Ion AmpliSeq™ Transcriptome Mouse Gene Expression Kit

Catalog Numbers A36553, A36554, and A36555

Pub. No. MAN0017424 **Rev.** B.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *Ion AmpliSeq™ Transcriptome Mouse Gene Expression Kit User Guide* (Pub. No. MAN0017343). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Guidelines for isolating and quantifying RNA

- A list of recommended RNA isolation kits is in the *Ion AmpliSeq™ Transcriptome Mouse Gene Expression Kit User Guide* (Pub. No. MAN0017343).
- We recommend using the Agilent™ Bioanalyzer™ for calculating the 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 you are using the ERCC RNA Spike-In Mix and the Ion AmpliSeq™ RNA ERCC Companion Panel proceed to "Add ERCC RNA Spike-In Mix 1 to RNA samples" on page 5.

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™ RT Reaction Mix	1.0 µL
10X SuperScript™ III Enzyme Mix	0.5 µL
DNase-treated total RNA (10 ng)[1]	≤ 3.5 µL
Nuclease-Free water	to 5 µL
Total	**5 µL**

[1] Input amount can range from 0.1–100 ng for high quality RNA. PCR cycles must be adjusted accordingly.

3. Seal the plate with MicroAmp™ adhesive film, vortex thoroughly, then centrifuge 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 Mouse 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 minutes
Cycle; (set number according to the following table)	99°C	15 seconds
	60°C	16 minutes
Hold	10°C	Hold[1]

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

Amount	Number of cycles
0.1 – 1 ng	16
10 ng	12
100 ng	10

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 minutes
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 the amplicons and purify

When sequencing multiple libraries on a single chip, you *must* ligate a different barcode adapter to each library. DNA and RNA libraries from the same sample also require different barcodes.

IonCode™ Adapters are provided at the appropriate concentration and include forward and reverse adapters in a single well. No further handling is necessary.

Ion Xpress™ adapters require handling and dilution as described in the *Ion AmpliSeq™ Library Kit 2.0 User Guide*.

Ion Xpress™ adapters only: Combine and dilute adapters

For each barcode X selected, prepare a mix of Ion P1 Adapter and Ion Xpress™ Barcode X at a final dilution of 1:4 for each adapter. For example, combine the volumes indicated in the following table. Scale volumes as necessary. Use 2 µL of this barcode adapter mix in step 3 below.

Component	Volume
Ion P1 Adapter	2 µL
Ion Xpress™ Barcode X[1]	2 µL
Nuclease-free Water	4 µL
Total	**8 µL**

[1] X = barcode chosen

Note: Store diluted adapters at –20°C.

Perform the ligation reaction

1. If there is visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.

2. Briefly centrifuge the plate to collect the contents.

3. Carefully remove the plate seal, then add the following components in the order that is listed to each well containing digested amplicons. If preparing multiple non-barcoded libraries, a master mix of Switch Solution and adapters can be combined before addition.

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	IonCode™ Adapters *or* diluted Ion Xpress™ barcode adapter mix (for barcoded libraries)	2 µL
3	DNA Ligase (blue cap)	2 µL
—	**Total volume**	**~30 µL**

4. Seal the plate with a new MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.

5. Place a MicroAmp™ Compression Pad on the plate, load in the thermal cycler, then run the following program:

Temperature	Time
22°C	30 minutes
72°C	5 minutes
10°C	Hold (up to 1 hour)

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.

Amount	Recommended dilutions
10 ng	1:2,500 or 1:10,000

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 minutes
Hold	95°C	20 seconds
Cycle (40 cycles)	95°C	1 second
	60°C	20 seconds

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

7. If the library concentration is greater than 70 pM, normalize the final library concentration to 70 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 <70 pM, dilute each library to the same concentration, and pool by combining an equal volume of each.

   Expected yield: 0.5–5.0 nM

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 Ion 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 Reagent.

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 minutes
5 cycles	98°C	15 seconds
	64°C	1 minute
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.

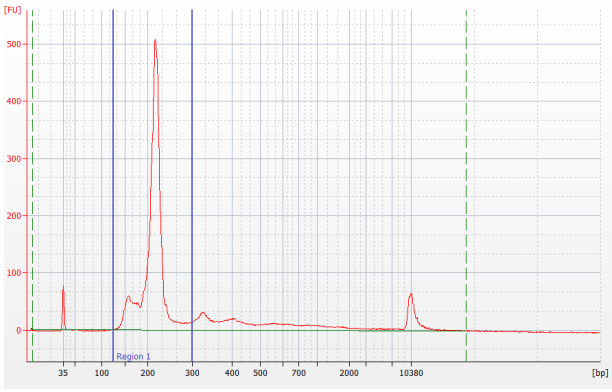
12. Place the plate on the magnet for at least 2 minutes. Transfer 45 µL of the supernatant to new a well on the same plate.

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. Ion 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 ~70 pM.

5. Dilute library to ~70 pM as described, pool barcoded libraries by combining an equal volume of each.

Supplemental procedures

Add ERCC RNA Spike-In Mix 1 to RNA samples

IMPORTANT! Ion AmpliSeq™ RNA panel size and content, as well as starting RNA quantity and quality, affect the percentage of ERCC mapped reads in the final libraries. We recommend using the following tables as starting points. Further adjustment of amount of ERCC RNA Spike-In Mix added to each sample may be needed.

1. Determine the amount of ERCC RNA Spike-In Mix 1 to add, using the following table as a guideline.

Amount of total RNA	Volume of diluted ERCC RNA Spike-In Mix 1
10 ng	1 µL (1:5,000 dilution)
20 ng	2 µL (1:5,000 dilution)
50 ng	1 µL (1:1,000 dilution)
100 ng	2 µL (1:1,000 dilution)

2. Prepare the appropriate dilution of ERCC RNA Spike-In Mix 1 needed using the following table. Scale the volumes accordingly if > 10 µL of the dilution is needed.

Dilution	ERCC RNA Spike-In Mix 1	Nuclease-free Water
1:10	1 µL undiluted	9 µL
1:100	1 µL of 1:10	9 µL
1:1,000	1 µL of 1:100	9 µL
1:5,000	2 µL of 1:1,000	8 µL
1:10,000	1 µL of 1:1,000	9 µL
1:50,000	1 µL of 1:5,000	9 µL

Note: Prepare a fresh dilution of the ERCC RNA Spike-In Mix for each procedure. Discard unused diluted Spike-In Mix.

3. Add the volume of the appropriate ERCC RNA Spike-In Mix 1 dilution, which is determined in step 1, to each RNA sample. Concentrate the RNA sample containing ERCC RNA Spike-In Mix 1 (centrifugal vacuum concentration recommended), if needed.

   Proceed to "Add the Ion AmpliSeq™ RNA ERCC Companion Panel to your RNA panel".

Add the Ion AmpliSeq™ RNA ERCC Companion Panel to your RNA panel

Note: Any Ion AmpliSeq™ Transcriptome Gene Expression panel can be modified through the addition of the Ion AmpliSeq™ RNA ERCC Companion Panel.

1. Add the Ion AmpliSeq™ RNA ERCC Companion Panel.

Reaction type	Amount
Manual reaction	Add 1 µL per reaction.
Automated (Ion Chef™ System)	Add 8 µL per pool (16 µL per Ion Chef™ Instrument run).

2. Mix thoroughly by vortexing, then centrifuge. The modified primer pools are ready to use.

3. Follow the standard library preparation protocol for Ion AmpliSeq™ RNA library preparation, using the change in panel volume specified in the table in step 1.

   Return to step 2 in "Reverse transcribe RNA" on page 1

Manufacturer: Life Technologies Corporation | 5781 Van Allen Way | Carlsbad, CA 92008

The information in this guide is subject to change without notice.

Revision history: Pub. No. MAN0017424

Revision	Date	Description
B.0	20 September 2018	• Protocol updates • Support added for the Ion AmpliSeq™ RNA ERCC Companion Panel
A.0	30 November 2017	New document