

# Ion Total RNA-Seq Kit v2 for Small RNA Libraries

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This quick reference covers library preparation for up to 200-base-read sequencing on the Ion PGM™, Ion Proton™, Ion S5™, and Ion S5™ XL Systems.

**Note:** For safety and biohazard guidelines, refer to the “Safety” section in the *Ion Total RNA-Seq Kit v2 User Guide* (Pub. No. MAN0010654). 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

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

Before you prepare the library, determine the quality of the total RNA sample. Use the NanoDrop™ Spectrophotometer and the Agilent™ 2100 Bioanalyzer™ instrument with the Agilent™ RNA 6000 Nano Kit and the Agilent™ Small RNA Kit to measure the fraction of small RNA in your sample.

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

**Note:** If you used the *mirVana™* miRNA Isolation Kit, the *mirVana™ PARIS™* RNA and Native Protein Purification Kit, or the *PureLink™* miRNA Isolation Kit to isolate small RNA from samples, you can skip to “Assess the quality and quantity of the small RNA-enriched sample” on page 3.

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

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

- a. Dilute the RNA to ~50–100 ng/μL.
- b. Run 1 μL of diluted RNA on the Agilent™ 2100 Bioanalyzer™ instrument with the Agilent™ RNA 6000 Nano chip to determine the concentration of total RNA. Follow the manufacturer’s instructions for performing the assay.
- c. Using the 2100 expert software, determine the mass of total RNA in the sample, then save the mass of total RNA for step 3c to calculate the miRNA content.
- d. Using the 2100 expert software, review the RNA Integrity Number (RIN). The highest-quality library mapping statistics are obtained from input RNA with higher RIN values.

3. Determine the percentage of small RNA in your sample:
  - a. Run 1 μL of diluted RNA on the Agilent™ 2100 Bioanalyzer™ instrument with the Small RNA chip. Follow the manufacturer’s instructions for performing the assay.
  - b. Using the 2100 expert software, determine the mass of total RNA (miRNA; 10–40 nt) from the Small RNA chip.
  - c. Calculate the miRNA content in your RNA sample using the formula:

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

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

Percentage of miRNA (10–40 nt) in the RNA sample	Recommendations for small RNA enrichment
≥0.5% miRNA	Use the total RNA in the ligation reaction, and small RNA enrichment is not needed. However, for optimal results, we recommend enrichment of all total RNA samples.  You can expect 5–15% more of rRNA and tRNA mapping in your sequencing data from total RNA, compared to sequencing data of libraries starting from enriched small RNA.  Proceed to the next section or skip to “Determine the input amount” on page 3.
<0.5% miRNA	Small RNA enrichment is strongly recommended. We recommend using the Magnetic Bead Cleanup Module for small RNA enrichment.  Proceed to the next section.

### Enrich the samples for small RNA

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 3.

## Before you begin

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

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

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## Enrich the samples for small RNA

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

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

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- e. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down ten times.
  - f. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.
3. Remove the supernatant from the beads:
    - a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

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

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

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4. Bind desired small RNA products to the beads:
  - a. Remove the Processing Plate from the magnetic stand.
  - b. Add 30 µL of Nuclease-free Water to the supernatant in the new sample well.
  - c. Set a P1000 pipettor at 570 µL. Attach a new 1000-µL tip to the pipettor, then pre-wet the new 1000-µL tip with 100% ethanol by pipetting the ethanol up and down three times.
  - d. Without changing tips, add 570 µL of 100% ethanol to each well.

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

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- e. Gently vortex the Nucleic Acid Binding Beads tube to resuspend the magnetic beads completely.
  - f. Add 7 µL of beads to the wells of the Processing Plate.
  - g. Set a single or multi-channel P200 pipettor at 150 µL. Attach new 200-µL tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down ten times.
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- IMPORTANT!** Because of the large volume in each well, use a P200 pipettor for mixing to avoid cross-well contamination.
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- Note:** The color of the mixture should be homogeneous after mixing.
- h. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

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

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

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

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

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

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- d. Use a P10 or P20 pipettor to remove remaining ethanol.
  - e. Air-dry the beads at room temperature for 1–2 minutes to remove all traces of ethanol.
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**IMPORTANT!** Do not overdry the beads (overdried beads appear cracked). Overdrying significantly decreases elution efficiency.

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

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**STOPPING POINT** Store the small RNA at  $-86^{\circ}\text{C}$  to  $-68^{\circ}\text{C}$ . After storage, proceed to the next section “Assess the quality and quantity of the small RNA-enriched sample”.

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## 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  $\mu\text{L}$  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 1), 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  $\mu\text{L}$ . If necessary, concentrate the small RNA with a SpeedVac™ centrifugal concentrator.

Input sample type	Amount of miRNA(10-40 nt) in 3 $\mu\text{L}$	Total RNA input <sup>[1]</sup>
Total RNA	5–100 ng	$\leq 1 \mu\text{g}$
Enriched small RNA	1–100 ng	$\leq 1 \mu\text{g}$

<sup>[1]</sup> The yield drops if you use more than 1  $\mu\text{g}$  of RNA for ligation.

## Note:

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

## Construct the amplified small RNA library

### Hybridize and ligate the RNA

1. On ice, prepare the hybridization master mix:

Contents	Volume per reaction <sup>[1]</sup>
Hybridization Solution	3 $\mu\text{L}$
Ion Adaptor Mix v2	2 $\mu\text{L}$
<b>Total Volume</b>	<b>5 <math>\mu\text{L}</math></b>

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

2. Add 5  $\mu\text{L}$  of hybridization master mix to 3  $\mu\text{L}$  of small RNA sample (1–100 ng of miRNA in  $\leq 1 \mu\text{g}$  of enriched small RNA).
3. Flick the tube or pipet up and down five times to mix, then centrifuge briefly to collect the liquid in the bottom of the tube.
4. Run the hybridization reaction in a thermal cycler:

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

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

Contents	Volume per reaction <sup>[1]</sup>
2X Ligation Buffer	10 $\mu\text{L}$
Ligation Enzyme Mix	2 $\mu\text{L}$
<b>Total Volume</b>	<b>12 <math>\mu\text{L}</math></b>

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

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**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.

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6. Gently vortex to mix, then centrifuge briefly to collect the liquid in the bottom of the tube.
7. Add 12  $\mu\text{L}$  of ligation master mix to each 8- $\mu\text{L}$  hybridization reaction, for a total of 20  $\mu\text{L}$  per reaction.
8. Flick the tube or pipet up and down five times to mix, then centrifuge briefly.

- Incubate the 20- $\mu$ L ligation reactions in a thermal cycler at 16°C for 2–16 hours.

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

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

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## Reverse transcribe the RNA

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

Contents	Volume per reaction <sup>[1]</sup>
Nuclease-free Water	2 $\mu$ L
10X RT Buffer	4 $\mu$ L
2.5 mM dNTP Mix	2 $\mu$ L
Ion RT Primer v2	8 $\mu$ L
<b>Total Volume</b>	<b>16 <math>\mu</math>L</b>

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

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

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**STOPPING POINT** The cDNA can be stored at –30°C to –10°C for 2 weeks, stored at –86°C to –68°C for long-term storage, or used immediately.

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## Purify and size-select the cDNA

Before you begin

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

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

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## Purify and size-select the cDNA

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

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

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  - Set a single or multi-channel P200 pipettor at 150  $\mu$ L. Attach new 200- $\mu$ L tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down ten times.
  - Incubate the samples for 5 minutes at room temperature off of the magnetic stand.
- Remove the supernatant from the beads:
  - Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.

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

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**IMPORTANT!** Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3  $\mu\text{L}$  of supernatant in the tube.

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4. Bind desired cDNA products to the beads:

- a. Remove the Processing Plate from the magnetic stand.
- b. Add 72  $\mu\text{L}$  of Nuclease-free Water to the supernatant in the new sample well.
- c. Set a P100 or P200 pipettor at 78  $\mu\text{L}$ . Attach a new 100- $\mu\text{L}$  or 200- $\mu\text{L}$  tip to the pipettor, then pre-wet the new 100- or 200- $\mu\text{L}$  tip with 100% ethanol by pipetting the ethanol up and down three times.
- d. Without changing tips, add 78  $\mu\text{L}$  of 100% ethanol to each well.

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

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- e. Gently vortex the Nucleic Acid Binding Beads tube to resuspend the magnetic beads completely.
- f. Add 7  $\mu\text{L}$  of beads to the wells of the Processing Plate.
- g. Take the samples off of the magnetic stand. Set a single or multi-channel P200 pipettor at 150  $\mu\text{L}$ . Attach new 200- $\mu\text{L}$  tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down ten times.
- h. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

5. Remove the supernatant from the beads.

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

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**IMPORTANT!** Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3  $\mu\text{L}$  of supernatant in the tube.

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6. Wash the beads with the Wash Solution Concentrate with ethanol:

- a. Leave the Processing Plate on the magnetic stand, then add 150  $\mu\text{L}$  of Wash Solution Concentrate with ethanol to each sample.
- b. Incubate the samples for 30 seconds.

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

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

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

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

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7. Elute the cDNA from the beads:

- a. Remove the Processing Plate from the magnetic stand.
- b. Add 12  $\mu\text{L}$  of pre-warmed (37°C) Nuclease-free Water to each sample.
- c. Mix thoroughly by pipetting up and down ten times.
- d. Incubate the samples at room temperature for 1 minute.
- e. For each sample, collect 12  $\mu\text{L}$  of the eluant.

## Amplify the cDNA

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

**IMPORTANT!** Use the appropriate primers.

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

Non-barcoded Library PCR Mix

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

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

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

- Transfer 6 µL of cDNA sample to a new PCR tube.
  - Transfer 47 µL of the Non-barcoded Library PCR Mix to each 6 µL of cDNA sample.
  - Proceed to step 2.
- For barcoded libraries:
    - Prepare the Barcoded Library PCR Mix according to the following table.

Barcoded Library PCR Mix

Component	Volume per reaction <sup>[1]</sup>
Platinum™ PCR SuperMix High Fidelity <sup>[2]</sup>	45 µL
Ion Xpress™ RNA 3" Barcode Primer	1 µL
<b>Total Volume</b>	<b>46 µL</b>

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

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

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

- Flick the tube or slowly pipet the solution up and down five times to mix well, then centrifuge briefly to collect the liquid in the bottom of the tube.
- Run the cDNA samples in a thermal cycler:

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

## Purify and size-select the amplified DNA

Before you begin

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

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

Purify the amplified cDNA

- Prepare beads for each sample:
  - Gently vortex the Nucleic Acid Binding Beads tube to resuspend the magnetic beads completely.
  - Add 7-µL beads to wells on the Processing Plate.
  - Add 140 µL Binding Solution Concentrate to each well, then mix the Concentrate and beads by pipetting up and down ten times.
- Bind larger amplified DNA to the beads:
  - Transfer each 53-µL PCR reaction to a well with beads of the Processing Plate.
  - Set a P200 pipettor at 110 µL. Attach a new 200-µL tip to the pipettor, then pre-wet the tip with 100% ethanol by pipetting the ethanol up and down three times.

- c. Without changing tips, add 110  $\mu\text{L}$  of 100% ethanol to each well.

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

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- d. Set a single or multi-channel P200 pipettor at 150  $\mu\text{L}$ . Attach new 200- $\mu\text{L}$  tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down ten times.
- e. Incubate the samples for 5 minutes at room temperature.

3. Remove the supernatant from the beads:

- a. Place the Processing Plate on a magnetic stand for 5 minutes to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
- b. Leave the Processing Plate on the magnetic stand, then transfer the supernatant to a new well on the plate or to a well on a new plate.

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**IMPORTANT!** Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3  $\mu\text{L}$  of supernatant behind.

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4. Bind desired cDNA products to the beads:

- a. Remove the Processing Plate from the magnetic stand.
- b. Add 35  $\mu\text{L}$  of Nuclease-free Water to the supernatant in the new sample well.
- c. Set a P100 or P200 pipettor at 35  $\mu\text{L}$ . Attach a new 100- or 200- $\mu\text{L}$  tip to the pipettor, then pre-wet the new 100- or 200- $\mu\text{L}$  tip with 100% ethanol by pipetting the ethanol up and down three times.
- d. Without changing tips, add 35  $\mu\text{L}$  of 100% ethanol to each well.

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

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- e. Gently vortex the Nucleic Acid Binding Beads tube to resuspend the magnetic beads completely.
- f. Add 7- $\mu\text{L}$  beads to the wells of the Processing Plate.
- g. Set a single or multi-channel P200 pipettor at 150  $\mu\text{L}$ . Attach new 200- $\mu\text{L}$  tips to the pipettor, then mix the suspension in each well thoroughly by pipetting the wells up and down ten times.
- h. Incubate the samples for 5 minutes at room temperature off of the magnetic stand.

5. Remove the supernatant from the beads:

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

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**IMPORTANT!** Do not disturb the magnetic beads. If any beads are aspirated, leave 2–3  $\mu\text{L}$  of supernatant in the tube.

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6. Wash the beads with Wash Solution Concentrate with ethanol:

- a. Leave the Processing Plate on the magnetic stand, then add 150  $\mu\text{L}$  of Wash Solution Concentrate with ethanol to each sample.
- b. Incubate the plate at room temperature for 30 seconds.
- c. Leave the Processing Plate on the magnetic stand, then aspirate and discard the supernatant from the plate.
- d. Use a P10 or P20 pipettor to remove remaining ethanol.

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

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

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

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7. Elute the cDNA from the beads:

- a. Remove the Processing Plate from the magnetic stand.
- b. Add 15  $\mu\text{L}$  of pre-warmed (37°C) Nuclease-free Water to each sample.
- c. Mix thoroughly by pipetting up and down ten times.
- d. Incubate the Processing Plate at room temperature for 1 minute.
- e. Place the Processing Plate on the magnetic stand for 1 minute to separate the beads from the solution. Wait for the solution to clear before proceeding to the next step.
- f. For each sample, collect the 15  $\mu\text{L}$  of eluant.

## Assess the yield and size distribution of the amplified DNA

- Run 1  $\mu\text{L}$  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.
- Using the 2100 expert software, perform a smear analysis to determine size distribution of the amplified DNA:
  - Measure the area for the DNA that is:
    - 50–300 bp (the size range for all the ligation products)
    - 86–106 bp for non-barcoded libraries or 94–114 bp for barcoded libraries (the size range for the desired miRNA ligation products)
  - Calculate the ratio of miRNA ligation products in total ligation products using the formula for:
    - Non-barcoded libraries:  

$$[\text{Area (86–106 bp)}] \div [\text{Area (50–300 bp)}]$$
    - Barcoded libraries:  

$$[\text{Area (94–114 bp)}] \div [\text{Area (50–300 bp)}]$$
  - 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 that is required for template preparation."

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

If the ratio is	Action
$\geq 50\%$	Proceed to the next section or "Determine the library dilution required for template preparation."
$< 50\%$	Proceed to the next section or "Determine the library dilution required for template preparation," but expect 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 required miRNA ligation products to overall products.

## 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".

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

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

- 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, select a concentration that is equal to or lower than the lowest concentration of the 3 libraries, such as 30 nM. Dilute all or part of each library to 30 nM.

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

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

## Determine the library dilution required for template preparation

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

Determine the dilution factor that gives a concentration of ~100 pM. This concentration is suitable for downstream template preparation. Use the following formula:  
 Dilution factor = (Library or pooled library concentration in pM)/100 pM

### Example:

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

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

Thus, 1  $\mu\text{L}$  of library or pooled library mixed with 149  $\mu\text{L}$  of Low TE (1:150 dilution) yields approximately 100 pM. Use this library dilution for template preparation.

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