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Ion Total RNA-Seq Kit for Small RNA Libraries

Note: For safety and biohazard guidelines, refer to the "Safety" section in the *Ion Total RNA-Seq Kit User Guide* (PN 4467098). For every chemical, read the Safety Data Sheet (SDS) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Prepare the starting material

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

Use the NanoDrop® Spectrophotometer and the Agilent® 2100 Bioanalyzer™ instrument with the Agilent® RNA 6000 Nano Kit and the Agilent® Small RNA Kit.

a. Quantitate the amount of RNA in the sample using the NanoDrop Spectrophotometer.

Note: If you used the PureLink® miRNA Isolation Kit to isolate the small RNA from samples, you can skip to "Assess the quality and quantity of the small RNA-enriched sample" on page 2.

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

1. Dilute the RNA to ~50 to 100 ng/µL.
2. Run 1 µL of diluted RNA on the Agilent 2100 Bioanalyzer instrument with the RNA 6000 Nano Kit to determine the concentration of total RNA.
3. Using the 2100 expert software, determine the **mass of total RNA** in the sample and review the RNA Integrity Number (RIN).

c. Determine the percentage of small RNA in your sample:

1. Run 1 µL of diluted RNA on the Agilent 2100 Bioanalyzer instrument with the Small RNA Kit chip.
2. Use the 2100 expert software to determine the **mass of small RNA (miRNA)** (10–40 nt).
3. Calculate the miRNA content in your RNA sample using the formula:

   % miRNA = (mass of miRNA ÷ mass of total RNA) × 100

d. 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
Greater than or equal to 0.5% miRNA	You can use the total RNA in the ligation reaction, and small RNA enrichment is not needed. However, for optimal results, we recommend enrichment of all total RNA samples. Proceed to "(If needed) Enrich the sample for small RNA" on page 1 or skip to "Determine the input amount" on page 2.
Less than or equal to 0.5% miRNA	Small RNA enrichment is strongly recommended. We recommend using the PureLink® miRNA Isolation Kit. Proceed to "(If needed) Enrich the sample for small RNA" on page 1.

2 (If needed) Enrich the sample for small RNA

If your RNA sample contains <0.5% miRNA, use the PureLink® miRNA Isolation Kit.

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

a. Run 1 µL of the purified and **enriched small RNA** sample on the Agilent 2100 Bioanalyzer instrument with the Small RNA Kit.

b. Use the 2100 expert software to determine the quality and quantity of recovered small RNA:

% miRNA = (mass of miRNA ÷ mass of enriched small RNA) × 100

c. Compare the bioanalyzer traces to those of the sample before enrichment, and determine whether the RNA is degraded. For enriched small RNA samples, peaks should be from 10–200 nt.

4 **Determine the input amount**

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

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

Construct the amplified small RNA library

1 **Hybridize and ligate the RNA**

Use components from the Ion Total RNA-Seq Kit.

a. On ice, prepare the hybridization master mix:

Component	Volume for one reaction†
Ion Adaptor Mix	2 µL
Hybridization Solution	3 µL
Total volume	**5 µL**

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

b. Add 5 µL of hybridization master mix to 3 µL of small RNA sample; flick the tube or pipet up and down to mix, then spin briefly:

- 5–100 ng of miRNA in ≤ 1 µg of total RNA
- 1–100 ng of miRNA in ≤ 1 µg of enriched small RNA

c. Run the hybridization reactions in a thermal cycler:

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

1 Hybridize and ligate the RNA (continued)

d. On ice, prepare the ligation master mix; gently vortex to mix, then spin briefly:

Component	Volume for one reaction†
2X Ligation Buffer	10 µL
Ligation Enzyme Mix	2 µL
Total volume	**12 µL**

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

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

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

f. Incubate the ligation reactions in a thermal cycler at 16°C for 16 hours.

IMPORTANT! Turn off the heated lid or leave the thermal cycler open during the incubation.

2 Perform reverse transcription

Use components from the Ion Total RNA-Seq Kit.

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

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

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

b. Incubate the RT master mix with the ligated RNA sample:

1. Add 19 µL of RT master mix to each 20-µL ligation reaction. Pipet up and down to mix, then spin briefly.
2. Incubate in a thermal cycler with a heated lid at 70°C for 5 minutes, then snap-cool on ice.

c. Perform reverse transcription:

1. Add 1 µL of ArrayScript™ Reverse Transcriptase to each ligated RNA sample. ***Gently*** vortex to mix thoroughly, then spin briefly.
2. Incubate in a thermal cycler with a heated lid at 42°C for 30 minutes.

STOPPING POINT The cDNA can be stored at –20°C for a few weeks, stored at –80°C for long-term storage, or used immediately.

3 Purify the cDNA

Use the MinElute® PCR Purification Kit.

Note: The kit may be supplied with Buffer PB (without pH Indicator) or Buffer PBI (with pH Indicator). Either buffer can be used as is; it is not necessary to add pH Indicator to Buffer PB before use.

a. Add Nuclease-free Water and Buffer PB or Buffer PBI to the cDNA:

1. Transfer all of the prepared cDNA (40 µL) to a clean 1.5-mL microfuge tube.
2. Add 60 µL of Nuclease-free Water.
3. Add 500 µL of Buffer PB or Buffer PBI, then mix well.

b. Purify the cDNA:

Step	Add	Spin	Flowthrough
Load the cDNA onto the MinElute® column	600 µL of the sample containing Buffer PB or Buffer PBI	13,000 × g for 1 min	Discard
Wash the cDNA	750 µL of Buffer PE	13,000 × g for 1 min	Discard
	–	13,000 × g for 1 min	Discard
Elute the cDNA in a clean microfuge tube	10 µL of Buffer EB	Wait 1 min, then 13,000 × g for 1 min	Save the cDNA

4 Size-select the cDNA

a. Prepare and assemble a Novex® 10% TBE-Urea Gel in an XCell *SureLock*™ Mini-Cell, using 1X TBE Running Buffer, as described in the *Novex® Pre-Cast Gel Electrophoresis Guide*.

b. Prepare a 10-bp DNA Ladder and the cDNA:

1. Dilute the 10-bp DNA Ladder to 40 ng/µL: 1 µL of 10-bp DNA Ladder + 24 µL of RNase-free water.
2. Mix 5 µL of the cDNA with 5 µL of 2X Novex® TBE-Urea Sample Buffer.
3. Mix 5 µL of the 40 ng/µL 10-bp DNA Ladder with 5 µL of 2X Novex TBE-Urea Sample Buffer.
4. Heat the cDNA and the DNA Ladder at 95°C for 3 minutes.
5. Snap-cool the tubes on ice. Leave the tubes on ice for less than 30 minutes.

c. Flush the wells of the gel several times with 1X TBE Running Buffer to remove urea, then load the cDNA samples and the DNA Ladder onto the gel.

d. Run the gel at 180 V until the second dye front just passes the middle of the gel (~45 minutes).

e. Add 5 µL of SYBR® Gold nucleic acid gel stain to 50 mL of 1X TBE Running Buffer, then stain the gel for 5–10 minutes.

f. Illuminate the stained gel, then excise the gel containing 60–80 nt of cDNA.

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

h. Place the two gel slices from the middle of the lane individually into clean 0.2-mL PCR tubes. Place the outside gel slices into a clean 1.5-mL microfuge tube for storage.

5 Amplify the cDNA

Use components from the Ion Total RNA-Seq Kit.

a. Prepare the PCR master mix for each gel slice:

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

† The volume for two reactions includes 10% excess to compensate for pipetting errors.

b. Add 100 µL of PCR master mix to each 0.2-mL PCR tube with a gel slice.

c. Run the PCRs in a thermal cycler:

Stage	Temp	Time
Hold	95°C	5 min
Cycle (16 cycles)†	95°C	30 sec
	62°C	30 sec
	72°C	30 sec
Hold	72°C	7 min

† If you started with total RNA and input 1–25 ng, run 18 cycles. If you started with enriched/purified small RNA and input 1–10 ng, run 18 cycles.

6 Purify the amplified DNA

Use the PureLink® PCR Micro Kit.

a. Combine the two 100-µL PCRs in a new 1.5-mLmicrofuge tube, add 800 µL of Binding Buffer (B2), then mix well.

b. Purify the amplified DNA:

Step	Add	Spin	Flowthrough
Load the sample onto a PureLink® Column that is placed in a clean Collection Tube	500 µL of the sample containing Binding Buffer (B2)	10,000 × g for 1 min	Discard
	500 µL of sample containing Binding Buffer (B2)	10,000 × g for 1 min	Discard
Wash the DNA	600 µL of Wash Buffer (W1)	10,000 × g for 1 min	Discard
	–	14,000 × g for 1 min	Discard
Elute the DNA into a clean PureLink® Elution Tube	12 µL of Elution Buffer	Wait 1 min, then 14,000 × g for 1 min	Save the DNA

7 Assess the yield and size distribution of the amplified DNA

a. Run 1 µL of the purified DNA on an Agilent® 2100 Bioanalyzer™ instrument with the Agilent® DNA 1000 Kit.

b. Use the 2100 expert software to perform a smear analysis:

1. Measure the area for the DNA that is 25–150 bp (ligation products with no insert and short inserts) and 78–98 bp (desired miRNA ligation products).
2. Calculate the ratio of 78–98-bp DNA : 25–150-bp DNA using the formula:
   [Area (78–98 bp)] ÷ [Area (25–150 bp)]

Next steps

If the ratio of 78–98-bp DNA : 25–150-bp DNA is...	Then...
Greater than 50%	Proceed to "Determine the Template Dilution Factor and proceed to Template Preparation" (below).
Less than 50%	Proceed to "Determine the Template Dilution Factor and proceed to Template Preparation" (below), but expect to see an increase in the number of filtered reads when compared to samples with greater than 50% ratio of desired miRNA ligation products to overall products.

8 Determine the Template Dilution Factor and proceed to Template Preparation

a. Determine the Template Dilution Factor. If your library concentration is greater than 20 nM, serially dilute 1:10 or 1:100 until your library concentration is less than or equal to 20 nM, then determine the Template Dilution Factor for the diluted library:

Template Dilution Factor =
(Library concentration in nM) × [(5 × 10^9 molecules/µL)/(8.3 nM)] × [18 µL/(280 × 10^6 molecules)]

b. Proceed to the Template Preparation procedure for clonal amplification of the library on Ion Sphere™ particles. Refer to the user documentation for the Ion Xpress™ Template Kit.

Materials

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

Item	Source
Ion Total RNA-Seq Kit, 12 reactions	Life Technologies 4466666
Ion Total RNA-Seq Kit, 48 reactions	Life Technologies 4468200
8-strip PCR Tubes & Caps, RNase-free, 0.2-mL	Applied Biosystems AM12230
Non-Stick RNase-free Microfuge Tubes (0.5 mL), 500	Applied Biosystems AM12350
Non-Stick RNase-free Microfuge Tubes (1.5 mL), 250	Applied Biosystems AM12450
Pipette tips, RNase-free	Major Laboratory Supplier (MLS)
Nuclease-free Water (not DEPC-treated), 100 mL	Applied Biosystems AM9938
PureLink® PCR Micro Kit, 50 preps	Invitrogen K310050
Agilent® DNA 1000 Kit	Agilent 5067-1504
Ethanol, 100%, ACS reagent grade or equivalent	MLS
MinElute® PCR Purification Kit (50)	Qiagen 28004
10-bp DNA Ladder	Invitrogen 10821-015
Novex® 10% TBE-Urea Gels 1.0 mm, 10 well	Invitrogen EC6875BOX
Novex® TBE-Urea Sample Buffer (2X), 10 mL	Invitrogen LC6876
Novex® TBE Running Buffer (5X), 1 L	Invitrogen LC6675
SYBR® Gold nucleic acid gel stain, 10,000X concentrate in DMSO, 500 µL	Invitrogen S-11494
Agilent® RNA 6000 Nano Kit	Agilent 5067-1511
Agilent® Small RNA Kit	Agilent 5067-1548

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