Ion AmpliSeq™ Transcriptome Human Gene Expression Kit

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Pub. No. MAN0010743 **Rev.** C.0

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *Ion AmpliSeq™ Transcriptome Human Gene Expression Kit User Guide* (Pub. no. MAN0010742). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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Guidelines for isolating and quantifying RNA

- A list of recommended RNA isolation kits is provided in the *Before you begin* section of the *Ion AmpliSeq™ Transcriptome Human Gene Expression Kit User Guide* (Pub. No. MAN0010742).
- We recommend using the Agilent™ Bioanalyzer™ for quantifying RNA from FFPE and calculating percentage of RNA fragments larger than 200 nt using smear analysis. Expect optimal performance and gene expression measurements from RNA (unfixed and fixed) that has > 30% of fragments larger than 200 nt in length. Expect to see lower library yield and lower on-target mapping when using RNA that has < 30% of fragments that are larger than 200 nt.

Reverse transcribe RNA

1. If RNA was prepared from FFPE tissue and not previously heat-treated, pre-heat at 80°C for 10 minutes, then cool on ice or leave tube to cool at room temperature.

2. For each sample, add the following components into a single well of a 96-well PCR plate on ice. Prepare a master mix for multiple reactions, adding the enzyme last.

Component	Volume
5X VILO™ Reaction Mix	1.0 µL
10X SuperScript™ III Enzyme Mix	0.5 µL
DNase-treated total RNA (10 ng)	≤ 3.5 µL
Nuclease-Free water	to 5 µL
Total	**5 µL**

3. Seal the plate with MicroAmp™ adhesive film, vortex thoroughly, then spin down to collect droplets.

4. Load the plate in the thermal cycler, then run the following program to synthesize cDNA.

Temperature	Time
42°C	30 minutes
85°C	5 minutes
4°C	Hold[1]

[1] Samples can be held at 4°C overnight.

STOPPING POINT Samples can be stored at 4°C overnight. For longer periods, store at –20°C.

Amplify targets

1. For each reaction, combine the following components on ice. Prepare a master mix for multiple reactions, adding the enzyme last.

Component	Volume per reaction
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel	8 µL
Nuclease-Free Water	3 µL
Total	**15 µL**

2. If a master mix was prepared, gently vortex PCR master mix, then centrifuge briefly to collect droplets.

3. Remove the plate seal from the reverse transcription reaction, then add 15 µL of PCR master mix to each reaction well of the plate.

4. Seal the plate, vortex thoroughly, then centrifuge to collect droplets.

5. Load the plate in the thermal cycler, then run the following program.

Stage	Temperature	Time
Hold	99°C	2 min
Cycle; (set number according to the following table)	99°C	15 sec
	60°C	16 min
Hold	10°C	Hold[1]

[1] You can hold samples at 4°C overnight.

Input RNA	Amount	Number of cycles
Unfixed RNA	0.1 – 1 ng	16
	10 ng	12
	100 ng	10
FFPE RNA	10 ng	16
	100 ng	13

STOPPING POINT You can store PCR products at 4°C overnight. For longer periods, store at –20°C.

Partially digest primer sequences

1. Carefully remove the plate seal, then add 2 µL of FuPa Reagent (brown cap) to each amplified sample.

2. Seal the plate, vortex thoroughly, then centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

3. Load the plate in the thermal cycler, then run the following program.

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

IMPORTANT! Do NOT freeze samples at this point. Proceed to next step within 1 hour.

Ligate adapters to amplicons and purify

Combine and dilute adapters

IMPORTANT! When handling barcoded adapters, be careful to avoid cross-contamination by changing gloves frequently and opening one tube at a time.

For each barcode X chosen, prepare a mix of Ion P1 Adapter and Ion Xpress™ Barcode X at a final dilution of 1:4 for each adapter.

Example barcode adapter mix for up to 4 reactions

Component [1]	Volume
Ion P1 Adapter (violet cap)	2 µL
Ion Xpress™ Barcode X (white cap)	2 µL
Nuclease-Free water	4 µL
Total	**8 µL**

[1] All components are part of theIon Xpress™ Barcode Adapters 1–16 Kit (4471250

Note: You can store combined and diluted barcodes at –20°C for future use.

Perform ligation reaction

IMPORTANT! If there is visible precipitate in the Switch Solution, vortex or pipet up and down at room temperature to resuspend.

1. Carefully remove the plate seal, then add the following components to each well containing digested sample.

IMPORTANT! Add the DNA Ligase last. Do not combine DNA Ligase and adapters before adding to digested amplicons.

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	Diluted barcode adapter mix (for barcoded libraries)	2 µL
3	DNA Ligase (blue cap)	2 µL
—	**Total** (includes 22 µL of digested amplicon)	**30 µL**

2. Seal the plate, vortex thoroughly, then centrifuge to collect droplets.

3. Load the plate in the thermal cycler, then run the following program.

Temperature	Time
22°C	30 minutes (for unfixed RNA) *or* 60 minutes (for FFPE RNA)
72°C	5 minutes
10°C	Hold (up to 1 hour)

STOPPING POINT Store samples at –20°C.

Purify the unamplified library

1. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each library, then pipet up and down 5 times to thoroughly mix the bead suspension with the DNA.

2. Incubate the mixture for 5 minutes at room temperature.

3. Place the plate in a magnetic rack such as the DynaMag™-96 Side Magnet (Cat. No. 12331D), then incubate for 2 minutes or until solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.

4. Add 150 µL of freshly prepared 70% ethanol and move the plate side-to-side in the two positions of the magnet to wash the beads, then remove and discard the supernatant without disturbing the pellet. Alternatively, remove the plate from the magnet and gently pipet up and down 5 times, then return the plate to the magnet for 2 minutes or until solution clears.

5. Repeat step 4 for a second wash.

6. Use a 10- or 20-µL pipettor to remove all ethanol droplets from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2 minutes.

Proceed immediately to "Quantify and dilute the library" on page 3.

Quantify and dilute the library

Option 1: Quantify library by qPCR

Elute the unamplified library

1. Remove the plate containing the Ion AmpliSeq™ Transcriptome library from the magnet, then add 50 µL of Low TE to the pellet to disperse the beads. Seal the plate, vortex thoroughly, then centrifuge down to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

2. Place the plate in the magnet for at least 2 minutes. Transfer 45 µL of the supernatant to new wells on the same plate.

Quantify library by qPCR and calculate dilution factor

1. Prepare five 10-fold serial dilutions of the *E. coli* DH10B Ion Control Library (~68 pM; from the Ion Library TaqMan® Quantitation Kit) at 6.8 pM, 0.68 pM, 0.068 pM, 0.0068 pM, and 0.00068 pM (standards 1–5). Mark these as standards, then use these concentrations in the qPCR instrument software.

2. Dilute each Ion AmpliSeq™ Transcriptome library using the following recommendations.

Input RNA	Amount	Recommended dilutions
Unfixed RNA	10 ng	1:10,000 or 1:100,000
FFPE RNA	10 ng	1:100 or 1:1,000

3. Prepare reaction mixtures for 3 wells for each library and standard sample. Use the following tables to calculate the required volume for the master mix.

Component	Volume per reaction	
	96-well plate	384-well plate
2X TaqMan® Master Mix	10 µL	5 µL
20X Ion TaqMan® Assay	1 µL	0.5 µL
Total	**11 µL**	**5.5 µL**

4. Perform one of the following actions based on your choice of plates:

Option	Action
96-well reaction plates	Dispense 11 µL of the master mix into each well, then add 9 µL of your diluted library and standards.
384-well reaction plates	Dispense 5.5 µL of the master mix into each well, then add 4.5 µL of your diluted library and standards.

5. Load the plate in the real-time instrument, then run the following program.

Stage	Temperature	Time
Hold	50°C	2 min
Hold	95°C	20 sec
Cycle (40 cycles)	95°C	1 sec
	60°C	20 sec

6. Following qPCR, calculate the average concentration of the undiluted AmpliSeq™ Transcriptome library by multiplying the concentration determined with qPCR by the library dilution used in the assay.

7. If the library concentration is greater than 100 pM, normalize the final library concentration to 100 pM, then pool barcoded libraries for templating and sequencing by combining an equal volume of each barcoded library. Alternatively, if one or more libraries is < 100 pM, dilute each library to the same concentration, and pool by combining an equal volume of each.

   Expected yield:

Input RNA	Yield (concentration)
Unfixed RNA	0.5–5.0 nM
FFPE RNA	40–500 pM

   Proceed to template preparation and sequencing. Detailed information is in the user guide for your template preparation kit.

Option 2: Quantify the library using Agilent™ 2100 Bioanalyzer™ Instrument

Note: We do not recommend this option for libraries prepared from RNA.

Amplify the library

1. Remove the plate containing the AmpliSeq™ Transcriptome library from the magnet, then add 50 µL of 1X Library Amp Mix and 2 µL of 25X Library Amp Primers to each bead pellet. Pipet the mixture up and down 5 times to mix thoroughly.

2. Place the plate back on the magnet for at least 2 minutes or until solution clears, then carefully transfer ~50 µL of supernatant from each well to clean plate without disturbing the pellet.

   Note: *(Optional)* Alternatively, amplify the library in the presence of the AMPure™ XP beads.

3. Seal the plate with MicroAmp™ Adhesive Film, place a MicroAmp™ Compression Pad on the plate, load in the thermocycler, then run the following program:

Stage	Temperature	Time
Hold	98°C	2 min
5 cycles	98°C	15 sec
	64°C	1 min
Hold	10°C	Hold (up to 1 hour)

STOPPING POINT *(Optional)* You can store samples at –20°C.

Purify the amplified library

1. Add 25 µL of Agencourt™ AMPure™ XP Reagent (at room temperature) to each plate well containing ~50 µL of sample, then pipet up and down 5 times to thoroughly mix the bead suspension with the DNA.

2. Incubate the mixture for 5 minutes at room temperature.

3. Place the plate in a DynaMag™-96 Side Magnet for at least 3 minutes or until solution is completely clear.

4. Carefully transfer the supernatant to a new well on the same plate without disturbing the pellet. Discard the pellet.

5. Remove the plate from the magnet. To the supernatant from previous step, add 60 µL of Agencourt™ AMPure™ XP Reagent, then pipet up and down 5 times to thoroughly mix the bead suspension with the DNA.

6. Incubate the mixture for 5 minutes at room temperature.

7. Place the plate in the magnet for 5 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.

8. Add 150 µL of freshly prepared 70% ethanol to each well, then move the plate side to side in the magnet to wash the beads. Remove, then discard the supernatant without disturbing the pellet.

9. Repeat step 8 for a second wash.

10. Use a 10- or 20-µL pipette to remove all ethanol droplets from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2 minutes.

11. Remove the plate containing the Ion AmpliSeq™ Transcriptome library from the magnet, then add 50 µL of Low TE to the pellet to disperse the beads. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge down to collect droplets.

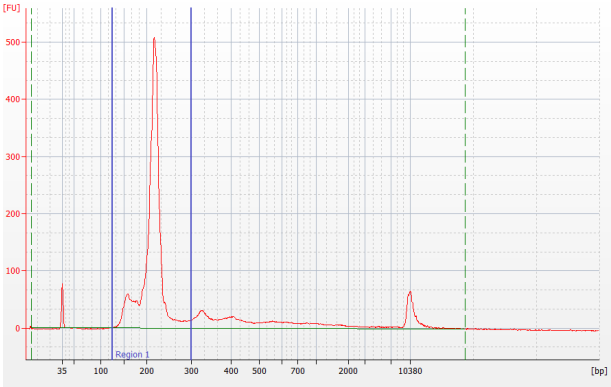
12. Place the plate on the magnet for at least 2 minutes. Transfer 45 µL of the supernatant to new a well on the same plate.

Quantify the library using the Agilent™ 2100 Bioanalyzer™ instrument and calculate dilution factor

1. Analyze 1 µL of amplified library on the Agilent™ 2100 Bioanalyzer™ instrument with the Agilent™ High Sensitivity DNA Kit (Cat. No. 5067-4626).

2. Determine the molar concentration of the amplified library using the Bioanalyzer™ software.

3. If the library concentration is > 20,000 pM, dilute the library 1:10, then repeat the quantification to obtain a more accurate measurement. AmpliSeq™ Transcriptome libraries typically have yields of 1,000–50,000 pM.



Example trace of amplified Ion AmpliSeq™ Transcriptome library.

4. Based on the calculated library concentration, determine the dilution that results in a concentration of ~100 pM.

5. Dilute library to ~100 pM as described, pool barcoded libraries by combining an equal volume of each, then proceed to template preparation.

   Proceed to template preparation and sequencing. Detailed information is in the user guide for your template preparation kit.