

SOLiD™ Total RNA-Seq Kit for Small RNA Libraries



Note: For safety and biohazard guidelines, refer to the “Safety” section in the *SOLiD™ Total RNA-Seq Kit Protocol* (PN 4452437). For every chemical, read the 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 with the RNA 6000 Nano Kit and the Small RNA Chip Kit.

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



Note: If you isolated the small RNA from samples using the PureLink™ miRNA Isolation Kit, 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 with the RNA 6000 Nano Kit.
3. Use the 2100 expert software to:
 - Determine the mass of total RNA in the sample. Save the value for [step c](#) (below).
 - Review the RNA Integrity Number (RIN). Proceed if the RIN is ≥6.



Note: If the RIN is <6, repeat RNA isolation.

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

1. Run 1 μL of diluted RNA on the Agilent® 2100 Bioanalyzer with the Small RNA Chip.
2. Use the 2100 expert software to determine the mass of small RNA (miRNA, 10–40 nts).
3. Calculate the miRNA content in your RNA sample using the following formula:

$$\% \text{ miRNA} = \left(\frac{\text{mass of miRNA (10–40 nts) from the Small RNA Chip}}{\text{mass of total RNA from the RNA 6000 Nano Chip}} \right) \times 100$$

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

% miRNA (10–40 nt)	Next steps
≥0.5% miRNA	Proceed with small RNA enrichment or purification or skip to “Determine the input amount” on page 2.
0.1–0.5% miRNA	Use the Invitrogen PureLink™ miRNA Isolation Kit, following the instructions in Appendix B of the <i>SOLiD™ Total RNA-Seq Kit Protocol</i> .
<0.1% miRNA	Use the Ambion flashPAGE™ Fractionator System and flashPAGE™ Clean-up Kit, following the instructions provided with the kit. You can load up to 100 μg total RNA on the flashPAGE Fractionator.

2 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 with the Small RNA Chip.
- b. Use the 2100 expert software to determine the quality and quantity of recovered small RNA.

$$\% \text{ miRNA} = \left(\frac{\text{mass of miRNA (10–40 nts) from the Small RNA Chip}}{\text{mass of enriched small RNA from the Small RNA Chip}} \right) \times 100$$

- c. Compare the bioanalyzer traces to those before enrichment and determine whether the RNA is degraded. Expected results:
 - flashPAGE-purified small RNA: Peaks from 10 to 40 nt and very low or no peaks after 40 nt
 - Enriched small RNA samples: Peaks from 10 to 200 nt

3 Determine the input amount

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

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

Construct the amplified whole transcriptome library

1 Hybridize and ligate the RNA

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

Component	Volume
Small RNA sample: <ul style="list-style-type: none"> • 5 to 100 ng of miRNA in ≤ 1 µg of total RNA • 1 to 100 ng of miRNA in ≤ 1 µg of enriched small RNA • 1 to 100 ng of purified small RNA 	3 µL
Hybridization Solution	3 µL
SOLiD™ Adaptor Mix	2 µL
Total volume per reaction	8 µL

- b. Run the 8-µL hybridization reactions in a thermal cycler:

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

- c. Add the RNA ligation reagents to each 8-µL hybridization reaction:

- 10 µL 2X Ligation Buffer
- 2 µL Ligation Enzyme Mix



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

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

2 Perform reverse transcription

a. Prepare RT master mix (*without* the ArrayScript™ Reverse Transcriptase) for all reactions:

Component	Volume for each reaction†
Nuclease-free Water	11 µL
10X RT Buffer	4 µL
dNTP Mix	2 µL
SOLiD™ RT Primer	2 µL
Total volume per reaction	19 µL

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

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

1. Transfer 19 µL of RT master mix to each 20-µL ligation reaction. Pipet up and down a few times 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 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.



Note: 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 (Qiagen).



Note: The kit may be supplied with Buffer PB (without pH Indicator) or Buffer PBI (with pH Indicator). 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 cDNA (40 µL) to a clean 1.5-mL microcentrifuge 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	Load	Spin	Flowthrough
Load the cDNA onto the MinElute column	600 µL of 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 microcentrifuge tube	10 µL of Buffer EB	Wait 1 min, then 13,000 × g for 1 min	Save the cDNA

4 Size select the cDNA

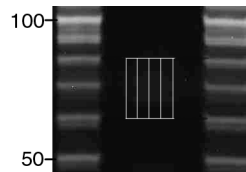
Use Novex® pre-cast gel products, 10 bp DNA Ladder, and SYBR® Gold nucleic acid gel stain (Invitrogen).

- 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* by Invitrogen.
- Prepare the cDNA and the 10 bp DNA ladder, then load the gel:
 - Dilute the 10 bp DNA Ladder to 40 ng/μL: 1 μL 10 bp DNA Ladder + 24 μL RNase-free water.
 - Mix 5 μL of the 40 ng/μL 10 bp DNA Ladder with 5 μL of 2X Novex TBE-Urea Sample buffer.
 - Mix 5 μL of the cDNA with 5 μL of 2X Novex TBE-Urea Sample Buffer.
 - Heat the samples at 95 °C for 3 minutes.
 - Snap-cool on ice, then load. Leave on ice for less than 30 minutes before loading.



Note: Immediately before you load each sample, flush the well of the gel several times with 1X TBE Running Buffer.

- Run the gel at 180 V until the second dye front just passes the middle of the gel (~45 minutes).
- 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.
- Illuminate the stained gel, then excise the gel containing 60–80 nt cDNA.
- 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.



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

5 Amplify the cDNA

- Prepare 98 μL PCR mix for each gel slice:

Component	Volume	
	One 100-μL reaction	Two 100-μL reactions†
Nuclease-free Water	76.8 μL	169.0 μL
10X PCR Buffer	10.0 μL	22.0 μL
dNTP Mix	8.0 μL	17.6 μL
SOLiD™ 5' PCR Primer	2.0 μL	4.4 μL
AmpliTaq® DNA Polymerase	1.2 μL	2.6 μL
Total volume	98.0 μL	215.6 μL

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

- Transfer 98 μL PCR mix into each 0.2-mL PCR tube with a gel slice.
- Add 2 μL SOLiD 3' PCR Primer to each tube.



Note: For multiplex SOLiD System sequencing, substitute with barcoded SOLiD™ 3' PCR Primers from a SOLiD™ RNA Barcoding Kit.

5 Amplify the cDNA
(continued)

d. Run the PCR reactions in a thermal cycler:

Stage	Temp	Time
Hold	95 °C	5 min
Cycle (15 cycles)	95 °C	30 sec
	62 °C	30 sec
	72 °C	30 sec
Hold	72 °C	7 min

6 Purify the amplified DNA Use the PureLink™ PCR Micro Kit (Invitrogen).

a. Prepare the sample:

- Combine the two 100-µL PCR reactions in a new 1.5-mL tube.

! **IMPORTANT!** If you used two different barcoded SOLiD 3' Primers for your sample, do not combine the PCRs at this step.

- Add 800 µL of Binding Buffer (B2) to the tube, then mix well.

b. Purify the amplified DNA:

Step	Load	Spin	Flowthrough
Load the sample onto the PureLink™ Micro Kit Column	500 µL of sample containing Binding Buffer (B2)	10,000 × g for 1 min	Discard
Load the remaining half of the sample onto the PureLink™ Micro Kit Column	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 with the DNA 1000 Kit (Agilent).

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

- Measure the area for the DNA that is 25–150 bp (ligation products with no insert and ligation products with short inserts) and 120–130 bp (desired miRNA ligation products).
- Calculate the ratio of 120–130-bp DNA: 25–150-bp DNA:
[Area (120–130 bp)] ÷ [Area (25–150 bp)]

Ratio of 120–130-bp DNA:25–150-bp DNA	Next steps
Greater than 50%	Proceed with SOLiD System templated bead preparation (below).
Less than 50%	Perform second-round size selection of amplified cDNA as described in the <i>SOLiD™ Total RNA-Seq Kit Protocol</i> .

8 Proceed with SOLiD System templated bead preparation

Emulsion PCR library concentrations (singleplex or multiplex sequencing pools) of 0.4 pM and 0.8 pM for ePCR are recommended for workflow analysis. Refer to the *Applied Biosystems SOLiD™ 4 System Templated Bead Preparation Guide* (PN 4448378).

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
SOLiD™ Total RNA-Seq Kit	Applied Biosystems PN 4445374
SOLiD RNA Barcoding Kits (optional) For a complete list of available kits, go to www.appliedbiosystems.com .	Applied Biosystems
8-strip PCR Tubes & Caps, RNase-free, 0.2-mL	Applied Biosystems PN AM12230
Non-Stick RNase-free Microfuge Tubes (0.5 mL), 500	Applied Biosystems PN AM12350
Non-Stick RNase-free Microfuge Tubes (1.5 mL), 250	Applied Biosystems PN AM12450
Nuclease-free Water (not DEPC-treated), 100 mL	Applied Biosystems PN AM9938
10 bp DNA Ladder	Invitrogen PN 10821-015
Novex® 10% TBE-Urea Gels 1.0 mm, 10 well	Invitrogen PN EC6875BOX
Novex® TBE Running Buffer [5X], 1 L	Invitrogen PN LC6675
Novex® TBE-Urea Sample Buffer [2X], 10 mL	Invitrogen PN LC6876
PureLink™ PCR Micro Kit, 50 preps	Invitrogen PN K310050
SYBR® Gold nucleic acid gel stain, 10,000X concentrate in DMSO, 500 µL	Invitrogen PN S-11494
Agilent DNA 1000 Kit	Agilent PN 5067-1504
RNA 6000 Nano Kit	Agilent PN 5067-1511
Small RNA Chip Kit	Agilent PN 5067-1548
Ethanol, 100%, ACS reagent grade or equivalent	MLS
MinElute® PCR Purification Kit (50)	Qiagen PN 28004
Pipette tips, RNase-free	MLS

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