iontorrent

Ion Total RNA-Seq Kit v2

USER GUIDE

for use with:
Ion PGM™ System
Ion Proton™ System
Ion S5™ XL System
Ion GeneStudio™ S5 System
Ion GeneStudio™ S5 Plus System
Ion GeneStudio™ S5 Prime System

Catalog Numbers 4475936, 4479789, 4475485
Publication Number MAN0010654
Revision D.0

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

Life Technologies Corporation | 5781 Van Allen Way | Carlsbad, CA 92008
For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

The information in this guide is subject to change without notice.

Revision history: Pub. No. MAN0010654

Revision	Date	Description of Change
D.0	1 October 2020	• Corrected errors in the listed kit configuration of the 48-reaction Magnetic Bead Cleanup Module • Deleted the methods chapter for preparing small RNA libraries • Updated to include use of Ion GeneStudio™ S5 Systems • General style updates and improvements for clarity and ease of use
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.

Contents

1 Product information

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

Product description

The Ion Total RNA-Seq Kit v2 (Cat. Nos. 4475936 and 4479789) includes the reagents needed to prepare representative cDNA libraries for strand-specific RNA sequencing on an Ion GeneStudio™ S5 Series System, Ion S5™ XL System, Ion Proton™ System, or Ion PGM™ System. Whole transcriptome RNA samples can be prepared for next-generation sequencing using the Ion Total RNA-Seq Kit v2. Key features of the kit include:

- High accuracy —SuperScript™ III Reverse Transcriptase and Platinum™ PCR SuperMix High Fidelity included for high template fidelity
- Barcode compatible—works with Ion Xpress™ RNA-Seq Barcode 1–16 Kit for multiplexing
- Automation friendly—magnetic bead-based purification simplifies automation of library construction
- Preserves strand information—all mapped reads are aligned in the direction of transcription relative to the chromosomal strand
- Compatible with rRNA-depleted total RNA and poly(A) RNA input

This user guide supports library preparation of whole transcriptome libraries for up to 200-base-read sequencing. To prepare whole transcriptome libraries, follow the procedures in Chapter 2, “Prepare whole transcriptome libraries”.

Preparing barcoded libraries

The Ion Total RNA-Seq Kit v2 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 1–16 Kit (Cat. No. 4475485). This guide provides procedures for both barcoded and non-barcoded library preparation options.

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 an Ion PGM™ System, Ion Proton™ System, Ion S5™ XL System, or Ion GeneStudio™ S5 Series System.

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	
10X RT Buffer (yellow cap)	56 µL	
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 Adapter 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
Binding Solution Concentrate	11 mL	

(continued)

Components[1]	Amount	Storage
Wash Solution Concentrate	11 mL	15°C to 30°C
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 an Ion PGM™ System, Ion Proton™ System, Ion S5™ XL System, or Ion GeneStudio™ S5 Series System.

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	
Hybridization Solution (green cap)	170 µL	
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 Adapter Mix v2 (green cap)	120 µL	–30°C to –10°C
Ion RT Primer v2 (yellow cap)	416 µL	

(continued)

Components[1]	Amount	Storage
Ion 5' PCR Primer v2 (white cap)	80 µL	–30°C to –10°C
Ion 3' PCR Primer v2 (blue cap)	80 µL	
Magnetic Bead Cleanup Module (Part No. 4479767)		
Processing Plate	4	15°C to 30°C (room temperature)
Binding Solution Concentrate	48 mL	
Wash Solution Concentrate	45 mL	
Nucleic Acid Binding Beads (clear cap)	3.4 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" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
Thermal cycler with heated lid, capable of holding 0.2-mL tubes; one of the following, or equivalent: · Veriti™ 96-Well Thermal Cycler · SimpliAmp™ Thermal Cycler · MiniAmp™ Plus Thermal Cycler · ProFlex™ 96-well PCR System	4375786 A24811 A37835 4484075
NanoDrop™ 2000/2000c Spectrophotometer	ND2000USCAN
Agilent™ 2100 Bioanalyzer™ instrument	Agilent G2938A
Agilent™ High Sensitivity DNA Kit	Agilent 5067-4626
DNA 1000 Kit	Agilent 5067-1504
Agilent™ RNA 6000 Pico Kit, 275 samples	50671513

(continued)

Item	Source
Qubit™ 4 Fluorometer[1]	Q33238
Qubit™ RNA HS Assay Kit, 100 assays	Q32852
(Optional) Qubit™ dsDNA HS Assay Kit, 100 assays	Q32851
Centrifugal vacuum concentrator (for example, Savant™ SpeedVac™ DNA130 Integrated Vacuum Concentrator System, or equivalent)	DNA130-115 or MLS
Microcentrifuge	MLS
Pipettors, positive-displacement or air-displacement	MLS
Magnetic stand – one of the following: · Magnetic Stand-96 · Magnetic-Ring Stand (96 well) · DynaMag™–96 Side Magnet	 AM10027 AM10050 12331D
(Optional) MicroAmp™ Clear Adhesive Film	4306311
(Optional) Multi-channel pipettor	MLS
Ethanol, 100%, ACS reagent grade or equivalent	MLS
PCR Tubes & Caps, RNase-free, 0.2 mL (8-strip format), 125 strips	AM12230
Nonstick, RNase-Free Microcentrifuge Tubes, 0.5 mL, 500	AM12350
Nonstick, RNase-Free Microfuge Tubes, 1.5 mL, 250	AM12450
Pipette tips, RNase-free	MLS
(Optional) ABgene™ 96–Well 1.2–mL Polypropylene Deepwell Storage Plate	AB1127
(Optional) Total RNA (available from various human tissues for use as controls at **thermofisher.com**)	(Various Cat. Nos.)

[1] The Qubit™ 3.0 Fluorometer and Qubit™ 2.0 Fluorometer are supported but no longer available for purchase.

Additional optional materials for whole transcriptome libraries

Item	Source
(Optional) ERCC RNA Spike-In Mix or ERCC ExFold RNA Spike-In Mixes **Note:** ERCC controls are highly recommended.	4456740 and 4456739
(Optional) Dynabeads™ mRNA DIRECT™ Micro Purification Kit *or* *(Optional)* Poly(A)Purist™ MAG Kit	61021 AM1922
(Optional) TaqMan™ Gene Expression Assays for ERCC Targets	(Various Cat. Nos.)
(Optional) TaqMan™ Gene Expression Master Mix	4369016
(Optional) RiboMinus™ Eukaryote System v2	A15026
(Optional) Low Input RiboMinus™ Eukaryote System v2	A15027
(Optional) RiboMinus™ Plant Kit for RNA-Seq	A1083808

(*Optional*) Ion Xpress™ RNA-Seq Barcode 1–16 Kit

Sufficient barcode adapters are supplied in the Ion Xpress™ RNA-Seq Barcode 1–16 Kit (Cat. No. 4475485) to prepare barcoded cDNA libraries from 12 samples for each barcode, for a total of 156 barcoded libraries. Up to16 sample libraries can be pooled before template preparation for sequencing on a single chip.

Note: This kit is ordered separately.

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

2 Prepare whole transcriptome libraries

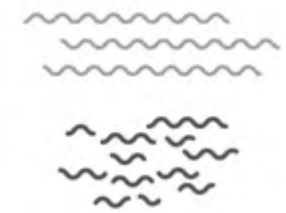
Workflow

(Optional) Add an ERCC RNA Spike-In Mix

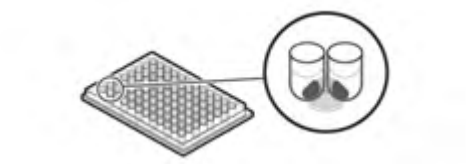
Add an ERCC RNA Spike-In Mix to RNA samples (page 15)

Fragment whole transcriptome RNA

Fragment the RNA using RNAse III (page 16)



Purify the fragmented RNA (page 17)



Assess the yield and size distribution of fragmented RNA (page 19)

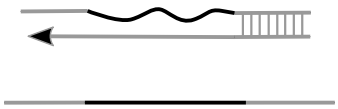
Construct the whole transcriptome library

Hybridize and ligate the RNA (page 21)

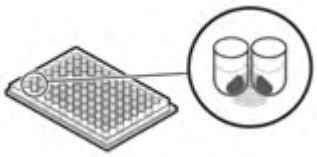
(continued)

Construct the whole transcriptome library

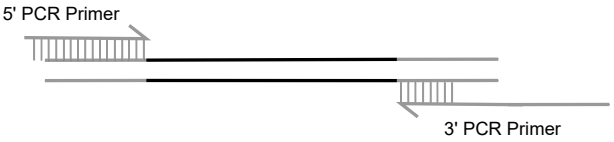
Perform reverse transcription (page 23)



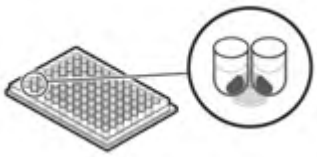
Purify the cDNA (page 24)



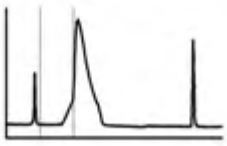
Amplify the cDNA (page 26)



Purify the amplified cDNA (page 28)



Assess the yield and size distribution of the amplified cDNA (page 30)



Pool barcoded whole transcriptome libraries (page 32)

Determine the library dilution required for template preparation (page 32)

Prepare templated Ion Sphere™ Particles

See the user guide for the Ion Torrent™ template preparation kit you are using.

Before first use of the kit

- If you are using the 12-reaction kit, add 44 mL of 100% ethanol to the Wash Solution Concentrate, then mix well.
- If you are using the 48-reaction kit, add 180 mL of 100% ethanol to the Wash Solution Concentrate, then mix well.
- Mark the label on the Wash Solution Concentrate bottle to indicate that you have added ethanol. Store the solution at room temperature (15°C to 30°C).

Note: The Wash Solution Concentrate with added ethanol is called "Wash Solution" in the procedures described in this user guide.

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 Purification Kit (Cat. No. 61021). See the *Dynabeads™ mRNA DIRECT™ Micro Purification 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 Poly(A)Purist™ MAG Kit (Cat. No. AM1922). 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.

Expected yields

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

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 13	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 21	<1–100 ng of fragmented RNA	>5 ng of cDNA

(Optional) Add an ERCC RNA Spike-In Mix to RNA samples

We strongly recommend that you add an ERCC RNA Spike-In Mix to the input total RNA before RNA depletion or poly(A) selection for whole transcriptome library generation. The ERCC RNA Spike-In Mix and ERCC ExFold RNA Spike-In Mixes provide sets of external RNA controls that enable performance assessment of a variety of technology platforms used for gene expression experiments.

Add one ERCC RNA Spike-In Mix or ERCC ExFold RNA Spike-In Mix to each RNA sample, then run these samples on your platform. Compare the spike-in mix data to known ERCC RNA Spike-In 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 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, ERCC ExFold RNA Spike-In Mix 1, or ERCC ExFold RNA 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

Required materials from the Ion RNA-Seq Core Kit v2:

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

1. Assemble a reaction for each RNA sample in a 0.2-mL PCR tube on ice:

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 minutes
	100–500 ng	10 minutes
rRNA-depleted RNA	10 to <100 ng	3 minutes
	100–500 ng	10 minutes
Total RNA	500 ng	10 minutes

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

IMPORTANT! Proceed immediately to "Purify the fragmented RNA" on page 17, or leave the fragmented RNA on ice for no longer than 1 hour.

Purify the fragmented RNA

Required materials from the Ion Total RNA-Seq Kit v2

From the Magnetic Bead Cleanup Module:

- Wash Solution (ethanol added to Wash Solution Concentrate)
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate

From the Ion RNA-Seq Core Kit v2:

- 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

- Ensure that you have added 100% ethanol to the Wash Solution Concentrate. For details, see “Before first use of the kit” on page 13.
- 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 at least 5 minutes.

Note: To reduce cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp™ Clear Adhesive Film. 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. Leave 2–3 µL of the supernatant behind to avoid accidentally aspirating beads.

4. Wash the beads with Wash Solution with ethanol:
   a. Leave the Processing Plate on the magnetic stand.

   b. Add 150 µL of Wash Solution 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 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, then transfer to a new low-bind tube.

Assess the yield and size distribution of fragmented RNA

Use the Qubit™ RNA HS 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 HS Assay Kit and Qubit™ Fluorometer. For increased accuracy, quantify the RNA concentration using the Qubit™ RNA HS 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 HS Assay Kit with the Qubit™ Fluorometer.

   Note: See the *Qubit™ RNA HS Assay Kits User Guide* (Pub. No. MAN0002327) or the *Qubit™ 4 Fluorometer User Guide* (Pub. No. MAN0017209) 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 Agilent™ 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" on page 20.

   Note:
   - For instructions on how to review the size distribution, see the *Agilent™ 2100 Bioanalyzer™ 2100 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" on page 21. Store the remaining RNA at –90°C to –70°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" on page 21.

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.

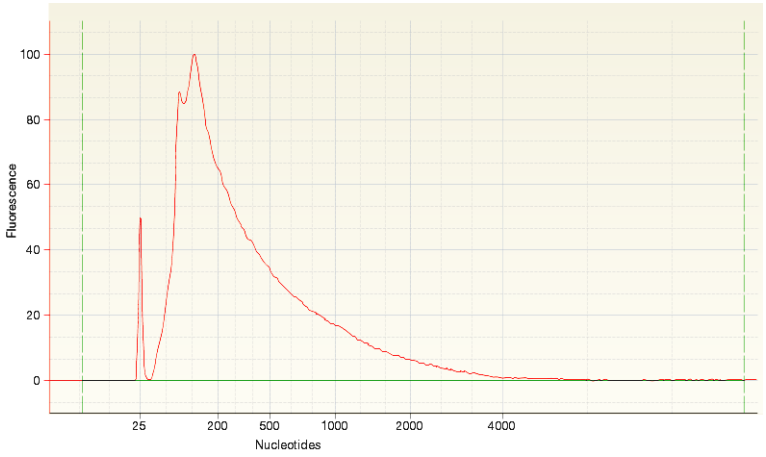


Figure 1 Size distribution of fragmented HeLa poly(A) RNA

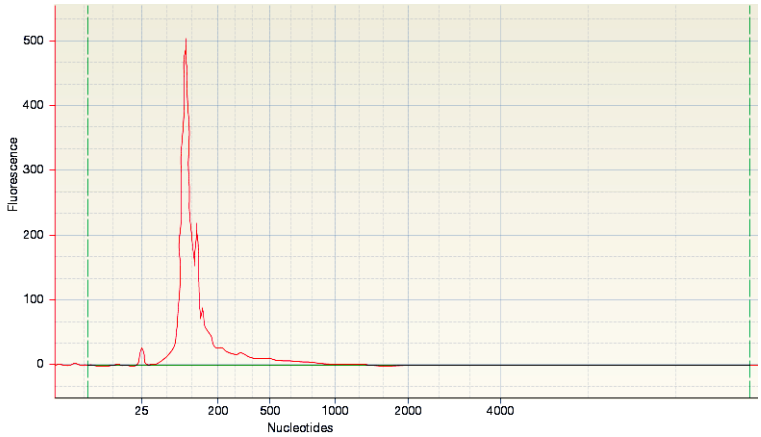


Figure 2 Size distribution of fragmented HeLa rRNA-depleted total RNA

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.

Hybridize and ligate the RNA

Required materials

From from the Ion RNA-Seq Core Kit v2:

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

From the Ion RNA-Seq Primer Set v2:

- Ion Adapter Mix v2

Hybridize and ligate the RNA

1. Prepare the Hybridization master mix on ice:

Component	Volume per reaction[1]
Ion Adapter 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 minutes
30°C	5 minutes

5. Add the RNA ligation reagents to the 8-µL hybridization reactions on ice:

Component	Volume per reaction[1]
Hybridization reaction (from step 4)	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 minutes
rRNA-depleted RNA	10–100 ng	1 hour
	>100 ng	30 minutes

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 RNA-Seq Core Kit v2:

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

From the Ion RNA-Seq Primer Set v2:

- Ion RT Primer v2

Reverse transcribe the RNA

1. Prepare the reverse transcription (RT) master mix on ice:

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 up to 2 weeks, store at –90°C to –70°C for long-term storage, or use immediately.

Purify the cDNA

Required materials from the Ion Total RNA-Seq Kit v2

From the Magnetic Bead Cleanup Module:

- Wash Solution (ethanol added to Wash Solution Concentrate)
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate

From the Ion RNA-Seq Core Kit v2:

- 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

- Ensure that you have added 100% ethanol to the Wash Solution Concentrate. For details, see “Before first use of the kit” on page 13.
- 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 at least 5 minutes.

Note: To reduce cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp™ Clear Adhesive Film. 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. Leave 2–3 µL of the supernatant behind to avoid accidentally aspirating beads.

4. Wash the beads with Wash Solution:
   a. Leave the Processing Plate on the magnetic stand.

   b. Add 150 µL of Wash Solution 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 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, then transfer to a new low-bind tube.

Amplify the cDNA

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

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

To prepare barcoded libraries, use the Platinum™ PCR SuperMix High Fidelity from the Ion RNA-Seq Core Kit v2, and use the following components from the Ion Xpress™ RNA-Seq Barcode 1–16 Kit:

- Ion Xpress™ RNA BC 01–BC 16 (select the barcodes you will use)
- 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 a Non-barcoded Library PCR Mix according to the following table.

Contents	Volume per reaction[1]
Platinum™ PCR SuperMix High Fidelity[2]	45.0 µL
Ion 5' PCR Primer v2	1.0 µL
Ion 3' PCR Primer v2	1.0 µL
Total Volume	**47.0 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 a 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.
  e. Proceed to step 2.

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

Purify the amplified cDNA

Required materials from the Ion Total RNA-Seq Kit v2

From the Magnetic Bead Cleanup Module:

- Wash Solution (ethanol added to Wash Solution Concentrate)
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- Processing Plate

From the Ion RNA-Seq Core Kit v2:

- 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

- Ensure that you have added 100% ethanol to the Wash Solution Concentrate. For details, see “Before first use of the kit” on page 13.
- 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 at least 5 minutes.

Note: To reduce cross-contamination, we strongly recommend sealing unused wells on the Processing Plate with MicroAmp™ Clear Adhesive Film. 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 substep 2b and substep 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. Leave 2–3 µL of the supernatant behind to avoid accidentally aspirating beads.

4. Leave the Processing Plate on the magnetic stand.
   a. Add 150 µL of Wash Solution 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 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, then transfer to a new low-bind tube.

Assess the yield and size distribution of the amplified cDNA

Quantify the yield using the NanoDrop™ Spectrophotometer or the Qubit™ dsDNA HS Assay Kit with the Qubit™ Fluorometer. You can also use the Agilent™ 2100 Bioanalyzer™ instrument with the DNA 1000 Kit or the Agilent™ High Sensitivity DNA Kit for quantification. Use the Agilent™ 2100 Bioanalyzer™ instrument to assess size distribution.

1. Measure the concentration of the purified cDNA with a NanoDrop™ Spectrophotometer or the Qubit™ 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 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 library: Use size range 50–1000 bp.

Note: For instructions on how to perform the smear analysis, see “Perform a smear analysis” on page 40, or see the *Agilent™ 2100 Bioanalyzer™ 2100 Expert User's Guide* (Pub. No. G2946-90004).

4. Take one of the actions in the following table, based on the percentage of cDNA in the 50–160 bp size range.

If the % of DNA in the 50–160 bp range is	Action
<50%	Proceed to the next section, "Pool barcoded whole transcriptome libraries" on page 32 or "Determine the library dilution required for template preparation" on page 32. Use the molar concentration of the cDNA in the 50–1000 bp range determined in substep 3b.
50–60%	Perform another round of purification on the amplified cDNA 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 28. *or* Proceed to the next section, "Pool barcoded whole transcriptome libraries" on page 32 or "Determine the library dilution required for template preparation" on page 32, but expect to see a slightly higher percentage of filtered reads in your sequencing data when compared to libraries with <50% of cDNA in the 50–160 bp 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 28.

Pool barcoded whole transcriptome libraries

Determine the molar concentration (nM) in the 50–1000 bp size range of each barcoded cDNA library with the NanoDrop™ Spectrophotometer, Qubit™ Fluorometer, or the Agilent™ 2100 Bioanalyzer™ instrument (see “Assess the yield and size distribution of the amplified cDNA” on page 30).

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

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

2. 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 needed to dilute the pooled library to a concentration appropriate for template preparation.

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 33) to prepare templated Ion Sphere™ Particles for sequencing.

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 30 nM, or 30,000 pM.

Dilution factor = 30,000 pM/100 pM = 300

Thus, 1 µL of library or pooled library mixed with 299 µL of Low TE (1:300 dilution) yields approximately 100 pM. Use this as the starting library dilution for template preparation.

Note: See the user guide for the Ion OneTouch™ or Ion Chef™ reagent kit you are using for the appropriate concentration to dilute your pooled library.

Proceed to template preparation

The library is ready for the template preparation procedure. In this procedure, library molecules are clonally amplified on Ion Sphere™ Particles using the Ion OneTouch™ 2 System or the Ion Chef™ System for sequencing on the Ion PGM™, Ion Proton™, Ion S5™ XL, or Ion GeneStudio™ S5 Series Systems. For detailed instructions, see the user guide for the Ion Torrent™ template preparation kit you are using.

You can find template preparation documentation at **thermofisher.com/ngs-template-preparation-support** or on the **thermofisher.com** product page for the template kit you are using.

Typical size distributions

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

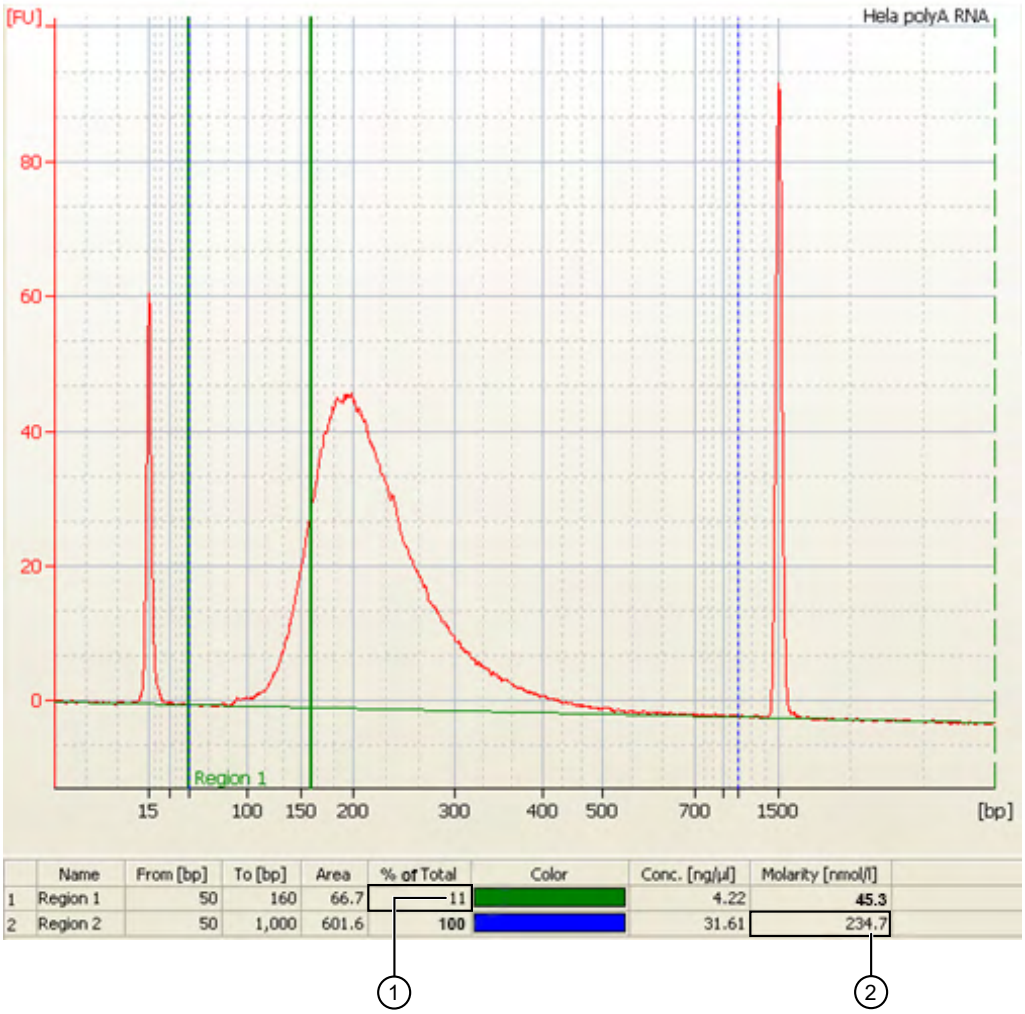


Figure 3 Molar concentration and size distribution of amplified library prepared from HeLa poly(A) RNA

① 11% amplified DNA falls within the designated range (the area under the curve in Region 1).
② The library concentration is 234.7 nM.

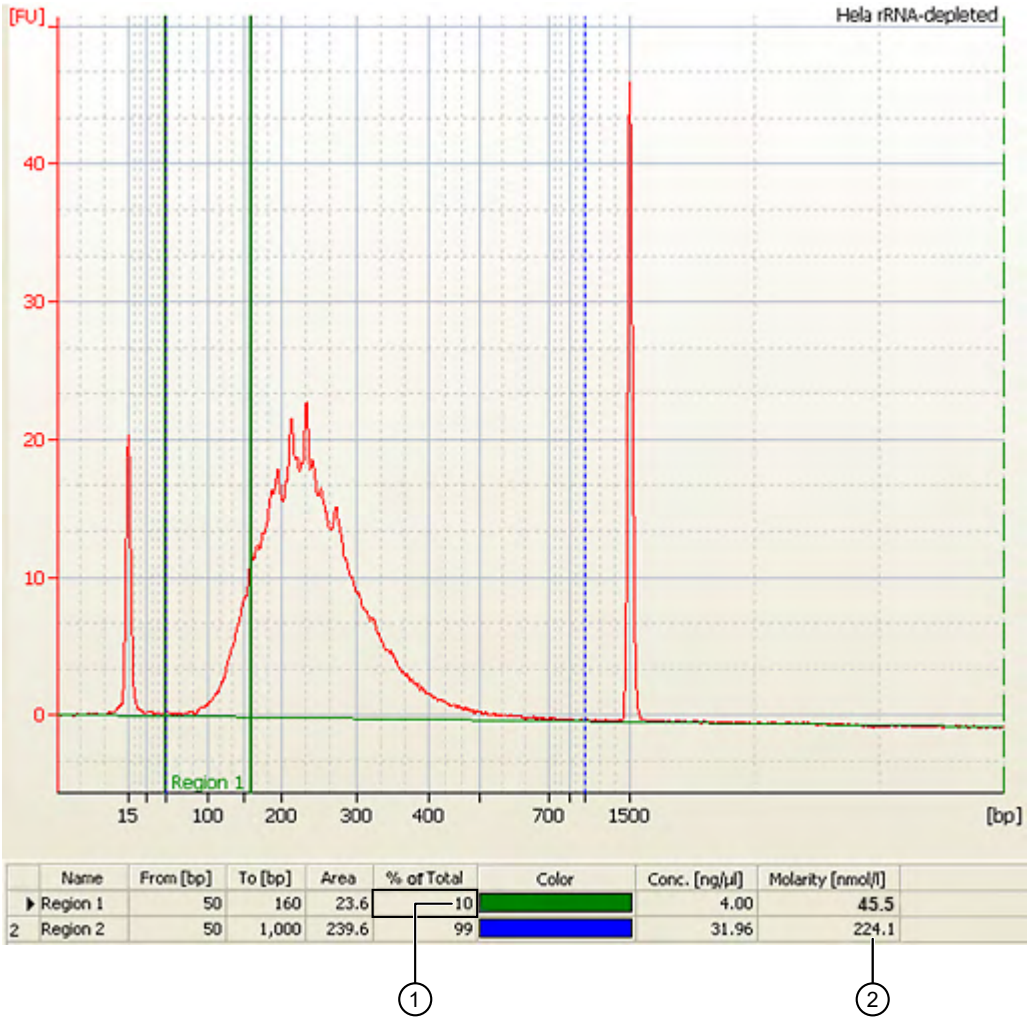


Figure 4 Molar concentration and size distribution of amplified library prepared from HeLa rRNA-depleted total RNA

(1) 10% ampified DNA falls within the designated range (the area under the curve in Region 1).
(2) The library concentration is 224.1 nM.

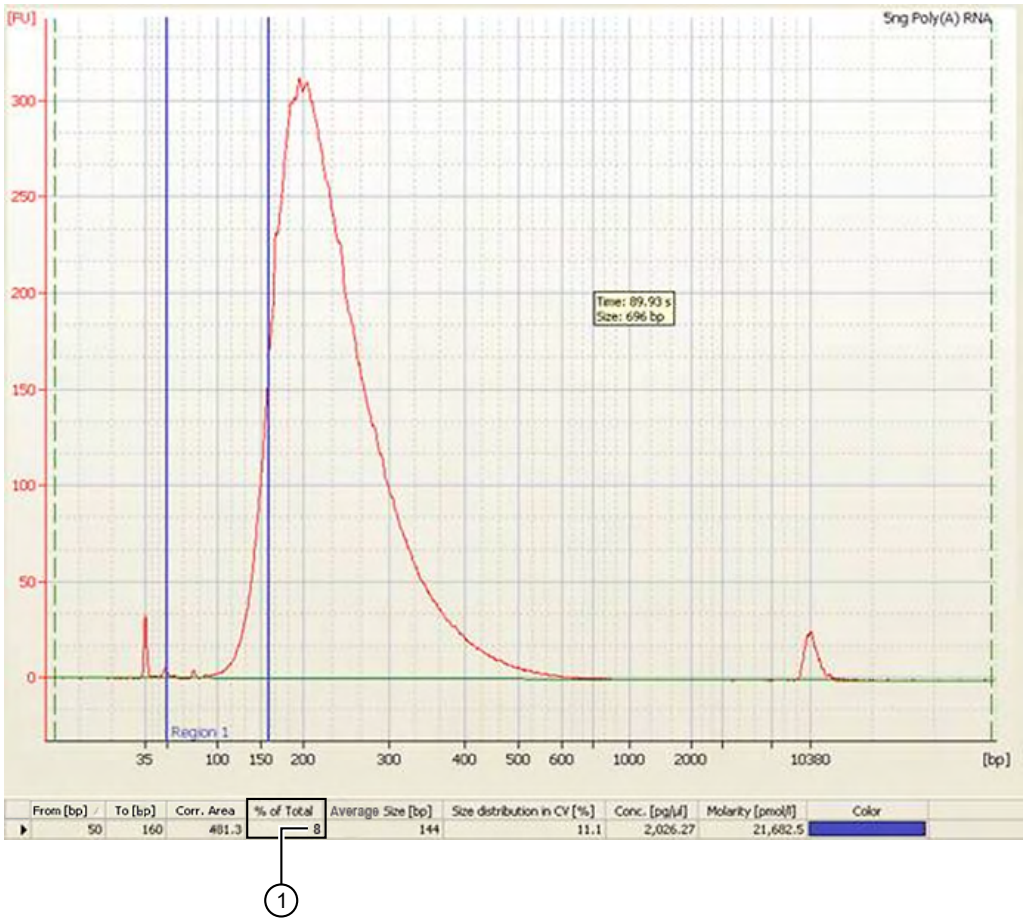


Figure 5 Size distribution of amplified library prepared using 5 ng of poly(A) RNA and isolated using the Dynabeads™ mRNA DIRECT™ Micro Purification Kit

For information for using higher RNA input, see the "Poly(A) selection from 100 ng–1 µg input total RNA samples" protocol in the *Dynabeads™ mRNA DIRECT™ Micro Purification Kit User Guide*.

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

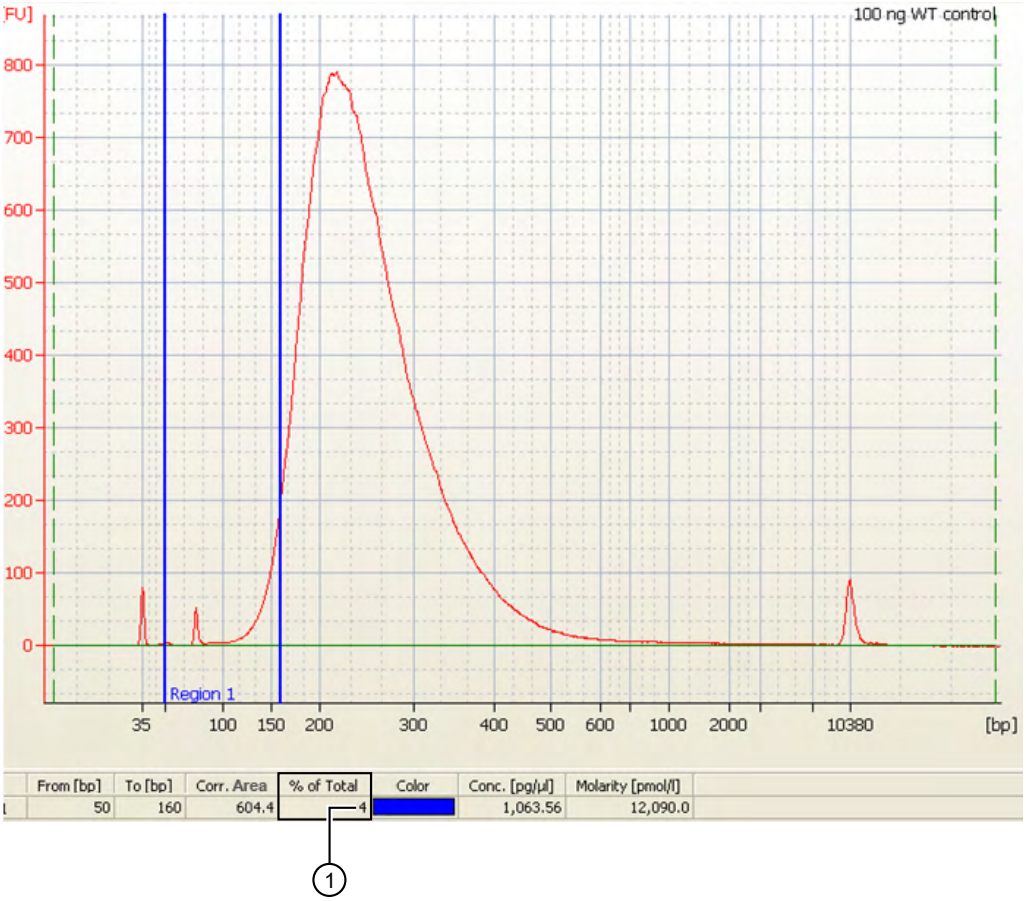


Figure 6 Size distribution of amplified library prepared using poly(A) RNA from 100 ng WT Control RNA

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

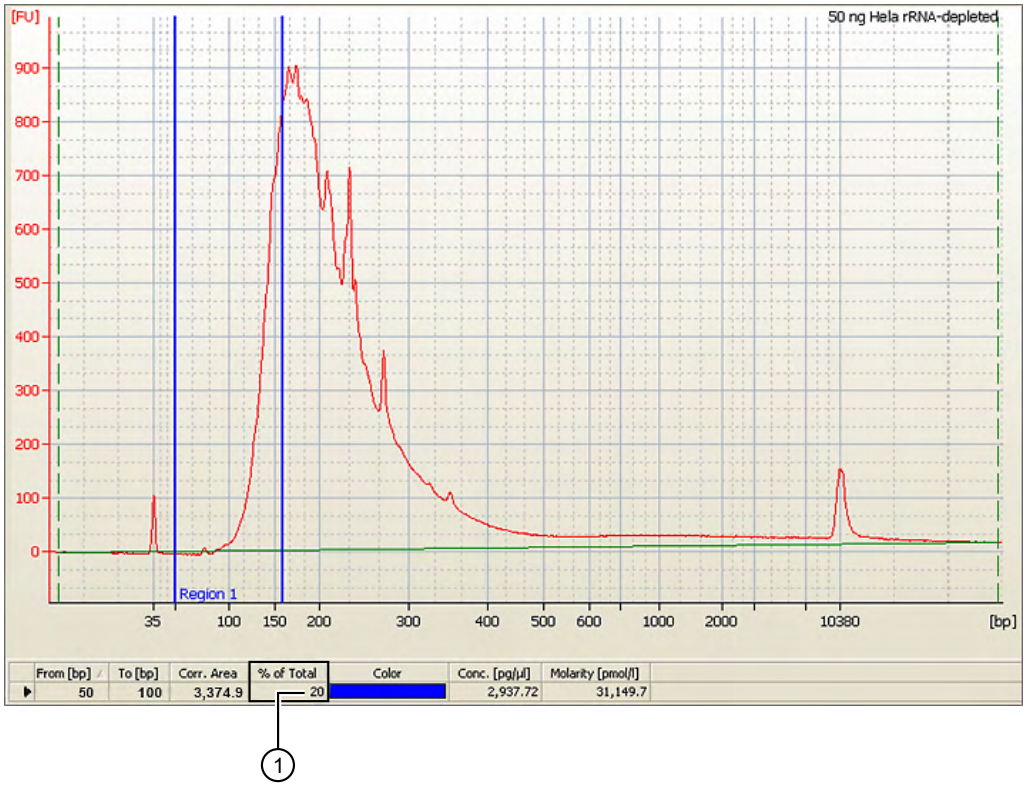


Figure 7 Size distribution of amplified library prepared from 50 ng of rRNA-depleted total RNA using the Agilent™ High Sensitivity DNA Kit

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

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 41.
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 page 16).
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 page 16).
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.

Using a positive control to troubleshoot

A general troubleshooting strategy is to perform the Ion Total RNA-Seq Kit v2 procedure using the WT Control RNA (HeLa total RNA) provided with the kit.

- Use 500 ng of WT Control RNA for the fragmentation procedure starting on page 16.
- Use 100 ng of fragmented WT Control RNA in the amplified library construction procedure starting on page 21.

The expected yields for the WT Control RNA should be the same as the RNA and cDNA yields listed on page 14.

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 Adapter Mix v2. The Ion Adapter 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 an Ion PGM™, Ion Proton™, Ion S5™ XL, or Ion GeneStudio™ S5 Series System. The Ion Adapter Mix v2 constrains the orientation of the RNA in the ligation reaction such that hybridization with the Ion Adapter Mix v2 yields template for sequencing from the 5′ end of the sense strand. Figure 8 illustrates the downstream emulsion PCR primer alignment and the resulting products of templated sphere preparation for sequencing.

Figure 8 Strand-specific RNA sequence information from Ion Total RNA-Seq Kit v2 product

Reverse transcription and size selection

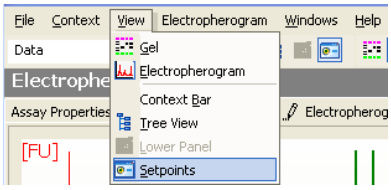
The RNA population with ligated adaptors is reverse transcribed to generate single-stranded cDNA copies of the fragmented RNA molecules. Library generation uses a magnetic bead-based, size-selection process, to enrich for library fragments within the desired size range.

Using 2100 Expert Software to assess whole transcriptome libraries

Perform a smear analysis

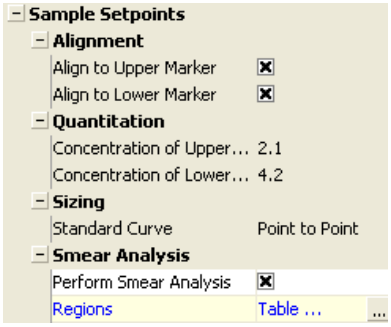
Perform a smear analysis to quantify the percentage of DNA in the 25–160 bp size range.

1. In the 2100 Expert Software, select **View** ▸ **Setpoints**.



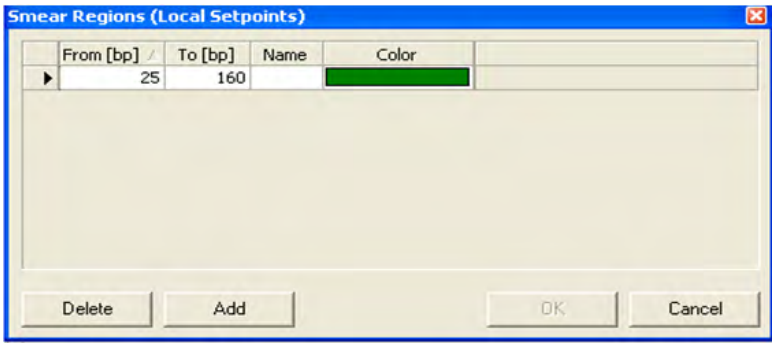
2. On the **Global** tab, select **Advanced** settings.

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



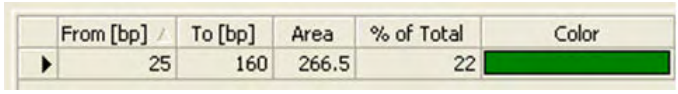
4. Set the smear regions in the **Smear Regions** dialog box: Click **Add**, then enter **25** bp and **160** bp for the lower and upper limits, respectively, for Region 1.

   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.

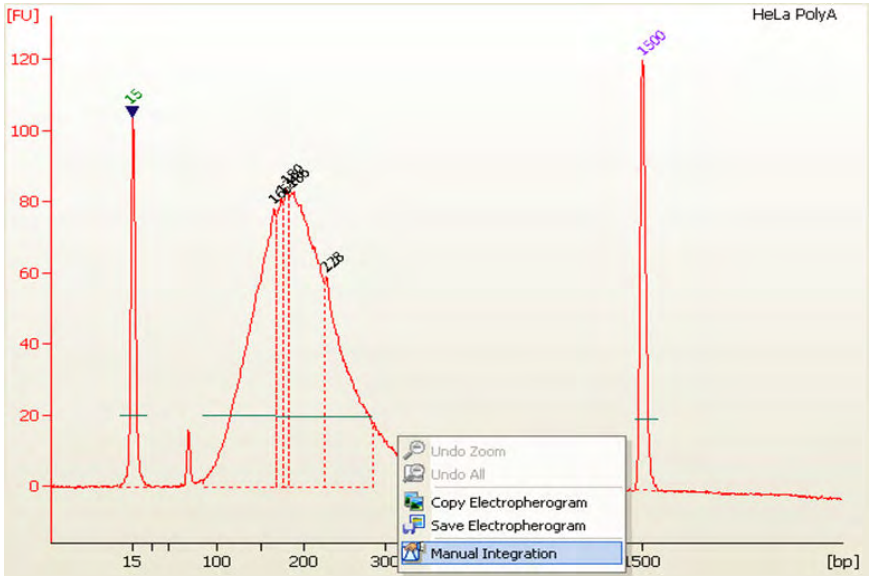


Analyze multiple peaks as one peak

In the **Peak Table** tab, the Agilent™ 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.

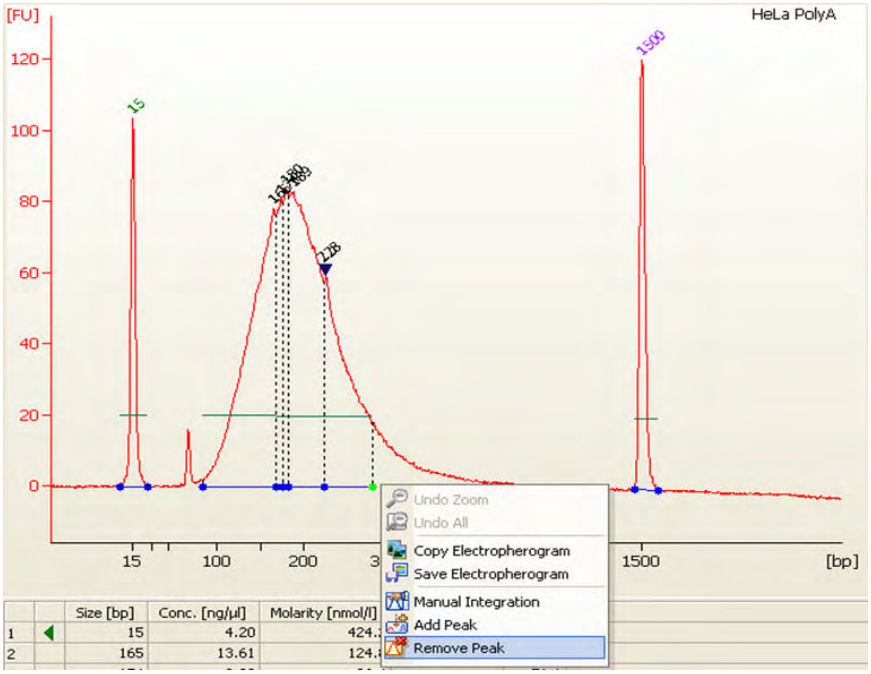
1. In the bottom-left corner of the software window, select the **Peak Table** tab.

2. Right-click anywhere on the electropherogram, then select **Manual Integration**.



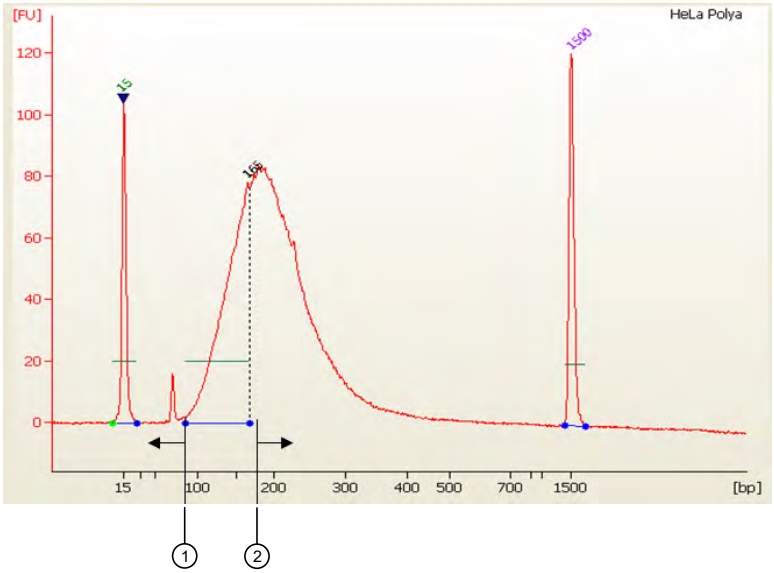
3. To remove multiple peaks:

   a. Place the cursor on the peak to remove, right-click, then select **Remove Peak**.



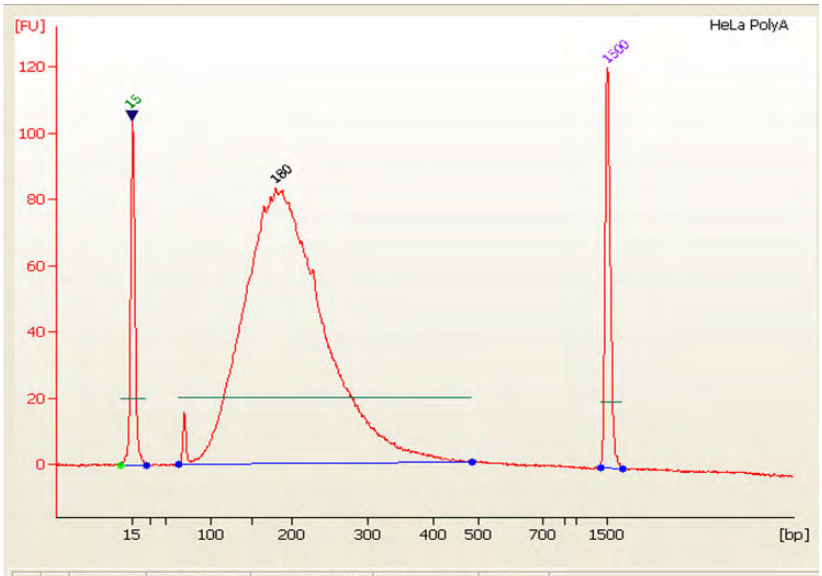
   b. Repeat until one peak remains within the region of interest.

   c. Drag the lower and upper limits of the region until the entire library is included.



① Drag the lower region limit toward the left.
② Drag the upper region limit toward the right.

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

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, and so on). 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:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.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

Documentation and support

Related documentation

Document	Publication number
Ion Total RNA-Seq Kit v2 Quick Reference	MAN0010655
Poly(A)Purist™ MAG Kit User Guide	1922M
RiboMinus™ Eukaryote System v2 User Guide	MAN0007159
Demonstrated Protocol: Bacterial Ribosomal RNA (rRNA) Depletion Workflow for RNA-Seq User Bulletin	MAN0009661
ERCC RNA Spike-In Control Mixes User Guide	4455352
ERCC_Analysis Plugin User Bulletin	4479068
Qubit™ 4 Fluorometer User Guide	MAN0017209
Qubit™ RNA HS Assay Kits User Guide	MAN0002327
Agilent™ 2100 Bioanalyzer™ 2100 Expert User's Guide	G2946-90004

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

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

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.