USER GUIDE

Ion Total RNA-Seq Kit for the AB Library Builder™ System

for use with:

Ion Personal Genome Machine® (PGM™) System

Ion Proton™ System

Catalog Number 4482416

Publication Number MAN0007498

Revision A.0

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

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

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

DISCLAIMER

LIFE TECHNOLOGIES CORPORATION AND/OR ITS AFFILIATE(S) DISCLAIM ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, OR NON-INFRINGEMENT. TO THE EXTENT ALLOWED BY LAW, IN NO EVENT SHALL LIFE TECHNOLOGIES AND/OR ITS AFFILIATE(S) BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF.

Important Licensing Information

This product may be covered by one or more Limited Use Label Licenses. By use of this product, you accept the terms and conditions of all applicable Limited Use Label Licenses.

Trademarks

All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. Bioanalyzer and Agilent are registered trademarks of Agilent Technologies, Inc. TaqMan is a registered trademark of Roche Molecular Systems, Inc. and is used under permission and license. Eppendorf LoBind is a registered trademark of Eppendorf AG.

Contents

About this guide

IMPORTANT! Before using this product, read and understand the information in Appendix B, "Safety" on page 83 in this document.

Revision history

Revision	Date	Description
A.0	1 May 2014	• Whole transcriptome libraries generated by the AB Library Builder™ System supported for use with the Ion Proton™ System. • Changed the recommendation for final library dilution to 100 pM to unify the guide with other Ion library and template user guides. • Minor errors corrected. • Version numbering reset to A.0 in conformance with internal document control.
1.0	14 February 2013	• First release

Other Ion library preparation kits and guides

Other library preparation kits and protocols are available. For guides and protocols, visit the Ion community at **http://ioncommunity.lifetechnologies.com/** and follow the links under **Protocols** > **Construct Library** > **Construct Library User Guides and Quick Reference**.

1 Product information

Purpose of the product

How to use the core kits and adapters

Use the Ion Total RNA-Seq Kit for the AB Library Builder™ System (Cat. no. 4482416) to automate conversion of 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™) System and the Ion Proton™ System. The Ion Total RNA-Seq Kit for the AB Library Builder™ System Protocol Card used with the kit directs the instrument to hybridize and ligate adapters to the RNA and then generate, purify, and size-select the cDNA. This user guide supports library preparation for up to 200-base-read sequencing:

- For whole transcriptome libraries, follow the procedures in Chapter 2 on page 13.
- For small RNA libraries, follow the procedures in Chapter 3 on page 43.

Whole transcriptome and small RNA libraries are compatible with both the Ion PGM™ and the Ion Proton™ Systems.

After automated library preparation, amplify and purify the library using reagents provided in the kit. Then quantitate the library prior to templated bead preparation using an appropriate Ion template preparation kit.

Preparing barcoded libraries

The Ion Total RNA-Seq Kit for the AB Library Builder™ System supports barcoded library preparation to enable sequencing of multiple samples in a single, multiplexed sequencing run. To prepare barcoded libraries, replace the adapters in the kit with primers from the Ion Xpress™ RNA-Seq Barcode 01–16 Kit (Cat. no. 4475485).

Related information

For complete site preparation and operating instructions of the AB Library Builder™ System, refer to the *AB Library Builder™ System Site Preparation Guide* (Pub. no. 4465106) and the *AB Library Builder™ System User Guide* (Pub. no. 4463421), available at: **http://www.lifetechnologies.com**.

For more information on preparing the RNA libraries for sequencing on the Ion PGM™ System, refer to the *Preparation and Sequencing of RNA Libraries with the Ion Personal Genome Machine® (PGM™) System User Bulletin* (Pub. no. 4478119).

Kit contents and storage

Ion Total RNA-Seq Kit for the AB Library Builder™ System

The Ion Total RNA-Seq Kit for the AB Library Builder™ System (Cat. no. 4482416) contains materials sufficient for preparing up to 13 whole transcriptome or small RNA libraries. Store the kit components as directed in the following table:

Ion Total RNA-Seq Kit for ABLB™ Core Reagents (Part no. 4482564)			
Box	**Components**	**Quantity**	**Storage**
RNA-Seq Cartridge for the ABLB™ System	Cartridge containing ready-to-use reagents	13 cartridges	–30°C to –10°C
RNA Fragmentation and Ligation Reagents for the ABLB™ System	2X RNA Ligase Buffer	13 tubes x 45 µL	
	RNAse III	1 tube x 20 µL	
	10X RNAse III Reaction Buffer	1 tube x 20 µL	
	DNAse/RNAse-free Water	1 tube x 1.7 mL	
RNA-Seq Amplification Reagents for the ABLB™ System	Platinum® PCR SuperMix High Fidelity	3 tubes x 1100 µL	
	Ion RNA 5' PCR Primer	1 tube x 20 µL	
	Ion RNA 3' PCR Primer	1 tube x 20 µL	
Magnetic Bead Cleanup Module (Part no. 4475486)			
Box	**Components**	**Quantity**	**Storage**
Magnetic Bead Cleanup Module	Wash Solution Concentrate	1 bottle x 11 mL	15°C to 30°C
	Binding Solution Concentrate	1 bottle x 11 mL	
	Processing Plate	1 plate	
	Nucleic Acid Binding Beads	1 tube x 0.7 mL	2°C to 8°C **IMPORTANT!** Do not freeze.
AB Library Builder™ System Plastics Module (Part no. 4465605)			
Box	**Components**	**Quantity**	**Storage**
AB Library Builder™ Plastics Module	Sample and elution tubes	52 tubes	15°C to 30°C
	Tips and holders	26 tips	

Materials and equipment required but not provided

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.

Ion Xpress™ RNA-Seq Barcode 01–16 Kit

The Ion Xpress™ RNA-Seq Barcode 01–16 Kit (Cat. no. 4475485) is available separately, and contains sufficient PCR primers to prepare barcoded cDNA libraries from 12 samples.

Components	Cap color	Quantity	Volume	Storage
Ion Xpress™ RNA BC 01–BC 16	White	1 each	12 µL	–30°C to –10°C
Ion Xpress™ RNA 3′ Barcode Primer	Blue	1 each	192 µL	

Required for library preparation

Item	Supplier	Cat. no.
Ion Total RNA-Seq Protocol Card for the AB Library Builder™ System	Life Technologies	4482563
AB Library Builder™ System The system includes: • AB Library Builder™ Device • Tip and Tube Tray • Reagent Cartridge Rack • Barcode Reader • RS232C Cable • CommViewer Barcode Software CD-ROM • 13 empty reagent cartridges • 52 sample/elution tubes	Life Technologies	4463592
AB Library Builder™ System with Service Installation The system includes: • AB Library Builder™ Device • Tip and Tube Tray • Reagent Cartridge Rack • Barcode Reader • RS232C Cable • CommViewer Barcode Software CD-ROM • 13 empty reagent cartridges • 52 sample/elution tubes	Life Technologies	4463794

Item	Supplier	Cat. no.
(*Optional*) AB Library Builder™ System replacement parts: • AB Library Builder™ Tips and Tip Holders • AB Library Builder™ Tip and Tube Rack • AB Library Builder™ Cartridge Rack • AB Library Builder™ D-Ring Tool • AB Library Builder™ Barcode Reader • AB Library Builder™ Sample Tubes • AB Library Builder™ D-Rings • AB Library Builder™ Plastics Module	Life Technologies	 • 4463781 • 4463776 • 4463782 • 4465603 • 4465657 • 4463779 • 4465602 • 4465605
Thermal cycler with heated lid, capable of holding 0.2-mL tubes: • Veriti® 96-Well Thermal Cycler • GeneAmp® PCR System 9700	Life Technologies	 • 4375786 • Various Cat. no.
Agilent® 2100 Bioanalzyer® instrument	Agilent	G2938A
NanoDrop® Spectrophotometer	Thermo Fisher Scientific	Various Cat. no.
Qubit® 2.0 Fluorometer	Life Technologies	Q32866
Centrifugal vacuum concentrator (for example, SpeedVac)	Major Laboratory Supplier (MLS)	—
Microcentrifuge	MLS	—
Pipettors and multi-channel pipettors, positive-displacement or air-displacement	MLS	—
DynaMag™-2 magnet (magnetic rack) ***or*** Magnetic stand – one of the following: • Magnetic Stand-96 • 96 well Magnetic-Ring Stand	Life Technologies	12321D • AM10027 • AM10050
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	Life Technologies	Q32851
8-strip PCR Tubes & Caps, RNase-free, 0.2-mL	Life Technologies	AM12230
Non-Stick RNase-Free Microfuge Tubes (0.5-mL), 500	Life Technologies	AM12350
Non-Stick RNase-Free Microfuge Tubes (1.5-mL), 250	Life Technologies	AM12450
Pipette tips, RNase-free	MLS	—
(*Optional*) MicroAmp® Clear Adhesive Film	Life Technologies	4306311
(*Optional*) 1.2-mL 96-well plates	Thermal Fisher Scientific	AB-1127
(*Optional*) FirstChoice® Total RNA	Life Technologies	Various Cat. no.

Materials for whole transcriptome libraries

Item	Supplier	Cat. no.
Qubit® RNA Assay Kit, 100 assays	Life Technologies	Q32852
Agilent® RNA 6000 Pico Kit	Agilent	5067-1513
(*Optional*) ERCC RNA Spike-In Control Mixes **Note:** ERCC controls are highly recommended.	Life Technologies	4456739 and 4456740
(*Optional*) iPrep™ PureLink® Total RNA Kit	Life Technologies	IS-10006
(*Optional*) Dynabeads® mRNA DIRECT™ Micro Kit	Life Technologies	61021
(*Optional*) MicroPoly(A)Purist™ Kit	Life Technologies	AM1919
(*Optional*) TaqMan® Gene Expression Assays for ERCC Targets	Life Technologies	Various Cat. no.
(*Optional*) TaqMan® Gene Expression Master Mix	Life Technologies	4369016 and 4369510
(*Optional*) RiboMinus™ Eukaryote System v2	Life Technologies	A15027
(*Optional*) RiboMinus™ Plant Kit for RNA-Seq	Life Technologies	A1083808

Materials for small RNA libraries

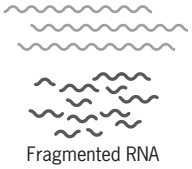
Item	Supplier	Cat. no.
Agilent® RNA 6000 Nano Kit	Agilent	5067-1511
Agilent® Small RNA Kit	Agilent	5067-1548
(*Optional*) *mir*Vana™ miRNA Isolation Kit, 40 purifications	Life Technologies	AM1560
(*Optional*) *mir*Vana™ PARIS™, 40 purification systems	Life Technologies	AM1556
(*Optional*) PureLink® miRNA Isolation Kit, 25 preps	Life Technologies	K1570-01

2 Prepare whole transcriptome libraries

Workflow

Fragment the whole transcriptome RNA

Start with poly(A) RNA or rRNA-depleted total RNA with ERCC RNA Spike-In Control Mix (page 16)



↓

Fragment the RNA using RNase III (page 17)

↓

Purify the fragmented RNA (page 18)

↓

Assess the yield and size distribution of the fragmented RNA (page 20)

↓

Construct the library using the Ion Total RNA-Seq Kit for the AB Library Builder™ System

Set up the AB Library Builder™ System (page 23)

↓

Insert the racks into the AB Library Builder™ Device (page 27)

↓

Start the run (page 29)

↓

Set up for a new run (page 30)

↓

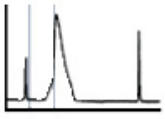
Amplify the cDNA (page 30)

↓

Purify the amplified cDNA (page 31)

↓

Assess the yield and size distribution of the amplified DNA (page 33)



↓

Prepare templated Ion Sphere™ Particles for sequencing on the Ion PGM™ System or the Ion Proton™ System

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

Procedural guidelines

- Use good laboratory practices (GLP) to minimize cross-contamination of products. If possible, prepare libraries in a separate location from template preparation.
- When handling barcoded primers, do not cross-contaminate. Change gloves frequently and open one tube at a time.
- Perform all steps requiring 0.5-mL and 1.5-mL tubes with Ambion® Non-Stick RNase-free Microfuge Tubes (Cat. nos. AM12350 and AM12450).
- When preparing to use reagents:
  - Store enzymes at –30°C to –10°C before use.
  - Thaw all reagents on ice or at room temperature before use.
  - Keep thawed reagents on ice when not in use.
- Mix reagents thoroughly before use, especially if reagents were frozen and thawed.

Guidelines for RNA sample type and amount

We strongly recommend using 5–500 ng of poly(A) RNA, or 10–500 ng of rRNA-depleted total RNA. You may 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 (Cat. no. 61021). Refer to the *Dynabeads® mRNA DIRECT™ Micro Kit User Guide*.
- 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™ Eukarytotic System v2, refer to the *Demonstrated Protocol: Bacterial Ribosomal RNA (rRNA) Depletion Workflow for RNA-Seq User Bulletin* (Pub. no. MAN0009661).

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. RNA extraction may be automated on the AB Library Builder™ System using the iPrep™ PureLink® Total RNA Kit (Cat. no. IF-10006). Quantitate 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, refer to 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)†	
	Total RNA	Poly(A) RNA
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)	—

† 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 or not the ERCC results indicate a problem with library preparation or the sequencing run.

For more information about the ERCC_Analysis Plugin, refer to the *ERCC_Analysis Plugin User Bulletin* (Pub. no. 4479068) available at: **http://www.lifetechnologies.com**.

Fragment the whole transcriptome RNA

Fragmenting the whole transcriptome RNA involves the following procedures:

Fragment the RNA using RNase III (page 17)

⬇

Purify the fragmented RNA (page 18)

⬇

Assess the yield and size distribution of the fragmented RNA (page 20)

Fragment the RNA using RNase III

Use components from the Ion Total RNA-Seq Kit for the AB Library Builder™ System:

- 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:

Component (add in the order shown)	Volume for One Reaction
1. RNA sample and Nuclease-free Water: • Poly(A) RNA: 5–500 ng • rRNA-depleted total RNA: 10–500 ng • Total 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.
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	5 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 "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
- (*Optional*) Processing Plate
- Nuclease-free Water

Note: 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

- Ethanol, 100%, ACS reagent grade or equivalent
- 37°C heat block or water bath
- Magnetic rack or stand
- Non-Stick RNAse-free Microfuge Tubes (1.5-mL)
- Pipettors or multi-channel pipettors, positive-displacement or air-displacement

Before you begin

- If you have not done so already, 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 (Life Technologies, Cat. no. 4306311). You can also skip a row between sample rows.

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

1. Gently vortex the Nucleic Acid Binding Beads tube, then add 5 µL of the beads to 90 µL Binding Solution Concentrate per sample in a 1.5-mL RNAse-free tube or the Processing Plate provided. Mix the beads with the Concentrate by pipetting up and down 10 times.
2. Transfer the 30-µL fragment RNA reaction to the beads in the tube or Processing Plate.

3. Set a P200 pipettor at 150 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the new 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times. Without changing tips, add 150 µL of 100% ethanol to each sample.

   IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the tube or well wall. Change the tip and repeat step 3 for the remaining samples only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

4. 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 should be homogeneous after mixing.

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

6. Place the tube or Processing Plate in a magnetic rack or stand for 5–6 minutes to clear the solution. Without removing the tube or plate from the magnet, carefully remove and discard the supernatant without disturbing the pellet.

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

7. Add 150 µL of Wash Solution Concentrate with ethanol to each sample on the magnet and incubate for 30 seconds. After the solution clears, carefully remove and discard the supernatant without disturbing the pellet.

8. Carefully remove any remaining supernatant with a P10 or P20 pipettor without disturbing the pellet.

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

9. Keeping the tube or plate on the magnet, air-dry the beads at room temperature for 1–2 minutes.

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

10. Remove the tube or plate from the magnetic rack, and add 12 µL of pre-warmed (37°C) Nuclease-free Water. Mix the Nuclease-free Water and beads by pipetting up and down 10 times.

11. Incubate the tube or plate at room temperature for 1 minute, then place the tube or plate on the magnet for an additional minute until the solution clears. Transfer the supernatant containing the eluted RNA to a new 1.5-mL RNAse-free tube without disturbing the pellet.

    IMPORTANT! The solution contains the fragmented RNA. Do not discard!

Assess the yield and size distribution of the 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 in place of the Qubit® RNA Assay Kit and Qubit® Fluorometer. However, for increased accuracy, quantitate the RNA concentration using the Qubit® RNA Assay Kit with the Qubit® Fluorometer.

1. Quantitate the yield of the fragmented RNA using the Qubit RNA Assay Kit with the Qubit Fluorometer.

   Refer to the *Qubit® RNA Assay Kit Protocol* (Pub. no. MAN0002327) or the *Qubit® Fluorometer Instruction Manual* (Pub. no. MAN0002328) for instructions.

2. Assess the size distribution of the fragmented RNA:

   a. If necessary, 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 should produce a distribution of RNA fragment sizes from 35 nt to several hundred or a few thousand nt, depending on your sample type. The average size should be 100–200 nt. See the figures starting on page 21.

      Note: For instructions on how to review the size distribution, refer to the *Agilent® 2100 Bioanalyzer® Expert User's Guide* (Pub. no. G2946-9000).

      If the profile for the fragmented RNA does not meet the typical results, see "Troubleshooting" on page 38 for guidance.

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

Amount of Fragmented RNA in 3 µL	Instructions
• ≥50 ng of poly(A) RNA • ≥100 ng of rRNA-depleted total RNA • ≥100 ng of Total RNA	Proceed to "Construct the library using the Ion Total RNA-Seq Kit for the AB Library Builder™ System" on page 22. Store the remaining RNA at –86°C to –68°C.
• <50 ng of poly(A) RNA • <100 of 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 should take 10–20 minutes. 2. Resuspend in 3 µL of Nuclease-free Water, then proceed to "Construct the library using the Ion Total RNA-Seq Kit for the AB Library Builder™ System" on page 22.

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

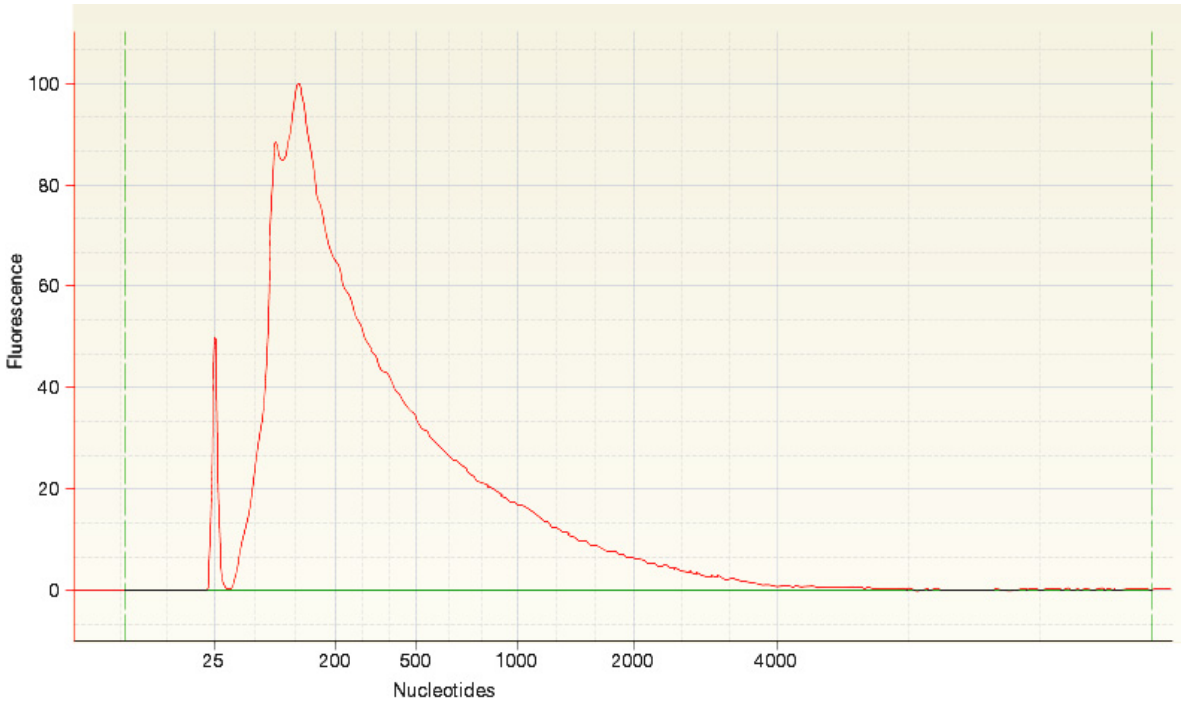
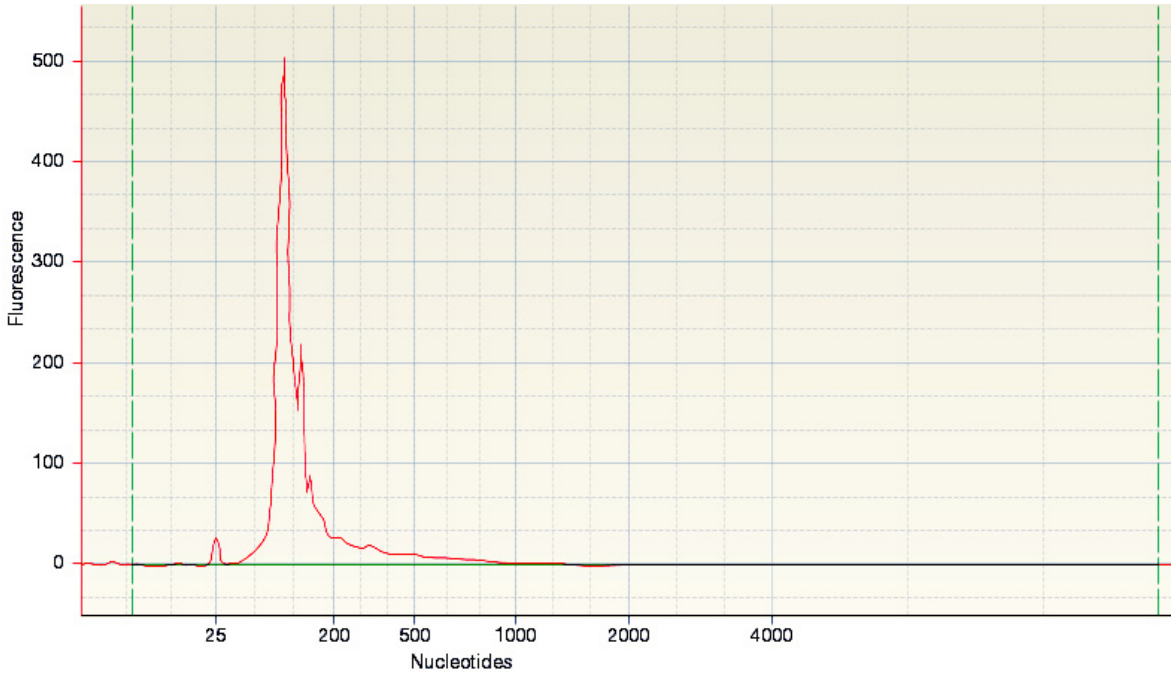
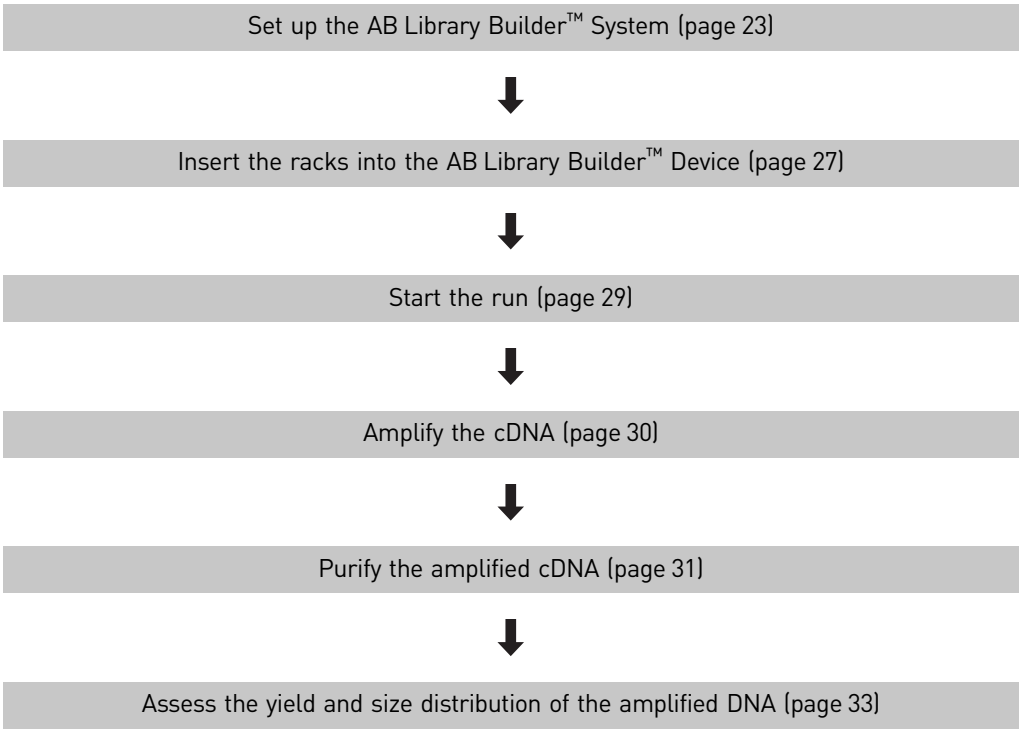


Figure 2 Size distribution of fragmented HeLa rRNA-depleted total RNA



Construct the library using the Ion Total RNA-Seq Kit for the AB Library Builder™ System

Constructing the whole transcriptome library involves the following procedures:



Guidelines for using the AB Library Builder™ System

Use the Ion Total RNA-Seq Kit for the AB Library Builder™ System to generate adaptor-ligated and purified cDNA libraries from the fragmented whole transcriptome RNA.

To install and set up the AB Library Builder™ System, see the *AB Library Builder™ System Site Preparation Guide* (Pub. no. 4465106) and the *AB Library Builder™ System User Guide* (Pub. no. 4463421).

IMPORTANT! To avoid data loss or run cancellation, always follow these practices:

- Before you insert or remove a protocol card, power off the instrument.
- Before you power on the instrument, insert the protocol card, then close the instrument door.
- To pause the instrument during a run, press **Stop** before you open the instrument door.
- When you are not performing a run or instrument test, you can open the instrument door with the power off or on.
- Do not move instrument components such as the platform, magnets, and syringes while the instrument is powered on.

Set up the AB Library Builder™ System

Required materials from the Ion Total RNA-Seq Kit for the AB Library Builder™ System

- Ion Total RNA-Seq Kit for the AB Library Builder™ System Cartridge
- 2X RNA Ligase Buffer (from the RNA Fragmentation and Ligation Reagents for the ABLB™ System)
- Nucleic Acid Binding Beads (from the Magnetic Bead Cleanup Module)
- Sample/elution tubes, tips, and tip holders (from the AB Library Builder™ Plastics Module)

Other materials and equipment

- Ion Total RNA-Seq Protocol Card for the AB Library Builder™ System
- AB Library Builder™ System and accessories
- Pipettors and pipette tips

Before you begin

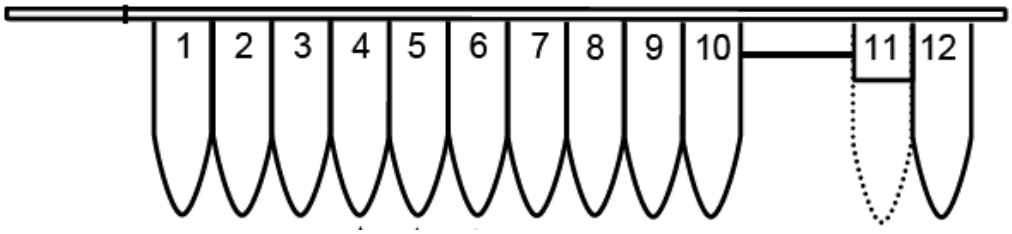
- Completely thaw an appropriate number of Ion Total RNA-Seq for the AB Library Builder™ System Cartridges for ≤2 hours at room temperature, 2°C to 8°C, or on ice. One cartridge is required per sample. Thawed cartridges do not require additional mixing.

  IMPORTANT! Avoid leaving the cartridges at room temperature for longer than necessary to completely thaw them. Avoid repeated freeze-thawing of unused cartridges.

- Thaw one tube of 2X RNA Ligase Buffer for each cartridge to be used. If you see a white precipitate in the Ligase Buffer, warm the solution at 37°C to resuspend the precipitate.
- Gently but thoroughly vortex the Nucleic Acid Binding Beads. In a separate tube, dilute the beads 1:2 into a sufficient volume of Nuclease-free Water to yield 40 µL of diluted beads per cartridge. For example, for a run with one cartridge, add 20 µL of Nucleic Acid Binding Beads to 20 µL of Nuclease-free Water.

  IMPORTANT! Do not add water directly to the stock tube of Nucleic Acid Binding Beads. Diluted Nucleic Acid Binding Beads are required for the cartridge only. Undiluted Nucleic Acid Binding Beads are required for other steps in the protocol.

- Inspect the cartridges. Each cartridge has 12 compartments for reagents:



Cartridge Compartment Numbers	Quantity
1	800 µL
2	600 µL
3	1000 µL
4	20 µL
5	25 µL
6	20 µL
7	Empty
8	20 µL
9	700 µL
10	Empty
11	Empty (user to insert 45 µL tube of 2X RNA Ligase Buffer)
12	Empty (user to add 40 µL of diluted Nucleic Acid Binding Beads)

IMPORTANT! Do not switch the supplied pre-filled reagents with any other buffers. The protocols are specifically optimized with the reagents supplied with the kit.

Insert or change the protocol card and power ON the instrument

IMPORTANT! Do not remove the protocol card while the instrument is on. Removing the card stops the run, which may cause instrument data file loss. To remove the card, see step 3 on page 25.

If you accidentally remove the protocol card during a run, power off the instrument immediately to minimize potential for instrument data loss.

For guidelines on handling protocol cards, see the *AB Library Builder™ System User Guide* (Part no. 4463421).

1. Confirm that the power switch is in the OFF position.

   Note: If you insert the card while the instrument is on, the instrument does not recognize the card.

2. Open the card slot.

3. To remove a card that is already installed in the slot, push the button located below the card slot (see the following photo), then pull the card out of the slot. Place the card in the plastic cover in the box.

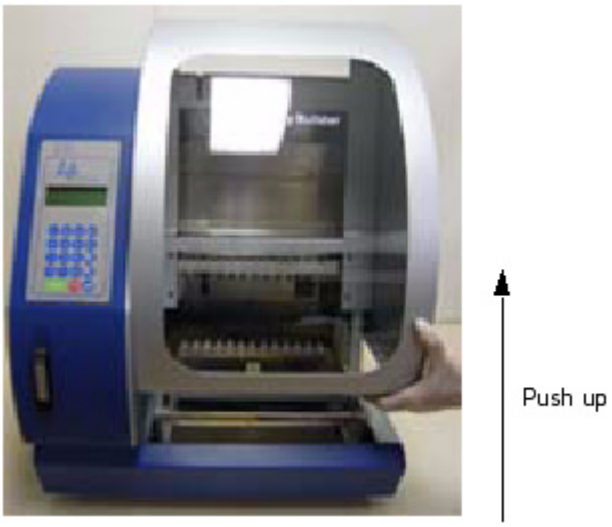
IMPORTANT! Do not remove the protocol card while the instrument is on.

4. Insert the appropriate protocol card in the slot with the arrow on the protocol card pointing toward the instrument and the label facing left.
5. Push the card completely into the card slot, then close the card slot.
6. Close the door to the AB Library Builder™ Device.
7. Power on the instrument.

   When the card is fully inserted in the correct orientation, the display briefly shows information including the instrument and protocol card version, then displays the Main menu.
8. Press **START.**

Load the racks and tubes

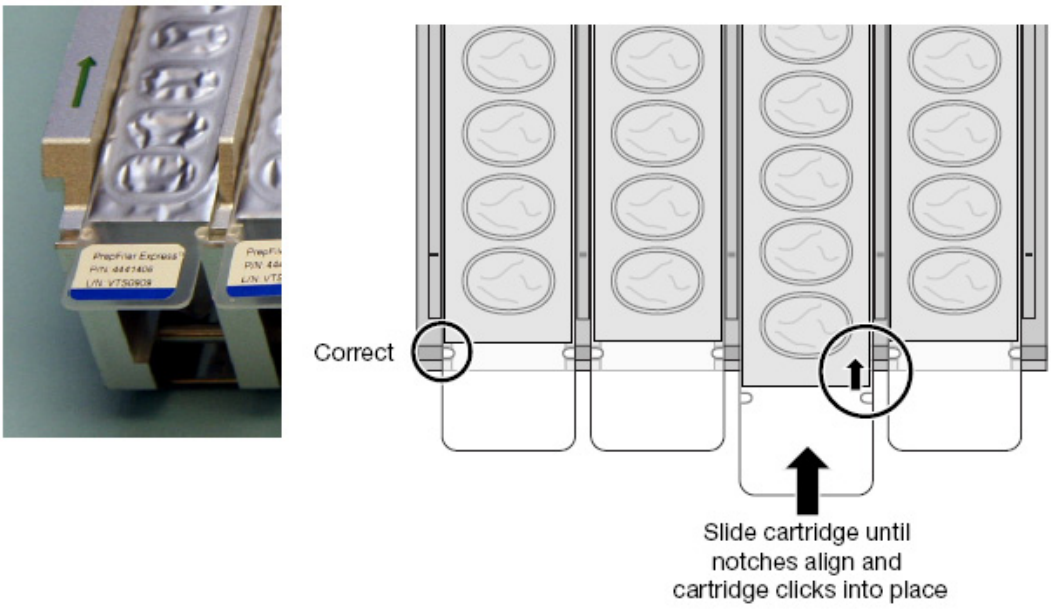
1. Open the instrument door (push up the door), then remove the tip and tube rack and the cartridge rack:



2. Gently tap each cartridge on the laboratory bench until any liquid droplets underneath the foil seal fall into the bottom of the wells.

3. Load the reagent cartridges into the cartridge rack by sliding each reagent cartridge along the groove in the direction of the arrow until the reagent cartridge clicks into place. Ensure that the notches in the cartridge align with the notches in the cartridge rack.

   Note: An incorrectly loaded cartridge rack may cause the instrument to stop during a run.



4. Pulse-spin a thawed tube of the 2X RNA Ligase Buffer.

5. Remove and discard the cap from the tube of 2X RNA Ligase Buffer. Place the tube into position 11 of the cartridge. Ensure that the tube is fully seated in the cartridge.

6. Carefully remove the seal covering *only* position 12 of the cartridge. Mix the diluted Nucleic Acid Binding Beads thoroughly, and add 40 µL to the well in position 12.

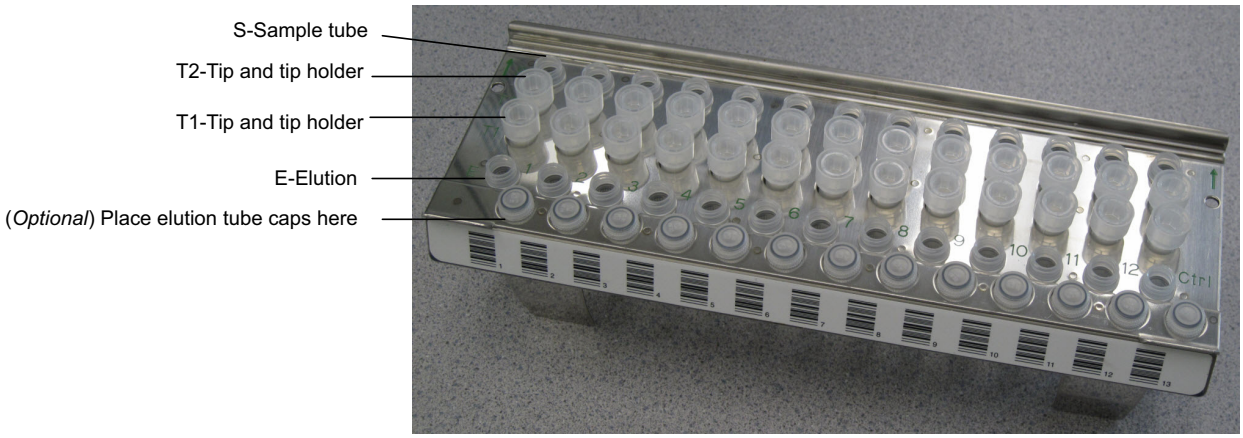
IMPORTANT! Do not pierce the seals covering any other positions in the cartridge. These seals are automatically pierced by the AB Library Builder™ System.

7. Load the tip and tube rack in the following order:

IMPORTANT! If you are processing fewer than 13 samples, load the tips and tubes in the same positions as the reagent cartridges in the cartridge rack.

   a. **Row S** (fourth row): Add 5 µL of the fragmented RNA to an empty tube with the cap removed and secured.
   b. **Rows T1 and T2** (second and third rows): Load with AB Library Builder™ Tips inserted into tip holders.

      Note: Two sets of tips and tip holders are required per sample.
   c. **Row E** (first row): Load with *labeled* sample/elution tubes, with the caps removed and secured. This tube will contain the final library at the end of the run:



Insert the racks into the AB Library Builder™ Device

IMPORTANT!

- Insert the cartridge rack first before inserting the tip and tube rack. Loading the racks in a different order can cause the instrument to stop during a run.
- Use only AB Library Builder™ Sample Tubes (sample/elution tubes). Other tubes may be picked up by the nozzle tips due to differences in tube height and shape, stopping the run.

1. Insert the loaded cartridge rack into the instrument.

WARNING! Do not touch the surface of the heat block. The temperature of the heat block can reach 95°C. Touching the block can cause burns.

2. Insert the loaded tip and tube rack into the instrument with row E in the front.

Start the run

1. Press **START** to select the AB Library Builder™ System Kit option.
2. Confirm that you have loaded and inserted the cartridge rack and tip and tube rack correctly.
3. Select Option 1 for WT (whole transcriptome protocol), then follow the on-screen prompts.
4. (*Optional*) Scan the sample, elution tube, and sample lane barcodes [refer to the *AB Library Builder™ System User Guide* (Pub. no. 4463421)].
5. Close the door to the AB Library Builder™ Device.
6. Press **START**.

   The screen shows the current step and the approximate incubation time remaining.

   IMPORTANT! Do not open the door during a protocol run. To pause or cancel the run, see *AB Library Builder™ System User Guide* (Pub. no. 4463421).

   Note: If you lose power or the power cord is unplugged, the run stops. When the power resumes, the digital display shows the Main menu. You cannot resume the run. If the tips are still on the syringe unit when the power resumes, return the tips to the original positions as described in *AB Library Builder™ System User Guide* (Pub. no. 4463421).

7. At the end of the run, the instrument beeps briefly and the digital display shows "Finished Protocol". To unload the instrument:
   a. Press  to return to the Main menu, then open the instrument door.
   b. Remove the elution tubes, confirm that they are properly labeled, then cap the elution tubes. The tubes will contain ~20–25 µL.

      Note: Libraries may have a brown tint, which may affect subsequent PCR steps. If desired, place each tube in a DynaMag™-2 magnetic rack for at least 1 minute until the solution is clear of brown tint when viewed at an angle, then transfer the supernatant to a new tube.

   c. Remove the tip and tube rack and cartridge rack.
   d. Properly dispose of the used reagent cartridges, tips, and tubes.

e. Close the instrument door.

f. Clean the tip and tube rack as needed.

Note: No cooling period is required between runs.

STOPPING POINT Store the cDNA in a supplied Sample Tube at 2°C to 8°C for short-term storage or at –30°C to –10°C for long-term storage. Otherwise, proceed directly to "Amplify the cDNA".

Set up for a new run

WARNING! If the instrument is used with any iPrep™, PrepFiler® Express, PrepFiler® Express BTA, or PrepSEQ® Express cartridges, do not clean the instrument with acids, or bases (such as bleach). Acids and bases can react with the guanidine thiocyanate in the lysis buffer and generate toxic gas.

1. Follow the set-up procedures for a new run (see "Set up the AB Library Builder™ System" on page 23).

   Note: To set up for a new run using the same protocol card, leave the instrument ON. To set up for a new run with a different protocol card, power OFF the instrument, then change the protocol card (see "Insert or change the protocol card and power ON the instrument" on page 24).

2. Start the run (see "Start the run" on page 29).

Amplify the cDNA

To prepare non-barcoded libraries, use the following components from the Ion Total RNA-Seq Kit for the AB Library Builder™ System:

- Ion RNA 5′ PCR Primer
- Ion RNA 3′ PCR Primer
- 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. Remove and cap the tube containing the cDNA library from position E of the tube and tip rack from the AB Library Builder System.

2. For each cDNA sample, prepare the PCR mix, according to the non-barcoded or barcoded library tables.

Non-barcoded Library	
Component	**Volume for one reaction†**
Platinum® PCR SuperMix High Fidelity‡	45 µL
Ion RNA 5′ PCR Primer	1 µL
Ion RNA 3′ PCR Primer	1 µL
Total volume	**47 µL**

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

‡ Platinum® PCR SuperMix High Fidelity contains a proofreading enzyme for high-fidelity amplification.

a. Transfer 10 µL of cDNA sample to a new PCR tube. Store the remaining 10 µL of library at –30°C to –10°C for future use, if needed.

b. Transfer 47 µL of the PCR mix to each 10 µL of cDNA sample.

c. Proceed to step 3.

Barcoded Library	
Component	**Volume for one reaction†**
Platinum® PCR SuperMix High Fidelity‡	45 µL
Ion Xpress™ RNA 3′ Barcode Primer	1 µL
Total volume	**46 µL**

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

‡ Platinum® PCR SuperMix High Fidelity contains a proofreading enzyme for high-fidelity amplification.

a. Transfer 10 µL of cDNA sample to a new PCR tube.

b. Transfer 46 µL of the PCR mix to each 10 µL of cDNA sample.

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

Note: Handle barcode primer stocks carefully to avoid cross-contamination.

d. Proceed to step 3.

3. 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.

4. Run the PCRs 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 (14 cycles)	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
- (*Optional*) Processing Plate
- Nuclease-free Water

Note: 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

- Ethanol, 100%, ACS reagent grade or equivalent
- 37°C heat block or water bath
- Magnetic rack or stand
- Non-Stick RNAse-Free Microfuge Tubes (1.5-mL)
- Pipettors or multi-channel pipettors, positive-displacement or air-displacement
- (*Optional*) MicroAmp® Clear Adhesive Film

Before you begin

- If you have not done so already, 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 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 (Life Technologies, Cat. no. 4306311). You can also skip a row between sample rows.

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

1. Gently vortex the Nucleic Acid Binding Beads tube, then add 10 µL of the beads to 180 µL Binding Solution Concentrate per sample in a 1.5-mL RNAse-free tube or the Processing Plate provided. Mix the beads and the Concentrate by pipetting up and down 10 times.
2. Transfer the 53-µL PCR reaction to the beads in the tube or Processing Plate.
3. Set a P200 pipettor at 130 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the new 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times. Without changing tips, add 130 µL of 100% ethanol to each sample.

   IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the tube or well wall. Change the tip and repeat step 3 for the remaining samples only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for best results.

4. 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 should be homogeneous after mixing.

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

6. Place the tube or Processing Plate in a magnetic rack or stand for 5–6 minutes to clear the solution. Without removing the tube or plate from the magnet, carefully remove and discard the supernatant without disturbing the pellet.

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

7. Add 150 µL of Wash Solution Concentrate with ethanol to each sample on the magnet and incubate for 30 seconds. After the solution clears, carefully remove and discard the supernatant without disturbing the pellet.
8. Carefully remove any remaining supernatant with a P10 or P20 pipettor without disturbing the pellet.

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

9. Keeping the tube or plate on the magnet, air-dry the beads at room temperature for 1–2 minutes.

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

10. Remove the tube or plate from the magnetic rack, and add 15 µL of pre-warmed (37°C) Nuclease-free Water. Mix the Nuclease-free Water and beads by pipetting up and down 10 times.
11. Incubate the tube or plate at room temperature for 1 minute, then place the tube or plate on the magnet for an additional minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL RNAse-free tube without disturbing the pellet.

    IMPORTANT! The solution contains the purified DNA. Do not discard!

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.

Molar concentration of the cDNA libraries should be used for "Pool barcoded whole transcriptome libraries" on page 34 and "Determine the library dilution required for template preparation" on page 35.

Note: For instructions on how to perform the smear analysis, see "Perform a smear analysis" on page 76, and refer to the *Agilent® 2100 Bioanalyzer® Expert User's Guide* (Pub. no. G2946-90000).

Next steps:

If the percent of DNA in the range is...	Then...
<50%	Proceed to "Determine the library dilution required for template preparation" on page 35 or "Pool barcoded whole transcriptome libraries".
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 31. *or* Proceed to "Determine the library dilution required for template preparation" on page 35 or "Pool barcoded whole transcriptome libraries", 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 31.

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" on page 35.

1. Determine the molar concentration (nM) of each of the barcoded cDNA libraries 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 necessary, adjust the range to include all the library peaks.

2. Dilute each barcoded cDNA library to the same molar concentration (nM). For example, if you have 3 different barcoded libraries that are 45, 55, 65 nM, choose 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 the library to 30 nM.

3. Mix an equal volume of each diluted library to prepare a pool of the barcoded libraries.

4. 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 library dilution factor. You can also 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 the amplified DNA" on page 33 and "Using 2100 expert software to assess whole transcriptome libraries" on page 76).

Determine the library dilution required for template preparation

With less than 50% of the amplified DNA in the correct range, you can proceed to the template preparation procedure (see "Proceed to template preparation") to prepare templated Ion Sphere™ Particles for sequencing on the Ion PGM™ System or the Ion Proton™ System.

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 for sequencing on the Ion PGM™ System or the Ion Proton™ System. For instructions, refer to the specific user guide for an appropriate Ion template preparation kit.

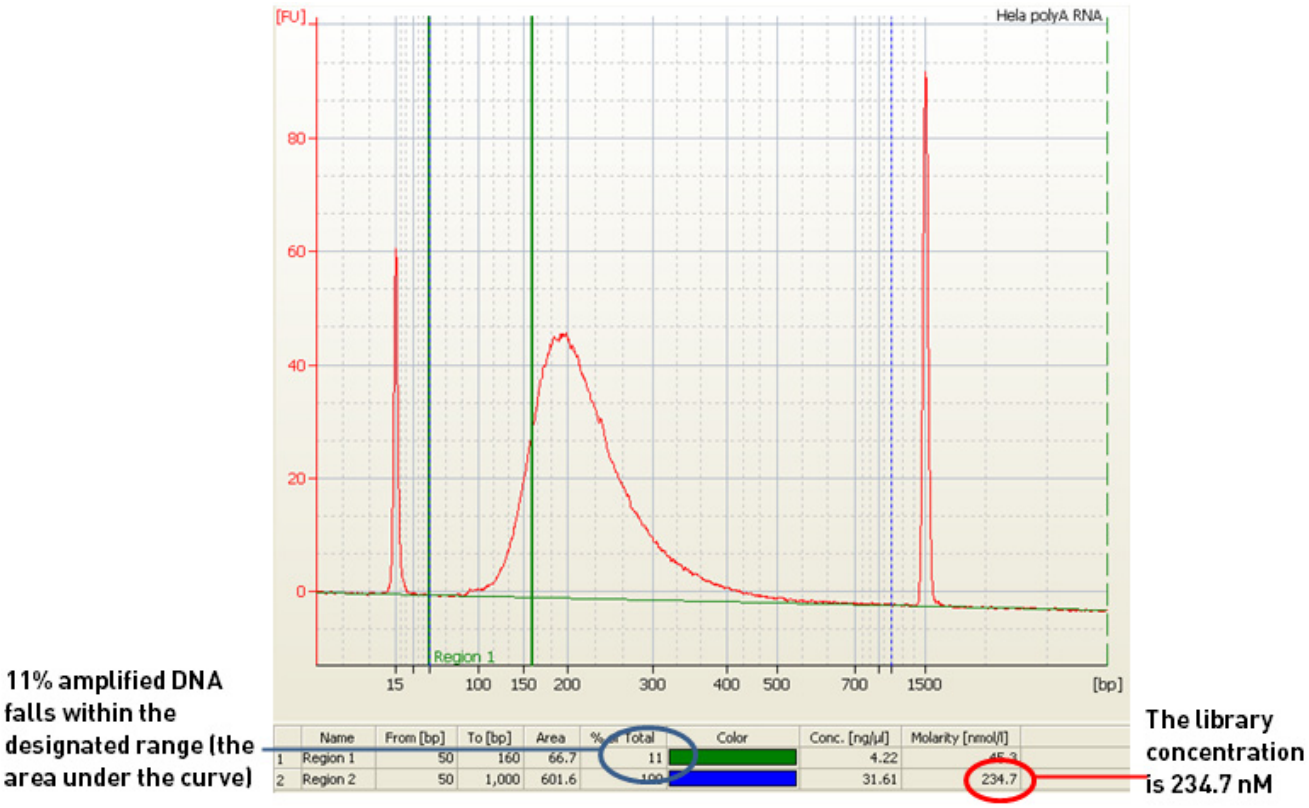
Template preparation documentation is available on the Ion Community at **http://ioncommunity.lifetechnologies.com/**. Follow the links under **Protocols** > **Prepare Template** > **Prepare Template User Guides and Quick Reference**.

Size distributions and yields

Typical size distributions

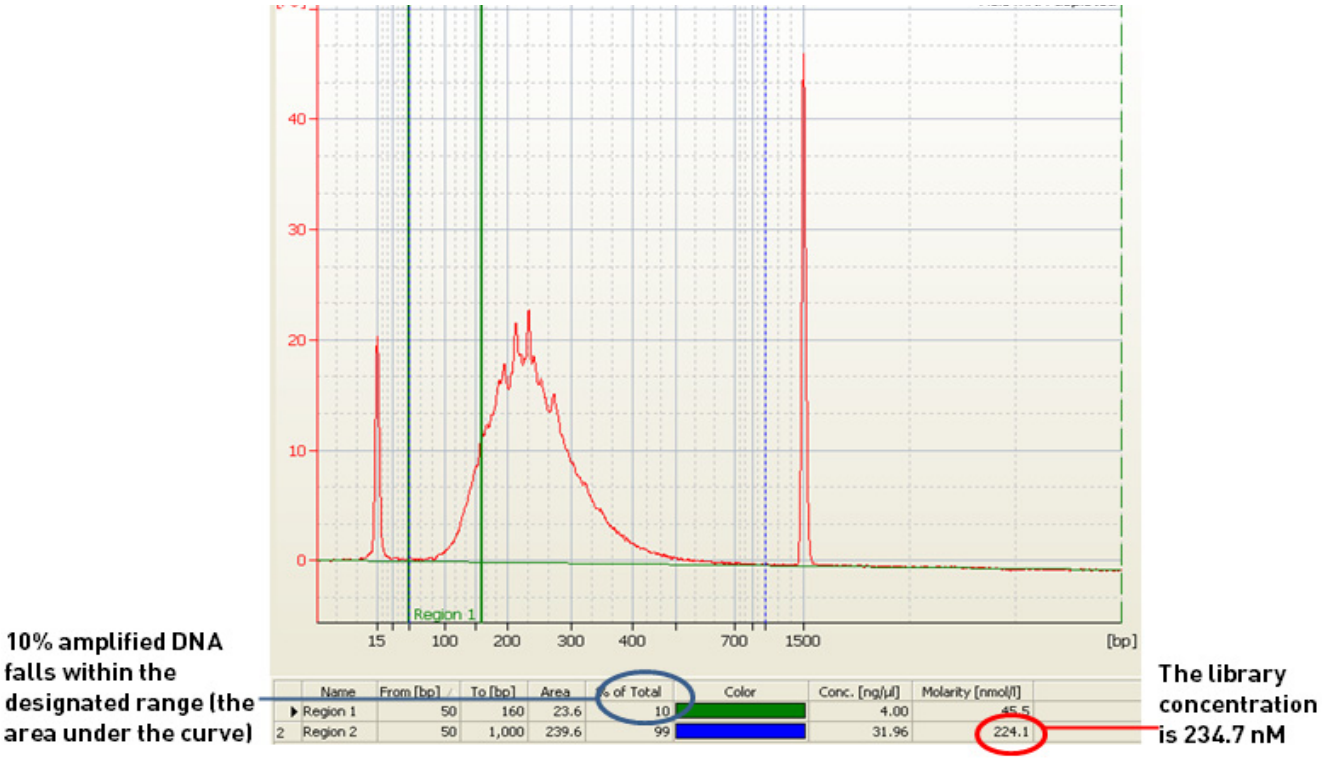
The highest quality libraries have less than 50% amplified DNA between 25–160 bp:

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



	Name	From [bp]	To [bp]	Area	% of Total	Color	Conc. [ng/µl]	Molarity [nmol/l]
1	Region 1	50	160	66.7	11		4.22	45.3
2	Region 2	50	1,000	601.6	100		31.61	234.7

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



Expected yields

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

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 (page 17)	500 ng of poly(A) RNA, total RNA, or rRNA-depleted total RNA	300–400 ng of RNA
Construct the library using the Ion Total RNA-Seq Kit for the AB Library Builder™ System (page 22)	<1–100 ng of fragmented RNA	>5 ng of cDNA

Troubleshooting

Troubleshooting library preparation

Observation	Possible Cause	Solution
The Agilent® software does not calculate one concentration and peak size	The software detects multiple peaks in the amplified cDNA profile	Refer to "Analyze multiple peaks as one peak" on page 77.
Low yield and poor size distribution in the amplified library	You recovered <20% of the input RNA after you fragmented and cleaned up the RNA	Decrease the RNase III digestion from 10 minutes to 5 minutes (step 3 on page 17).
Low yield in the amplified library and very few differences in the Agilent® 2100 Bioanalyzer® instrument traces before and after you fragment the RNA	RNA fragmentation failed	Purify the RNA sample again to remove the extra salts that may affect the RNase III activity. If RNA fragmentation still fails, increase the RNase III digestion from 10 minutes to 20 minutes (step 3 on page 17).
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.

Troubleshooting the AB Library Builder™ System

Observation	Possible Cause	Recommended Action
Before loading the cartridges in the cartridge rack		
Precipitate in RNA Ligase Buffer tubes	Buffer was exposed to low temperatures during shipping or storage.	To dissolve precipitate that may have formed during shipping or storage, incubate the tubes at 37°C for 5 minutes or until precipitate is no longer visible.
During the automated run		
No power (the digital display is blank and the fan does not turn on when you power ON)	AC power cord is not connected.	Check AC power cord connections at both ends. Use the correct cords.
	Fuse has blown.	Check the integrity of the fuse and replace it if necessary (refer to the *AB Library Builder™ System User Guide*, Part no. 4463421). If the problem persists after connecting the correct power cord and replacing the fuse, contact Technical Support (see page 87).
The digital display is blank, but the fan turns on when you power ON	Protocol card is not inserted correctly.	Power off the instrument and re-insert the protocol card in the proper orientation into the card slot (see page 25). Insert it completely into the slot by manually pushing the card.
	Protocol card was inserted when the instrument was powered ON.	Power off the instrument, then power on the instrument.
Error code displayed	—	See "Instrument error codes" on page 41.

Observation	Possible Cause	Recommended Action
Reagent cartridges, tips, or tubes are not inserted in the correct positions	—	Press **STOP** to pause the run. Open the door, add the missing items, then press **START** to resume the run. Do not open the door without pausing the run.
Run stops after an initial start (you may also see an error code)	• Instrument door opened during the run. • Reagent cartridges, tips, or tubes incorrectly loaded in the rack. • Racks incorrectly loaded on the instrument.	**IMPORTANT!** If you open the instrument door while the instrument is running, the run stops, and it cannot be restarted. If you need to open the instrument door during a run, first press **Stop** to pause the run, then open the door. 1. Follow the procedure in "Instrument error codes" on page 41. 2. Before starting a new run, make sure that the reagent cartridges, tips, and tubes are correctly loaded: • Slide the reagent cartridges into the cartridge rack as described on page 26. • Load the cartridge rack before the tip and tube rack for proper positioning. • Do not cap the tubes. 3. If the instrument continues to stop during the run, contact Technical Support.
	Reagent cartridges not completely thawed.	1. Stop the run. 2. Remove the tip and tube rack, then remove the cartridge rack. 3. Inspect cartridge wells 2 and 3 for ice. 4. If any well is frozen, close the door to the AB Library Builder™ Device, then thaw the cartridges completely. 5. Replace the tips in position T2. 6. Insert the cartridge rack then the tip and tube rack onto the AB Library Builder™ Device. 7. Restart the run.
No DNA yield	No sample added to tube	Add samples to tubes, load new reagent cartridges, then perform the run again.
No liquid in tip, or liquid in tip not moving	No sample added to tube, leading to wet filter barrier on the tip and blockage of nozzles.	Add samples to tubes, load new reagent cartridges, then perform the run again.
Buffer in the bottom tray	Motor movements are not smooth.	Schedule preventive maintenance annually to ensure proper motor movements.
	Reagent cartridges, tips, or tubes incorrectly loaded in the rack.	If you are processing fewer than 13 samples, make sure to load the tips and tubes in the same positions as the reagent cartridges that are loaded in the cartridge rack. See next row for recommended action when experiencing leakage from tips.
Leakage from tips or uneven liquid handling between nozzles	D-Rings are not greased regularly or they need replacement.	You can continue the run, but maintain the D-rings as scheduled. To prevent leakage, maintain or replace the D-rings (refer to the (*AB Library Builder™ System User Guide*).

Observation	Possible Cause	Recommended Action
Blockage of tips	Too much starting material causing clumps or aggregates.	Contact Technical Support (see page 87). In future runs, use the sample volume recommended in the user guide for the kit you are using.
After the automated run		
No elution volume	Sample volume is lower than the recommended volume, leading to wet filter barrier on the tip and blockage of nozzles.	In future runs, use the recommended sample volume for the protocol you are using. Long-term operation with lower-than-recommended sample volumes can lead to issues with liquid handling performance.
No amplifiable library	Enzymes or buffer not at bottom of wells.	Tap the wells down against a hard surface to move enzymes and buffer to bottom of wells, then inspect the wells.
Final library is brownish	Beads in final library.	1. Place the tube with the final library in a DynaMag™-2 magnetic rack for at least 1 minute until the solution is clear of brown tint when viewed at an angle. 2. Without disturbing the pellet, carefully transfer the **supernatant**, which contains the final library, to a new 1.5-mL Eppendorf LoBind® Tube.

Instrument error codes

If an extraction run is interrupted by an error, you cannot resume the interrupted run. Follow the following procedure to resolve the error before you start a new run.

If you observe an error code:

1. Make a note of the error code, including the line number. Common error codes are listed in the following table:

Code	Problem	Code	Problem
10	Failed return to origins, protocol cannot run	21	P axis time out, protocol in run
11	Limit error, protocol can not run	22	M axis time out, protocol in run
12	Failed to return to Z Axis, protocol in run	23	Y axis time out, protocol in run
13	Failed to return to P axis, protocol in run	24	Open door in motion
14	Failed to return to M axis, protocol in run	25	Abnormal input from bottom sensor in motion
15	Failed to return to Y axis, protocol in run	26	Failed to initialize heating block
16	Z axis limit error, protocol in run	27	Failed to initialize motion control board
19	Y axis end limit, protocol in run	110	System error; (Assigned greater than 10)
20	Z axis time out, protocol in run		

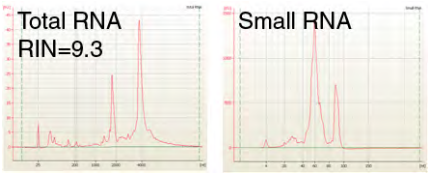
2. Press **ESC** to return to the Main menu.
3. If there are tips attached to the nozzles, press **1** to select the Manual screen, then press **2** to return the tips to the original position.
4. Power OFF the instrument, remove the protocol card, wait 5 minutes, insert the protocol card, then power ON the instrument.
5. Run the axis test (refer to the *AB Library Builder™ System User Guide*, Pub. no. 4463421).
6. If the axis test:
   - Is successful, start a new extraction run. Use new samples and plastics where required.
   - Is *not* successful, contact Technical Support (see page 87).

3 Prepare small RNA libraries

Workflow

Prepare the starting material

Assess the amount and quality of small RNA in your total RNA samples (page 45)



↓

Enrich the samples for small RNA (page 47)

↓

Assess the quality and quantity of the small RNA-enriched sample (page 50)

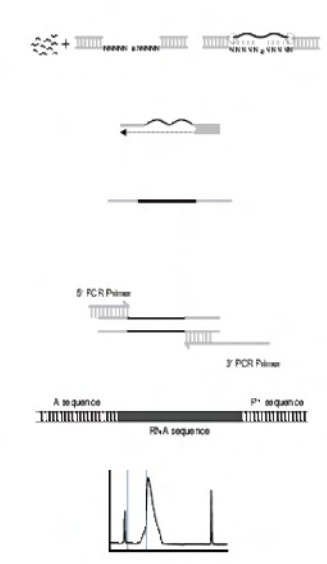
↓

Determine the input amount (page 50)

↓

Construct the library using the Ion Total RNA-Seq Kit for the AB Library Builder™ System

Set up the AB Library Builder™ System (page 51)



↓

Insert the racks into the AB Library Builder™ Device (page 56)

↓

Start the run (page 58)

↓

Set up for a new run (page 59)

↓

Amplify the cDNA (page 59)

↓

Purify and size-select the amplified cDNA (page 61)

↓

Assess the yield and size distribution of the amplified DNA (page 63)

↓

Prepare templated Ion Sphere™ particles for sequencing on the Ion PGM™ System or the Ion Proton™ System

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

Procedural guidelines

- Use good laboratory practices (GLP) to minimize cross-contamination of products. If possible, prepare libraries in a separate location from template preparation.
- When handling barcoded primers, do not cross-contaminate. Change gloves frequently and open one tube at a time.
- Perform all steps requiring 0.5-mL and 1.5-mL tubes with Ambion® Non-Stick RNase-free Microfuge Tubes (Cat. nos. AM12350 and AM12450).
- When preparing to use reagents:
  - Store enzymes at –30°C to –10°C before use.
  - Thaw all reagents on ice or at room temperature before use.
  - Keep thawed reagents on ice when not in use.
- Mix reagents thoroughly before use, especially if reagents were frozen and thawed.

Prepare the starting material

Preparing the starting material involves the following procedures:

Assess the amount and quality of small RNA in your total RNA samples (page 45)

⬇

Enrich the samples for small RNA (page 47)

⬇

Assess the quality and quantity of the small RNA-enriched sample (page 50)

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 mirVana™ miRNA Isolation Kit or the mirVana™ PARIS™ 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.

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

   Note: If you used the mirVana™ miRNA Isolation Kit, the mirVana™ PARIS™ 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 50.

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

   Note: For instructions on using the software, refer to the *Agilent® 2100 Bioanalyzer® Expert User's Guide* (Pub. no. G2946-9000).

   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, and 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 Kit chip. Follow the manufacturer's instructions for performing the assay.

   b. Using the 2100 expert software, determine the mass of small RNA (miRNA; 10–40 nt) from the Small RNA Kit chip.

   c. Calculate the miRNA content in your RNA sample using the formula:

   $$\%\ miRNA = (mass\ of\ miRNA \div mass\ of\ total\ RNA) \times 100$$

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

How much miRNA (10–40 nt) is in your RNA sample?	Recommendations for small RNA enrichment and next steps
≥0.5% miRNA	You can use the total RNA in the ligation reaction, and small RNA enrichment is not needed. However, for optimal results, we recommend enrichment of all total RNA samples. You can expect to see 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 "Enrich the samples for small RNA" or skip to "Determine the input amount" on page 50.

How much miRNA (10–40 nt) is in your RNA sample?	Recommendations for small RNA enrichment and next steps
<0.5% miRNA	Small RNA enrichment is strongly recommended. We recommend using the Magnetic Bead Cleanup Module for small RNA enrichment. Proceed to "Enrich the samples for small RNA".

Enrich the samples for small RNA

Note: We recommend enriching all total RNA samples for small RNA for optimal results. However, if the tissue or cell lines contain sufficient small RNA to allow efficient library preparation, skip to "Assess the quality and quantity of the small RNA-enriched sample" on page 50.

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.

If the tissues or cell lines that you are using contain a small fraction of small RNA, we recommend enrichment of these RNA samples for small RNA. Use an Agilent® Small RNA chip to measure the concentration of miRNA (10-40 nts) in your total RNA or enriched small RNA sample.

This protocol uses magnetic beads to enrich for small RNA. Use the Magnetic Bead Cleanup Module twice with the same sample to size-select the desired small RNA products. 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-warmed (80°C) Nuclease-free Water.

Required materials from the Magnetic Bead Cleanup Module

- Wash Solution Concentrate
- Binding Solution Concentrate
- Nucleic Acid Binding Beads
- (*Optional*) Processing Plate
- Nuclease-free Water

Note: 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

- Ethanol, 100%, ACS reagent grade or equivalent
- Magnetic rack or stand
- 80°C heat block or water bath
- Non-Stick RNAse-free Microfuge Tubes (1.5-mL)
- Pipettors or multi-channel pipettors, positive-displacement or air-displacement
- (*Optional*) MicroAmp® Clear Adhesive Film

Before you begin

- If you have not done so already, add 44 mL of 100% ethanol to bottle of 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 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 (Life Technologies, 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.

Remove larger RNA from total RNA

1. Gently vortex the Nucleic Acid Binding Beads tube, then add 7 µL of the beads to 120 µL Binding Solution Concentrate per sample in a 1.5-mL RNAse-free tube or the Processing Plate provided. Mix the beads with the Concentrate by pipetting up and down 10 times.
2. Resuspend 0.5–20 µg of total RNA in 75 µL Nuclease-free Water, then transfer the RNA to the beads in the tube or Processing Plate.
3. Set a P200 pipettor at 105 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the new 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times. Without changing tips, add 105 µL of 100% ethanol to each sample.

   IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the tube or well wall. Change the tip and repeat step 3 for the remaining samples only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for size-selection. Follow the instructions exactly for best results.

4. 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 should be homogeneous after mixing.

5. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.
6. Place the tube or Processing Plate in a magnetic rack or stand for 5–6 minutes to clear the solution. Without removing the tube or plate from the magnet, transfer the supernatant to a new tube or well.

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

Bind and elute small RNA

1. Remove the tube or plate from the magnetic rack, and add 30 µL of Nuclease-free Water to the supernatant containing the small RNA.
2. 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. Without changing tips, add 570 µL of 100% ethanol to each sample.

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

3. Gently vortex the Nucleic Acid Binding Beads tube, then add 7 µL of the beads to each sample.
4. Set a P200 pipettor at 150 µL. Attach new 200-µL tips to the pipette, then mix the suspension in each well thoroughly by pipetting the wells up and down 10 times.

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

5. Incubate the samples for 5 minutes at room temperature.
6. Place the tube or Processing Plate in a magnetic rack or stand for 5–6 minutes to clear the solution. Without removing the tube or plate from the magnet, carefully remove and discard the supernatant without disturbing the pellet.

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

7. Add 150 µL of Wash Solution Concentrate with ethanol to each sample on the magnet and incubate for 30 seconds. After the solution clears, carefully remove and discard the supernatant without disturbing the pellet.
8. Carefully remove any remaining supernatant with a P10 or P20 pipettor without disturbing the pellet.

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

9. Keeping the tube or plate on the magnet, air-dry the beads at room temperature for 1–2 minutes.

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

10. Remove the tube or plate from the magnetic rack, and add 30 µL of pre-warmed (80°C) Nuclease-free Water. Mix the Nuclease-free Water and beads by pipetting up and down 10 times.

11. Incubate the tube or plate at room temperature for 1 minute, then place the tube or plate on the magnet for an additional minute until the solution clears. Transfer the supernatant containing the eluted RNA to a new 1.5-mL RNAse-free tube without disturbing the pellet.

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 45), 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†
Total RNA	5–100 ng	≤1 µg
Enriched small RNA	5–100 ng	≤1 µg

† 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 could be over-estimated on the Agilent® Small RNA chip, so more input into ligation is recommended. Ideally, use >25 ng of miRNA.

Construct the library using the Ion Total RNA-Seq Kit for the AB Library Builder™ System

Constructing the small RNA library involves the following procedures:

Set up the AB Library Builder™ System (page 51)

⬇

Insert the racks into the AB Library Builder™ Device (page 56)

⬇

Start the run (page 58)

⬇

Amplify the cDNA (page 59)

⬇

Purify and size-select the amplified cDNA (page 61)

⬇

Assess the yield and size distribution of the amplified DNA (page 63)

Guidelines for using the AB Library Builder™ System

To install and set up the AB Library Builder™ System, see the *AB Library Builder™ System Site Preparation Guide* (Pub. no. 4465106) and the *AB Library Builder™ System User Guide* (Pub. no. 4463421).

IMPORTANT! To avoid data loss or run cancellation, always follow these practices:

- Before you insert or remove a protocol card, power off the instrument.
- Before you power on the instrument, insert the protocol card, then close the instrument door.
- To pause the instrument during a run, press **Stop** before you open the instrument door.
- When you are not performing a run or instrument test, you can open the instrument door with the power off or on.
- Do not move instrument components such as the platform, magnets, and syringes while the instrument is powered on.

Set up the AB Library Builder™ System

Required materials from the Ion Total RNA-Seq Kit for the AB Library Builder™ System

- Ion Total RNA-Seq Kit for the AB Library Builder™ System Cartridge
- 2X RNA Ligase Buffer (from the RNA Fragmentation and Ligation Reagents for the ABLB™ System)

- Nucleic Acid Binding Beads (from the Magnetic Bead Cleanup Module)
- Sample/elution tubes, tips, and tip holders (from the AB Library Builder™ Plastics Module)

Other materials and equipment

- Ion Total RNA-Seq Protocol Card for the AB Library Builder™ System
- AB Library Builder™ System and accessories
- Pipettors and pipette tips

Before you begin

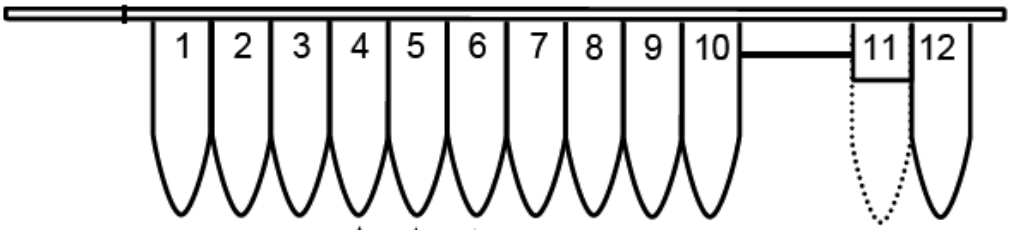
- Completely thaw an appropriate number of Ion Total RNA-Seq for the AB Library Builder™ System Cartridges for ≤2 hours at room temperature, 2°C to 8°C, or on ice. One cartridge is required per sample. Thawed cartridges do not require additional mixing.

  IMPORTANT! Avoid leaving the cartridges at room temperature for longer than necessary to completely thaw them. Avoid repeated freeze-thawing of unused cartridges.

- Thaw one tube of 2X RNA Ligase Buffer for each cartridge to be used. If you see a white precipitate in the Ligase Buffer, warm the solution at 37°C to resuspend the precipitate.
- Gently but thoroughly vortex the Nucleic Acid Binding Beads. In a separate tube, dilute the beads 1:2 into a sufficient volume of Nuclease-free Water to yield 60 µL of diluted beads per cartridge. For example, for a run with one cartridge, add 30 µL of Nucleic Acid Binding Beads to 30 µL of Nuclease-free Water.

  IMPORTANT! Do not add water directly to the stock tube of Nucleic Acid Binding Beads. Diluted Nucleic Acid Binding Beads are required for the cartridge only. Undiluted Nucleic Acid Binding Beads are required for other steps in the protocol.

- Inspect the cartridges. Each cartridge has 12 compartments for reagents:



Cartridge Compartment Numbers	Quantity
1	800 µL
2	600 µL
3	1000 µL
4	20 µL
5	25 µL
6	20 µL
7	Empty
8	20 µL
9	700 µL
10	Empty
11	Empty (user to insert 45 µL tube of 2X RNA Ligase Buffer)
12	Empty (user to add 60 µL diluted Nucleic Acid Binding Beads)

IMPORTANT! Do not switch the supplied pre-filled reagents with any other buffers. The protocols are specifically optimized with the reagents supplied with the kit.

Insert or change the protocol card and power ON the instrument

IMPORTANT! Do not remove the protocol card while the instrument is on. Removing the card stops the run, which may cause instrument data file loss. To remove the card, see step 3 on page 54.

If you accidentally remove the protocol card during a run, power off the instrument immediately to minimize potential for instrument data loss.

For guidelines on handling protocol cards, see the *AB Library Builder™ System User Guide* (Part no. 4463421).

1. Confirm that the power switch is in the OFF position.

   Note: If you insert the card while the instrument is on, the instrument does not recognize the card.

2. Open the card slot.

3. To remove a card that is already installed in the slot, push the button located below the card slot (see the following photo), then pull the card out of the slot. Place the card in the plastic cover in the box.

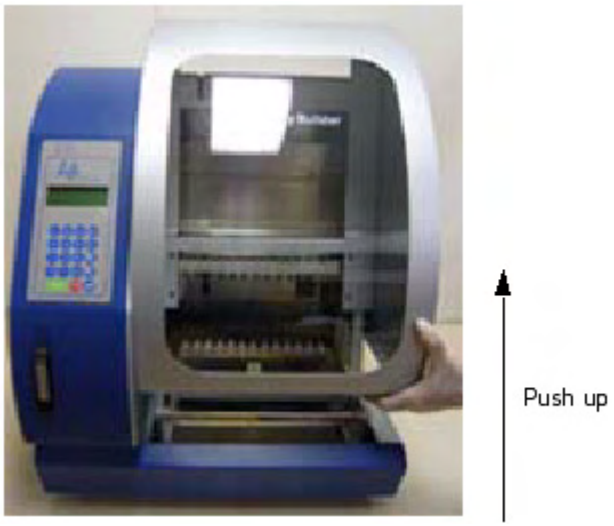
IMPORTANT! Do not remove the protocol card while the instrument is on.

4. Insert the appropriate protocol card in the slot with the arrow on the protocol card pointing toward the instrument and the label facing left.
5. Push the card completely into the card slot, then close the card slot.
6. Close the door to the AB Library Builder™ Device.
7. Power on the instrument.

   When the card is fully inserted in the correct orientation, the display briefly shows information including the instrument and protocol card version, then displays the Main menu.
8. Press **START.**

Load the racks and tubes

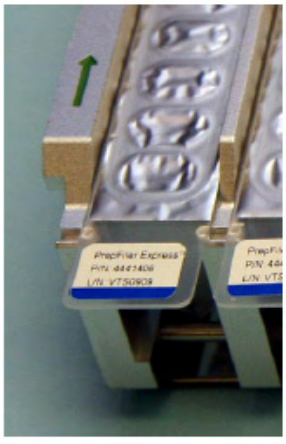
1. Open the instrument door (push up the door), then remove the tip and tube rack and the cartridge rack:

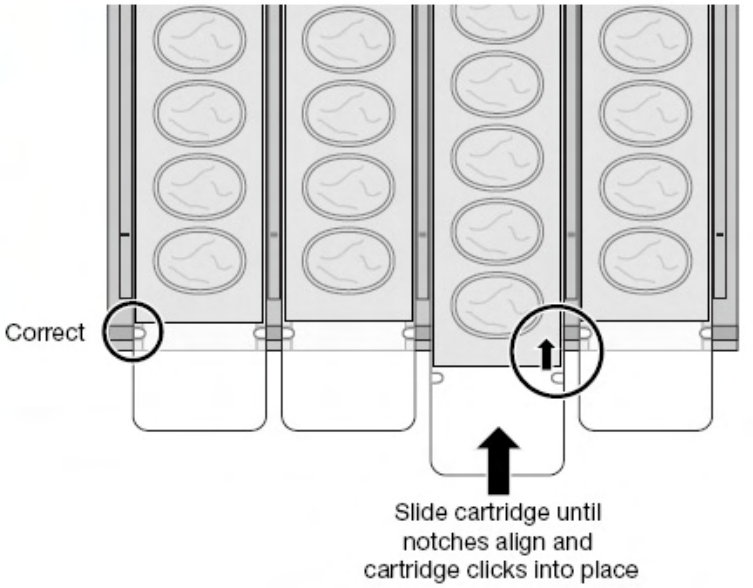


2. Gently tap each cartridge on the laboratory bench until any liquid droplets underneath the foil seal fall into the bottom of the wells.

3. Load the reagent cartridges into the cartridge rack by sliding each reagent cartridge along the groove in the direction of the arrow until the reagent cartridge clicks into place. Ensure that the notches in the cartridge align with the notches in the cartridge rack.

   Note: An incorrectly loaded cartridge rack may cause the instrument to stop during a run.





4. Pulse-spin a thawed tube of the 2X RNA Ligase Buffer.

5. Remove and discard the cap from the tube of 2X RNA Ligase Buffer. Place the tube into position 11 of the cartridge. Ensure that the tube is fully seated in the cartridge.
6. Carefully remove the seal covering *only* position 12 of the cartridge. Mix the diluted Nucleic Acid Binding Beads thoroughly, and add 60 µL to the well in position 12.

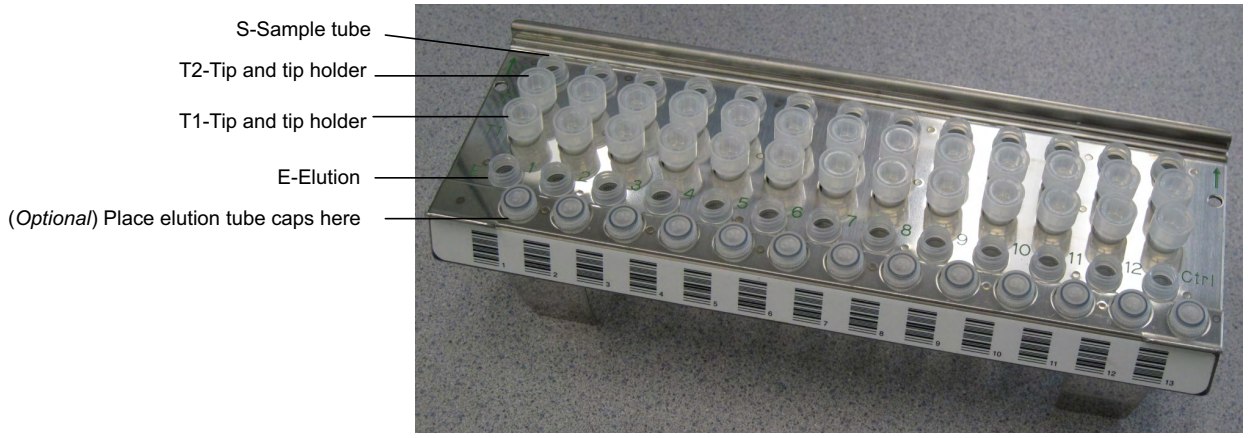
IMPORTANT! Do not pierce the seals covering any other positions in the cartridge. These seals are automatically pierced by the AB Library Builder™ System.

7. Load the tip and tube rack in the following order:

IMPORTANT! If you are processing fewer than 13 samples, load the tips and tubes in the same positions as the reagent cartridges in the cartridge rack.

   a. **Row S** (fourth row): Add 5 µL of the fragmented RNA to an empty tube with the cap removed and secured.
   b. **Rows T1 and T2** (second and third rows): Load with AB Library Builder™ Tips inserted into tip holders.

      Note: Two sets of tips and tip holders are required per sample.
   c. **Row E** (first row): Load with *labeled* sample/elution tubes, with the caps removed and secured. This tube will contain the final library at the end of the run:



Insert the racks into the AB Library Builder™ Device

IMPORTANT!

- Insert the cartridge rack first before inserting the tip and tube rack. Loading the racks in a different order can cause the instrument to stop during a run.
- Use only AB Library Builder™ Sample Tubes (sample/elution tubes). Other tubes may be picked up by the nozzle tips due to differences in tube height and shape, stopping the run.

1. Insert the loaded cartridge rack into the instrument.

WARNING! Do not touch the surface of the heat block. The temperature of the heat block can reach 95°C. Touching the block can cause burns.

2. Insert the loaded tip and tube rack into the instrument with row E in the front.

Start the run

1. Press **START** to select the AB Library Builder™ System Kit option.
2. Confirm that you have loaded and inserted the cartridge rack and tip and tube rack correctly.
3. Select Option 2 for the small RNA protocol, then follow the on-screen prompts.
4. (*Optional*) Scan the sample, elution tube, and sample lane barcodes [refer to the *AB Library Builder™ System User Guide* (Pub. no. 4463421)].
5. Close the door to the AB Library Builder™ Device.
6. Press **START**.

   The screen shows the current step and the approximate incubation time remaining.

   IMPORTANT! Do not open the door during a protocol run. To pause or cancel the run, see *AB Library Builder™ System User Guide* (Pub. no. 4463421).

   Note: If you lose power or the power cord is unplugged, the run stops. When the power resumes, the digital display shows the Main menu. You cannot resume the run. If the tips are still on the syringe unit when the power resumes, return the tips to the original positions as described in *AB Library Builder™ System User Guide* (Pub. no. 4463421).

7. At the end of the run, the instrument beeps briefly and the digital display shows "Finished Protocol". To unload the instrument:
   a. Press  to return to the Main menu, then open the instrument door.
   b. Remove the elution tubes, confirm that they are properly labeled, then cap the elution tubes. The tubes will contain ~20–25 µL.

      Note: Libraries may have a brown tint, which may affect subsequent PCR steps. If desired, place each tube in a DynaMag™-2 magnetic rack for at least 1 minute until the solution is clear of brown tint when viewed at an angle, then transfer the supernatant to a new tube.

   c. Remove the tip and tube rack and cartridge rack.
   d. Properly dispose of the used reagent cartridges, tips, and tubes.

e. Close the instrument door.

f. Clean the tip and tube rack as needed.

Note: No cooling period is required between runs.

STOPPING POINT Store the cDNA in a supplied Sample Tube at 2°C to 8°C for short-term storage or at –30°C to –10°C for long-term storage. Otherwise, proceed directly to "Amplify the cDNA".

Set up for a new run

WARNING! If the instrument is used with any iPrep™, PrepFiler® Express, PrepFiler® Express BTA, or PrepSEQ® Express cartridges, do not clean the instrument with acids, or bases (such as bleach). Acids and bases can react with the guanidine thiocyanate in the lysis buffer and generate toxic gas.

1. Follow the set-up procedures for a new run (see "Set up the AB Library Builder™ System" on page 51).

   Note: To set up for a new run using the same protocol card, leave the instrument ON. To set up for a new run with a different protocol card, power OFF the instrument, then change the protocol card (see "Insert or change the protocol card and power ON the instrument" on page 53).

2. Start the run (see "Start the run" on page 58).

Amplify the cDNA

To prepare non-barcoded libraries, use the following components from the Ion Total RNA-Seq Kit for the AB Library Builder™ System:

- Ion RNA 5′ PCR Primer
- Ion RNA 3′ PCR Primer
- 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. Remove and cap the tube containing the cDNA library from position E of the tube and tip rack from the AB Library Builder System.
2. For each cDNA sample, prepare the PCR mix, according to the non-barcoded or barcoded library tables.

Non-barcoded library	
Component	**Volume for one reaction†**
Platinum® PCR SuperMix High Fidelity‡	45 µL
Ion RNA 5′ PCR Primer	1 µL
Ion RNA 3′ PCR Primer	1 µL
Total volume	**47 µL**

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

‡ Platinum® PCR SuperMix High Fidelity contains a proofreading enzyme for high-fidelity amplification.

a. Transfer 10 µL of cDNA sample to a new PCR tube. Store the remaining 10 µL of library at –30°C to –10°C for future use, if needed.

b. Transfer 47 µL of the PCR mix to each 10 µL of cDNA sample.

c. Proceed to step 3.

Barcoded library	
Component	**Volume for one reaction†**
Platinum® PCR SuperMix High Fidelity‡	45 µL
Ion Xpress™ RNA 3′ Barcode Primer	1 µL
Total volume	**46 µL**

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

‡ Platinum® PCR SuperMix High Fidelity contains a proofreading enzyme for high-fidelity amplification.

a. Transfer 10 µL of cDNA sample to a new PCR tube.

b. Transfer 46 µL of the PCR mix to each 10 µL of cDNA sample.

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

Note: Handle barcode primer stocks carefully to avoid cross-contamination.

d. Proceed to step 3.

3. 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.

4. Run the PCRs 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 (14 cycles)	94°C	30 sec
	62°C	30 sec
	68°C	30 sec
Hold	68°C	5 min

Purify and size-select the amplified cDNA

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
- (*Optional*) Processing Plate
- Nuclease-free Water

Note: 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

- Ethanol, 100%, ACS reagent grade or equivalent
- Magnetic rack or stand
- 80°C heat block or water bath
- Non-Stick RNAse-Free Microfuge Tubes (1.5-mL)
- Pipettors or multi-channel pipettors, positive-displacement or air-displacement
- (*Optional*) MicroAmp® Clear Adhesive Film

Before you begin

- If you have not done so already, add 44 mL of 100% ethanol to the bottle of 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 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 (Life Technologies, 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.

Remove larger amplified DNA from the cDNA

1. Gently vortex the Nucleic Acid Binding Beads tube, then add 7 µL of the beads to 140 µL Binding Solution Concentrate per sample in a 1.5-mL RNAse-free tube or the Processing Plate provided. Mix the beads with the Concentrate by pipetting up and down 10 times.

2. Transfer 53 µL of each PCR reaction to the beads in the tube or Processing Plate. Save the remaining liquid in the PCR reaction for troubleshooting if the library size selection does not perform as expected.
3. Set a P200 pipettor at 110 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the new 200-µL tip with 100% ethanol by pipetting the ethanol up and down 3 times. Without changing tips, add 110 µL of 100% ethanol to each sample.

   IMPORTANT! While dispensing the ethanol, do not force out the last drops. Remove the last drop by touching the drop to the tube or well wall. Change the tip and repeat step 3 for the remaining samples only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for size-selection. Follow the instructions exactly for best results.

4. 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. Incubate for 5 minutes at room temperature.

   Note: The color of the mixture should be homogeneous after mixing.

5. Place the tube or Processing Plate in a magnetic rack or stand for 5–6 minutes to clear the solution. Without removing the tube or plate from the magnet, transfer the supernatant to a new tube or well.

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

Bind and elute small cDNA products

1. Remove the tube or plate from the magnetic rack, and add 35 µL of Nuclease-free Water to each sample.
2. 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. Without changing tips, add 35 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 tube or well wall. Change the tip and repeat step 2 for the remaining samples only if the tip touches the wall. Accurate pipetting of 100% ethanol is critical for size-selection. Follow the instructions exactly for best results.

3. Gently vortex the Nucleic Acid Binding Beads tube, then add 7 µL of the beads to each sample. 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 should be homogeneous after mixing.

4. Incubate the samples for 5 minutes at room temperature off of the magnet.

5. Place the tube or Processing Plate in a magnetic rack or stand for 5–6 minutes to clear the solution. Without removing the tube or plate from the magnet, carefully remove and discard the supernatant without disturbing the pellet.

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

6. Add 150 µL of Wash Solution Concentrate with ethanol to each sample on the magnet and incubate for 30 seconds. After the solution clears, carefully remove and discard the supernatant without disturbing the pellet.

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

7. Carefully remove any remaining supernatant with a P10 or P20 pipettor without disturbing the pellet.
8. Keeping the tube or plate on the magnet, air-dry the beads at room temperature for 1–2 minutes.

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

9. Remove the tube or plate from the magnetic rack, and add 15 µL of pre-warmed (37°C) Nuclease-free Water. Mix the Nuclease-free Water and beads by pipetting up and down 10 times.
10. Incubate the tube or plate at room temperature for 1 minute, then place the tube or plate on the magnet for an additional minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL RNAse-free tube without disturbing the pellet.

    IMPORTANT! The solution contains the purified DNA. Do not discard!

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 of 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 mRNA 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" and "Determine the library dilution required for template preparation" on page 65.

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

Note: For instructions on how to perform the smear analysis, see "Perform a smear analysis" on page 76, and refer to the *Agilent® 2100 Bioanalyzer® Expert User's Guide* (Pub. no. G2946-90000).

Next steps

If the ratio is...	Then...
≥50%	Proceed to "Determine the library dilution required for template preparation" on page 65 or "Pool barcoded small RNA libraries".
<50%	Proceed to "Determine the library dilution required for template preparation" on page 65, or "Pool barcoded small RNA libraries" 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 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 of the barcoded cDNA libraries 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 necessary, adjust the range to include all of the library peaks.

2. Dilute each barcoded cDNA library to the same molar concentration (nM). For example, if you have 3 different barcoded libraries that are 45, 55, 65 nM, choose 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 the library to 30 nM.

3. Mix an equal volume of each diluted library to prepare a pool of the barcoded libraries.

4. 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 library dilution factor. You can also 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 the amplified DNA" on page 63 and "Using 2100 expert software to assess small RNA libraries" on page 80).

Determine the library dilution required for template preparation

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 for sequencing on the Ion PGM™ System or Ion Proton™ System. For instructions, refer to the specific user guide for an appropriate Ion template preparation kit.

Template preparation documentation is available on the Ion Community at **http://ioncommunity.lifetechnologies.com/**. Follow the links under **Protocols** > **Prepare Template** > **Prepare Template User Guides and Quick Reference**.

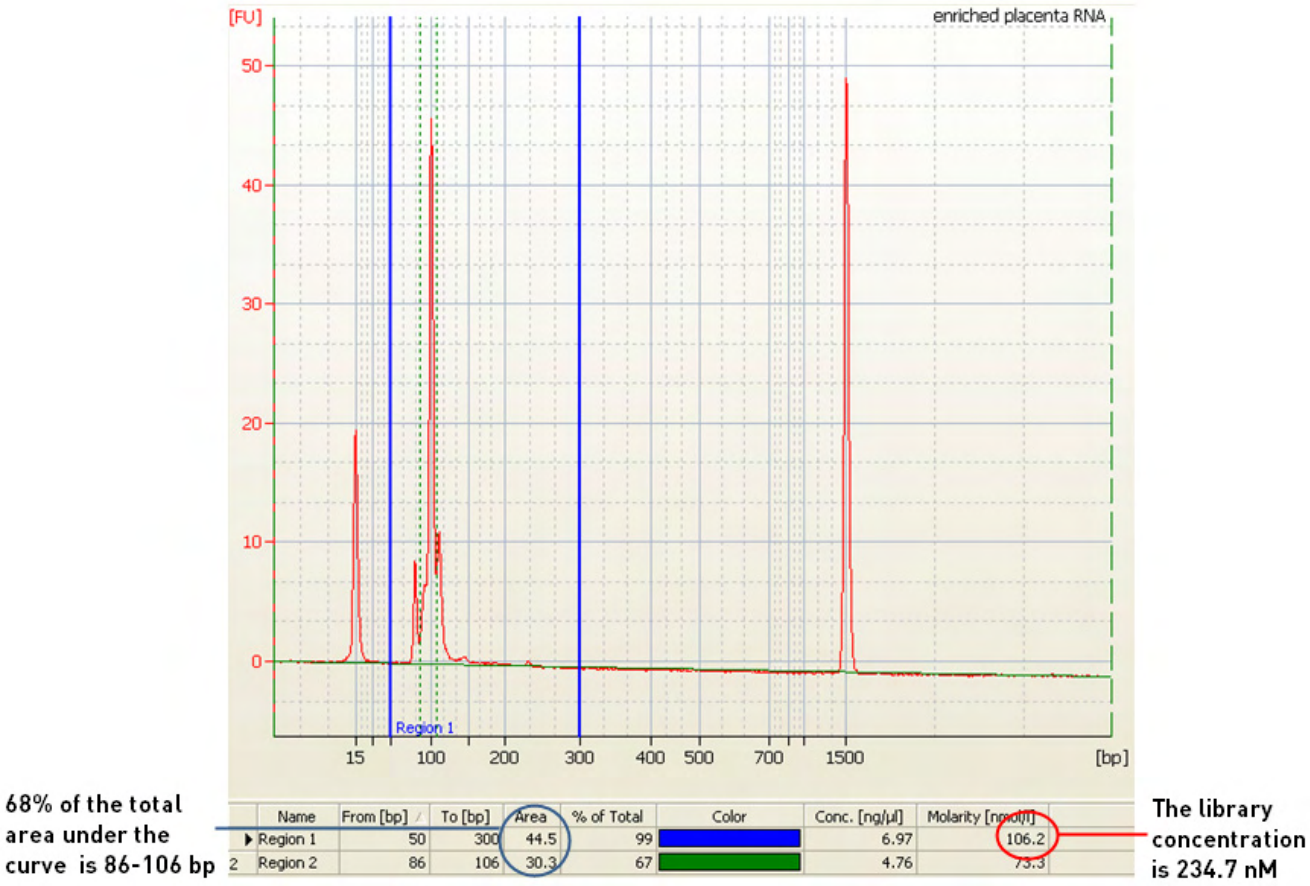
Typical size distribution

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

Plotted size distributions

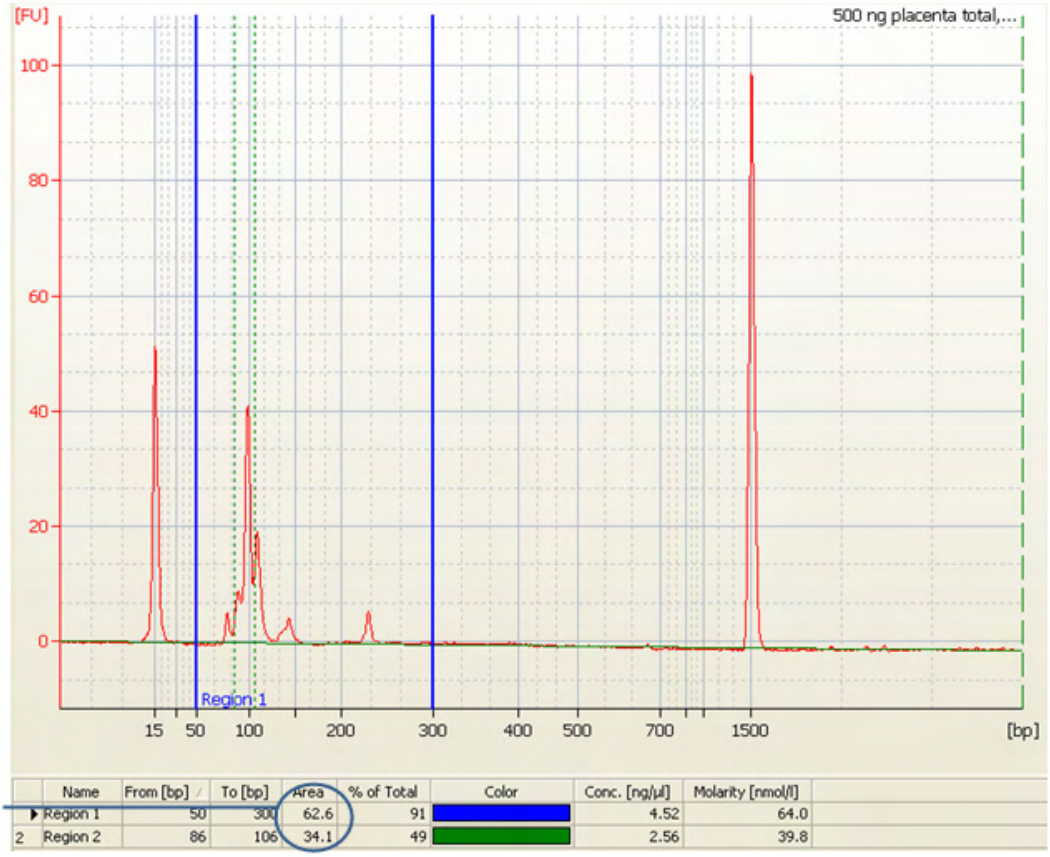
Figure 5 shows the size distribution of non-barcoded, small RNA library from enriched placenta. Figure 6 on page 67 illustrates a typical size distribution of placenta total RNA library (Agilent® 2100 Bioanalyzer® instrument profile). For the highest quality libraries, the ratio of 86–106-bp DNA 50–300-bp DNA is greater than 50%. Figure 7 on page 68 illustrates the size distribution of a barcoded small RNA library prepared from placenta total RNA.

Figure 5 Molar concentration and size distribution of non-barcoded library prepared from enriched placenta small RNA



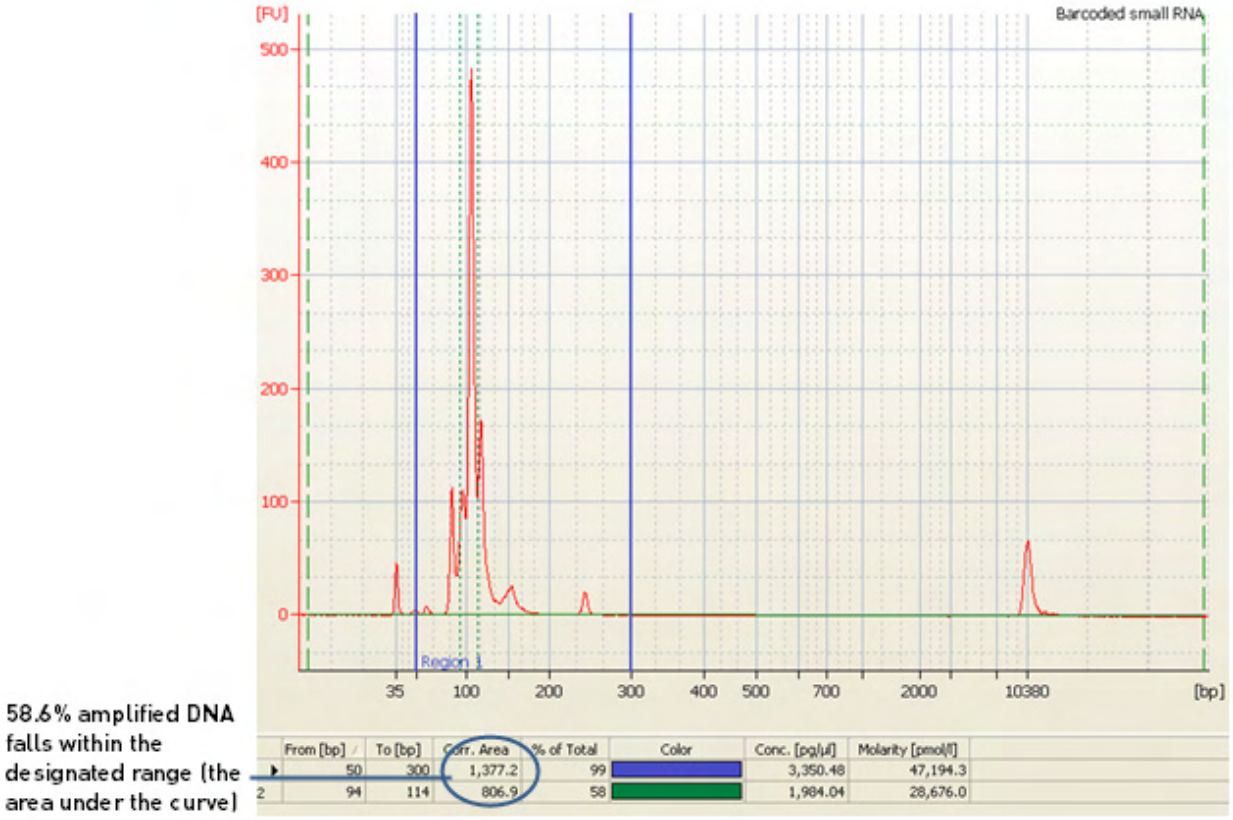
Note: The amount of tRNA in the final library (reflected by the height of the peak that is about 108 bp on the bioanalyzer trace) varies depending on the lot of placenta you use. Be expected to see differences in the ratio of 86–106-bp DNA/ 50–300-bp DNA when different lot of placenta control RNA is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

Figure 6 Size distribution of non-barcoded library prepared from placenta total RNA without enriching small RNA



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

Figure 7 Size distribution of barcoded small RNA library prepared from enriched small RNA



Size distributions compared

Insert Length	Size of Non-barcoded Library on the Bioanalyzer® Instrument	Size of Barcoded Library on the 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

Troubleshooting

Troubleshooting library preparation

Observation	Possible Cause	Solution
The Agilent® software does not calculate one concentration and peak size.	The software detects multiple peaks in the amplified cDNA profile.	Refer to "Analyze multiple peaks as one peak" on page 77.
Low yield in the desired size range and high background of smaller <86 bp non-barcoded library or <94 bp for barcoded small RNA library; or >106 bp for non-barcoded library or >114 bp for 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 pipette. 4. Using the remaining half of cDNA, repeat PCR and PCR cleanup.
Low yield or only self-ligation products are visible on Bioanalyzer® instrument traces.	Your 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.
	Contaminating ethanol is inhibiting the PCR.	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 larger than 150 bp.	Too many PCR cycles resulted in overamplification.	Decrease the number of PCR cycles (step 4 on page 60).

Troubleshooting the AB Library Builder™ System

Observation	Possible Cause	Recommended Action
Before loading the cartridges in the cartridge rack		
Precipitate in RNA Ligase Buffer tubes	Buffer was exposed to low temperatures during shipping or storage.	To dissolve precipitate that may have formed during shipping or storage, incubate the tubes at 37°C for 5 minutes or until precipitate is no longer visible.
During the automated run		
No power (the digital display is blank and the fan does not turn on when you power ON)	AC power cord is not connected.	Check AC power cord connections at both ends. Use the correct cords.
	Fuse has blown.	Check the integrity of the fuse and replace it if necessary (refer to the *AB Library Builder™ System User Guide*, Part no. 4463421). If the problem persists after connecting the correct power cord and replacing the fuse, contact Technical Support (see page 87).
The digital display is blank, but the fan turns on when you power ON	Protocol card is not inserted correctly.	Power off the instrument and re-insert the protocol card in the proper orientation into the card slot (see page 54). Insert it completely into the slot by manually pushing the card.
	Protocol card was inserted when the instrument was powered ON.	Power off the instrument, then power on the instrument.
Error code displayed	—	See "Instrument error codes" on page 73.
Reagent cartridges, tips, or tubes are not inserted in the correct positions	—	Press **STOP** to pause the run. Open the door, add the missing items, then press **START** to resume the run. Do not open the door without pausing the run.

Observation	Possible Cause	Recommended Action
Run stops after an initial start (you may also see an error code)	• Instrument door opened during the run. • Reagent cartridges, tips, or tubes incorrectly loaded in the rack. • Racks incorrectly loaded on the instrument.	**IMPORTANT!** If you open the instrument door while the instrument is running, the run stops, and it cannot be restarted. If you need to open the instrument door during a run, first press **Stop** to pause the run, then open the door. 1. Follow the procedure in "Instrument error codes" on page 73. 2. Before starting a new run, make sure that the reagent cartridges, tips, and tubes are correctly loaded: • Slide the reagent cartridges into the cartridge rack as described on page 55. • Load the cartridge rack before the tip and tube rack for proper positioning. • Do not cap the tubes. 3. If the instrument continues to stop during the run, contact Technical Support.
	Reagent cartridges not completely thawed.	1. Stop the run. 2. Remove the tip and tube rack, then remove the cartridge rack. 3. Inspect cartridge wells 2 and 3 for ice. 4. If any well is frozen, close the door to the AB Library Builder™ Device, then thaw the cartridges completely. 5. Replace the tips in position T2. 6. Insert the cartridge rack then the tip and tube rack onto the AB Library Builder™ Device. 7. Restart the run.
No DNA yield	No sample added to tube	Add samples to tubes, load new reagent cartridges, then perform the run again.
No liquid in tip, or liquid in tip not moving	No sample added to tube, leading to wet filter barrier on the tip and blockage of nozzles.	Add samples to tubes, load new reagent cartridges, then perform the run again.
Buffer in the bottom tray	Motor movements are not smooth.	Schedule preventive maintenance annually to ensure proper motor movements.
	Reagent cartridges, tips, or tubes incorrectly loaded in the rack.	If you are processing fewer than 13 samples, make sure to load the tips and tubes in the same positions as the reagent cartridges that are loaded in the cartridge rack. See next row for recommended action when experiencing leakage from tips.
Leakage from tips or uneven liquid handling between nozzles	D-Rings are not greased regularly or they need replacement.	You can continue the run, but maintain the D-rings as scheduled. To prevent leakage, maintain or replace the D-rings (refer to the (*AB Library Builder™ System User Guide*).
Blockage of tips	Too much starting material causing clumps or aggregates.	Contact Technical Support (see page 87). In future runs, use the sample volume recommended in the user guide for the kit you are using.

Observation	Possible Cause	Recommended Action
After the automated run		
No elution volume	Sample volume is lower than the recommended volume, leading to wet filter barrier on the tip and blockage of nozzles.	In future runs, use the recommended sample volume for the protocol you are using. Long-term operation with lower-than-recommended sample volumes can lead to issues with liquid handling performance.
No amplifiable library	Enzymes or buffer not at bottom of wells.	Tap the wells down against a hard surface to move enzymes and buffer to bottom of wells, then inspect the wells.
Final library is brownish	Beads in final library.	1. Place the tube with the final library in a DynaMag™-2 magnetic rack for at least 1 minute until the solution is clear of brown tint when viewed at an angle. 2. Without disturbing the pellet, carefully transfer the **supernatant**, which contains the final library, to a new 1.5-mL Eppendorf LoBind® Tube.

Instrument error codes

If an extraction run is interrupted by an error, you cannot resume the interrupted run. Follow the following procedure to resolve the error before you start a new run.

If you observe an error code:

1. Make a note of the error code, including the line number. Common error codes are listed in the following table:

Code	Problem	Code	Problem
10	Failed return to origins, protocol cannot run	21	P axis time out, protocol in run
11	Limit error, protocol can not run	22	M axis time out, protocol in run
12	Failed to return to Z Axis, protocol in run	23	Y axis time out, protocol in run
13	Failed to return to P axis, protocol in run	24	Open door in motion
14	Failed to return to M axis, protocol in run	25	Abnormal input from bottom sensor in motion
15	Failed to return to Y axis, protocol in run	26	Failed to initialize heating block
16	Z axis limit error, protocol in run	27	Failed to initialize motion control board
19	Y axis end limit, protocol in run	110	System error; (Assigned greater than 10)
20	Z axis time out, protocol in run		

2. Press **ESC** to return to the Main menu.
3. If there are tips attached to the nozzles, press **1** to select the Manual screen, then press **2** to return the tips to the original position.
4. Power OFF the instrument, remove the protocol card, wait 5 minutes, insert the protocol card, then power ON the instrument.
5. Run the axis test (refer to the *AB Library Builder™ System User Guide*, Pub. no. 4463421).
6. If the axis test:
   - Is successful, start a new extraction run. Use new samples and plastics where required.
   - Is *not* successful, contact Technical Support (see page 87).

A Supplemental information

Amplified library construction concepts

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

Hybridization and ligation to the Adaptor Mix

The RNA samples are hybridized and ligated with the Ion Adapters. The Ion Adapters are a set of oligonucleotides with a single-stranded degenerate sequence at one end and a defined sequence required for the Ion PGM™ System and Ion Proton™ System at the other end. The Ion Adapters constrains the orientation of the RNA in the ligation reaction such that hybridization with the Ion Adapters 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 products

Reverse transcription and size-selection

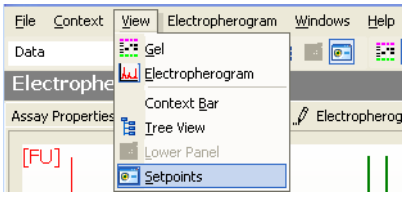
The RNA population with ligated adaptors is reverse transcribed to generate single-stranded cDNA copies of the fragmented RNA molecules. Library generation uses a magnetic bead-based, size-selection process, to enrich for library fragments within the desired size range.

Using 2100 expert software to assess whole transcriptome libraries

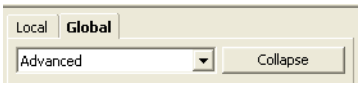
Perform a smear analysis

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

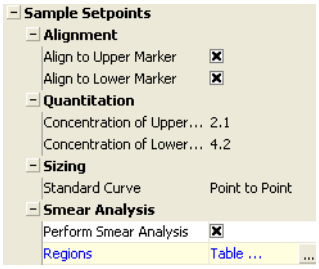
1. In the 2100 expert software, select **View** ▸ **Setpoints**.



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

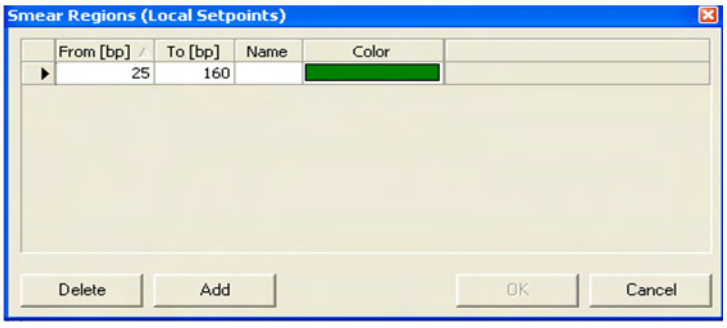


3. In the Sample Setpoints section of the Advanced settings, select the **Perform Smear Analysis** checkbox, then double-click **Table**.



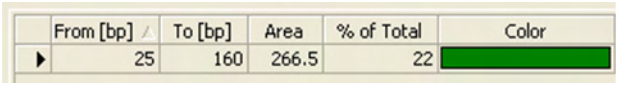
4. Set the smear regions in the Smear Regions dialog box: Click **Add**, then enter **25** bp and **160** bp for the lower and upper limits, respectively.

   These settings are used to 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.

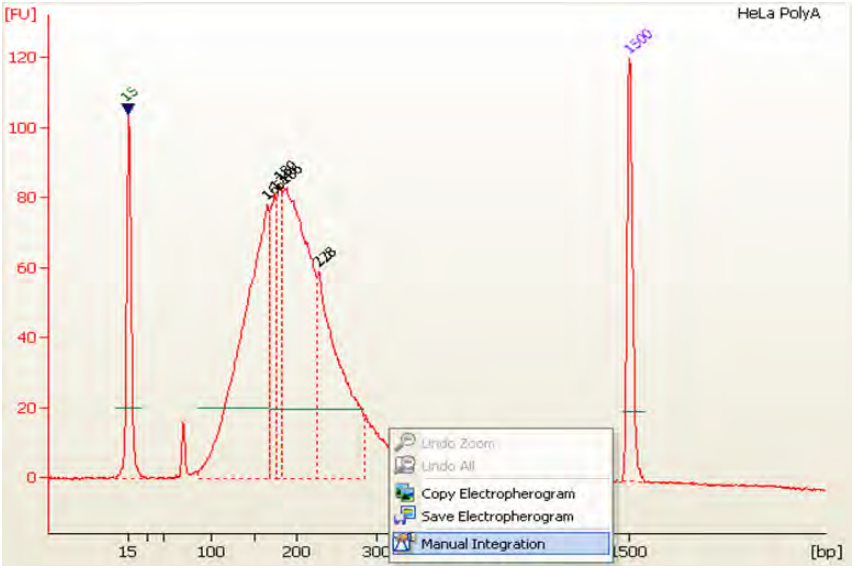


Analyze multiple peaks as one peak

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

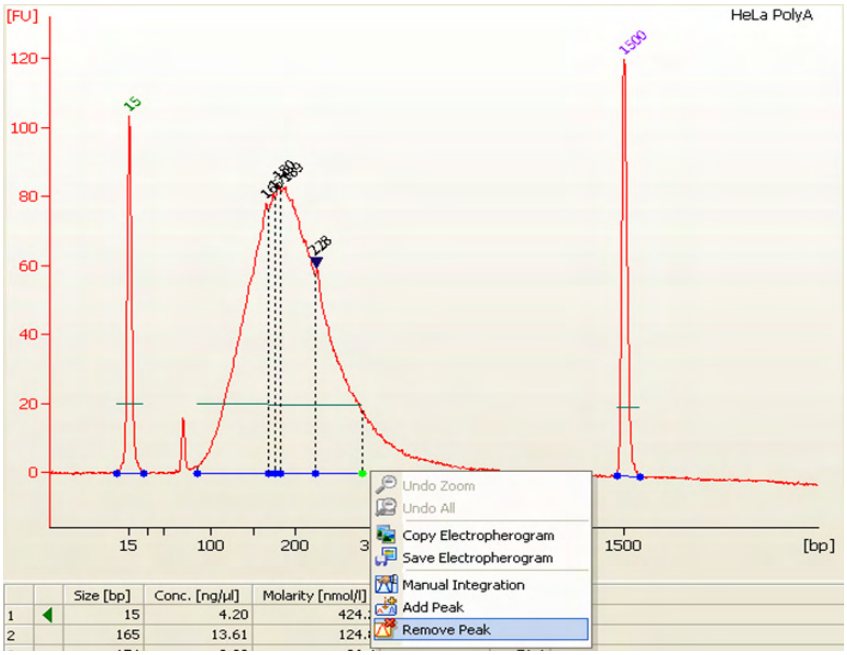
1. In the bottom-left corner of the software window, select the **Peak Table** tab.

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



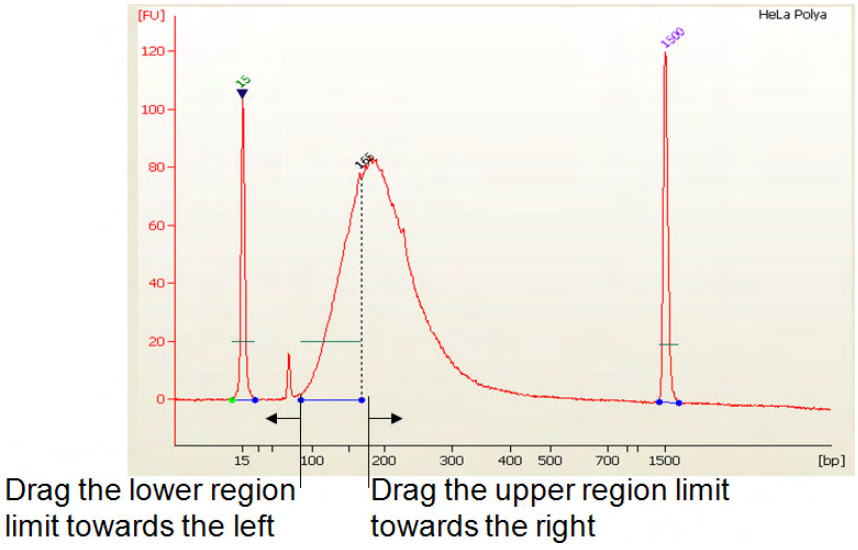
3. To remove multiple peaks:

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

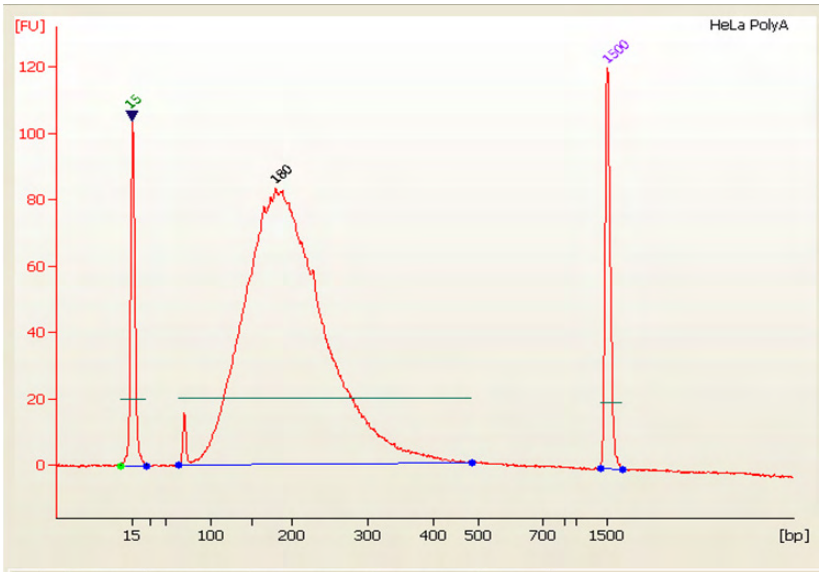


b. Repeat until one peak remains within the region of interest.

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



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



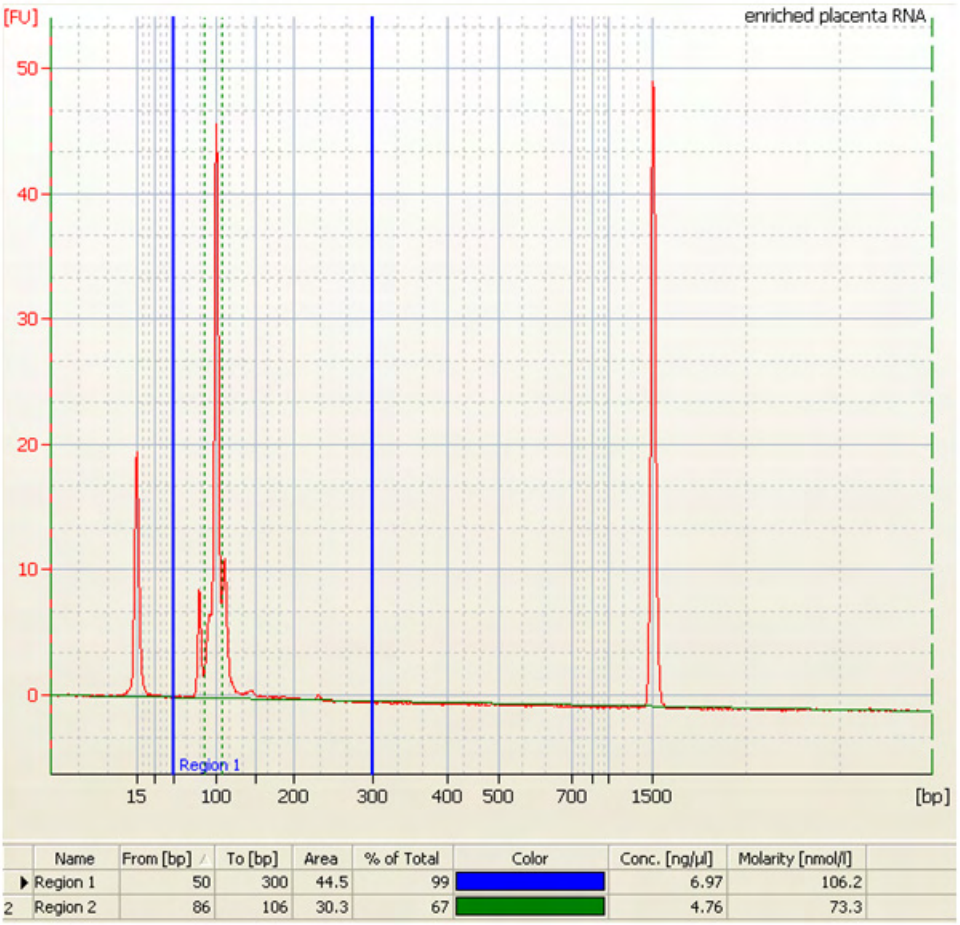
		Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations	Area
1	◀	15	4.20	424.2	Lower Marker	70.5
2		180	43.48	366.7		1,038.2
3	▶	1,500	2.10	2.1	Upper Marker	101.0

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 should be ~87–91 bp.

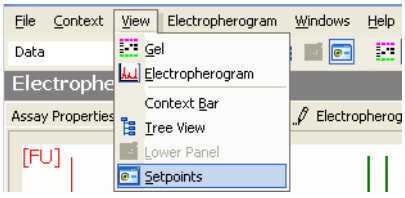


	Name	From [bp]	To [bp]	Area	% of Total	Color	Conc. [ng/µl]	Molarity [nmol/l]
▸	Region 1	50	300	44.5	99		6.97	106.2
2	Region 2	86	106	30.3	67		4.76	73.3

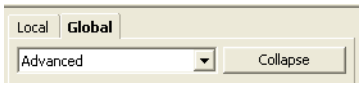
Perform a smear analysis

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.

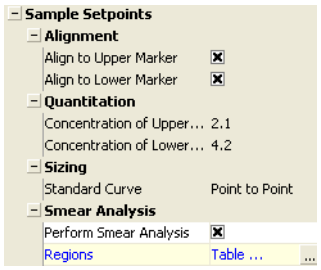
1. In the 2100 expert software, select **View** ▸ **Setpoints**.



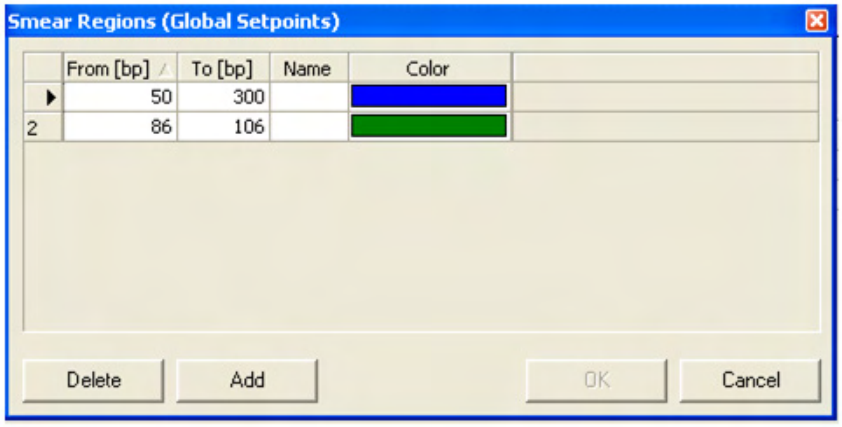
2. On the **Global** tab, select **Advanced** settings.



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.

	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

Determine the % miRNA library

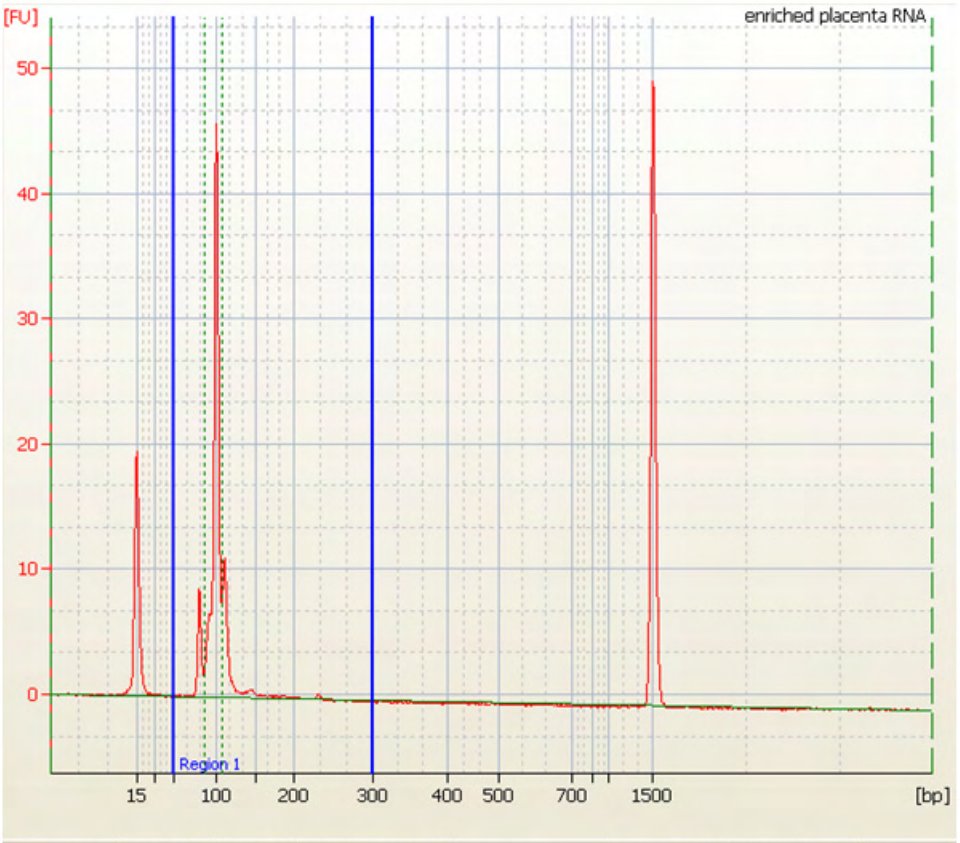
Using the area values from the Region Table, calculate the % miRNA library in the 86–106 bp region as a fraction of the 50–300 bp region using the formula:

% 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

B

Safety

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

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

Chemical safety

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

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

Biological hazard safety

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

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **www.cdc.gov**

In the EU:

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

Documentation and support

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**.

Note: For the SDSs of chemicals not distributed by Thermo Fisher Scientific, contact the chemical manufacturer.

Obtaining Certificates of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

Obtaining support

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

www.lifetechnologies.com/support

At the website, you can:

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

Ion contact information

Website: lifetechnologies.com/iontorrent

Ion community: ioncommunity.lifetechnologies.com

Support email: ionsupport@lifetech.com

Phone numbers
In North America: 1-87-SEQUENCE (1-877-378-3623)
Outside of North America: +1-203-458-8552

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 found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.