iontorrent

Ion AmpliSeq™ Transcriptome Human Gene Expression Kit

USER GUIDE

for use with:
Ion PGM™ System
Ion OneTouch™ 2 System
Ion Chef™ System
Ion Proton™ System
Ion S5™ and Ion S5™ XL Systems

Catalog Number A26325, A26326, and A26327
Publication Number MAN0010742
Revision C.0

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Revision history: Pub. No. MAN0010742

Revision	Date	Description
C.0	20 January 2017	• Simplified chapter structure • Added notes for use on the Ion Chef™
B.0	12 February 2015	Fixed broken link in "Tips and troubleshooting"
A.0	08 August 2014	New guide covers library construction using Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel

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Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

About this guide

This guide describes the procedure for using the Ion AmpliSeq™ Human Gene Expression Core Panel from the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit to prepare libraries from RNA.

Product description

The Ion AmpliSeq™ Human Gene Expression Core Panel provides gene-level expression information from a single multiplexed panel targeting over 20,000 genes (> 95% of the RefSeq gene database). The small amplicon designs enable the use of the panel with RNA isolated from fixed tissues, such as formalin-fixed paraffin-embedded (FFPE) samples. Library preparation with the kit requires 10 ng of total RNA input, without the need for poly (A) selection or ribosomal RNA depletion.

Note: For preparing automated libraries with the Ion Chef System, use the Ion AmpliSeq™ Transcriptome Human Gene Expression Panel: Chef-ready Kit (Cat. No. A31446, ordered separately). For more information, see the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432).

Compatible kits

The panel is optimized to work with the following kits:

- Ion AmpliSeq™ Library Kit Plus
- Ion Xpress™ Barcode Adapters
- IonCode™ Barcode Adaptors
- Templating kits for:
  - Ion Chef™ System
  - Ion OneTouch™ 2 System
- Sequencing kits for:
  - Ion PGM™ System
  - Ion Proton™ Systems
  - Ion S5™/Ion S5™ XL Systems

Contents and storage

The Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (Cat. Nos. A26325, A26326, and A26327) provides reagents for 24, 96, and 384 libraries, respectively.

Component	Number of kits		
	Cat. No. A26325 (24 reactions)	Cat. No. A26326 (96 reactions)	Cat. No. A26327 (384 reactions)
Ion AmpliSeq™ Library Kit Plus	1	4	16
SuperScript™ VILO™ cDNA Synthesis Kit	1	2	8
Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel	1	4	16

The kit components for each kit are shown in the following table. The amount of each component is indicated for a single kit.

Contents	Amount	Storage
Ion AmpliSeq™ Library Kit Plus [1]		
5X Ion AmpliSeq™ HiFi Mix (red cap)	130 µL	-30°C to -10°C
FuPa Reagent (brown cap)	60 µL	
Switch Solution (yellow cap)	130 µL	
DNA Ligase (blue cap)	60 µL	
25X Library Amp Primers (white cap)	60 µL	
1X Library Amp Mix (black cap)	1.5 mL	
Low TE (clear cap)	1.7 mL	Room temp (15–30°C)
SuperScript™ VILO™ cDNA Synthesis Kit[2, 3]		
5X VILO™ Reaction Mix	200 µL	-30°C to -10°C
10X SuperScript™ III Enzyme Mix	100 µL	
Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel[3]		
Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel	192 µL	-30°C to -10°C

[1] Kit is shipped on frozen gel packs. Store as indicated.
[2] May also be ordered separately (Cat. No. 11754-050).
[3] Shipped on dry ice. Store as indicated.

Required materials not supplied

Unless otherwise specified, all materials are available from Thermo Fisher Scientific (**www.thermofisher.com**). MLS: Fisher Scientific (**www.fisherscientific.com**) or major laboratory suppliers.

Item	Source
Recommended **RNA isolation kits**	
MagMAX™-96 Total RNA Isolation Kit	4463365
PureLink™ RNA Mini Kit	12183020
For FFPE: RecoverAll™ Total Nucleic Acid Isolation Kit for FPPE	AM1975
Recommended **for quantification**	
Qubit™ RNA HS Assay Kit	Q32852
Agilent™ 2100 Bioanalyzer™ Instrument	G2939AA
Agilent™ High Sensitivity DNA Kit	5067-4626
Other materials	
Ion Xpress™ Barcode Adapters 1-16 Kit	4471250
MicroAmp™ Optical 96-well Reaction Plates	N8010560 4306737 (with barcode)
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical Film Compression Pad	4312639
Ion Library Quantitation Kit	4468802
Agencourt™ AMPure™ XP Magnetic Beads	A63882
100% Ethanol	MLS
Nuclease-Free Water	AM9932
1.5-mL Eppendorf LoBind™ Tubes	022431021
Qubit™ 3.0 Fluorometer or equivalent Qubit™ 2.0 Fluorometer[1]	Q33216
Magnetic Stand-96 or DynaMag™-96 Side Magnet	AM10027, 12331D

Item	Source
One of the following: • SimpliAmp™ Thermal Cycler • AB™ 2720 Thermal Cycler • Veriti™ 96-well Thermal Cycler • ProFlex™ 96-Well PCR System • GeneAmp™ PCR System 9700 Single or Dual 96-well Thermal Cycler[1]	Various
Microcentrifuge (for quick 2000 × *g* centrifugations)	MLS

[1] Supported but no longer available for purchase.

Ion AmpliSeq™ Transcriptome Human Gene Expression Panel: Chef-ready Kit

The Ion AmpliSeq™ Transcriptome Human Gene Expression Panel: Chef-ready Kit (Cat. No. A31446, ordered separately) provides reagents for using the Ion AmpliSeq™ Transcriptome Human Gene Expression Panel on the Ion Chef™ System. The Ion AmpliSeq™ Transcriptome Human Gene Expression Panel: Chef-ready Kit consists of Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel tubes, which are ready to load into an Ion AmpliSeq™ Chef Reagents DL8 cartridge, and the Ion AmpliSeq™ Kit for Chef DL8, which contains all the reagents and supplies sufficient for preparing 32 libraries. See the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432) for more information.

Ion AmpliSeq™ Transcriptome Human Gene Expression Panel: Chef-ready Kit (Cat. No. A31446, ordered separately)

Component	Quantity	Storage
Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel[1]		
Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel	8 × 150 µL	–30°C to –10°C
Ion AmpliSeq™ Kit for Chef DL8		
Ion AmpliSeq™ Chef Reagents DL8	4 cartridges	–30°C to –10°C
Ion AmpliSeq™ Chef Solutions DL8	4 cartridges	2°C to 8°C[2]
Ion AmpliSeq™ Chef Supplies DL8 (per insert) • Ion AmpliSeq™ Tip Cartridge L8 • PCR Frame Seal • Enrichment Cartridge	1 box with 4 inserts	15°C to 30°C
IonCode™ 0101–0132 in 96 Well PCR Plates (dried) Set includes 4 PCR plates: • IonCode™ 0101–0108 in 96 Well PCR Plate (red) • IonCode™ 0109–0116 in 96 Well PCR Plate (yellow) • IonCode™ 0117–0124 in 96 Well PCR Plate (green) • IonCode™ 0125–0132 in 96 Well PCR Plate (blue)	1 set of 4 plates	15°C to 30°C

[1] Shipped on dry ice. Store as indicated.

[2] Ion AmpliSeq™ Chef Solutions DL8 cartridges are shipped at ambient temperature, but need to be stored at 2°C to 8°C upon arrival.

2 Methods

Before you begin

- Thaw components that contain enzymes—such as 5X Ion AmpliSeq™ HiFi Mix, FuPa Reagent, and DNA Ligase—on ice, and keep on ice during procedure. All other components, including primer pools, may be thawed at room temperature. Gently vortex and centrifuge before use.
- 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.
- Minimize freeze-thawing of Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel and 5X VILO™ Reaction Mix by aliquoting as needed for your experiments.
- Use good laboratory practices to minimize cross-contamination of products. If possible, perform PCR setup in an area or room that is separate from template preparation. Always change pipette tips between samples.
- Do not reuse MicroAmp™ Clear Adhesive Films.
- Pipet viscous solutions slowly and ensure complete mixing by vigorous vortexing or pipetting up and down several times.

Guidelines for isolating and quantifying RNA

- A list of recommended RNA isolation kits is provided in the "Required materials not supplied" on page 7 section.

IMPORTANT! We strongly recommend DNase treatment of RNA samples. Follow the DNase treatment instructions in RNA isolation kit manual, or use TURBO DNA-*free*™ Kit (Cat. No. AM1907) for DNase treatment. Although DNase-treated total RNA is the recommended input, you can also start from poly(A)+ RNA or rRNA-depleted RNA. When poly (A)+ RNA is used, amplicons corresponding to genes without poly(A) tail will not be amplified.

- A list of recommended kits and instruments for quantifying RNA can be found in the "Required materials not supplied" on page 7. We recommend the Qubit™ RNA HS Assay Kit (Cat. No. Q32852) for quantifying unfixed RNA.

  Note: 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™ 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 may range from 0.1–100 ng for high quality RNA and 10–100 ng for FFPE 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 min
85°C	5 min
4°C	Hold[1]

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

STOPPING POINT Samples may 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.

   Note: Use a new adhesive film to avoid cross-contamination. Due to the long PCR incubation time and small reaction volumes, be sure to seal the plate well and/or use a compression pad to minimize evaporation.

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

IMPORTANT! Use recommended input amount and number of PCR cycles to avoid bias in gene expression levels due to PCR saturation.

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

If you are running multiple libraries on a single chip, you must assign a unique barcode to each library. Additionally, we recommend barcodes to verify sample identity and to track potential sources of contamination.

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.

For example, combine the volumes indicated in the following table. Scale volumes as necessary.

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 thelon 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

IMPORTANT! Bring the AMPure™ XP reagent to room temperature, then vortex thoroughly to disperse the beads before use. Pipet the suspension slowly.

IMPORTANT! Use freshly prepared 70% ethanol for the next steps. Combine 230 µL of 100% ethanol with 100 µL of Nuclease-Free Water per sample.

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.

   Note: If your magnet does not have two positions for shifting the beads, remove the plate from the magnet and gently pipet up and down 5 times (with the pipettor set at 100 µL), then return the plate to the magnet and incubate for 2 minutes or until the 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 16.

Quantify and dilute the library

Determine library quantification method

Use the following table to determine recommended library quantification method for your Ion AmpliSeq™ Transcriptome libraries:

Input RNA	Recommended quantification method
Unfixed RNA	Ion Library TaqMan® Quantitation Kit or Agilent™ 2100 Bioanalyzer™ Instrument
FFPE RNA	Ion Library TaqMan® Quantitation Kit

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.

   Note: Use a smaller volume (45 µL instead of 50 µL) to avoid bead carryover during transferring. You may also leave the plate on the magnet before transferring final libraries for quantification or template preparation.

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.

    Note: Use slightly smaller volume (45 µL instead of 50 µL) to avoid bead carryover during transfer. You can also leave the plate on the magnet before transferring final libraries for quantification or template preparation.

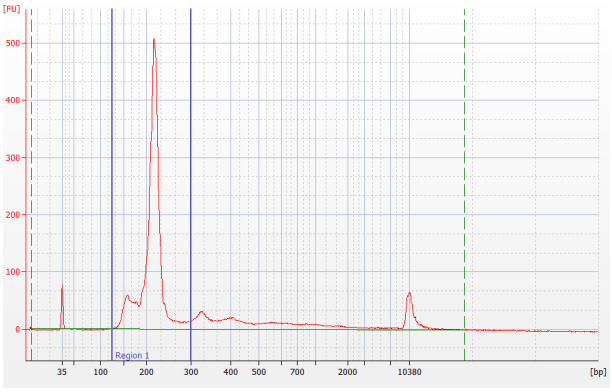
Quantify the library using the Agilent™ 2100 Bioanalyzer™ instrument and calculate dilution factor

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.

    Note: Ensure that the upper and lower marker peaks are identified and assigned correctly. Follow the manufacturer's instructions to perform a region analysis (smear analysis) in the 125–300 bp size range.

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.

A Tips and troubleshooting

Troubleshooting with control RNA

You can use Universal Human Reference RNA (Agilent™ Cat. No. 740000) with the Ion AmpliSeq™ Transcriptome Human Gene Expression Core Panel as a general troubleshooting strategy. Use 10 ng of total RNA to follow the procedures outlined in this user guide. When 8 libraries are sequenced on an Ion PI™ Chip and analyzed using the ampliSeqRNA analysis plugin, you can expect about 90% of the reads on target for this control RNA library. Additionally you can expect 65–70% of the targets in this panel to be detected at ≥ 10 reads, representing gene expression levels covering 5 log units of dynamic range.

Observation	Possible cause	Recommended action
Library yield is low	Input RNA was incorrectly quantified.	Re-quantify input RNA using Qubit™ 2.0 Fluorometer or Agilent™ RNA LabChip™ Kit. If neither is available, quantify with a NanoDrop™ Spectrophotometer, a less accurate alternative.
	Input RNA was less than 10 ng.	Add more RNA or increase target amplification cycles (See the table of recommendations in step 5 of "Amplify targets" on page 12 for cycle numbers based on input amounts).
	RT reaction was inefficient.	Make master mix if possible. For individual reaction setup, make sure correct volume of 5X VILO™ buffer and 10X SuperScript™ III enzyme mix is added into each reaction.
	PCR, digestion, or ligation reactions were inefficient.	Ensure proper dispensing and mixing of viscous components at each step.
	AMPure™ Beads were overdried.	Do not dry the AMPure™ Beads for more than 5 minutes.
Library yield is high	Input RNA was incorrectly quantified.	Re-quantify input RNA using Qubit™ 2.0 Fluorometer or Agilent™ RNA LabChip™ Kit. If neither is available, quantify with NanoDrop™ as a less accurate alternative.
	Input RNA was more than 10 ng.	Add less RNA or decrease target amplification cycles. **Note:** Do not use more than 100 ng of input RNA in the reverse transcription reaction; this can cause non-linear target amplification.

Observation	Possible cause	Recommended action
Number of on-target reads is lower than expected	Either the input RNA was less than 10 ng, or the PCR amplification cycles were less than optimal.	Add more RNA or increase target amplification cycles.
	RNA is degraded.	Use highest quality RNA possible. For degraded RNA, use up to 16 PCR cycles.
Barcode representation is uneven	Library was inaccurately quantitated.	Use correct dilution factor when calculating concentration.
	Library was inaccurately combined.	Dilute libraries to 100 pM, then combine equal volumes. If library concentration is less than 100 pM, dilute to a fixed concentration, for example, 50 pM, then combine equal volumes. Re-quantify the library pool to confirm the expected concentration.
The fraction of polyclonal ISPs (>40%) is high	Library was over-seeded.	Decrease amount of library added to the template preparation reaction by 50%.
	Library was incorrectly quantified.	Ensure that library was accurately quantified.
Library yields from replicate RNA samples are inconsistent	Sample evaporated in thermal cycler.	Seal the 96-well MicroAmp™ plates well with MicroAmp™ Adhesive Film Applicator (Cat. No. 4333183) and use a MicroAmp™ Compression Pad (Cat. No. 4312639).

B

Data analysis

Analysis overview

Ion AmpliSeq™ Transcriptome library analysis has two components:

- Mapping of sequence reads to the

  `hg19_AmpliSeq_Transcriptome_ERCC_v1.fasta` reference
- Quantifying matches per amplicon using defined amplicon regions from the

  `hg10_AmpliSeq_Transcriptome_21K_v1.bed` file

Torrent Suite™Software (TSS) aligns reads to the reference and the *ampliSeqRNA* plugin determines valid matches to amplicon target regions in the panel using the BED file. The following are some brief instructions to set up Ion AmpliSeq™ Transcriptome analysis within the TSS.

Install BED files on Torrent Server

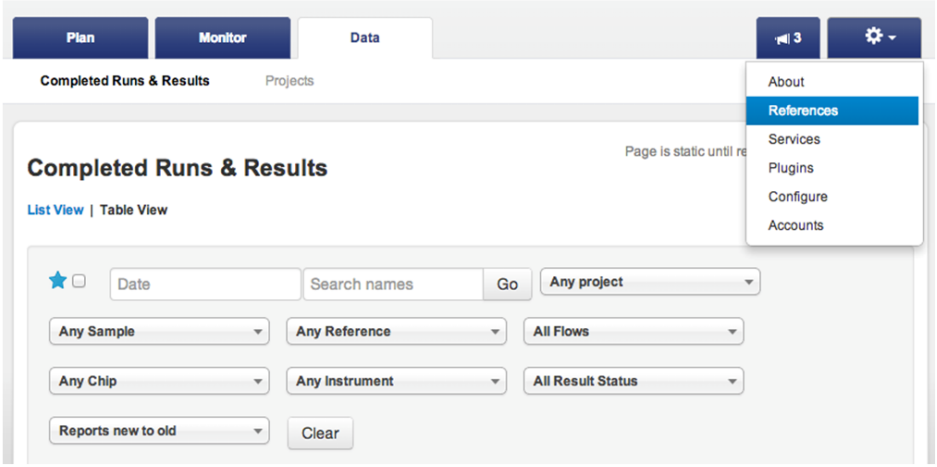
1. Download the BED file for the target regions of the Ion AmpliSeq™ Transcriptome panel product page at **www.thermofisher.com**.

2. Upload the files to your Torrent Server by following the instructions for BED file upload in the latest Torrent Suite™ software documentation, available at **https://www.thermofisher.com/us/en/home/technical-resources/technical-reference-library/next-generation-sequencing-support-center.html** .

Install the Ion AmpliSeq™ Transcriptome mapping reference

1. Download the hg19_AmpliSeq_Transcriptome_ERCC_v1.fasta FASTA file from **https://www.thermofisher.com/us/en/home/technical-resources/technical-reference-library/next-generation-sequencing-support-center/ngs-library-preparation-support.html**.

2. In the TSS click **Settings** on the right side of the screen, then select **References** under the admin gear icon.



3. Click **Add Reference Sequence** and enter the information in the fields. In the Short Name field, enter a short form name for the mapping reference hg19_AmpliSeq_Transcriptome_ERCC_v1.fasta. The transcript index creation takes a few minutes.

Install ampliSeqRNA plugin

Proceed to **https://www.thermofisher.com/plugins/** for instructions to download the ampliSeqRNA plugin.

Run ampliSeqRNA plugin

1. Set up an Ion AmpliSeq™ Transcriptome run in your Ion Torrent™ Browser according to the table. Click on each tab and make the appropriate selection:

Tab	Action
Plan	Select Template Plan, then AmpliSeq RNA application.
References	Select AmpliSeq Transcriptome mapping reference. Select AmpliSeq Transcriptome BED file of targeted regions.
Plugins	Check the box next to **ampliSeqRNA** plugin.

2. Complete all other run setup parameters as appropriate.

   If the run is completed without setting up the run plan:

   1. If hg19_RNA_CanTran was not previously selected as the reference, reanalyze the run using this reference.
   2. Return to the sequencing run report, click **Select plugins to run** at the bottom of the page, and select the **ampliSeqRNA plugin**.

   Note: The plugin can be set to auto-run within a run plan so that the plugin executes automatically after a sequencing run has completed. If desired, the plugin can be run manually using the following steps:

   1. On the ampliSeqRNA plugin setup page, confirm that hg19_AmpliSeq_Transcriptome_ERCC_v1 is selected as the Reference Genome. If hg19_AmpliSeq_Transcriptome_ERCC_v1 was not selected, reanalyze the sequencing run using this reference.
   2. Click **Submit**.

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

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  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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