Kit (Part No. 4474810)		
Ion Adaptor Mix v2 (green cap)	30 µL	–30°C to –10°C
Ion RT Primer v2 (yellow cap)	104 µL	
Ion 5' PCR Primer v2 (white cap)	20 µL	
Ion 3' PCR Primer v2 (blue cap)	20 µL	
Magnetic Bead Cleanup Module (Part No. 4475486)		
Processing Plate	1	15°C to 30°C (room temperature)
Binding Solution Concentrate	11 mL	
Wash Solution Concentrate	11 mL	
Nucleic Acid Binding Beads (clear cap)	0.7 mL	2°C to 8°C **IMPORTANT!** Do not freeze.

[1] We verified this protocol using this specific material. Substitution may adversely affect performance.

Ion Total RNA-Seq Kit v2, 48-Reaction Kit

Sufficient reagents are supplied in the Ion Total RNA-Seq Kit v2, 48-Reaction Kit (Cat. No. 4479789) to prepare cDNA libraries from 48 samples for sequencing analysis with the Ion PGM™, Ion Proton™, Ion S5™, or Ion S5™ XL Systems.

Components[1]	Amount	Storage
Ion RNA-Seq Core Kit v2, 48-Reaction Kit (Part No. 4475482)		
Nuclease-free Water	10 mL	15°C to 30°C (room temperature)
10X RNase III Reaction Buffer (red cap)	60 µL	–30°C to –10°C
RNase III (red cap)	60 µL	

Components[1]	Amount	Storage
Hybridization Solution (green cap)	170 µL	–30°C to –10°C
2X Ligation Buffer (green cap)	650 µL	
Ligation Enzyme Mix (green cap)	110 µL	
10X RT Buffer (yellow cap)	224 µL	
2.5 mM dNTP Mix (white cap)	120 µL	
10X SuperScript™ III Enzyme Mix (yellow cap)	224 µL	
Platinum™ PCR SuperMix High Fidelity (blue cap)	2 × 1800 µL	
WT Control RNA (1 µg/µL HeLa total RNA; clear cap)	50 µL	
Small RNA Control (1 µg/µL human placenta total RNA; purple cap)	10 µL	
Ion RNA-Seq Primer Set v2, 48-Reaction Kit (Part No. 4475481)		
Ion Adaptor Mix v2 (green cap)	120 µL	–30°C to –10°C
Ion RT Primer v2 (yellow cap)	416 µL	
Ion 5' PCR Primer v2 (white cap)	80 µL	
Ion 3' PCR Primer v2 (blue cap)	80 µL	
Magnetic Bead Cleanup Module (Part No. 4475486)		
Processing Plate	1	15°C to 30°C (room temperature)
Binding Solution Concentrate	11 mL	
Wash Solution Concentrate	11 mL	
Nucleic Acid Binding Beads (clear cap)	0.7 mL	2°C to 8°C **IMPORTANT!** Do not freeze.

[1] We verified this protocol using this specific material. Substitution may adversely affect performance.

Required materials not supplied

For the Safety Data Sheet (SDS) of any chemical not distributed by Thermo Fisher Scientific, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Required for library preparation

Unless otherwise indicated, all materials are available through **thermofisher.com** MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Thermal cycler with heated lid, capable of holding 0.2-mL tubes: • Veriti™ 96-Well Thermal Cycler • GeneAmp™ PCR System 9700	• 4375786 • 4310899
Agilent™ 2100 Bioanalyzer™ instrument	Agilent G2938A
NanoDrop™ Spectrophotometer	ND 2000
Qubit™ 3.0 Fluorometer[1]	Q33216
Centrifugal vacuum concentrator (for example, SpeedVac)	MLS
Microcentrifuge	MLS
Pipettors, positive-displacement or air-displacement	MLS
Magnetic stand – one of the following: • Magnetic Stand-96 • 96 well Magnetic-Ring Stand	• AM10027 • AM10050
(Optional) Multi-channel pipettor	MLS
Agilent™ DNA 1000 Kit	Agilent 5067-1504
Agilent™ High Sensitivity DNA Kit	Agilent 5067-4626
Ethanol, 100%, ACS reagent grade or equivalent	MLS
(Optional) Qubit™ dsDNA HS Assay Kit, 100 assays	Q32851
8-strip PCR Tubes & Caps, RNase-free, 0.2-mL	AM12230
Non-Stick RNase-Free Microfuge Tubes (0.5-mL), 500	AM12350
Non-Stick RNase-Free Microfuge Tubes (1.5-mL), 250	AM12450
Pipette tips, RNase-free	MLS
(Optional) 1.2-mL 96-well plates	AB-1127
(Optional) FirstChoice™ Total RNA	(Various Cat. Nos.)

[1] The Qubit™ 2.0 Fluorometer is supported but no longer available for purchase.

Materials for whole transcriptome libraries

Item	Source
Qubit™ RNA Assay Kit, 100 assays	Q32852
Agilent™ RNA 6000 Pico Kit	5067-1513
(Optional) ERCC RNA Spike-In Control Mixes **Note:** ERCC controls are highly recommended.	4456739 and 4456740
(Optional) Dynabeads™ mRNA DIRECT™ Micro Kit Micro Kit	61021
(Optional) MicroPoly(A)Purist™ Kit	AM1919
(Optional) TaqMan® Gene Expression Assays for ERCC Targets	(Various Cat. Nos.)
(Optional) TaqMan® Gene Expression Master Mix	4369016 and 4369510
(Optional) RiboMinus™ Eukaryote System v2	A15026
(Optional) Low Input RiboMinus™ Eukaryote System v2	A15027
(Optional) RiboMinus™ Plant Kit for RNA-Seq	A1083808

Materials for small RNA libraries

Item	Source
Agilent™ RNA 6000 Nano Kit	5067-1511
Agilent™ Small RNA Kit	5067-1548
*(Optional) mir*Vana™ miRNA Isolation Kit, with phenol, 40 purifications	AM1560
*(Optional)mir*Vana™ miRNA Isolation Kit, without phenol, 40 purifications	AM1561
*(Optional) mir*Vana™ PARIS™ RNA and Native Protein Purification Kit, 40 purifications	AM1556
(Optional) PureLink™ miRNA Isolation Kit, 25 preps	K157001

(*Optional*) Ion Xpress™ RNA-Seq Barcode 01-16 Kit

Sufficient PCR primers are supplied in the Ion Xpress™ RNA-Seq Barcode 01–16 Kit (Cat. No. 4475485) to prepare barcoded cDNA libraries from 12 samples.

Note: This kit is ordered separately.

Contents	Amount	Storage
Ion Xpress™ RNA BC 01–BC 16 (white cap)	12 µL	–30°C to –10°C
Ion Xpress™ RNA 3′ Barcode Primer (blue cap)	192 µL	

2 Prepare whole transcriptome libraries

Workflow

Fragment the whole transcriptome RNA

Start with RNA with "ERCC RNA Spike-In control mixes" on page 13

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"Fragment the RNA using RNase III" on page 14

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"Purify the fragmented RNA" on page 15

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"Assess the yield and size distribution of fragmented RNA" on page 17

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Construct the whole transcriptome library

"Hybridize and ligate the RNA" on page 19

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"Perform reverse transcription (RT)" on page 21

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"Purify the cDNA" on page 21

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"Amplify the cDNA" on page 24

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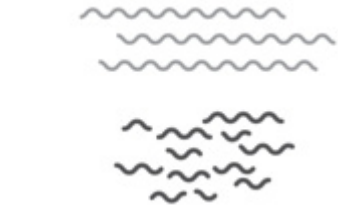
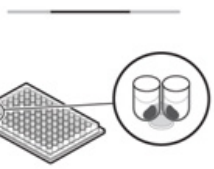


Fragmented RNA



"Purify the amplified cDNA" on page 26

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"Assess the yield and size distribution of the amplified DNA" on page 28

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"Pool barcoded whole transcriptome libraries" on page 30

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"Determine the library dilution required for template preparation" on page 30

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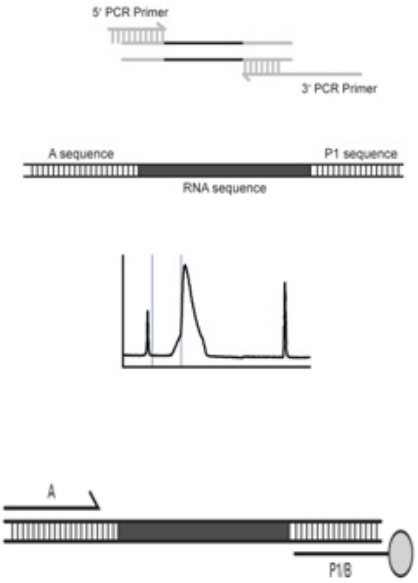
Prepare templated Ion Sphere™ Particles

Refer to the specific user guide for an Ion template preparation kit



Fragment the whole transcriptome RNA

Guidelines for RNA sample type and amount

We strongly recommend using 1–500 ng of poly(A) RNA, or 10–500 ng of rRNA-depleted total RNA. You can also use high-quality total RNA.

Guidelines for using poly(A) RNA

To prepare poly(A) RNA from:

- 100 ng–50 µg total RNA, we recommend using the Dynabeads™ mRNA DIRECT™ Micro Kit Micro Kit (Cat. No. 61021). See the *Dynabeads™ mRNA DIRECT™ Micro Kit User Guide* for more information.
- 50–400 µg total RNA, we recommend performing two rounds of oligo(dT) selection of the poly(A) RNA using the MicroPoly(A)Purist™ Kit (Cat. No. AM1919). 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.

Guidelines for using rRNA-depleted total RNA

To prepare rRNA-depleted total RNA from:

- 1–5 µg total RNA, we recommend that you remove rRNA using the RiboMinus™ Eukaryote System v2 (Cat. No. A15026).
- 100 ng–1 µg total RNA, we recommend that you remove rRNA using the Low Input RiboMinus™ Eukaryote System v2 (Cat. No. A15027).

Note: For depletion of bacterial rRNA from total bacterial RNA using the RiboMinus™ Eukaryotic System v2, see the *Demonstrated Protocol: Bacterial Ribosomal RNA (rRNA) Depletion Workflow for RNA-Seq User Bulletin* (Pub. No. MAN0009661) for more information.

Guidelines for using total RNA

- Best results are obtained when using RNA with an RNA integrity number (RIN) greater than 7. FirstChoice™ Total RNA provides high-quality, intact RNA isolated from a variety of sources.
- Quantify the amount of RNA in the sample using the NanoDrop™ Spectrophotometer.

ERCC RNA Spike-In control mixes

We strongly recommend that you add ERCC RNA Spike-In Control Mixes to the input total RNA before RNA depletion or poly(A) selection for whole transcriptome library generation. The ERCC RNA Spike-In Control Mixes provide a set of external RNA controls that enable performance assessment of a variety of technology platforms used for gene expression experiments. Add one Spike-In Control Mix to each RNA sample, and run these samples on your platform; compare the Spike-In Control Mix data to known Spike-In Control Mix concentrations and ratios to assess the dynamic range, lower limit of detection, and fold-change response of your platform. The following table provides guidelines for how much Spike-In Control Mix to add to the input RNA for whole transcriptome library preparation. For detailed information, see the *ERCC RNA Spike-In Control Mixes User Guide* (Pub. No. 4455352).

Amount of Sample RNA	Volume of Spike-In Mix 1 or Mix 2 (dilution)[1]	
	Total RNA	Poly (A) RNA
1 ng	—	1 µL (1:1000)
5 ng	—	5 µL (1:1000)
10 ng	—	1 µL (1:100)
50 ng	—	5 µL (1:100)
100 ng	2 µL (1:1000)	1 µL (1:10)
500 ng	1 µL (1:100)	5 µL (1:10)
1000 ng	2 µL (1:100)	—
5000 ng	1 µL (1:10)	—

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

ERCC_Analysis plugin

The ERCC_Analysis plugin is intended to help with ERCC RNA Spike-in Controls. It enables you to quickly determine whether the ERCC results indicate a problem with library preparation or the sequencing run.

For more information about the ERCC_Analysis Plugin, see the *ERCC_Analysis Plugin User Bulletin* (Pub. No. 4479068).

Fragment the RNA using RNase III

Use components from the Ion Total RNA-Seq Kit v2:

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

1. On ice, assemble a reaction for each RNA sample in a 0.2-mL PCR tube:

Order	Component (add in order shown)	Volume per reaction
1	RNA sample and Nuclease-free Water: • Poly(A) RNA: 1–500 ng • rRNA-depleted total RNA: 10–500 ng • WT Control RNA: 500 ng	8–10 µL
2	10X RNase III Reaction Buffer	1 µL
3	RNase III	1 µl
—	**Total Volume**	**10–12 µL**

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

2. Flick the tube or pipet up and down 5 times to mix, then centrifuge briefly to collect the liquid in the bottom of the tube.

3. Incubate the reaction in a thermal cycler at 37°C according to library and input quantity:

RNA type	Amount	Reaction time
Poly(A) RNA	1 to <100 ng	3 min
	100–500 ng	10 min
rRNA-depleted RNA	10 to <100 ng	3 min
	100–500 ng	10 min
Total RNA	500 ng	10 min

4. *Immediately* after the incubation, add 20 µL of Nuclease-free Water, then place the fragmented RNA on ice.

IMPORTANT! Proceed immediately to the next section, "Purify the fragmented RNA," or leave the fragmented RNA on ice for less than 1 hour.

Purify the fragmented RNA

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate
- Nuclease-free Water

Other materials and equipment

- 100% ethanol or 200 proof (absolute) ethanol, ACS-grade or higher quality
- Magnetic stand for 96-well plates (Cat. No. AM10027 or AM10050)
- 37°C heat block or water bath
- *(Optional)* MicroAmp™ Clear Adhesive Film (Cat. No. 4306311)

Before you begin

- Add 44 mL of 100% ethanol to the bottle of Wash Solution Concentrate and mix well. Mark the label on the bottle to indicate that you added ethanol. Store the solution at room temperature (15°C to 30°C).
- If you see a white precipitate in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Nuclease-free Water at 37°C for ≥5 minutes.

Note: To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp™ Clear Adhesive Film (Cat. No. 4306311). You can also skip a row between sample rows.

IMPORTANT! Accurate pipetting of bead cleanup reagents is critical for best results.

Purify the fragmented RNA

1. Prepare beads for each sample:
   a. Gently vortex the Nucleic Acid Binding Beads tube to resuspend completely the magnetic beads.
   b. Add 5 µL beads to wells on the Processing Plate.
   c. Add 90 µL Binding Solution Concentrate to each well, then mix the Concentrate and beads by pipetting up and down 10 times.

2. Bind the fragment RNA products to the beads:
   a. Transfer each 30-µL fragment RNA reaction to a bead-containing well on the Processing Plate.
   b. Set a P200 pipettor at 150 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the tip with 100% ethanol by pipetting the ethanol up and down 3 times.
   c. Without changing tips, add 150 µL of 100% ethanol to each well.

      IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 2b and 2c for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

   d. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

      Note: If the color of the mixture is not the same throughout, mix again.

   e. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

3. Remove the supernatant from the beads:
   a. Place the Processing Plate on a magnetic stand for 5–6 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
   b. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.

      IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 µL of supernatant behind.

4. Wash the beads with Wash Solution Concentrate with ethanol:
   a. Leave the Processing Plate on the magnetic stand.
   b. Add 150 µL of Wash Solution Concentrate with ethanol to each sample.
   c. Incubate the samples at room temperature for 30 seconds.

5. Remove the supernatant from the beads:
   a. Aspirate and discard the supernatant from the plate.
   b. Use a P10 or P20 pipettor to remove any residual liquid.

      IMPORTANT! Do not disturb the separated magnetic beads. Remove all of the Wash Solution Concentrate from each well.

c. Air-dry the beads at room temperature to remove all traces of ethanol by leaving the Processing Plate on the magnetic stand for 1–2 minutes.

IMPORTANT! Do not overdry the beads (overdried beads appear cracked). Overdrying significantly decreases elution efficiency.

6. Elute the fragmented RNA from the beads:
   a. Remove the Processing Plate from the magnetic stand.
   b. Add 12 µL of pre-warmed (37°C) Nuclease-free Water to each sample, then mix the Nuclease-free Water and beads by pipetting up and down 10 times.
   c. Incubate at room temperature for 1 minute.
   d. Place the Processing Plate on the magnetic stand for 1 minute to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
   e. For each sample, collect the eluant.

Assess the yield and size distribution of fragmented RNA

Use the Qubit™ RNA Assay Kit with the Qubit™ Fluorometer and the Agilent™ RNA 6000 Pico Kit with the Agilent™ 2100 Bioanalyzer™ instrument.

Note: You can use a NanoDrop™ Spectrophotometer instead of the Qubit™ RNA Assay Kit and Qubit™ Fluorometer. For increased accuracy, quantify the RNA concentration using the Qubit™ RNA Assay Kit with the Qubit™ Fluorometer.

Note: We do not recommend evaluating the yield and size for poly(A) fragmented RNA samples from <5-ng poly(A) RNA due to low input amount.

1. Quantify the yield of the fragmented RNA using the Qubit™ RNA Assay Kit with the Qubit™ Fluorometer.
   See the *Qubit™ RNA Assay Kit Protocol* (Pub. No. MAN0002327) or the *Qubit™ 3.0 Fluorometer User Guide* (Pub. No. MAN0010866) for instructions.

2. Evaluate the size distribution of the fragmented RNA:
   a. If needed, dilute 1 µL of the sample to 50–5000 pg/µL with Nuclease-free Water.
   b. Run the diluted sample on an Agilent™ 2100 Bioanalyzer™ instrument with the RNA 6000 Pico Kit. Follow the manufacturer's instructions for performing the assay.
   c. Using the 2100 expert software, review the size distribution. The fragmentation procedure produces 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 is 100–200 nt.
      See the figures in "Typical results of fragmentation of whole transcriptome RNA".

   Note: For instructions on how to review the size distribution, see the *Agilent™ 2100 Bioanalyzer™ Expert User's Guide* (Pub. No. G2946-90004).
   If the profile for the fragmented RNA does not meet the typical results, see Appendix A, "Troubleshooting" for guidance.

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

Amount of fragmented RNA in 3 µL	Action
• ≥50 ng of poly(A) RNA • ≥100 ng of rRNA-depleted total RNA • ≥100 ng of WT Control RNA	Proceed to "Construct the whole transcriptome library." Store the remaining RNA at –86°C to –68°C.
• <50 ng of poly(A) RNA • <100 ng rRNA-depleted total RNA	1. Dry all of the RNA completely in a centrifugal vacuum concentrator at low or medium heat (≤40°C); this takes 10–20 minutes. 2. Resuspend in 3 µL of Nuclease-free Water, then proceed to "Construct the whole transcriptome library."

Typical results of fragmentation of whole transcriptome RNA

The figures in this section 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 HeLa rRNA-depleted total RNA.

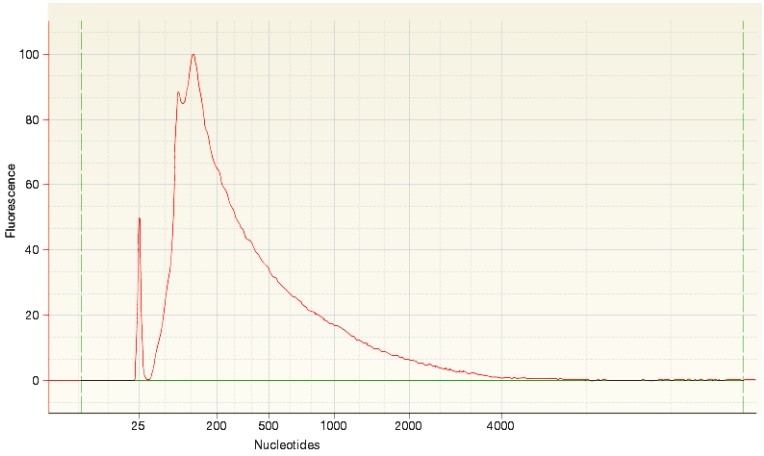


Figure 1 Size distribution of fragmented HeLa poly(A) RNA

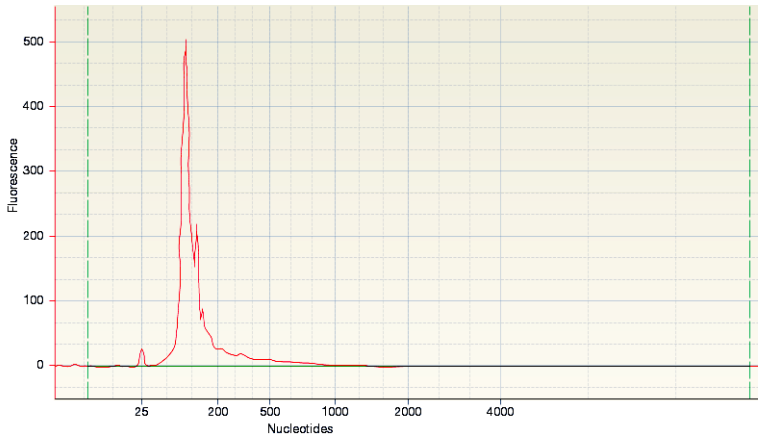


Figure 2 Size distribution of fragmented HeLa rRNA-depleted total RNA

Construct the whole transcriptome library

IMPORTANT! The Ion Adaptor Mix v2, Ion RT Primer v2, and Ion PCR primers are unique to the Ion Total-RNA Seq Kit v2. Do *not* use the reagents from the Ion Total-RNA Seq Kit (first version) to prepare libraries with this user guide.

Required materials

Use components from the Ion Total RNA-Seq Kit v2:

- Ion Adaptor Mix v2
- Hybridization Solution
- Nuclease-free Water
- 2X Ligation Buffer
- Ligation Enzyme Mix

Hybridize and ligate the RNA

1. On ice, prepare the hybridization master mix:

Component	Volume per reaction[1]
Ion Adaptor Mix v2	2 µL
Hybridization solution	3 µL
Total Volume	**5 µL**

[1] Include 5–10% excess volume to compensate for pipetting error when preparing the master mix.

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

   Note: If <50 ng of fragmented poly(A) RNA or <100 ng rRNA-depleted total RNA is recovered after fragmentation, we recommend using all fragmented RNA for ligation.

3. Slowly pipet the solution up and down 10 times to mix, then centrifuge briefly.

4. Run the hybridization reaction in a thermal cycler:

Temperature	Time
65°C	10 min
30°C	5 min

5. On ice, add the RNA ligation reagents to the 8-µL hybridization reactions:

Component	Volume per reaction[1]
Hybridization reaction	8 µL
2X Ligation Buffer	10 µL
Ligation Enzyme Mix	2 µL
Total volume	**20 µL**

[1] Include 5–10% excess volume to compensate for pipetting error when preparing the master mix.

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

6. Flick the tube or slowly pipet the solution up and down 5 times to mix well, then centrifuge briefly to collect the liquid in the bottom of the tube.

7. Incubate the 20-µL ligation reactions in a thermal cycler at 30°C according to input type and amount:

RNA type	Amount into fragmentation	Reaction time
Poly(A) RNA	1–5 ng	1 hour
	>5 ng	30 min
rRNA-depleted RNA	10–100 ng	1 hour
	>100 ng	30 min

IMPORTANT! Set the temperature of the thermal cycler lid to match the block temperature; turn OFF the heated lid; or leave the thermal cycler open during the incubation.

Required materials

Use components from the Ion Total RNA-Seq Kit v2:

- Nuclease-free Water
- 10X RT Buffer
- 2.5 mM dNTP Mix
- Ion RT Primer v2
- 10X SuperScript™ III Enzyme Mix

Perform reverse transcription (RT)

1. On ice, prepare the RT master mix:

Component	Volume per reaction[1]
Nuclease-free Water	2 µL
10X RT Buffer	4 µL
2.5 mM dNTP Mix	2 µL
Ion RT Primer v2	8 µL
Total Volume	**16 µL**

[1] Include 5–10% excess volume to compensate for pipetting error when preparing the master mix.

2. Incubate the RT master mix with the ligated RNA sample:
   a. Add 16 µL of the RT master mix to each 20-µL ligation reaction.
   b. Gently vortex the reaction to mix thoroughly, then centrifuge the reaction briefly to collect the liquid in the bottom of the tube.
   c. Incubate in a thermal cycler with a heated lid at 70°C for 10 minutes, then snap-cool on ice.
3. Perform the reverse transcription reaction:
   a. Add 4 µL of 10X SuperScript™ III Enzyme Mix to each ligated RNA sample.
   b. Gently vortex to mix thoroughly, then centrifuge briefly.
   c. Incubate in a thermal cycler with a heated lid at 42°C for 30 minutes.

STOPPING POINT Store the cDNA at –30°C to –10°C for 2 weeks, store at –86°C to –68°C for long-term storage, or use immediately.

Purify the cDNA

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate
- Nuclease-free Water

Other materials and equipment

- 100% ethanol or 200 proof (absolute) ethanol, ACS-grade or higher quality
- Magnetic stand for 96-well plates (Cat. No. AM10027 or AM10050)
- 37°C heat block or water bath
- *(Optional)* MicroAmp™ Clear Adhesive Film (Cat. No. 4306311)

Before you begin

- If you have not done so already, add 44 mL of 100% ethanol to the Wash Solution Concentrate and mix well. Mark the label on the bottle to indicate that you added ethanol. Store the solution at room temperature (15°C to 30°C).
- If you see a white precipitate in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Nuclease-free Water at 37°C for ≥5 minutes.

Note: To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp™ Clear Adhesive Film (Cat. No. 4306311). You can also skip a row between sample rows.

IMPORTANT! Accurate pipetting of bead cleanup reagents is critical for best results.

Purify the cDNA

1. Prepare beads for each sample:
   a. Gently vortex the Nucleic Acid Binding Beads tube to completely resuspend the magnetic beads.
   b. Add 5 µL beads to wells on the Processing Plate.
   c. Add 120 µL Binding Solution Concentrate to each well, then mix the Binding Solution Concentrate and beads by pipetting up and down 10 times.
2. Bind the cDNA to the beads:
   a. Add 60 µL of Nuclease-free Water to each of the 40-µL RT reaction.
   b. Transfer each 100-µL RT reaction to a bead-containing well on the Processing Plate.
   c. Set a P200 pipettor at 125 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the tip with 100% ethanol by pipetting the ethanol up and down 3 times.
   d. Without changing tips, add 125 µL of 100% ethanol to each well.

      IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 2c–2d for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

   e. Set a single or multi-channel pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

      Note: If the color of the mixture is not the same throughout, mix again.

   f. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

3. Remove the supernatant from the beads:
   a. Place the Processing Plate on the magnetic stand for 5–6 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
   b. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.

      IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 µL of supernatant behind.

4. Wash the beads with Wash Solution Concentrate with ethanol:
   a. Leave the Processing Plate on the magnetic stand.
   b. Add 150 µL of Wash Solution Concentrate with ethanol to each sample.
   c. Incubate the samples at room temperature for 30 seconds.
5. Remove the supernatant from the beads:
   a. Aspirate and discard the supernatant from the plate.
   b. Use a P10 or P20 pipettor to remove residual ethanol.

      IMPORTANT! Do not disturb the separated magnetic beads. Remove all of the Wash Solution Concentrate from each well.

   c. Air-dry the beads at room temperature to remove all traces of ethanol by leaving the Processing Plate on the magnetic stand for 1–2 minutes.

   IMPORTANT! Do not overdry the beads (overdried beads appear cracked). Overdrying significantly decreases elution efficiency.

6. Elute the cDNA from the beads:
   a. Remove the Processing Plate from the magnetic stand.
   b. Add 12 µL of pre-warmed (37°C) Nuclease-free Water to each sample, then mix the Nuclease-free Water and beads by pipetting up and down 10 times.
   c. Incubate at room temperature for 1 minute.
   d. Place the Processing Plate on the magnetic stand for 1 minute to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
   e. For each sample, collect the eluant.

Amplify the cDNA

To prepare non-barcoded libraries, use the following components from the Ion Total RNA-Seq Kit v2:

- Ion 5′ PCR Primer v2
- Ion 3′ PCR Primer v2
- Platinum™ PCR SuperMix High Fidelity

To prepare barcoded libraries, plan the barcodes that you want to use, then select the PCR primers from Ion Xpress™ RNA-Seq Barcode 01–16 Kit:

- Ion Xpress™ RNA-Seq Barcode BC 01–BC 16
- Ion Xpress™ RNA 3′ Barcode Primer

1. For each cDNA sample, set up the PCR reaction, according to the non-barcoded or barcoded library tables.

   IMPORTANT! Use the appropriate primers.

   - For non-barcoded libraries:
     a. Prepare the Non-barcoded Library PCR Mix according to the following table.

Contents	Volume per reaction[1]
Platinum™ PCR SuperMix High Fidelity[2]	45 µL
Ion 5' PCR Primer v2	1 µL
Ion 3' PCR Primer v2	1 µL
Total Volume	**47 µL**

[1] Include 5–10% excess volume to compensate for pipetting error when preparing master mix.
[2] Platinum™ PCR SuperMix High Fidelity contains a proofreading enzyme for high-fidelity amplification.

     b. Transfer 6 µL of cDNA to a new PCR tube.
     c. Transfer 47 µL of the Non-barcoded Library PCR Mix to each 6 µL of cDNA sample.
     d. Proceed to step 2.

   - For barcoded libraries
     a. Prepare the Barcoded Library PCR Mix according to the following table.

Contents	Volume per reaction[1]
Platinum™ PCR SuperMix High Fidelity[2]	45 µL
Ion Xpress™ RNA 3' Barcode Primer	1 µL
Total Volume	**46 µL**

[1] Include 5–10% excess volume to compensate for pipetting error when preparing master mix.
[2] Platinum™ PCR SuperMix High Fidelity contains a proofreading enzyme for high-fidelity amplification.

     b. Transfer 6 µL of cDNA sample to a new PCR tube.

c. Transfer 46 µL of the Barcoded Library PCR Mix to each 6 µL of cDNA sample.

d. Add 1 µL of the selected Ion Xpress™ RNA-Seq Barcode BC primer (choose from BC01–BC16) to each PCR tube.

2. Flick the tube or slowly pipet the solution up and down 5 times to mix well, then centrifuge briefly to collect the liquid in the bottom of the tube.

3. Run the reactions in a thermal cycler:

Stage	Temp	Time
Hold	94°C	2 min
Cycle (2 cycles)	94°C	30 sec
	50°C	30 sec
	68°C	30 sec
Cycle • 16 cycles for 1–5 ng poly(A) RNA or 10–100 ng rRNA-depleted RNA • 14 cycles for >5 ng poly(A) RNA or >100 ng rRNA-depleted RNA	94°C	30 sec
	62°C	30 sec
	68°C	30 sec
Hold	68°C	5 min

Purify the amplified cDNA

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate
- Nuclease-free Water

Other materials and equipment

- 100% ethanol or 200 proof (absolute) ethanol, ACS-grade or higher quality
- Magnetic stand for 96-well plates (Cat. No. AM10027 or AM10050)
- 37°C heat block or water bath
- *(Optional)* MicroAmp™ Clear Adhesive Film (Cat. No. 4306311)

Before you begin

- If you have not done so already, add 44 mL of 100% ethanol to the Wash Solution Concentrate and mix well. Mark the label on the bottle to indicate that you added ethanol. Store the solution at room temperature (15°C to 30°C).
- If you see a white precipitate in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Nuclease-free Water at 37°C for ≥5 minutes.

Note: To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp™ Clear Adhesive Film (Cat. No. 4306311). You can also skip a row between sample rows.

IMPORTANT! Accurate pipetting of bead cleanup reagents is critical for best results.

Purify the amplified cDNA

1. Prepare beads for each sample:
   a. Gently vortex the Nucleic Acid Binding Beads tube to resuspend the magnetic beads completely.
   b. Add 5 µL of bead suspension to wells on the Processing Plate.
   c. Add 180 µL Binding Solution Concentrate to each well, then mix the Concentrate and beads by pipetting up and down 10 times.
2. Bind the amplified cDNA to the beads:
   a. Transfer 53 µL of each amplified cDNA sample to a bead-containing well on the Processing Plate.
   b. Set a P200 pipettor at 130 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the tip with 100% ethanol by pipetting the ethanol up and down 3 times.

c. Without changing tips, add 130 µL of 100% ethanol to each well.

IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 2b and 2c for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

d. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

Note: The color of the mixture is homogeneous after mixing.

e. Incubate the samples for 5 minutes at room temperature.

3. Remove the supernatant from the beads:
   a. Place the Processing Plate on a magnetic stand for 5–6 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
   b. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.

IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 µL of supernatant behind.

4. Leave the Processing Plate on the magnetic stand.
   a. Add 150 µL of Wash Solution Concentrate with ethanol to each sample.
   b. Incubate the samples at room temperature for 30 seconds.
5. Remove the supernatant from the beads:
   a. Aspirate, then discard the supernatant from the plate.
   b. Use a P10 or P20 pipettor to remove remaining ethanol.

IMPORTANT! Do not disturb the separated magnetic beads. Remove all the Wash Solution Concentrate from each well.

   c. To remove all traces of ethanol, air-dry the beads at room temperature by leaving the Processing Plate on the magnetic stand for 1–2 minutes.

IMPORTANT! Do not overdry the beads (overdried beads appear cracked). Overdrying significantly decreases elution efficiency.

6. Elute the cDNA from the beads:
   a. Remove the Processing Plate from the magnetic stand.
   b. Add 15 µL of pre-warmed (37°C) Nuclease-free Water to each sample, then mix the Nuclease-free Water and beads by pipetting up and down 10 times.
   c. Incubate at room temperature for 1 minute.

d. Place the Processing Plate on a magnetic stand for 1 minute to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

e. For each sample, collect the eluant.

Assess the yield and size distribution of the amplified DNA

Use a NanoDrop™ Spectrophotometer or the dsDNA HS Assay Kit with the Qubit™ Fluorometer. Also use the Agilent™ 2100 Bioanalyzer™ instrument with the Agilent™ DNA 1000 Kit or Agilent™ High Sensitivity DNA Kit.

1. Measure the concentration of the purified DNA with a NanoDrop™ Spectrophotometer or the dsDNA HS Assay Kit with the Qubit™ Fluorometer.

2. Analyze 1 µL of the library using the appropriate chip on the Agilent™ 2100 Bioanalyzer™ instrument. If the library concentration is:
   - 1–50 ng/µL: Use the Agilent™ DNA 1000 Kit.
   - 5–1000 pg/µL: Use the Agilent™ High Sensitivity DNA Kit.

3. Using the 2100 expert software, perform a smear analysis to:
   a. Quantify the percentage of DNA that is ≤160 bp: Use size range 50–160 bp.

   b. Determine the molar concentration (nM) of the cDNA libraries: Use size range 50–1000 bp.

   Note: For instructions on how to perform the smear analysis, see "Perform a smear analysis" on page 62, and see the *Agilent™ 2100 Bioanalyzer™ Expert User's Guide* (Pub. No. G2946-90004).

4. Use molar concentration of the cDNA libraries from 3b of "Pool barcoded whole transcriptome libraries" on page 30 and "Determine the library dilution required for template preparation" on page 30.

If the percent of DNA in 50–160 bp the range is	Action
<50%	Proceed to the next section, "Pool barcoded whole transcriptome libraries," or "Determine the library dilution required for template preparation."
50–60%	Perform another round of purification on the amplified DNA using components from the Magnetic Bead Cleanup Module: 1. Bring the sample volume to 53 µL with Nuclease-free Water. 2. Follow the steps in "Purify the amplified cDNA" on page 26. *or* Proceed to the next section, "Pool barcoded whole transcriptome libraries," or "Determine the library dilution required for template preparation," but expect to see a slightly higher percentage of filtered reads in your sequencing data when compared to libraries with less than 50% of DNA in the range.
>60%	We recommend that you perform another round of purification on the amplified DNA using components from the Magnetic Bead Cleanup Module: 1. Bring the sample volume to 53 µL with Nuclease-free Water. 2. Follow the steps in "Purify the amplified cDNA" on page 26.

Pool barcoded whole transcriptome libraries

Note: If you are not pooling libraries, skip this section and proceed to "Determine the library dilution required for template preparation".

1. Determine the molar concentration (nM) of each barcoded cDNA library with the Agilent™ DNA 1000 Kit or the Agilent™ High Sensitivity DNA Kit.

   Note: 50–1000 bp size range is typically used to determine library concentration. If needed, adjust the range to include all the library peaks.

2. Dilute each barcoded cDNA library to the same molar concentration.
   For example, if you have 3 different barcoded libraries that are 45, 55, 65 nM, select a concentration that is equal or lower than the lowest concentration of the 3 libraries, such as 30 nM. Dilute all or part of each library to 30 nM.

3. Mix an equal volume of each diluted library to prepare a pool of the barcoded libraries.
   The final molar concentration of the pooled library is the same for each diluted library. For example, if you dilute each library to 30 nM, the concentration of the pooled library is 30 nM.
   Use the final molar concentration to determine the Template Dilution Factor. Alternatively, you can determine the molar concentration of the pooled libraries with the Agilent™ DNA 1000 Kit or the Agilent™ High Sensitivity DNA Kit (see "Assess the yield and size distribution of fragmented RNA" on page 17).

Determine the library dilution required for template preparation

With less than 50% of the amplified DNA in the 50–160 bp range, you can proceed to the template preparation procedure (see "Proceed to template preparation" on page 31) to prepare templated beads for sequencing on the Ion PGM™, Ion Proton™, Ion S5™, or Ion S5™ XL Systems.

Determine the dilution factor that gives a concentration of ~100 pM. This concentration is suitable for downstream template preparation. Use the following formula:

Dilution factor = (Library or pooled library concentration in pM)/100 pM

Example:

The library or pooled library concentration is 15,000 pM.

Dilution factor = 15,000 pM/100 pM = 150

Thus, 1 µL of library or pooled library mixed with 149 µL of Low TE (1:150 dilution) yields approximately 100 pM. Use this as the starting library dilution for template preparation.

Proceed to template preparation

The library is ready for the template preparation procedure. In this procedure, each library template is clonally amplified on Ion Sphere™ Particles using the Ion OneTouch™ 2 or Ion Chef™ Systems for sequencing on the Ion PGM™, Ion Proton™, Ion S5™, or Ion S5™ XL Systems. For instructions, see the specific user guide for an appropriate Ion template preparation kit.

You can find template preparation documentation at **thermofisher.com/ngs-template-preparation-support**.

Size distributions and yields

Typical size distributions

The highest quality libraries have less than 50% amplified DNA between 25–160 bp:

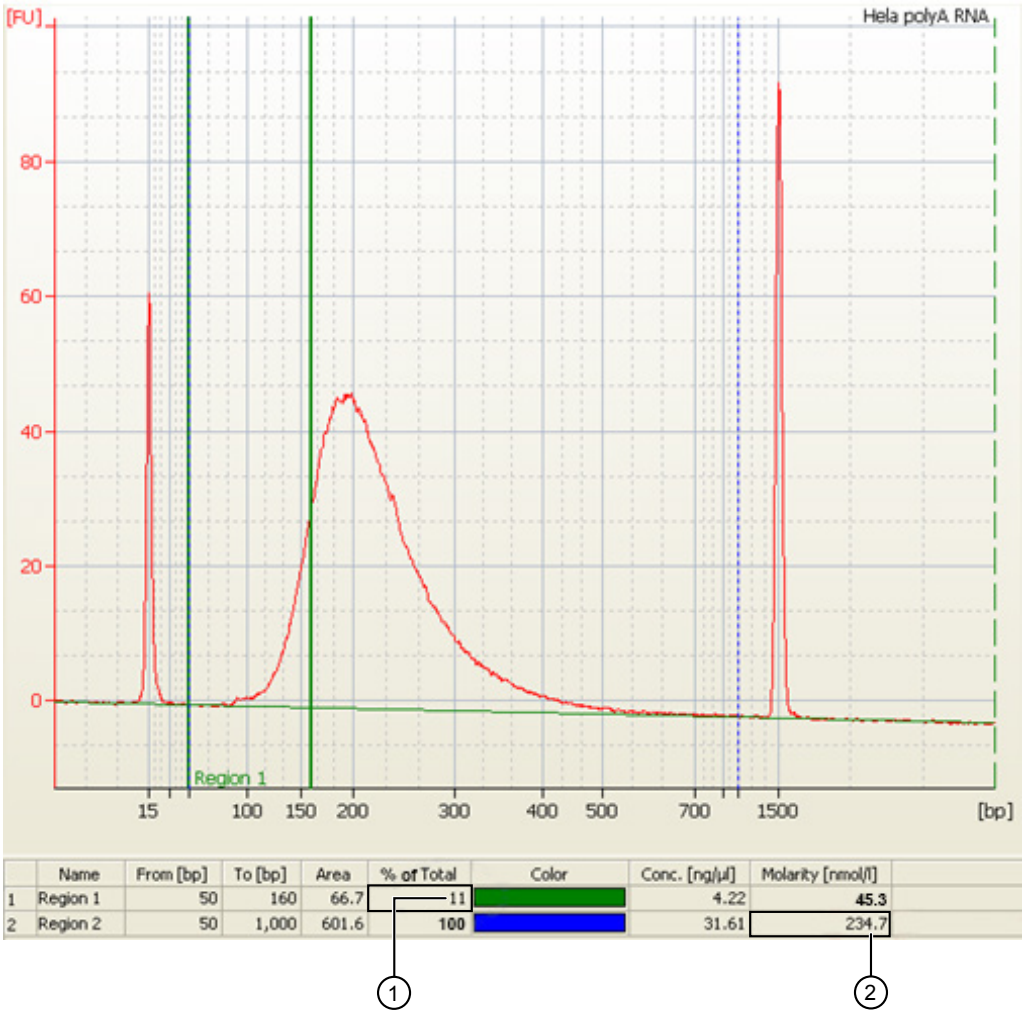


Figure 3 Molar concentration and size distribution of amplified library prepared from HeLa poly(A) RNA

① 11% amplified DNA falls within the designated range (the area under the curve).

② The Library concentration is 234.7 nM.

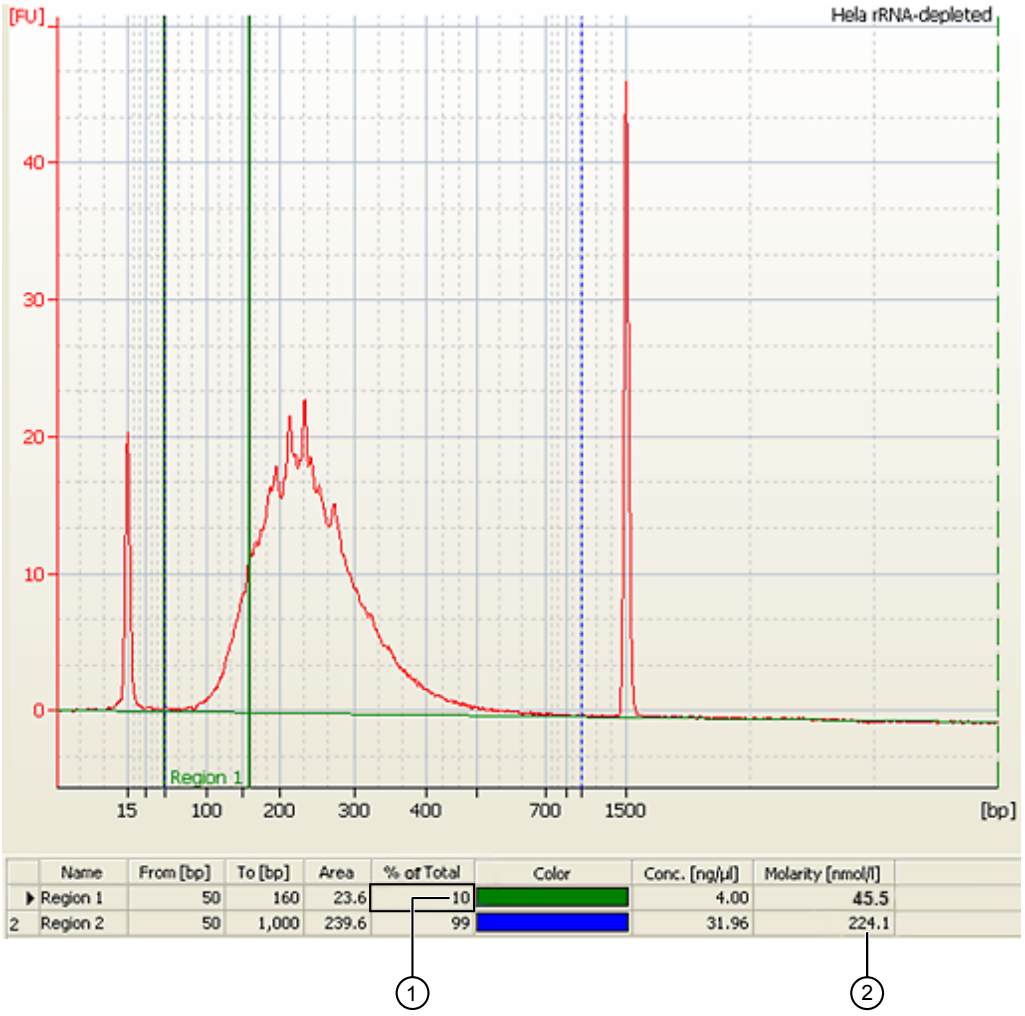


Figure 4 Molar concentration and size distribution of amplified library prepared from HeLa rRNA-depleted total RNA

① 10% ampified DNA falls within the designated range (the area under the curve)

② The Library concentration is 224.1 nM.

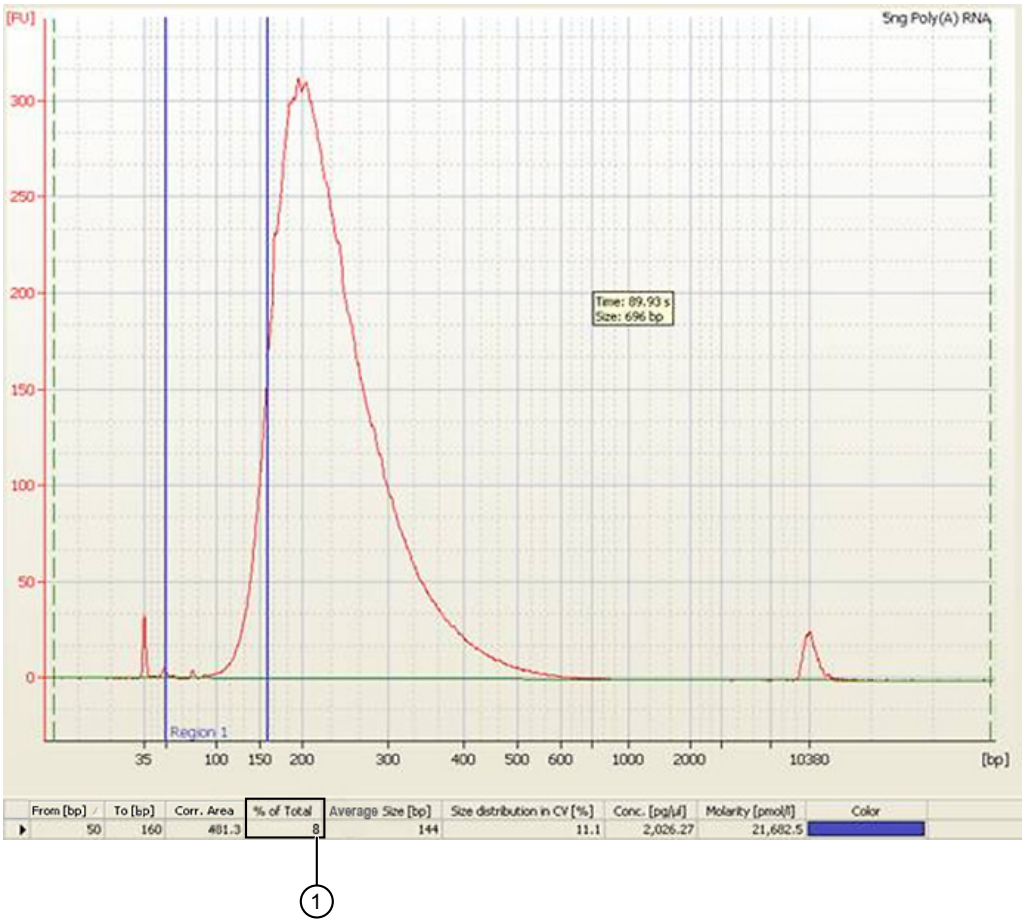


Figure 5 Size distribution of amplified library prepared using 5 ng of poly(A) RNA and isolated using the Dynabeads™ mRNA DIRECT™ Micro Kit Micro Kit. Refer to "Poly(A) selection from 100 ng– µg input Total RNA samples" protocol in the *Micro Kit User Guide*

① 8% amplified DNA falls within the designated range (the area under the curve).

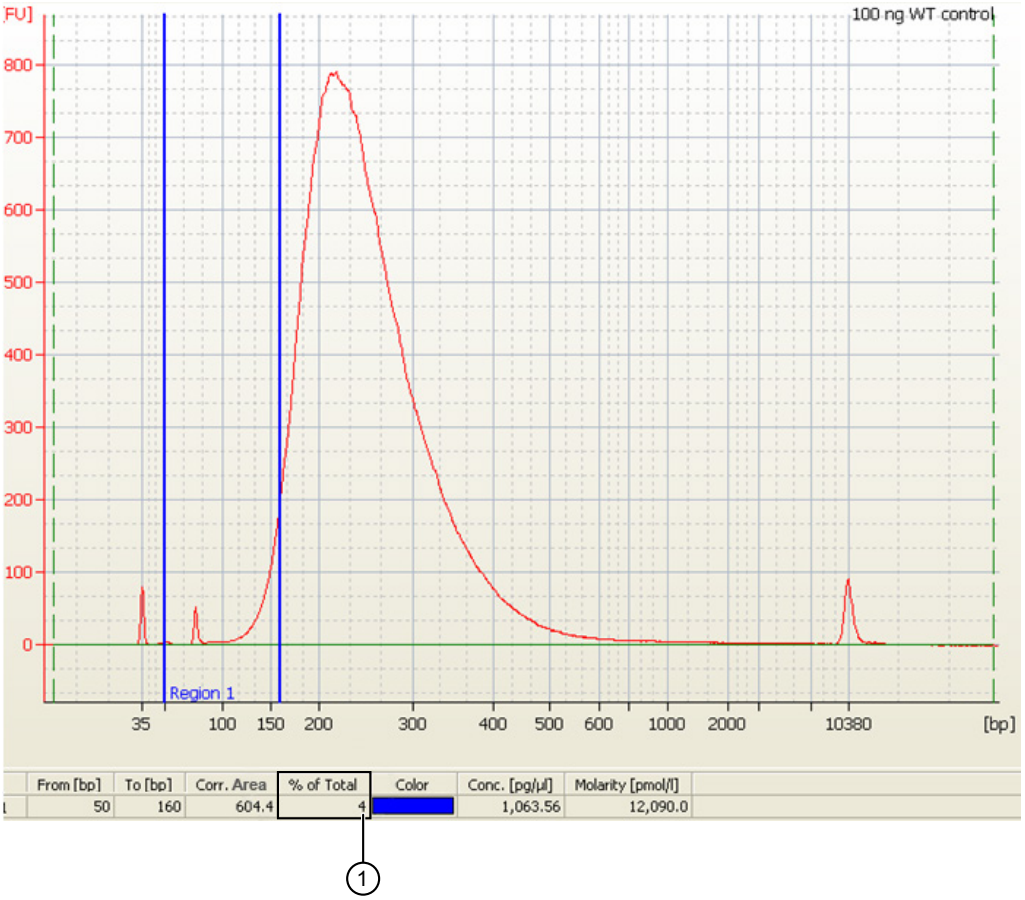


Figure 6 Size distribution of amplified library prepared using poly(A) RNA from 100 ng WT Control RNA

(1) 4% amplified DNA falls within the designated range (the area under the curve).

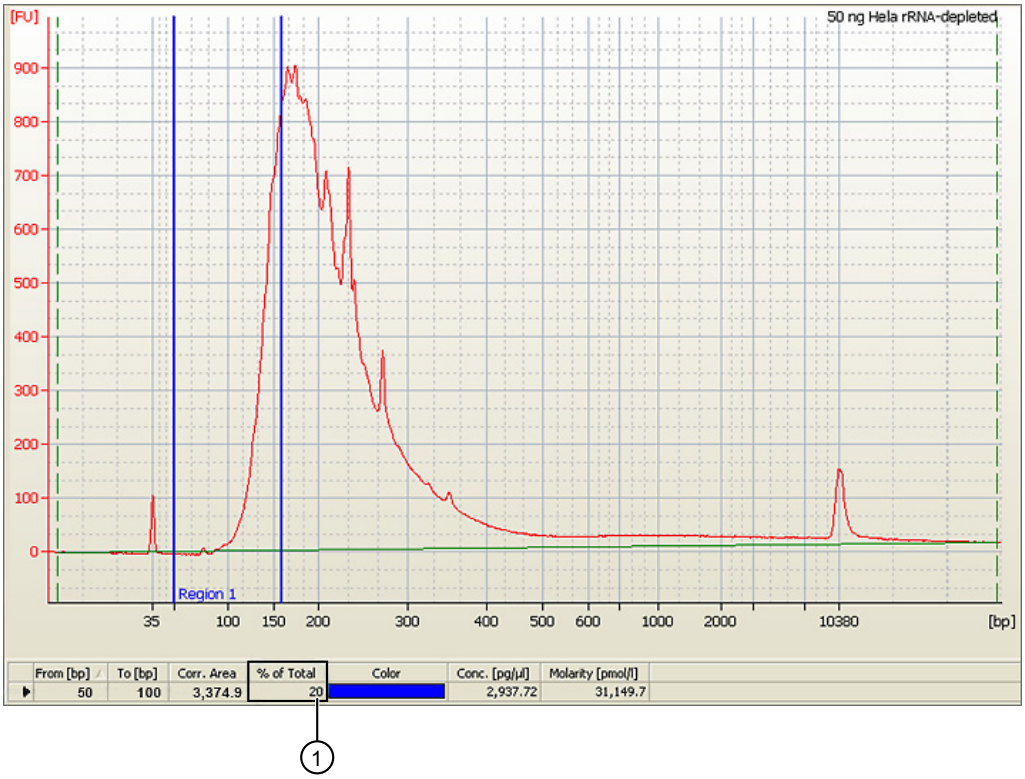


Figure 7 Size distribution of amplified library prepared from 50 ng of rRNA-depleted total RNA using the Agilent™ High Sensitivity DNA Kit

① 20% amplified DNA falls within the designated range (the area under the curve).

Expected yields

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

Note: Typical amplified DNA yields for HeLa poly(A) RNA and HeLa rRNA-depleted total RNA are greater than 200 ng in a 15-µL final volume.

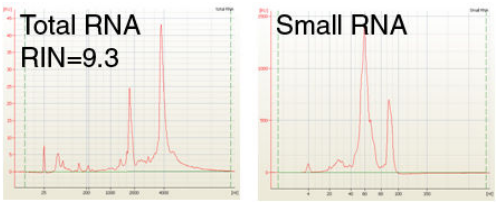
Workflow	Input amount	Typical recovery amount
"Fragment the whole transcriptome RNA" on page 12	500 ng of poly(A) RNA, total RNA, or rRNA-depleted total RNA	300–400 ng of RNA
"Construct the whole transcriptome library" on page 19	<1–100 ng of fragmented RNA	>5 ng of cDNA

3 Prepare small RNA libraries

Workflow

Prepare the starting material

"Assess the amount and quality of small RNA in your total RNA samples" on page 38



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"Enrich the samples for small RNA" on page 39

▼

"Assess the quality and quantity of the small RNA-enriched sample" on page 43

"Determine the input amount" on page 43

▼

Construct the small RNA library

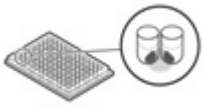
"Hybridize and ligate the RNA" on page 44

▼

"Perform reverse transcription" on page 45

▼

"Purify and size-select the cDNA" on page 46



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"Amplify the cDNA" on page 49

▼

"Purify and size-select the amplified DNA" on page 51

▼

"Assess the yield and size distribution of the amplified DNA" on page 54

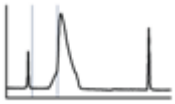
▼

"Pool barcoded small RNA libraries" on page 55

▼

"Determine the library dilution required for template preparation" on page 55

▼



Prepare templated Ion Sphere™ Particles

Refer to the specific user guide for an Ion template preparation kit

Prepare the starting material

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 small RNA.

We recommend the following products:

- RNA source: Best results are obtained using high quality total RNA with an RNA integrity number (RIN) >6 as your starting material.
- RNA isolation kits: Use the *mir*Vana™ miRNA Isolation Kit, with phenol, the *mir*Vana™ miRNA Isolation Kit, without phenol, or the *mir*Vana™ PARIS™ RNA and Native Protein Purification Kit to isolate total RNA that includes the small RNA fraction or small RNA from tissue or cells. Use the PureLink™ miRNA Isolation Kit to isolate small RNA from tissues or cells.

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

Before you prepare the library, determine the quality of the total RNA sample. Use the NanoDrop™ Spectrophotometer and the Agilent™ 2100 Bioanalyzer™ instrument with the Agilent™ RNA 6000 Nano Kit and the Agilent™ Small RNA Kit to measure the fraction of small RNA in your sample. Based on their source and the RNA isolation method, RNA samples vary widely in small RNA content. In some tissues, the proportion of small RNA is high enough to allow efficient library preparation from total RNA. For example, the control RNA provided in this kit is total RNA isolated from placenta. Many tissues and most cell lines, however, contain a much smaller fraction of small RNA.

1. Quantify the amount of RNA in the sample using the NanoDrop™ Spectrophotometer.

   Note: If you used the *mir*Vana™ miRNA Isolation Kit, the *mir*Vana™ PARIS™ RNA and Native Protein Purification Kit, or the PureLink™ miRNA Isolation Kit to isolate small RNA from samples, you can skip to "Assess the quality and quantity of the small RNA-enriched sample" on page 43.

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

   Note: For instructions on using the software, see the *Agilent™ 2100 Bioanalyzer™ Expert User's Guide* (Pub. No. G2946-90004).

   a. Dilute the RNA to ~50–100 ng/µL.

   b. Run 1 µL of diluted RNA on the Agilent™ 2100 Bioanalyzer™ instrument with the Agilent™ RNA 6000 Nano chip 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, then save the mass of total RNA for step 3c to calculate the miRNA content.

   d. Using the 2100 expert software, review the RNA Integrity Number (RIN). The highest-quality library mapping statistics are obtained from input RNA with higher RIN values.

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 total RNA (miRNA; 10–40 nt) from the Small RNA chip.

   c. Calculate the miRNA content in your RNA sample using the formula:
   % miRNA = (mass of miRNA ÷ mass of total RNA) × 100

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

Percentage of miRNA (10–40 nt) in the RNA sample	Recommendations for small RNA enrichment
≥0.5% miRNA	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. You can expect 5–15% more of rRNA and tRNA mapping in your sequencing data from total RNA, compared to sequencing data of libraries starting from enriched small RNA. Proceed to the next section or skip to "Determine the input amount" on page 43.
<0.5% miRNA	Small RNA enrichment is strongly recommended. We recommend using the Magnetic Bead Cleanup Module for small RNA enrichment. Proceed to the next section.

Enrich the samples for small RNA

This protocol uses magnetic beads to enrich for small RNA. To size-select the desired small RNA products, use the Magnetic Bead Cleanup Module twice with the same sample. During the first bead binding, magnetic beads capture larger RNA species such as mRNA and rRNA. During the second binding and with increased ethanol concentration, desired small RNA products (miRNA and other small RNA) in the supernatant re-bind to the magnetic beads. After washing the beads, the desired small RNA products are eluted with pre-heated (80°C) Nuclease-free Water.

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate
- Nuclease-free Water

Note: The 1.5-mL Non-Stick RNAse-Free Microfuge Tubes (Cat. No. AM12450), may be used in place of the Processing Plate.

Other materials and equipment

- 100% ethanol or 200 proof (absolute) ethanol, ACS-grade or higher quality
- Magnetic rack or stand
- 80°C heat block or water bath
- *(Optional)* MicroAmp™ Clear Adhesive Film

Before you begin

- Add 44 mL of 100% ethanol to the bottle of Wash Solution Concentrate, then mix well. To indicate that you added ethanol, mark the label on the bottle. Store the solution at room temperature (15°C–30°C).
- If you see a white precipitate in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Nuclease-free Water at 80°C for ≥5 minutes.

Note: To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp™ Clear Adhesive Film (Cat. No. 4306311). You can also skip a row between sample rows.

IMPORTANT! Accurate pipetting of bead cleanup reagents is critical to successful size-selection. For optimal size-selection, perform the following bead cleanup steps exactly.

Enrich the samples for small RNA

1. Prepare beads for each sample:
   a. Gently vortex the Nucleic Acid Binding Beads tube to resuspend the magnetic beads completely.
   b. Add 7-µL beads to the wells of the Processing Plate.
   c. Add 120 µL Binding Solution Concentrate to each well, then mix the Concentrate and beads by pipetting up and down 10 times.
2. Bind larger RNA to the beads:
   a. Resuspend 0.5–20 µg of total RNA in 75-µL Nuclease-free Water.
   b. Transfer 75 µL of each RNA sample to a well with beads of the Processing Plate.
   c. Set a P200 pipettor at 105 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the tip with 100% ethanol by pipetting the ethanol up and down 3 times.
   d. Without changing tips, add 105 µL of 100% ethanol to each well.

      IMPORTANT! When dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 2c and 2d for the remaining wells only if the tip touches the wall Accurate pipetting of 100% ethanol is critical for size-selection. Follow the instructions exactly for best results.

   e. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

      Note: If the color of the mixture is not the same throughout, mix again.

f. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

3. Remove the supernatant from the beads:
   a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

   b. Leave the Processing Plate on the magnetic stand, then transfer the supernatant to a new well on the plate or to a well on a new plate.

   IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 µL of supernatant behind.

4. Bind desired small RNA products to the beads:
   a. Remove the Processing Plate from the magnetic stand.

   b. Add 30 µL of Nuclease-free Water to the supernatant in the new sample well.

   c. Set a P1000 pipettor at 570 µL. Attach a new 1000-µL tip to the pipettor, then pre-wet the new 1000-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times.

   d. Without changing tips, add 570 µL of 100% ethanol to each well.

   IMPORTANT! When dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 4c and 4d for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for size-selection. Follow the instructions exactly for best results.

   e. Gently vortex the Nucleic Acid Binding Beads tube to resuspend the magnetic beads completely.

   f. Add 7 µL of beads to the wells of the Processing Plate.

   g. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

   IMPORTANT! Because of the large volume in each well, use a P200 pipettor for mixing to avoid cross-well contamination.

   Note: If the color of the mixture is not the same throughout, mix again.

   h. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

5. Remove the supernatant from the beads:
   a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

b. Leave the Processing Plate on the magnetic stand, then carefully aspirate and discard the supernatant from the plate.

IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 µL of supernatant in the tube.

6. Wash the beads with the Wash Solution Concentrate with ethanol:
   a. Leave the Processing Plate on the magnetic stand, then add 150 µL of Wash Solution Concentrate with ethanol to each sample.
   b. Incubate the samples for 30 seconds.
   c. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.

      IMPORTANT! Do not disturb the separated magnetic beads. Remove all the Wash Solution Concentrate from each well.

   d. Use a P10 or P20 pipettor to remove remaining ethanol.
   e. Air-dry the beads at room temperature for 1–2 minutes to remove all traces of ethanol.

IMPORTANT! Do not overdry the beads (overdried beads appear cracked). Overdrying significantly decreases elution efficiency.

7. Elute the small RNA from the beads:
   a. Remove the Processing Plate from the magnetic stand.
   b. Add 30 µL of pre-warmed (80°C) Nuclease-free Water to each sample.
   c. Mix thoroughly by pipetting up and down 10 times.
   d. Incubate the samples at room temperature for 1 minute.
   e. Place the Processing Plate on the magnetic stand for 1 minute to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
   f. For each sample, collect 30 µL of the eluant.

STOPPING POINT Store the small RNA at –86°C to –68°C. After storage, proceed to the next section "Assess the quality and quantity of the small RNA-enriched sample".

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 Agilent™ Small RNA Kit.

1. Run 1 µL of purified and *enriched small RNA* sample on the Agilent™ 2100 Bioanalyzer™ instrument with the Small RNA Kit chip. Follow the manufacturer's instructions for performing the assay.

2. Compare the bioanalyzer traces to those of the sample before enrichment (see step 2 in "Assess the amount and quality of small RNA in your total RNA samples" on page 38), and determine whether the RNA is degraded. For enriched small RNA samples, peaks should be from 10–200 nt.

Determine the input amount

Using the results from the Agilent™ 2100 Bioanalyzer™ instrument and the Small RNA Kit, determine the amount of total RNA to use according to the type of RNA you ran and the amount of miRNA in 3 µL. If necessary, concentrate the small RNA with a SpeedVac™ centrifugal concentrator.

Input sample type	Amount of miRNA(10-40 nt) in 3 µL	Total RNA input[1]
Total RNA	5–100 ng	≤1 µg
Enriched small RNA	1–100 ng	≤1 µg

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

Note:
When starting from total RNA with low RIN, the miRNA quantity may be overestimated on the Agilent™ Small RNA chip, so more input into ligation is recommended. Ideally, use >10 ng of miRNA.

Construct the small RNA library

Hybridize and ligate the RNA

Required materials from the Ion Total RNA-Seq Kit v2

- Ion Adaptor Mix v2
- Hybridization Solution
- 2X Ligation Buffer
- Ligation Enzyme Mix

Hybridize and ligate the RNA

1. On ice, prepare the hybridization master mix:

Contents	Volume per reaction[1]
Hybridization Solution	3 µL
Ion Adaptor Mix v2	2 µL
Total Volume	**5 µL**

[1] Include 5–10% excess volume to compensate for pipetting error when preparing the master mix.

2. Add 5 µL of hybridization master mix to 3 µL of small RNA sample (1–100 ng of miRNA in ≤1 µg of enriched small RNA).

3. Flick the tube or pipet up and down 5 times to mix, then centrifuge briefly to collect the liquid in the bottom of the tube.

4. Run the hybridization reaction in a thermal cycler:

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

5. On ice, prepare the ligation master mix. Combine in a 0.5-mL or 1.5-mL Non-Stick RNase-Free Microfuge Tube:

Contents	Volume per reaction[1]
2X Ligation Buffer	10 µL
Ligation Enzyme Mix	2 µL
Total Volume	**12 µL**

[1] Include 5–10% excess volume to compensate for pipetting error when preparing the master mix.

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

6. Gently vortex to mix, then centrifuge briefly to collect the liquid in the bottom of the tube.

7. Add 12 µL of ligation master mix to each 8-µL hybridization reaction, for a total of 20 µL per reaction.

8. Flick the tube or pipet up and down 5 times to mix, then centrifuge briefly.

9. Incubate the 20-µL ligation reactions in a thermal cycler at 16°C for 2–16 hours.

IMPORTANT! If the starting enriched small RNA is <5 ng, we strongly recommend overnight incubation (16 hours) at 16°C. For a set of experiments, we recommend using the same ligation time for all samples to minimize variation.

IMPORTANT! Set the temperature of the thermal cycler lid to match the block temperature; turn OFF the heated lid; or leave the thermal cycler open during the incubation.

Perform reverse transcription

Required materials from the Ion Total RNA-Seq Kit v2

- Nuclease-free Water
- 10X RT Buffer
- 2.5 mM dNTP Mix
- Ion RT Primer v2
- 10X SuperScript™ III Enzyme Mix

Reverse transcribe the RNA

1. On ice, prepare the RT master mix with the ligated RNA sample:

Contents	Volume per reaction[1]
Nuclease-free Water	2 µL
10X RT Buffer	4 µL
2.5 mM dNTP Mix	2 µL
Ion RT Primer v2	8 µL
Total Volume	**16 µL**

[1] Include 5–10% excess volume to compensate for pipetting error when preparing the master mix.

2. Incubate the RT master mix with the ligated RNA sample:
   a. Add 16 µL of RT master mix to each 20-µL ligation reaction.
   b. Pipet up and down 5 times to mix, then centrifuge briefly to collect the liquid in the bottom of the tube.
   c. Incubate in a thermal cycler with a heated lid at 70°C for 10 minutes, then snap-cool on ice.

3. Perform the reverse transcription reaction:
   a. Add 4 µL of 10X SuperScript™ III Enzyme Mix to each ligated RNA sample.
   b. Gently vortex to mix thoroughly, then centrifuge briefly.

c. Incubate in a thermal cycler with a heated lid at 42°C for 30 minutes.

STOPPING POINT Store the cDNA at –30°C to –10°C for 2 weeks, store at –86°C to –68°C for long-term storage, or use immediately.

Purify and size-select the cDNA

Use the Magnetic Bead Cleanup Module twice with the same sample to size-select the desired cDNA products. During the first bead binding, magnetic beads capture larger cDNA species such as tRNA and rRNA. During the second binding and with increased ethanol concentration, desired cDNA products (miRNA and other small RNA) in the supernatant re-bind to the magnetic beads. After washing the beads, the desired cDNA products are eluted with pre-warmed (37°C) Nuclease-free Water.

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate
- Nuclease-free Water

Other materials and equipment

- 100% ethanol or 200 proof (absolute) ethanol, ACS-grade or higher quality
- Magnetic stand for 96-well plates (Cat. No. AM10027 or AM10050)
- 37°C heat block or water bath
- *(Optional)* MicroAmp™ Clear Adhesive Film (Cat. No. 4306311)

Before you begin

- If you have not done so already, add 44 mL of 100% ethanol to the Wash Solution Concentrate and mix well. Mark the label on the bottle to indicate that you added ethanol. Store the solution at room temperature (15°C–30°C).
- If you see a white precipitate in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Nuclease-free Water at 37°C for ≥5 minutes.

Note: To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp™ Clear Adhesive Film (Cat. No. 4306311). You can also skip a row between sample rows.

IMPORTANT! Accurate pipetting of bead cleanup reagents is critical for best results.

Purify and size-select the cDNA

1. Prepare beads for each sample:
   a. Gently vortex the Nucleic Acid Binding Beads tube to resuspend the magnetic beads completely.
   b. Add 7-µL beads to the wells of the Processing Plate.
   c. Add 140 µL Binding Solution Concentrate to each well, then mix the Concentrate and beads by pipetting up and down 10 times.
2. Bind larger cDNA products to the beads:
   a. Transfer each 40-µL RT reaction to a well with beads of the Processing Plate.
   b. Set a P200 pipettor at 120 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the tip with 100% ethanol by pipetting the ethanol up and down 3 times.
   c. Without changing tips, add 120 µL of 100% ethanol to each well.

      IMPORTANT! When dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 2b and 2c for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

   d. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

      Note: If the color of the mixture is not the same throughout, mix again.

   e. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.
3. Remove the supernatant from the beads:
   a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
   b. Leave the Processing Plate on the magnetic stand, then transfer the supernatant to a new well on the plate or to a well on a new plate.

      IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 µL of supernatant in the tube.

4. Bind desired cDNA products to the beads:
   a. Remove the Processing Plate from the magnetic stand.
   b. Add 72 µL of Nuclease-free Water to the supernatant in the new sample well.
   c. Set a P100 or P200 pipettor at 78 µL. Attach a new 100-µL or 200-µL tip to the pipettor, then pre-wet the new 100- or 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times.

d. Without changing tips, add 78 µL of 100% ethanol to each well.

IMPORTANT! When dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 4c and 4d for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

e. Gently vortex the Nucleic Acid Binding Beads tube to resuspend the magnetic beads completely.

f. Add 7 µL of beads to the wells of the Processing Plate.

g. Take the samples off of the magnetic stand. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

Note: If the color of the mixture is not the same throughout, mix again.

h. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

5. Remove the supernatant from the beads.
   a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

   b. Leave the Processing Plate on the magnetic stand, then carefully aspirate and discard the supernatant from the plate.

   IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 µL of supernatant in the tube.

6. Wash the beads with the Wash Solution Concentrate with ethanol:
   a. Leave the Processing Plate on the magnetic stand, then add 150 µL of Wash Solution Concentrate with ethanol to each sample.

   b. Incubate the samples for 30 seconds.

   c. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.

   IMPORTANT! Do not disturb the separated magnetic beads. Remove all the Wash Solution Concentrate from each well.

   d. Use a P10 or P20 pipettor to remove remaining ethanol.

   e. Air-dry the beads at room temperature for 1–2 minutes to remove all traces of ethanol.

   IMPORTANT! Do not overdry the beads (overdried beads appear cracked). Overdrying significantly decreases elution efficiency.

7. Elute the cDNA from the beads:
   a. Remove the Processing Plate from the magnetic stand.
   b. Add 12 µL of pre-warmed (37°C) Nuclease-free Water to each sample.
   c. Mix thoroughly by pipetting up and down 10 times.
   d. Incubate the samples at room temperature for 1 minute.
   e. For each sample, collect 12 µL of the eluant.

Amplify the cDNA

To prepare non-barcoded libraries, use the following components from the Ion Total RNA-Seq Kit v2:

- Ion 5′ PCR Primer v2
- Ion 3′ PCR Primer v2
- Platinum™ PCR SuperMix High Fidelity

To prepare barcoded libraries, plan the barcodes that you want to use, then select the PCR primers from Ion Xpress™ RNA-Seq Barcode 01–16 Kit:

- Ion Xpress™ RNA-Seq Barcode BC 01–BC 16
- Ion Xpress™ RNA 3′ Barcode Primer

1. For each cDNA sample, set up the PCR reaction, according to the preparation of a non-barcoded or barcoded library:

   IMPORTANT! Use the appropriate primers.

   - For non-barcoded libraries:
     a. Prepare the Non-barcoded Library PCR Mix according to the following table.

     Non-barcoded Library PCR Mix

Component	Volume per reaction[1]
Platinum™ PCR SuperMix High Fidelity[2]	45 µL
Ion 5' PCR Primer v2	1 µL
Ion 3' PCR Primer v2	1 µL
Total Volume	**47 µL**

     [1] Include 5–10% excess volume to compensate for pipetting error when preparing master mix.
     [2] Platinum™ PCR SuperMix High Fidelity contains a proofreading enzyme for high-fidelity amplification.

     b. Transfer 6 µL of cDNA sample to a new PCR tube.
     c. Transfer 47 µL of the Non-barcoded Library PCR Mix to each 6 µL of cDNA sample.
     d. Proceed to step 2.

- For barcoded libraries:
  a. Prepare the Barcoded Library PCR Mix according to the following table.

     Barcoded Library PCR Mix

Component	Volume per reaction[1]
Platinum™ PCR SuperMix High Fidelity[2]	45 µL
Ion Xpress™ RNA 3′ Barcode Primer	1 µL
Total Volume	**46 µL**

[1] Include 5–10% excess volume to compensate for pipetting error when preparing master mix.

[2] Platinum™ PCR SuperMix High Fidelity contains a proofreading enzyme for high-fidelity amplification.

  b. Transfer 6 µL of cDNA sample to a new PCR tube.
  c. Transfer 46 µL of the Barcoded Library PCR Mix to each 6 µL of cDNA sample.
  d. Add 1 µL of the selected Ion Xpress™ RNA-Seq Barcode BC primer (select from BC01–BC16) to each PCR tube.

2. Flick the tube or slowly pipet the solution up and down 5 times to mix well, then centrifuge briefly to collect the liquid in the bottom of the tube.

3. Run the cDNA samples in a thermal cycler:

Stage	Temp	Time
Hold	94°C	2 minutes
Cycle (2 cycles)	94°C	30 seconds
	50°C	30 seconds
	68°C	30 seconds
Cycle (14 cycles)	94°C	30 seconds
	62°C	30 seconds
	68°C	30 seconds
Hold	68°C	5 minutes

Purify and size-select the amplified DNA

Use the Magnetic Bead Cleanup Module twice with the same sample to size-select the desired cDNA products. During the first round of bead binding, magnetic beads capture larger cDNA species such as tRNA and rRNA. During the second round of bead binding and with increased ethanol concentration, desired cDNA products (miRNA and other small RNA) in the supernatant re-bind to the magnetic beads. After washing the beads, the desired cDNA products are eluted with pre-warmed (37°C) Nuclease-free Water.

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate
- Nuclease-free Water

Other materials and equipment

- 100% ethanol or 200 proof (absolute) ethanol, ACS-grade or higher quality
- *(Optional)* Orbital shaker for 96-well plates
- Magnetic stand for 96-well plates (Cat. No. AM10027 or AM10050)
- 37°C heat block or water bath
- *(Optional)* MicroAmp™ Clear Adhesive Film (Cat. No. 4306311)

Before you begin

- If you have not done so already, add 44 mL of 100% ethanol to the Wash Solution Concentrate and mix well. Mark the label on the bottle to indicate that you added ethanol. Store the solution at room temperature.
- If you see a white precipitate in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Nuclease-free Water at 37°C for ≥5 minutes.

Note: To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp™ Clear Adhesive Film (Cat. No. 4306311). You can also skip a row between sample rows.

IMPORTANT! Accurate pipetting of bead cleanup reagents is critical for best results.

Purify the amplified cDNA

1. Prepare beads for each sample:
    a. Gently vortex the Nucleic Acid Binding Beads tube to resuspend the magnetic beads completely.
    b. Add 7-µL beads to wells on the Processing Plate.
    c. Add 140 µL Binding Solution Concentrate to each well, then mix the Concentrate and beads by pipetting up and down 10 times.

2. Bind larger amplified DNA to the beads:
   a. Transfer each 53-µL PCR reaction to a well with beads of the Processing Plate.
   b. Set a P200 pipettor at 110 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the tip with 100% ethanol by pipetting the ethanol up and down 3 times.
   c. Without changing tips, add 110 µL of 100% ethanol to each well.

      IMPORTANT! When dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 2b and 2c for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

   d. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

      Note: If the color of the mixture is not the same throughout, mix again.

   e. Incubate the samples for 5 minutes at room temperature.
3. Remove the supernatant from the beads:
   a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
   b. Leave the Processing Plate on the magnetic stand, then transfer the supernatant to a new well on the plate or to a well on a new plate.

      IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 µL of supernatant behind.
4. Bind desired cDNA products to the beads:
   a. Remove the Processing Plate from the magnetic stand.
   b. Add 35 µL of Nuclease-free Water to the supernatant in the new sample well.
   c. Set a P100 or P200 pipettor at 35 µL. Attach a new 100- or 200-µL tip to the pipettor, then pre-wet the new 100- or 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times.
   d. Without changing tips, add 35 µL of 100% ethanol to each well.

      IMPORTANT! When dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the well wall. Change the tip and repeat steps 4c–4d for the remaining wells only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

   e. Gently vortex the Nucleic Acid Binding Beads tube to resuspend the magnetic beads completely.

f. Add 7-µL beads to the wells of the Processing Plate.

g. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

Note: If the color of the mixture is not the same throughout, mix again.

h. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

5. Remove the supernatant from the beads:
   a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

   b. Leave the Processing Plate on the magnetic stand, then carefully aspirate and discard the supernatant from the plate.

   IMPORTANT! Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3 µL of supernatant in the tube.

6. Wash the beads with Wash Solution Concentrate with ethanol:
   a. Leave the Processing Plate on the magnetic stand, then add 150 µL of Wash Solution Concentrate with ethanol to each sample.

   b. Incubate the plate at room temperature for 30 seconds.

   c. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.

   d. Use a P10 or P20 pipettor to remove remaining ethanol.

   IMPORTANT! Do not disturb the separated magnetic beads. Remove all of the Wash Solution Concentrate from each well.

   e. Air-dry the beads at room temperature for 1–2 minutes to remove all traces of ethanol.

   IMPORTANT! Do not overdry the beads (overdried beads appear cracked). Overdrying significantly decreases elution efficiency.

7. Elute the cDNA from the beads:
   a. Remove the Processing Plate from the magnetic stand.

   b. Add 15 µL of pre-warmed (37°C) Nuclease-free Water to each sample.

   c. Mix thoroughly by pipetting up and down 10 times.

   d. Incubate the Processing Plate at room temperature for 1 minute.

   e. Place the Processing Plate on the magnetic stand for 1 minute to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

f. For each sample, collect the 15 µL of eluant.

Assess the yield and size distribution of the amplified DNA

Use the Agilent™ 2100 Bioanalyzer™ instrument with the Agilent™ DNA 1000 Kit.

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

2. Using the 2100 expert software, perform a smear analysis to determine size distribution of the amplified DNA:

   a. Measure the area for the DNA that is:
      - 50-300 bp (the size range for all the ligation products)
      - 86-106 bp for non-barcoded libraries or 94-114 bp for barcoded libraries (the size range for the desired miRNA ligation products)

   b. Calculate the ratio of miRNA ligation products in total ligation products using the formula for:
      - Non-barcoded libraries: *[Area (86–106 bp)] ÷ [Area (50–300 bp)]*
      - Barcoded libraries: *[Area (94–114 bp)] ÷ [Area (50–300 bp)]*

   c. Determine the molar concentration of cDNA libraries using size range 50-300 bp. Use this concentration for "Pool barcoded small RNA libraries" on page 55 and "Determine the library dilution required for template preparation" on page 55.

      Note: Adjust the size range to include all library peaks, if needed.

      Note: For instructions on how to perform the smear analysis, see "Perform a smear analysis" on page 62, and the *Agilent™ 2100 Bioanalyzer™ Expert User's Guide* (Pub. No. G2946-90004).

If the ratio is	Action
≥50%	Proceed to "Determine the library dilution required for template preparation" on page 55 or "Pool barcoded small RNA libraries" on page 55.
<50%	Proceed to "Determine the library dilution required for template preparation" on page 55, or "Pool barcoded small RNA libraries" on page 55 but expect to see an increase in the number of filtered reads (no insert, tRNA, or rRNA mapped reads) when compared to samples with greater than 50% ratio of desired miRNA ligation products to overall products.

Note: Samples that are run on a 2100 Bioanalyzer™ instrument typically show 5-8 bp larger than their actual size.

Pool barcoded small RNA libraries

Note: If you are not pooling libraries, skip this section and proceed to "Determine the library dilution required for template preparation".

1. Determine the molar concentration (nM) of each barcoded cDNA library with the Agilent™ DNA 1000 Kit or the Agilent™ High Sensitivity DNA Kit.

   Note: 50–300 bp size range is typically used to determine the library concentration. If needed, adjust the range to include all the library peaks.

2. Dilute each barcoded cDNA library to the same molar concentration.
   For example, if you have 3 different barcoded libraries that are 45, 55, 65 nM, select a concentration that is equal to or lower than the lowest concentration of the 3 libraries, such as 30 nM. Dilute all or part of each library to 30 nM.

3. Mix an equal volume of each diluted library to prepare a pool of the barcoded libraries.
   The final molar concentration of the pooled library is the same for each diluted library. For example, if you dilute each library to 30 nM, the concentration of the pooled library is 30 nM. Use the final molar concentration to determine the dilution factor required for template preparation. You can also determine the molar concentration of the pooled libraries with the Agilent™ DNA 1000 Kit or the Agilent™ High Sensitivity DNA Kit.

Determine the library dilution required for template preparation

With more than 50% of the amplified DNA in the correct range, you can proceed to the template preparation procedure to prepare templated beads for sequencing on the Ion PGM™, Ion Proton™, and Ion S5™ Systems.

Determine the dilution factor that gives a concentration of ~100 pM. This concentration is suitable for downstream template preparation. Use the following formula:

Dilution factor = (Library or pooled library concentration in pM)/100 pM

Example:

The library or pooled library concentration is 15,000 pM.

Dilution factor = 15,000 pM/100 pM = 150

Thus, 1 µL of library or pooled library mixed with 149 µL of Low TE (1:150 dilution) yields approximately 100 pM. Use this library dilution for template preparation.

Proceed to template preparation

The library is ready for the template preparation procedure. In this procedure, each library template is clonally amplified on Ion Sphere™ Particles using the Ion OneTouch™ 2 or Ion Chef™ Systems for sequencing on the Ion PGM™, Ion Proton™, Ion S5™, or Ion S5™ XL Systems. For instructions, see the specific user guide for an appropriate Ion template preparation kit.

You can find template preparation documentation at **thermofisher.com/ngs-template-preparation-support**.

Typical size distribution

Review the plotted and tabulated size distributions in the following sections.

Plotted size distributions

Figure 8 shows the size distribution of non-barcoded, small RNA library from enriched placenta. Figure 9 illustrates a typical size distribution of placenta total RNA library (Agilent™ 2100 Bioanalyzer™ instrument profile). For the highest quality libraries, the ratio of 86–106-bp DNA to 50–300-bp DNA is greater than 50%. Figure 10 illustrates the size distribution of a barcoded small RNA library prepared from placenta total RNA.

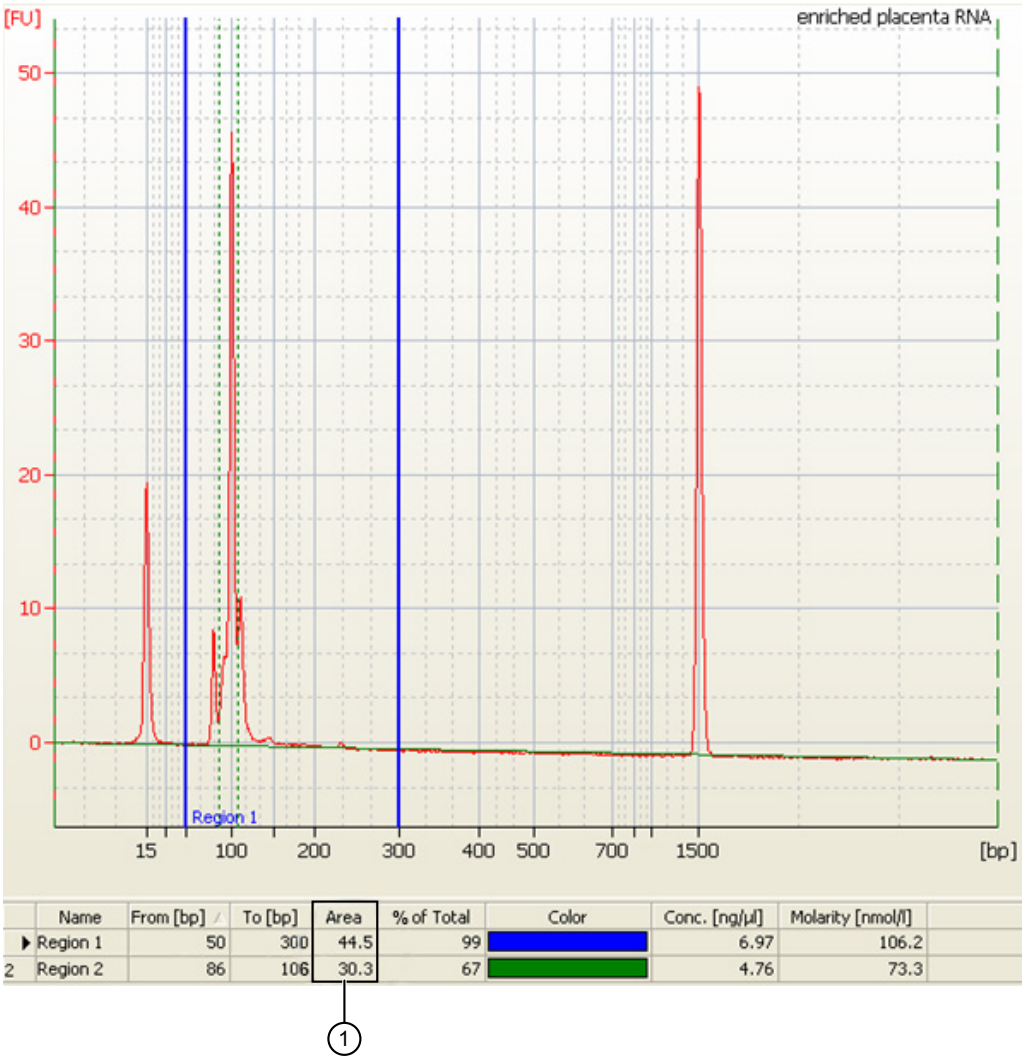


Figure 8 Molar concentration and size distribution of non-barcoded library prepared from enriched placenta small RNA

① 68% amplified DNA falls within the designated range (the area under the curve).

Note: The amount of tRNA in the final library (reflected by the height of the peak that is about 108 bp on the bioanalyzer trace) varies depending on the lot of placenta you use. Expect to see differences in the ratio of 86-106-bp DNA/50–300-bp DNA when different lots of placenta control RNA are used.

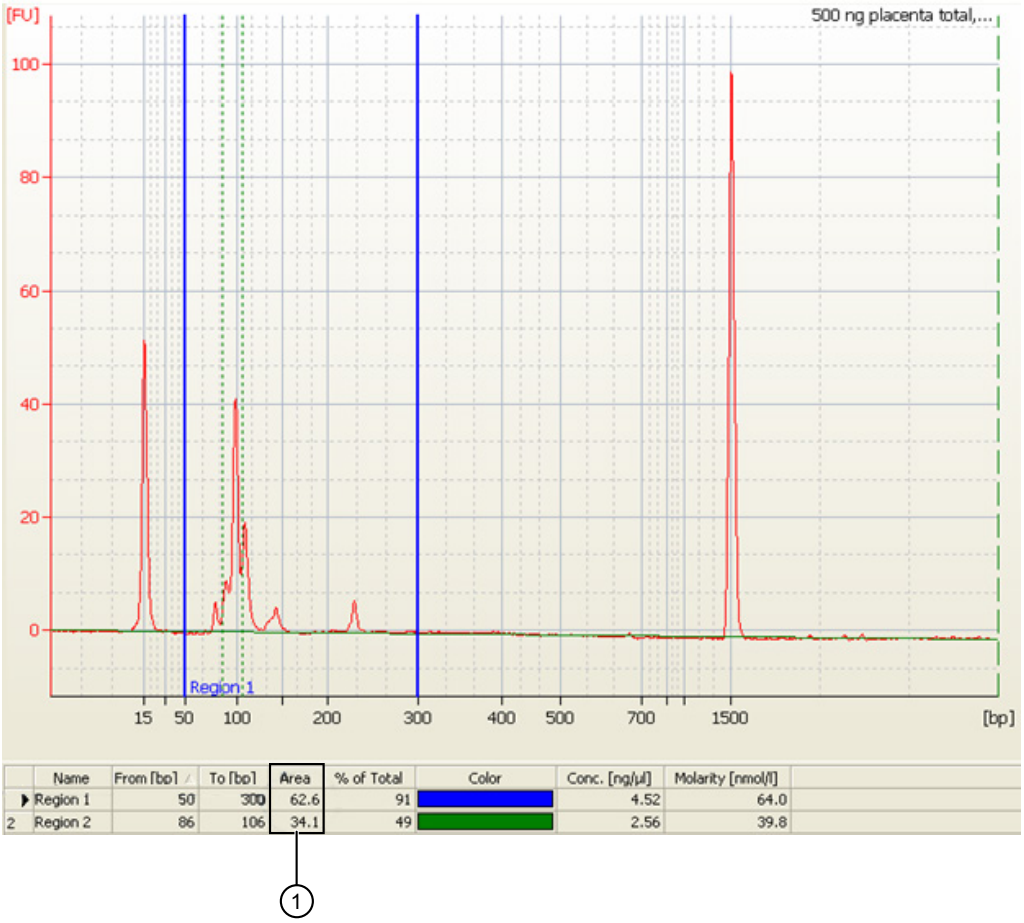


Figure 9 Size distribution of non-barcoded library prepared from placenta total RNA without enriching small RNA

① 54% amplified DNA falls within the designated range (the area under the curve).

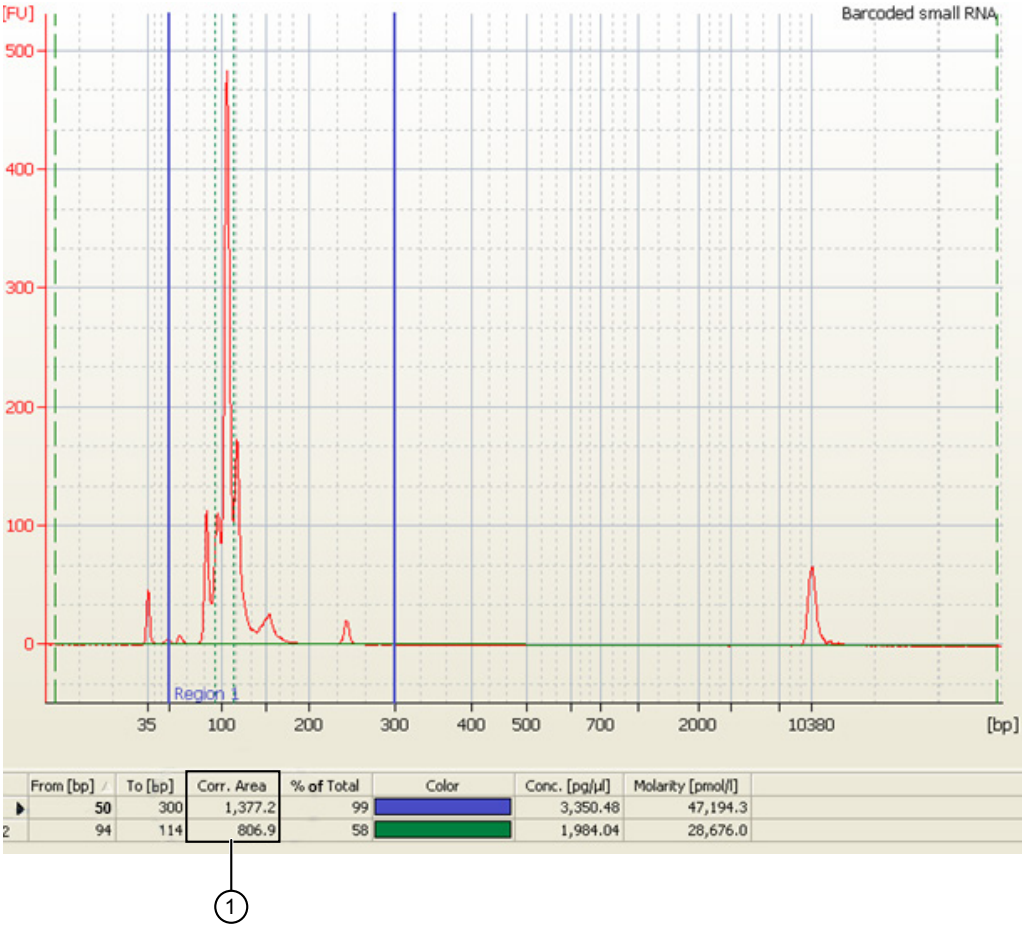


Figure 10 Size distribution of barcoded small RNA library prepared from enriched small RNA

① 58.6% amplified DNA falls within the designated range (the area under the curve).

Size distributions compared

Insert length	Size of *non-barcoded* library on the 2100 Bioanalyzer™ instrument	Size of *Barcoded* Library on the 2100 Bioanalyzer™ instrument
0 bp	~77 bp	~85 bp
10 bp	~87 bp	~95 bp
20 bp	~97 bp	~105 bp
50 bp	~127 bp	~135 bp

A

Troubleshooting

Whole transcriptome libraries

Observation	Possible cause	Recommended action
The Agilent™ software does not calculate one concentration and peak size	The software detects multiple peaks in the amplified cDNA profile.	See "Analyze multiple peaks as one peak" on page 64.
Low yield and poor size distribution obtained 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 14).
Low yield obtained in the amplified library, and little difference observed in the Agilent™ 2100 Bioanalyzer™ instrument traces after the RNA is fragmented	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 14).
Low yield and no PCR products	An enzymatic reaction or purification performed after RNase III treatment failed.	Repeat the ligation with more fragmented RNA, and run a parallel ligation reaction with fragmented Control RNA.

Small RNA libraries

Observation	Possible cause	Recommended action
The Agilent™ software does not calculate one concentration and peak size	The software detects multiple peaks in the amplified cDNA profile.	See "Analyze multiple peaks as one peak" on page 64.
Low yield in the desired size range, and high background of <86 bp inserts in non-barcoded library, or <94 bp inserts in barcoded small RNA library; or high background of >106 bp inserts in non-barcoded library, or >114 bp inserts in barcoded library	Ethanol concentration is incorrect during bead size-selection.	1. Ensure that the ethanol is 100% or 200 proof (absolute). Sub-optimal library size selection with lower ethanol percentage could generate libraries with larger RNA species, such as tRNA and 5S, 5.8S rRNA. 2. Follow the protocol exactly. Some of the steps, such as pre-wetting the tip, are critical for accurate pipetting and correct size selection. 3. Calibrate your pipettor. 4. Using the remaining half of cDNA, repeat PCR and PCR cleanup.

Observation	Possible cause	Recommended action
Low yield or only self-ligation products are visible on Bioanalyzer™ instrument traces	RNA input amount is too low.	Use enriched or purified small RNA instead of total RNA for ligation or increase the ligation time to 16 hours.
	An enzymatic reaction is not optimal.	Before eluting cDNA from the Nucleic Acid Binding Beads, ensure that the beads are completely dry before adding elution buffer. Residual ethanol can inhibit PCR.
Normal or high yield, but PCR products are larger than 150 bp	Too many PCR cycles resulted in overamplification.	Decrease the number of PCR cycles (step 3 on page 50).

Using a positive control to troubleshoot

A general troubleshooting strategy is to perform the Ion Total RNA-Seq Kit v2 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 44.

You can also use the enrichment procedure included in this guide to enrich small RNA from control RNA, then use that as the input for ligation ("Enrich the samples for small RNA" on page 39). If you are starting from total RNA, the yield will be low; we strongly recommend that you use purified or enriched small RNA.

B Supplemental information

Amplified library construction concepts

The procedures in this protocol are based on our Ligase-Enhanced Genome Detection (LEGenD) technology (patented).

Hybridization and ligation to the Adaptor Mix

The RNA samples are hybridized and ligated with the Ion Adaptor Mix v2. The Ion Adaptor Mix v2 is a set of oligonucleotides with a single-stranded degenerate sequence at one end and a defined sequence at the other end required for sequencing on Ion PGM™, Ion Proton™, and Ion S5™/Ion S5™ XL Systems. The Ion Adaptor Mix v2 constrains the orientation of the RNA in the ligation reaction such that hybridization with the Ion Adaptor Mix v2 yields template for sequencing from the 5′ end of the sense strand. Figure 11 illustrates the downstream emulsion PCR primer alignment and the resulting products of templated sphere preparation for sequencing.

Figure 11 Strand-specific RNA sequence information from Ion Total RNA-Seq Kit product

Reverse transcription and size selection

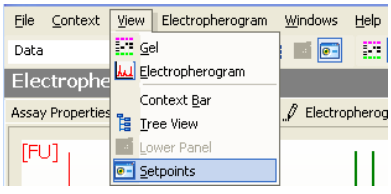
The RNA population with ligated adaptors is reverse transcribed to generate single-stranded cDNA copies of the fragmented RNA molecules. Library generation uses a magnetic bead-based, size-selection process, to enrich for library fragments within the desired size range.

Using 2100 expert software to assess whole transcriptome libraries

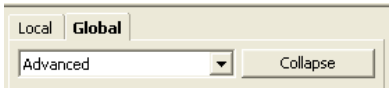
Perform a smear analysis

Perform a smear analysis to quantify the percentage of DNA in the 25–160 bp size range.

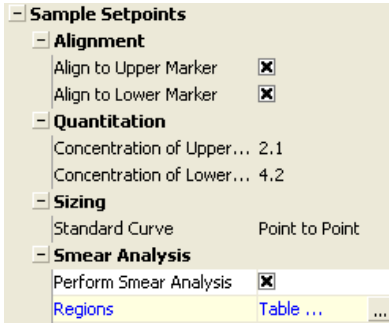
1. In the 2100 expert software, select **View** ▸ **Setpoints.**



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

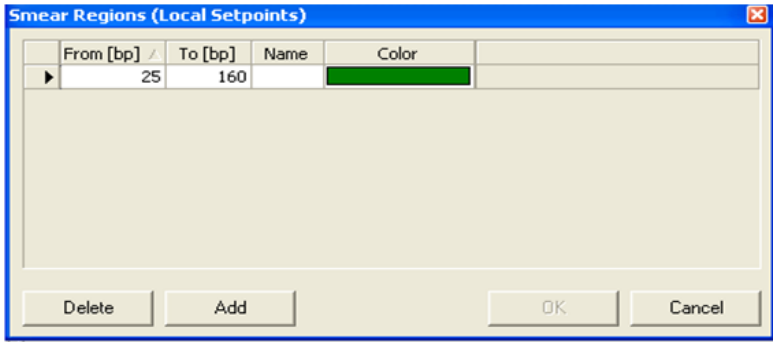


3. In the Sample Setpoints section of the Advanced settings, select the **Perform Smear Analysis** checkbox, then double-click **Table.**



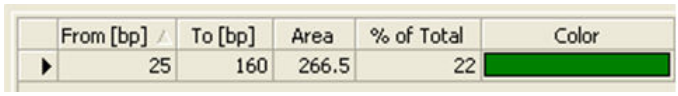
4. Set the smear regions in the Smear Regions dialog box: Click **Add**, then enter **25** bp and **160** bp for the lower and upper limits, respectively.

   These settings determine the percentage of total product that is 25–160 bp in length.



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

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

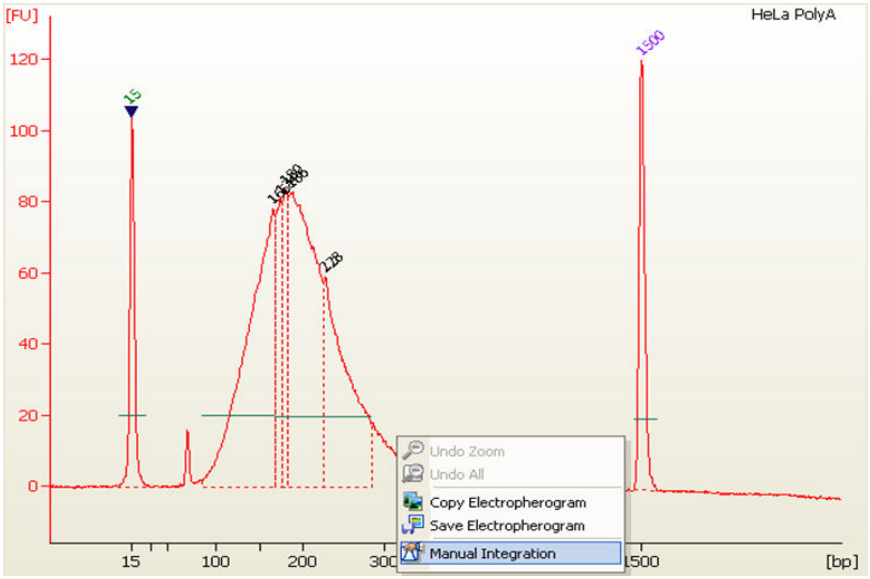


Analyze multiple peaks as one peak

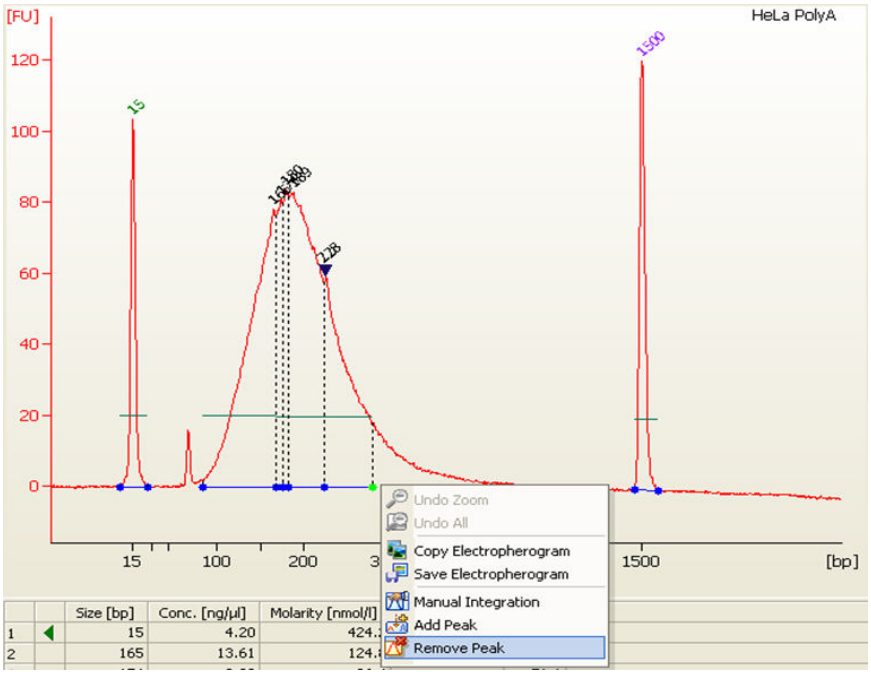
On the Peak Table tab, the 2100 Bioanalyzer™ software identifies multiple peaks that you can consider as one peak. To obtain one concentration and automatically determine the median size for a peak region, manually set the size range of the desired peak region.

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**.

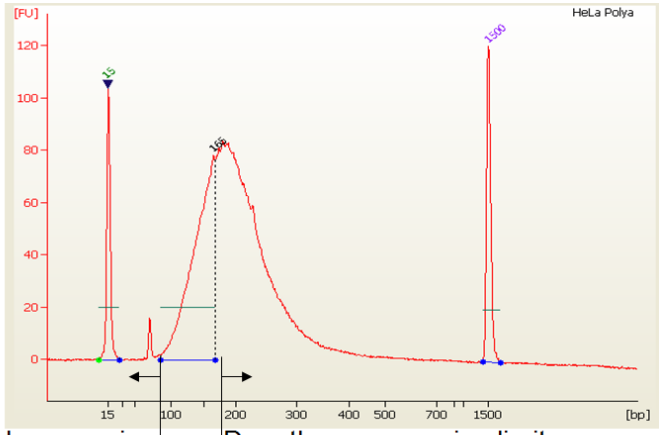


3. To remove multiple peaks:
   a. Place the cursor on the peak to remove, right-click, then select **Remove Peak**.

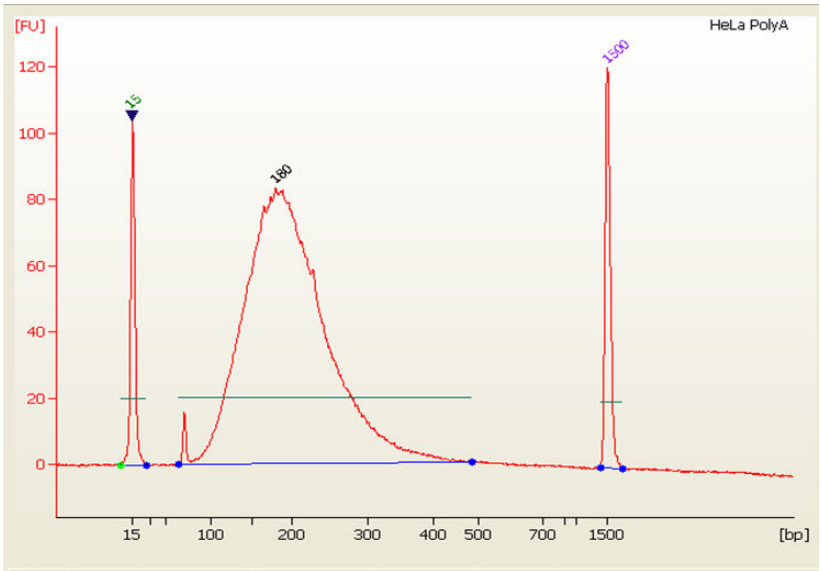


   b. Repeat until one peak remains within the region of interest.

   c. Drag the lower and upper 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.



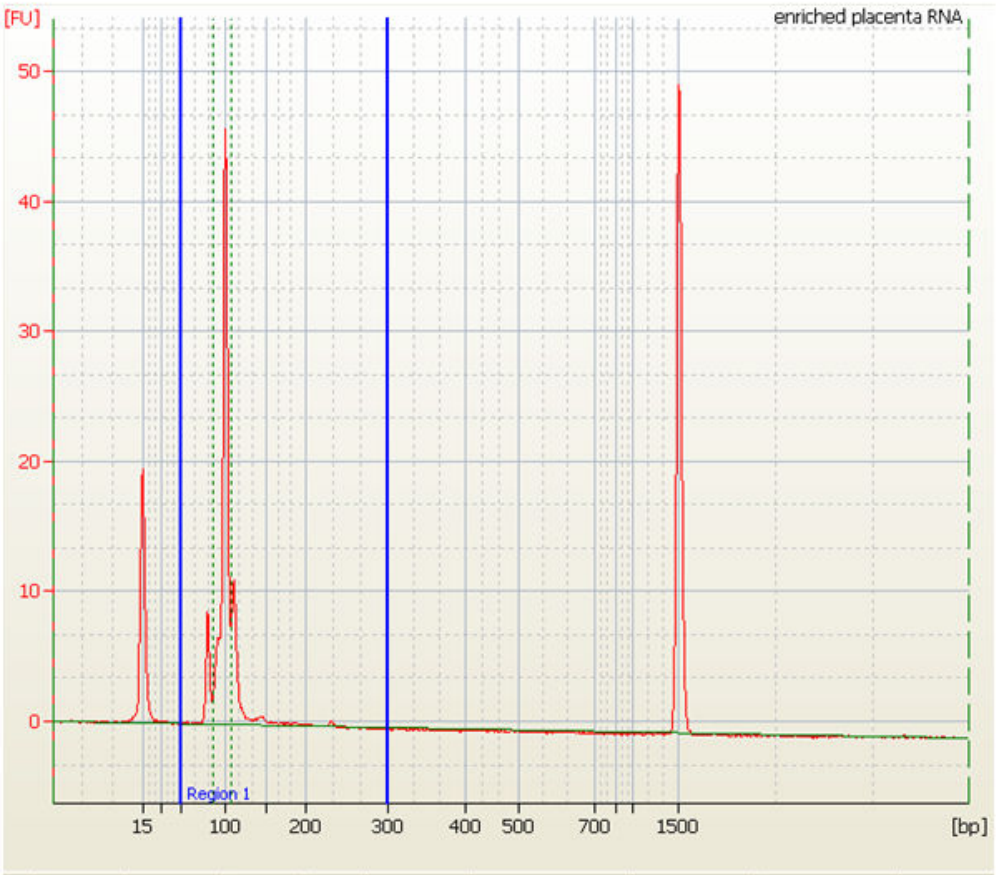
		Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations	Area
1	◀	15	4.20	424.2	Lower Marker	70.5
2		180	43.48	366.7		1,038.2
3	▶	1,500	2.10	2.1	Upper Marker	101.0

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 is ~87-91 bp.

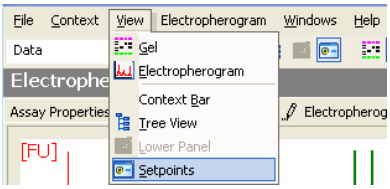


	Name	From [bp]	To [bp]	Area	% of Total	Color	Conc. [ng/µl]	Molarity [nmol/l]
▸	Region 1	50	300	44.5	99		6.97	106.2
2	Region 2	86	106	30.3	67		4.76	73.3

Perform a smear analysis

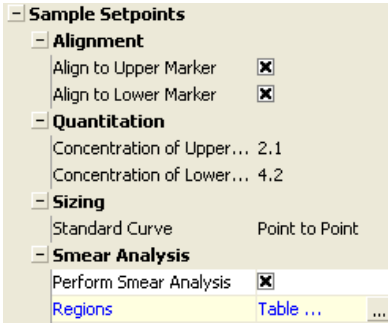
Perform a smear analysis to quantify the percentage of DNA in the 50–300-bp and 86–106-bp size range. The desired size range for miRNA ligation products is 86–106 bp.

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

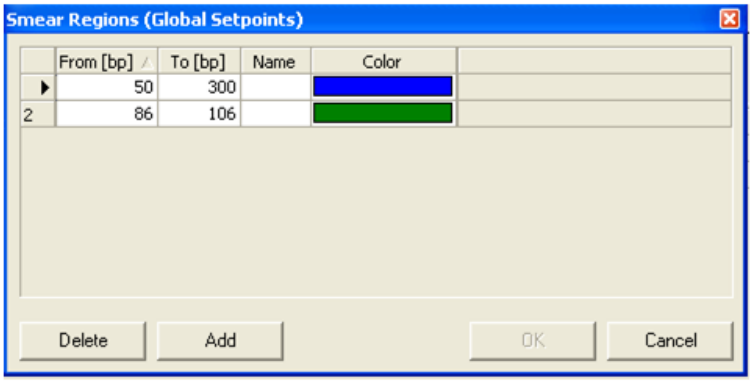


2. On the **Global** tab, select **Advanced** settings.

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

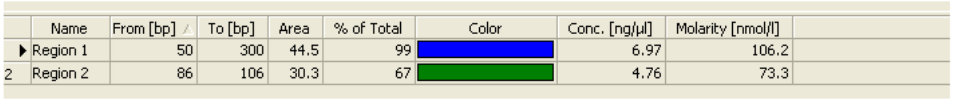


4. Set the smear regions in the Smear Regions dialog box:
   a. Click **Add**, then enter **50** bp and **300** bp for the lower and upper limits, respectively.
   b. Click **Add**, enter **86** bp and **106** bp, then click **OK**.



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

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



Determine the % miRNA library

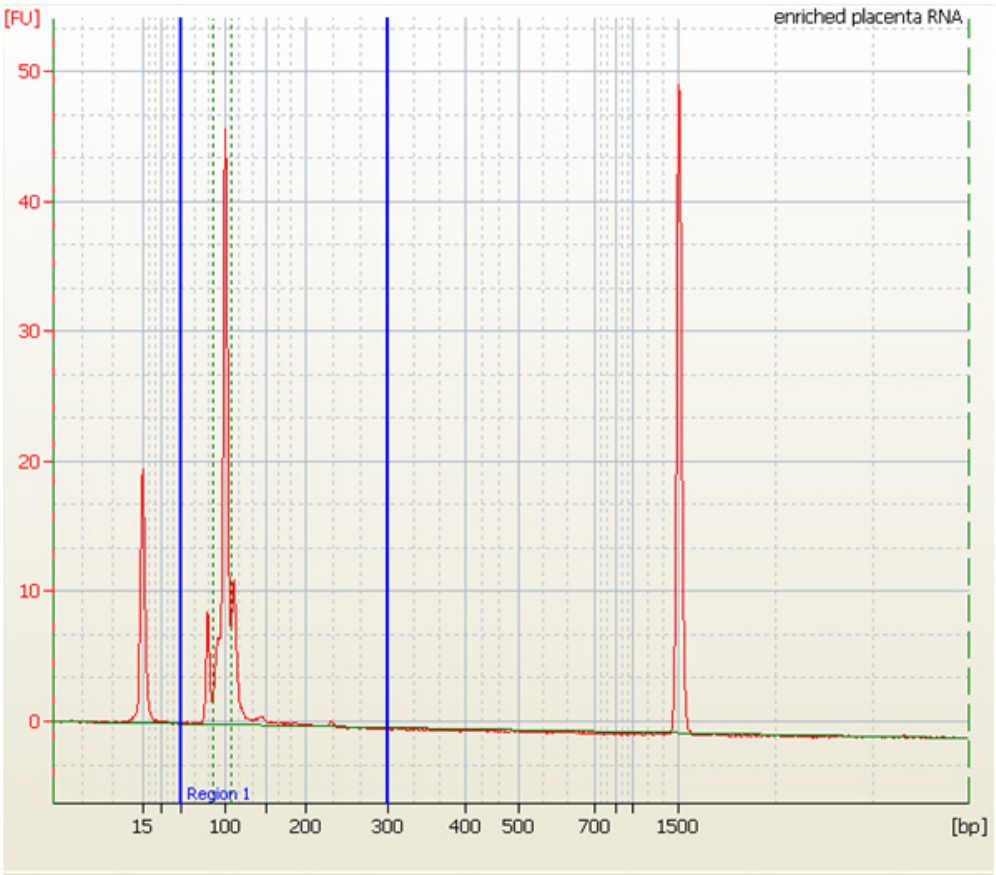
Using the area values from the Region Table, calculate the % miRNA library in the 86–106 bp region as a fraction of the 50–300 bp region using the formula:

% miRNA library = (Area from 86–106 bp ÷ Area from 50–300 bp) × 100

Example % miRNA library calculation

In the example below, the % miRNA library is 68%:

% miRNA library = (30.3 ÷ 44.5) × 100 = 68%



	Name	From [bp]	To [bp]	Area	% of Total	Color	Conc. [ng/µl]	Molarity [nmol/l]
▶	Region 1	50	300	44.5	99		6.97	106.2
2	Region 2	86	106	30.3	67		4.76	73.3

C

Safety

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

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

Chemical safety

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

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

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

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  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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