QUICK REFERENCE

ion torrent
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Ion AmpliSeq™ RNA Library Kit

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Pub. No. MAN0007451 **Rev.** 2.0

Note: For safety and biohazard guidelines, refer to the "Safety" appendix in the *Ion AmpliSeq™ RNA Library Kit User Guide* (Pub. no. MAN0007450). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

This quick reference is intended for experienced users of the Ion AmpliSeq™ RNA Library Kit. For detailed instructions and troubleshooting information, refer to the *Ion AmpliSeq™ RNA Library Kit User Guide* (Pub. no. MAN0007450).

Reverse transcribe the RNA

1. Pre-heat RNA prepared from FFPE tissue at 80°C for 10 minutes, then cool on ice or leave the tube at room temperature.
2. Combine the following components in a tube on ice.

Component	Volume per rxn.
5X VILO™ RT Reaction Mix	2 µL
10X SuperScript® III Enzyme Mix	1 µL
Total RNA, 10 ng	≤6 µL
Nuclease-free Water	to 10 µL

3. Gently mix the tube contents, and centrifuge the tube briefly.
4. Load the tube in the thermal cycler, and run the following program:

Temperature	Time
42°C	30 min
85°C	5 min
4°C	∞ [1]

[1] Remove samples when ready to proceed to the next step.

Amplify targets

IMPORTANT! Primers and 5X Ion AmpliSeq™ HiFi Master Mix may be viscous. Pipet slowly and mix thoroughly. We recommend PCR setup on ice or a cold block.

1. Combine the following components on ice, in a PCR strip tube or plate.

Component	Volume per 10-µL rxn.
5X Ion AmpliSeq™ HiFi Master Mix (red cap)	4 µL
5X Ion RNA Panel	4 µL
Nuclease-free Water	to 10 µL

2. Pipet the mixture up and down 5 times, or gently vortex, to mix.
3. Add the entire contents of the reverse transcription reaction, and gently mix again.
4. Seal the tubes or plate, load the samples in the thermal cycler, and run the following program:

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	2 min
Cycle; set number according to the following table	Denature	99°C	15 sec
	Anneal and extend	60°C	4 min
Hold	—	10°C	∞[1]

[1] Remove samples when ready to proceed to the next step.

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Number of amplification cycles		
Primer pairs per pool (see following table for Ready-to-use Panels)	Unfixed RNA	FFPE (fixed) RNA
12–24	21	24
25–48	20	23
49–96	19	22
97–192	18	21
193–384	17	20
385–768	16	19
769–1200	15	18

Ready-to-use Panels	Primer pairs per pool
Ion AmpliSeq™ RNA Apoptosis Panel	267
Ion AmpliSeq™ RNA Cancer Panel	50

STOPPING POINT (*Optional*) Store amplification products at 10°C overnight. For longer periods, store at –20°C.

Partially digest primer sequences

1. Carefully remove the cap or plate seal, and add 2 µL of FuPa Reagent (brown cap) to each amplified sample.
2. Pipet the mixture up and down 5 times, or gently vortex.
3. Seal the tubes or plate, load the samples in the thermal cycler, and run the following program.

Temperature	Time
50°C	10 min
55°C	10 min
60°C	20 min
10°C	Hold (for up to 1 hour)

Ligate adapters to the amplicons and purify

Barcoded libraries only: Combine and dilute adapters

Scale volumes as necessary.

IMPORTANT! Avoid cross-contamination of barcoded adapters.

Example barcode adapter mix for up to 4 reactions (8 µL total)	
Component	Volume
Ion P1 Adapter (violet cap)	2 µL
Ion AmpliSeq™ Barcode X[1] (white cap)	2 µL
Nuclease-free Water	4 µL

[1] X = barcode chosen

Set up and run the ligation reaction

1. If there is visible precipitate in the Switch Solution, pipet up and down at room temperature to resuspend.
2. Uncap the tubes or remove the plate seal, and add the following components to each tube or well containing digested sample.

Component	Volume
(Digested sample)	(22 µL)
Switch Solution (yellow cap)	4 µL
Ion AmpliSeq™ Adapters (for non-barcoded libraries; green cap) *or* Diluted barcode adapter mix (for barcoded libraries)	2 µL
Total volume	**28 µL**

3. Add 2 µL of DNA Ligase (blue cap) to each well.
4. Pipet up and down 5 times, or gently vortex, to mix.
5. Seal the tubes or plate, load the samples in the thermal cycler, and run the following program.

Temperature	Time	
	Unfixed samples	Fixed (FFPE) samples
22°C	30 min	60 min
72°C	10 min	10 min
10°C	Hold[1]	Hold[1]

[1] Remove samples when ready to proceed to the next step.

STOPPING POINT (*Optional*) Samples may be stored at –20°C.

Purify the unamplified library (two-round purification)

Reagent preparation

- Ensure that the Wash Solution has been completed.
- Dissolve any precipitate in the Binding Solution Concentrate.
- Incubate the Dynabeads® Magnetic Beads at room temperature for ≥10 minutes. Vortex to mix thoroughly immediately before pipetting.
- Pre-heat the elution solution—Nuclease-free Water or Low TE—at 37°C for ≥5 minutes.

First-round purification

1. Add 5 µL of thoroughly mixed Dynabeads® Magnetic Beads to each designated well in the 96-well Processing Plate.

2. Add 72 µL of Binding Solution Concentrate to each bead-containing well, and pipet up and down 10 times.
3. Add 30 µL of Nuclease-free Water to each 30-µL ligation reaction (unamplified library), and mix gently.
4. Add the 60-µL ligation reaction/unamplified library to the beads and Binding Solution mixture from step 2, and mix by pipetting up and down 5 times.
5. Pre-wet a pipette tip, and add 32 µL of 100% ethanol to each sample well and mix by pipetting up and down 10 times.
6. Incubate the samples for 5 minutes at room temperature.
7. Place the plate on a magnetic stand for 1–2 minutes until the solution is clear.
8. Keeping the plate on the magnetic stand, carefully remove the supernatant and transfer to a fresh well in the plate.

IMPORTANT! Keep the supernatant.

Second-round purification: Bind the library to the beads

1. Add 5 µL of thoroughly mixed Dynabeads® Magnetic Beads to the supernatant (≈162 µL) from the first-round purification.
2. Add 25 µL of Binding Solution Concentrate to each bead-containing well, and pipet up and down 5 times to mix.
3. Pre-wet a pipette tip by pipetting 32 µL of 100% ethanol up and down 3 times.
4. Without changing tips, add 32 µL of 100% ethanol to each well, and pipet up and down 10 times to mix.
5. Incubate the samples for 5 minutes at room temperature.
6. Place the plate on a magnetic stand for 5 minutes until the solution is clear.
7. Keeping the plate on the magnetic stand, carefully remove and discard the supernatant.

IMPORTANT! Keep the beads.

Second-round purification: Wash the beads and elute the library

1. While the plate is on the magnetic stand, add 150 µL of completed Wash Solution to each sample well, and incubate the samples for 30 seconds.
2. Keeping the plate on the magnetic stand, carefully aspirate and discard the supernatant.
3. Air dry the beads at room temperature for 3 minutes.
4. Remove the plate from the magnetic stand, add 10 µL of pre-heated (37°C) Nuclease-free Water to each well, and pipet up and down 10 times to mix.
5. Incubate for 1 minute at room temperature.
6. Place the plate on the magnetic stand for 1 minute.
7. Keeping the plate on the magnetic stand, carefully remove the supernatant and transfer to a fresh well or tube.

IMPORTANT! Keep the supernatant.

Amplify the library and purify

Amplify the library

1. On ice, combine the following in a 0.2-mL PCR strip tube or 96-well reaction plate:

Component	Volume per rxn.
Platinum® PCR SuperMix HiFi (black cap)	50 µL
Library Amplification Primer Mix (white cap)	2 µL
Purified, unamplified library	10 µL

2. Seal the tubes or plate, load the samples in the thermal cycler, and run the following program.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	98°C	2 min
5 cycles	Denature	98°C	15 sec
	Anneal and extend	60°C	1 min
Hold	—	10°C	∞[1]

[1] Remove samples when ready to proceed to the next step.

STOPPING POINT (*Optional*) Store the amplified library at –20°C.

Purify the amplified library

Reagent preparation

- Ensure that the Wash Solution has been completed.
- Dissolve any precipitate in the Binding Solution Concentrate.
- Incubate the Dynabeads® Magnetic Beads at room temperature for ≥10 minutes. Vortex to mix thoroughly immediately before pipetting.
- Pre-heat the elution solution—Nuclease-free Water or Low TE—at 37°C for ≥5 minutes.

Bind the library to the beads

1. Add 10 µL of thoroughly mixed Dynabeads® Magnetic Beads to each designated well in the 96-well Processing Plate.
2. Add 72 µL of Binding Solution Concentrate to each bead-containing well, and pipet up and down 10 times.
3. Add 62 µL of the amplification reaction/amplified library to the beads and Binding Solution mixture from step 2, and mix by pipetting up and down 5 times.
4. Pre-wet a pipette tip, add 45 µL of 100% ethanol to each sample well, and mix by pipetting up and down 10 times.
5. Incubate the samples for 5 minutes at room temperature.

6. Place the plate on a magnetic stand for 1–2 minutes until the solution is clear.
7. Keeping the plate on the magnetic stand, carefully remove and discard the supernatant.

> **IMPORTANT!** Keep the beads.

Wash the beads and elute the library

1. While the plate is on the magnetic stand, add 150 µL of completed Wash Solution to each sample well, and incubate the samples for 30 seconds.
2. Carefully aspirate and discard the supernatant without disturbing the beads.
3. Air dry the beads at room temperature for 3 minutes.
4. Remove the plate from the magnetic stand, add 15 µL of pre-heated (37°C) Low TE to each well, and pipet up and down 10 times to mix.
5. Incubate for 1 minute at room temperature.
6. Place the plate on the magnetic stand for 1 minute.
7. Keeping the plate on the magnetic stand, carefully remove the supernatant and transfer to a fresh well or tube.

> **IMPORTANT!** Keep the supernatant, which contains the final library.

Quantify the library and dilute for template preparation

Refer to the *Ion AmpliSeq™ RNA Library Kit User Guide* for information on library quantification and dilution for template preparation.

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