USER GUIDE

# Ion AmpliSeq™ RNA Library Kit

for use with:
Ion AmpliSeq™ RNA Ready-to-use Panels
Ion AmpliSeq™ RNA Custom Panels

**Catalog Number** 4482335, 4482340, 4482752
**Publication Number** MAN0007450
**Revision** A.0

**For Research Use Only. Not for use in diagnostic procedures.**

**For Research Use Only. Not for use in diagnostic procedures.**

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

# Contents

# About This Guide

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

## Revision history

| Revision | Date | Description |
|---|---|---|
| A.0 | December 2013 | • Recommend use of the Magnetic Stand-96<br>• Updated "Proceed to template preparation"<br>• Version numbering changed to alphanumeric format and reset to A.0 in conformance with internal document control procedures |
| 2.0 | August 2013 | • Added amplification parameters for up to 1200 primer pairs per pool<br>• Updated library dilution recommendation to 100 pm<br>• Updated "Proceed to template preparation" |
| 1.0 | February 2013 | New document |

# Product information

## Product description

The Ion AmpliSeq™ RNA Library Kit provides a complete set of library preparation reagents for targeted RNA-Seq on the Ion Personal Genome Machine® (PGM™) and the Ion Proton™ Systems. The kit is designed for use with predesigned or custom pools of PCR primers targeting cDNA for transcripts of genes of interest.

This guide covers the following products:

- Ion AmpliSeq™ RNA Library Kit (Cat. nos. 4482335, 4482340, 4482752)
- Ion AmpliSeq™ RNA Ready-to-use Panels (various Cat. nos.)
- Ion AmpliSeq™ RNA Custom Panels (ordered at **ampliseq.com**)
- Ion Xpress™ Barcode Adapters 1–96 (various Cat. nos.)

The **Ion AmpliSeq™ RNA Library Kit** contains reagents for rapid preparation of libraries using Ion AmpliSeq™ Ready-to-use and Custom Panels. The kit requires 10 ng of input RNA per target amplification reaction. RNA from formalin-fixed, paraffin-embedded (FFPE) tissue or from unfixed samples can be used as the starting material.

**Ion AmpliSeq™ RNA Ready-to-use Panels** contain predesigned PCR primers for multiplex PCR amplification of cDNA for transcripts from targeted genomic regions. In addition, they contain proprietary modifications that enable removal of primer sequences during library preparation, for efficient target assessment during sequencing.

**Ion AmpliSeq™ RNA Custom Panels** are designed and synthesized according to your experimental needs, and they contain the same proprietary modifications as the Ion AmpliSeq™ RNA Ready-to-use Panels.

**Ion Xpress™ Barcode Adapters 1–96** enable the preparation of barcoded libraries using the Ion AmpliSeq™ RNA Library Kit. Multiple barcoded libraries can be combined and loaded onto a single Ion Chip to minimize sequencing run time and cost and to allow for accurate sample-to-sample comparisons.

**Note:** Gene targets that are very highly expressed in your sample can decrease the sensitivity of a sequencing run for detecting genes expressed at low levels. This varies per sample. The Ion AmpliSeq™ designer may alert you to this situation in the **Needs Attention** tab. For more information about the effects on sensitivity and dynamic range, see "Considerations for optimal gene detection sensitivity" on page 29.

# Ion AmpliSeq™ RNA Library Kit contents and storage

| Component | Cap color | Quantity | | | Storage |
|---|---|---|---|---|---|
| | | 4482335 (8 rxns) | 4482340 (96 rxns) | 4482752 (384 rxns) | |
| **Ion AmpliSeq™ RNA RT Module** | | | | | |
| 5X VILO™ RT Reaction Mix | Red | 1 X 32 µL | 1 X 384 µL | 4 X 384 µL | –25°C to –15°C |
| 10X SuperScript® III Enzyme Mix | Red | 1 X 16 µL | 1 X 192 µL | 4 X 192 µL | |
| **Ion AmpliSeq™ RNA Dynabeads® Cleanup Module**[1] | | | | | |
| Binding Solution Concentrate | Clear | 1 X 2 mL | 1 X 16 mL | 4 X 16 mL | Ambient |
| Wash Solution Concentrate<br>Add the indicated volume of 100% ethanol before use. | Clear | 1 X 2 mL | 1 X 6 mL | 4 X 6 mL | |
| | | 8 mL | 24 mL | 24 mL to each bottle | |
| Dynabeads® Magnetic Beads | Amber | 1 X 200 µL | 1 X 2.4 mL | 4 X 2.4 mL | 2 to 8°C<br>**Do not freeze.** |
| Nuclease-free Water | Clear | 2 X 1.75 mL | 1 X 10 mL | 4 X 10 mL | –25°C to –15°C , 2 to 8°C , or room temp. (15°C to 30°C) |
| Processing Plate and Lid | N/A | 1 | 4 | 16 | Room temp. (15°C to 30°C) |
| **Ion AmpliSeq™ Library Kit 2.0**[2] | | | | | |
| 5X Ion AmpliSeq™ HiFi Master Mix | Red | 1 X 32 µL | 1 X 384 µL | 4 X 384 µL | –30°C to –10°C |
| FuPa Reagent | Brown | 1 X 16 µL | 1 X 192 µL | 4 X 192 µL | |
| Switch Solution | Yellow | 1 X 32 µL | 1 X 384 µL | 4 X 384 µL | |
| DNA Ligase | Blue | 1 X 16 µL | 1 X 192 µL | 4 X 192 µL | |
| Ion AmpliSeq™ Adapters | Green | 1 X 16 µL | 1 X 192 µL | 4 X 192 µL | |
| Platinum® PCR SuperMix HiFi | Black | 1 X 400 µL | 3 X 1.6 mL | 12 X 1.6 mL | |
| Library Amplification Primer Mix | White | 1 X 16 µL | 1 X 192 µL | 4 X 192 µL | |

| Component | Cap color | Quantity | | | Storage |
|---|---|---|---|---|---|
| | | 4482335 (8 rxns) | 4482340 (96 rxns) | 4482752 (384 rxns) | |
| Low TE | Clear | 1 X 1 mL | 1 X 12 mL | 4 X 12 mL | Room temp. (15°C to 30°C) or –30°C to –10°C |

[1] The Ion AmpliSeq™ RNA Dynabeads® Cleanup Module is **not** interchangeable with the Magnetic Bead Purification Module (Cat. no. 4475486) included in the Ion Total RNA-Seq Kit v2.

[2] Included, but available separately (Cat. no. 4475345, 8 libraries; Cat. no. 4480441, 96 libraries; Cat. no. 4480442, 384 libraries)

# Ion AmpliSeq™ RNA Ready-to-use Panels

The Ion AmpliSeq™ RNA Library Kit supports all Ion AmpliSeq™ RNA Ready-to-use Panels, including the following:

| Name (Cat. no.) | Conc. | Cap color | Quantity | No. of primer pairs | Storage |
|---|---|---|---|---|---|
| Ion AmpliSeq™ RNA Apoptosis Panel (Cat. no. 4482571) | 5X | Clear | 1 X 96 µL (24 rxns) | 267 | –30°C to –10°C |
| Ion AmpliSeq™ RNA Cancer Panel (Cat. no. 4482572) | 5X | Clear | 1 X 96 µL (24 rxns) | 50 | |

# Ion AmpliSeq™ RNA Custom Panels

Ion AmpliSeq™ RNA Custom Panels are designed and synthesized according to your experimental needs, for use with the Ion AmpliSeq™ RNA Library Kit.

| Number of primer pairs per pool | Conc. | Cap color | Number of tubes per pool | Storage |
|---|---|---|---|---|
| 12–1200 | 5X | Clear | 3 tubes (~375 rxns/tube) | –30°C to –10°C |

# (*Optional*) Ion Xpress™ Barcode Adapters

One or more Ion Xpress™ Barcode Adapters Kits are required for preparing barcoded libraries. Each kit includes reagents sufficient for preparing up to 40 Ion AmpliSeq™ libraries per barcode (40 X 16 libraries). Substitute Ion Xpress™ Barcode Adapters for standard Ion AmpliSeq™ Adapters as described in this user guide.

- Ion Xpress™ Barcode Adapters 1–16 Kit (Cat. no. 4471250)
- Ion Xpress™ Barcode Adapters 17–32 Kit (Cat. no. 4474009)
- Ion Xpress™ Barcode Adapters 33–48 Kit (Cat. no. 4474518)
- Ion Xpress™ Barcode Adapters 49–64 Kit (Cat. no. 4474519)
- Ion Xpress™ Barcode Adapters 65–80 Kit (Cat. no. 4474520)

- Ion Xpress™ Barcode Adapters 81–96 Kit (Cat. no. 4474521)

And the complete set of adapters:

- Ion Xpress™ Barcode Adapters 1-96 (Cat. no. 4474517)

| Ion Xpress™ Barcode Adapters Kits (each kit includes 16 individually numbered barcodes) | | | | |
|---|---|---|---|---|
| **Component** | **Cap color** | **Quantity** | **Volume** | **Storage** |
| Ion Xpress™ P1 Adapter | Violet | 1 tube | 320 µL | –30°C to –10°C |
| Ion Xpress™ Barcode <X> | White | 16 tubes (1 per barcode) | 20 µL each | |

# Required materials and equipment

| Description | Supplier | Cat. no. | Quantity |
|---|---|---|---|
| **For target and library amplification, one of the following:**<br>• GeneAmp® PCR System 9700 Single or Dual 96-well Thermal Cycler<br>• AB 2720 Thermal Cycler<br>• Veriti® 96-well Thermal Cycler | Life Technologies | See web product pages | 1 |
| **For library quantitation, one of the following:** | | | |
| Ion Library Quantitation Kit<br>*and*<br>A real-time PCR instrument (e.g., Applied Biosystems® 7900HT, 7300, 7500, StepOne™, StepOnePlus™, ViiA™ 7 Systems, or QuantStudio™ 12K Flex Real-Time PCR System) | Life Technologies | 4468802<br><br>See website for real-time PCR instruments | 1 |
| Agilent® 2100 Bioanalyzer® instrument<br>*and one of the following:*<br>• Agilent® High Sensitivity DNA Kit<br>• Agilent® DNA 1000 Kit | Agilent | G2939AA<br><br>5067-4626<br>5067-1504 | 1 |
| • Qubit® 2.0 Fluorometer<br>• Qubit® dsDNA HS Assay Kit<br>• Qubit® Assay Tubes | Life Technologies | Q32866<br>Q32851, Q32854<br>Q32856 | 1 |
| **Other materials required for library preparation** | | | |
| MicroAmp® Optical 8-Tube Strip, 0.2 mL<br>*and*<br>MicroAmp® Optical 8-Cap Strip | Life Technologies | 4316567<br><br>4323032 | 1000 tubes<br><br>300 caps |

| Description | Supplier | Cat. no. | Quantity |
|---|---|---|---|
| MicroAmp® Optical 96-well Reaction Plates | Life Technologies | N8010560<br>4306737 (with barcode) | 10 plates<br>20 plates |
| MicroAmp® Clear Adhesive Film<br>*or*<br>MicroAmp® Optical Adhesive Film Kit | Life Technologies | 4306311<br>4313663 | 1 |
| MicroAmp® Optical Film Compression Pad | Life Technologies | 4312639 | 1 |
| Magnetic Stand-96 | Life Technologies | AM10027 | 1 each |
| Nuclease-free Water | Life Technologies | AM9932 | 1000 mL |
| Absolute ethanol | MLS[1] | N/A | According to kit size (~10–100 mL) |
| Pipettors, 2–1000 µL, and low-retention filtered pipette tips | MLS | N/A | 1 each |
| **Materials for sample preparation** | | | |
| (*Optional*) PureLink® RNA Mini Kit | Life Technologies | 12183020<br>12183018A<br>12183025 | 10 preps<br>50 preps<br>250 preps |
| (*Optional*) *mir*Vana™ miRNA Isolation Kit | Life Technologies | AM1560 | 40 isolations |
| (*Optional*) MagMAX™ FFPE Total Nucleic Acid Isolation Kit | Life Technologies | 4463365 | 96 preps |
| (*Optional*) RecoverAll™Total Nucleic Acid Isolation Kit for FFPE | Life Technologies | AM1975 | 40 preps |

[1] MLS: Major laboratory supplier

# Procedure overview

The procedure is illustrated in the following workflow diagram.

First, RNA from fixed (FFPE) or unfixed samples is reverse transcribed. The resulting cDNA undergoes PCR amplification using an Ion AmpliSeq™ RNA Ready-to-use or Custom Panel for targets of interest. The resulting amplicons are treated with FuPa Reagent to partially digest the primers and phosphorylate the amplicons. The amplicons are then ligated to Ion Adapters and purified using a two-round purification procedure with the included Dynabeads® Magnetic Beads. Ion Xpress™ Barcode Adapters can be substituted at the ligation step to generate barcoded libraries.

The libraries are then amplified using Library Amplification Primer Mix and purified in one round using the Dynabeads® Magnetic Beads. The amplified libraries can be quantified using the Ion Library Quantitation Kit (a qPCR method), the Agilent® 2100

Bioanalyzer® instrument, or the Qubit® 2.0 Fluorometer. The procedure can be completed in about 5.5 hours.

Barcoded libraries may be combined before clonal amplification on Ion Sphere™ Particles (ISPs) and sequencing. Combining libraries maximizes chip use while minimizing cost and labor.

## Workflow

Reverse transcription (~30 min)

▼

Amplify targets (~90 min)

▼

Partially digest primer sequences (~40 min)

▼

Ligate adaptors to the amplicons (~40 min) and purify (~35 min)

▼

Amplify the library (~10 min) and purify (~20 min)

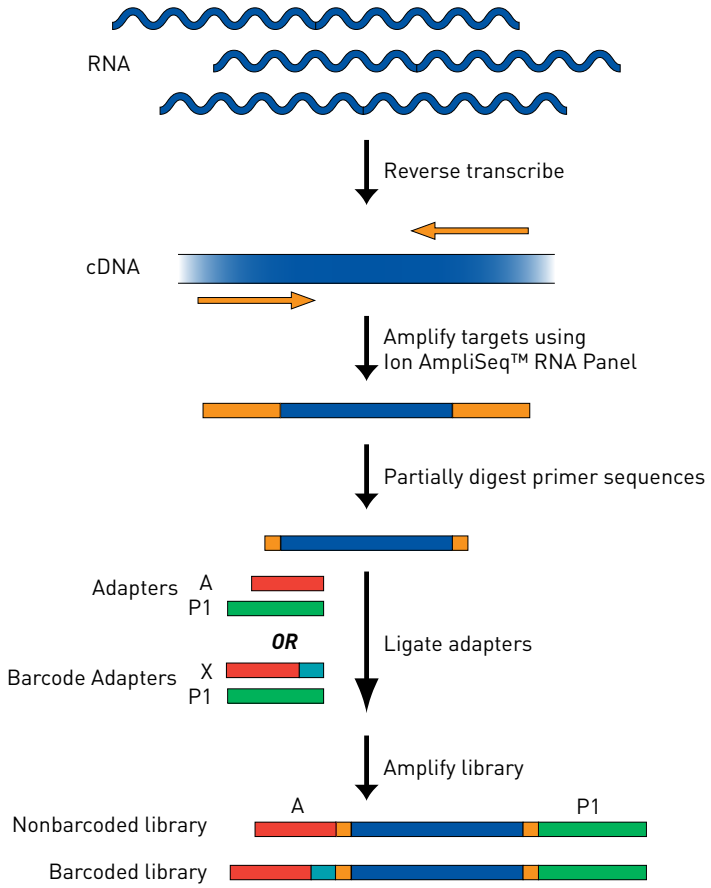
▼

Quantify the library:

Ion Library Quantitation Kit (~45–60 min) *or*

Agilent® Bioanalyzer® instrument (~35 min) *or*

Qubit® 2.0 Fluorometer (~10 min)



# Methods

## Procedure guidelines

- Thaw components that contain enzymes—such as 5X Ion AmpliSeq™ HiFi Master Mix, FuPa Reagent, DNA Ligase, and Platinum® PCR SuperMix HiFi—on ice, and keep on ice during the procedure. All other components, including primer pools, may be thawed at room temperature. Gently vortex and spin down before use.
- If there is visible precipitate in the Switch Solution after thawing, pipet up and down at room temperature to resuspend.
- Minimize freeze-thawing of Ion AmpliSeq™ Primer Pools by aliquoting the pools 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.
- Use a calibrated thermal cycler specified in "Required materials and equipment" on page 9.
- Pipet viscous solutions slowly and ensure complete mixing.
- Always seal the reaction tubes or plate before loading into the thermal cycler, for consistency in replicate libraries.
  - For PCR strip tubes, cap the tubes completely to prevent evaporation.
  - For 96-well PCR plates, seal the plate with MicroAmp® adhesive film and place a MicroAmp® Optical Film Compression Pad on the plate.

### Input RNA requirements

Each reverse transcription reaction requires 10 ng RNA, prepared from unfixed or FFPE tissue. Libraries have been successfully generated from as little as 500 pg of RNA from unfixed samples, and from 5 ng of fixed/FFPE samples.

In general, the library yield from high-quality RNA is higher than from degraded samples. However, libraries from degraded RNA samples will still give good sequencing results. Yield is not indicative of sequencing performance. See "Required materials and equipment" on page 9 for kits recommended for isolating high-quality RNA.

**Note:** RNA prepared from FFPE tissue must be heated before reverse transcription, as described in step 1 of the procedure.

### Before using the kit for the first time

Complete the Wash Solution: add the indicated amount of 100% ethanol to each bottle of Wash Solution Concentrate and mix well. Mark the label on the bottle to indicate that you added ethanol. Store the completed Wash Solution at room temperature.

| Kit size (Cat. no.) | Volume Wash Solution Concentrate per bottle | Volume 100% ethanol |
|---|---|---|
| 8 rxn (Cat. no. 4482335) | 2 mL | 8 mL |
| 96 rxn (Cat. no. 4482340)<br>384 rxn (Cat. no. 4482752) | 6 mL | 24 mL |

## Reverse transcribe the RNA

1. If the input RNA was prepared from FFPE tissue, pre-heat the RNA at 80°C for 10 minutes, then cool on ice or leave the tube at room temperature, before assembling the reverse transcription mix in the next step.
2. Combine the following components in a tube on ice. For multiple reactions, prepare a master mix without RNA.

| 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 to collect the contents at the bottom.
4. Load the tube in the thermal cycler, and run the following program to synthesize cDNA.

| 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 gently but thoroughly.

3. Add the entire contents of the reverse transcription reaction (the total volume is now 20 µL), and gently mix again as in the previous step.

4. Seal the tubes or plate, load the samples in the thermal cycler, and run the following program to amplify target cDNA regions.

| 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.

| 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. The total volume is 22 µL.
2. Pipet the mixture up and down 5 times, or gently vortex, to mix gently but thoroughly.
3. Seal the tubes or plate, load the samples in the thermal cycler, and run the following program to partially digest the primer sequences.

| 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

If you are running multiple sample libraries on a single chip, you can assign a unique barcode to each library. 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. Substitute 2 µL of this barcode adapter mix for the Ion AmpliSeq™ Adapters in the ligation reaction. Store diluted adapters at –20°C.

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

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

| 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. Switch Solution and Adapters can be combined before addition.

| Component | Volume |
|---|---|
| (Digested sample) | (22 µL) |
| Switch Solution (yellow cap) | 4 µL |
| Ion AmpliSeq™ Adapters (for non-barcoded libraries; green cap)<br>*or*<br>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 (30 µL total volume).
4. Pipet up and down 5 times, or gently vortex, to mix gently but thoroughly.
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 | ≤24 hours[1] | ≤24 hours[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)

Perform a two-round purification process with Dynabeads® Magnetic Beads to remove high molecular-weight DNA and primers from the library.

- **First round:** High molecular-weight DNA is bound to beads, while the amplicons and primers remain in solution. **Save the supernatant.**
- **Second round:** Amplicons are bound to beads, and primers remain in solution. **Save the bead pellet, and elute the amplicons from the beads.**

### Important procedure guidelines and reagent preparation

- Ensure that the Wash Solution has been completed.
- If a white precipitate is present in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Dynabeads® Magnetic Beads at room temperature for ≥10 minutes. Vortex to mix thoroughly immediately before pipetting.

  Do not substitute components from the Magnetic Bead Purification Module (Cat. no. 4475486) in this procedure.
- Pre-heat the elution solution—Nuclease-free Water or Low TE—at 37°C for ≥5 minutes.
- To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the 96-well Processing Plate with MicroAmp® Clear Adhesive Film.

  Alternatively, skip a row between sample rows on the 96-well plate.

- If the guidelines for reducing cross-contamination are followed, the same 96-well Processing Plate may be used for both bead purification procedures.
- Accurate pipetting of bead cleanup reagents is critical to successful size selection. Pre-wet pipette tips with 100% ethanol as described.

  For optimal size selection, perform the bead cleanup steps exactly.

### First-round purification

1. Vortex the Dynabeads® Magnetic Beads, and add 5 µL of 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 to mix the beads and buffer.
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 by pipetting 32 µL of 100% ethanol up and down 3 times.
6. Without changing tips, add 32 µL of 100% ethanol to each sample well and mix by pipetting up and down 10 times.
7. Incubate the samples for 5 minutes at room temperature (off the magnetic stand).
8. Place the plate on a magnetic stand for 1–2 minutes until the solution is clear, to capture the beads.
9. Keeping the plate on the magnetic stand, carefully remove the supernatant and transfer to a fresh well in the plate.

**IMPORTANT!** Keep the supernatant! It contains the desired library.

### Second-round purification: Bind the library to the beads

1. Vortex the Dynabeads® Magnetic Beads thoroughly, and add 5 µL of 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 (off the magnetic stand).
6. Place the plate on a magnetic stand for 5 minutes until the solution is clear, to capture the beads.

7. Keeping the plate on the magnetic stand, carefully remove and discard the supernatant.

**IMPORTANT!** Keep the beads! The library is bound to 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 without disturbing the beads.
3. Air dry the beads at room temperature for 3 minutes to remove all traces of ethanol.
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. Off the magnetic stand, incubate for 1 minute at room temperature.
6. Place the plate on the magnetic stand for 1 minute to capture the beads.
7. Keeping the plate on the magnetic stand, carefully remove the supernatant, which contains the purified library, and transfer to a fresh well or tube, without disturbing the beads.

**IMPORTANT!** Keep the supernatant! It contains the desired library.

## 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 (the SuperMix and Primer Mix may be combined before addition):

| 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 | ≤24 hours[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

A single-round purification with Dynabeads® Magnetic Beads removes high molecular-weight DNA and primers from the amplified library.

### Important procedure guidelines and reagent preparation

- Ensure that the Wash Solution has been completed.
- If a white precipitate is present in the Binding Solution Concentrate, warm the solution at 37°C, then shake the solution to dissolve any precipitate before use.
- Incubate the Dynabeads® Magnetic Beads at room temperature for ≥10 minutes. Vortex to mix thoroughly immediately before pipetting.

  Do not substitute components from the Magnetic Bead Purification Module (Cat. no. 4475486) in this procedure.
- Pre-heat the elution solution—Nuclease-free Water or Low TE—at 37°C for ≥5 minutes.
- To reduce the chance of cross-contamination, we strongly recommend sealing unused wells on the 96-well Processing Plate with MicroAmp® Clear Adhesive Film.

  Alternatively, skip a row between sample rows on the 96-well plate.
- If the guidelines for reducing cross-contamination are followed, the same 96-well Processing Plate may be used for both bead purification procedures.
- Accurate pipetting of bead cleanup reagents is critical to successful size selection. Pre-wet pipette tips with 100% ethanol as described.

  For optimal size selection, perform the bead cleanup steps exactly.

### Bind the library to the beads

1. Vortex the Dynabeads® Magnetic Beads thoroughly, and add 10 µL of 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 to mix thoroughly.

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. (Total volume 144 µL.)

4. Pre-wet a pipette tip by pipetting 45 µL of 100% ethanol up and down 3 times.

5. Without changing tips, add 45 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 (off the magnetic stand).
7. Place the plate on a magnetic stand for 1–2 minutes until the solution is clear, to capture the beads.
8. Keeping the plate on the magnetic stand, carefully remove and discard the supernatant.

**IMPORTANT!** Keep the beads! The library is bound to 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. Keeping the plate on the magnetic stand, carefully aspirate and discard the supernatant without disturbing the beads.
3. Air dry the beads at room temperature for 3 minutes to remove all traces of ethanol.
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. Off the magnetic stand, incubate for 1 minute at room temperature.
6. Place the plate on the magnetic stand for 1 minute to capture the beads. Place the plate on the magnetic stand for 1 minute.
7. Keeping the plate on the magnetic stand, carefully remove the supernatant, containing the purified library, and transfer to a fresh well or tube, without disturbing the beads.

**IMPORTANT!** Keep the supernatant! It contains the desired library.

## Quantify the library and dilute for template preparation

Quantify the amplified library by one of the following methods, then dilute the library for downstream template preparation (clonal amplification on ISPs).

The concentration of amplified libraries is typically up to 15 nM for unfixed RNA input, and is typically less than 1 nM for FFPE RNA input.

- Use the Ion Library Quantitation Kit to quantify the library by qPCR.
- Use the Agilent® Bioanalyzer® instrument with the Agilent® High Sensitivity DNA Kit.
- Use the Qubit® 2.0 Fluorometer with the Qubit® dsDNA HS Assay Kit.

In practice, the chip-to-chip variation in library quantification by the Bioanalyzer® instrument is higher than the variation seen by qPCR. The qPCR method may be more accurate for low-input or FFPE-source libraries.

## Quantify the library by qPCR and dilute

Determine the concentration of each Ion AmpliSeq™ library by qPCR with the Ion Library Quantitation Kit using the following procedure. Each sample, standard, and negative control should be analyzed in duplicate 20-µL reactions.

1. Prepare five 10-fold serial dilutions of the *E. coli* DH10B Ion Control Library (~68 pM; included in the Ion Library 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 and use these concentrations in the qPCR instrument.

2. Prepare serial dilutions of the sample library as follows:
   a. Prepare an initial 1:10 dilution of the sample library in Nuclease-free Water, in a non-stick microcentrifuge tube, on ice.

   b. Prepare serial dilutions at 1:1000 and 1:5000 for use in the qPCR:

| Dilution | Library | Nuclease-free Water[1] |
|---|---|---|
| 1:1000 | 1 µL of 1:10 | 99 µL |
| 1:5000 | 10 µL of 1:1000 | 40 µL |

[1] Not DEPC-treated

   Higher dilutions of libraries prepared from unfixed RNA may be necessary to achieve a dilution that falls between standards 3 and 4 of the DH10B standard curve. Conversely, lower dilutions may be necessary for libraries prepared from FFPE or low-input samples.

3. Prepare a reaction mix and dispense 11 µL to the wells of a PCR plate.
   Set up two wells for each library sample, DHB10 library standard, and negative control.

| Component | Volume per sample (duplicate reactions) |
|---|---|
| 2X TaqMan® MasterMix | 20 µL |
| 20X Ion TaqMan® Assay | 2 µL |

4. Add 9 µL of each library dilution or 9 µL of each control dilution to each well (two wells per sample as noted before), for a total reaction volume of 20 µL.

5. Program the real-time instrument as follows:
   - Enter the concentrations of the control library standards.
   - Use ROX™ Reference Dye as the passive reference dye.
   - Select a reaction volume of 20 µL.
   - Select FAM™ dye/MGB as the TaqMan® probe reporter/quencher
   - The Ion Library TaqMan® qPCR Mix can be used on a variety of instruments, as listed below. The fast cycling program was developed using the StepOnePlus™ System in Fast mode.

| Real-time PCR system | Stage | Temp | Time |
|---|---|---|---|
| 7900 HT System<br>7900 HT Fast System (Fast 96-Well, Standard 96-Well, or 384-Well Block Modules)<br>ViiA™ 7 System<br>StepOne™ System<br>StepOnePlus™ System | Hold | 50°C | 2 min |
| | Hold | 95°C | 20 sec |
| | Cycle (40 cycles) | 95°C | 1 sec |
| | | 60°C | 20 sec |
| 7500 Fast System<br>7500 System<br>7300 System | Hold | 50°C | 2 min |
| | Hold | 95°C | 20 sec |
| | Cycle (40 cycles) | 95°C | 3 sec |
| | | 60°C | 30 sec |

6. Calculate the average concentration of the undiluted library by multiplying the concentration determined with qPCR by the library dilution used in the assay.

   Use the library dilution that falls between standards 3 and 4 of the DH10B standard curve.

7. Based on the calculated concentration of the undiluted library, determine the dilution factor (Template Dilution Factor) that results in a library concentration of 100 pM.

   For example:

   - The undiluted library concentration is 300 pM.
   - The library dilution factor is 300 pM/100 pM = 3.
   - Therefore, 1 µL of library mixed with 2 µL of Low TE (1:3 dilution) yields approximately 100 pM.

8. Prior to combining libraries or proceeding to template preparation, dilute an aliquot of each library to a concentration of approximately 100 pM.

   Store diluted libraries at 4–8°C and use within 48 hours. Store undiluted libraries at 4–8°C for 48 hours; store at –20°C for long-term storage.

## Quantify the library with the Agilent® Bioanalyzer® instrument and dilute

Analyze 1 µL of amplified library on the Agilent® Bioanalyzer® instrument with the Agilent® High Sensitivity DNA Kit. Amplicon libraries should have multiple peaks in the 125–300 bp size range. If the library concentration is over 20,000 pM, dilute the library 1:10 and repeat the quantification to obtain a more accurate measurement. Alternatively, use the Agilent® DNA 1000 Kit.

1. Determine the molar concentration of the amplified library using the Bioanalyzer® software. 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). Briefly:
   a. Select the **Data** icon in the Contexts panel, and view the electropherogram of the sample to be quantified.
   b. Select the **Region Table** tab below, and create a region spanning the desired amplicon peaks. Correct the baseline if needed.
   c. The molarity is automatically calculated and displayed in the table in pM (pmol/L).
2. Determine the library dilution that gives a concentration of 100 pM.

For example:

- The library concentration is 3000 pM.
- The library dilution is 3000 pM/100 pM = 30.
- Therefore, 1 µL of library mixed with 29 µL of Low TE (1:30 dilution) yields approximately 100 pM.

3. Prior to combining libraries or template preparation as described in the following sections, dilute an aliquot of each library to a concentration of approximately 100 pM.

   Store diluted libraries at 4–8°C and use within 48 hours. Store undiluted libraries at 4–8°C for 48 hours; store at –20°C for long-term storage.

## Quantify the library and dilute: Qubit® 2.0 Fluorometer

Analyze each amplified library using the Qubit® 2.0 Fluorometer (Cat. no. Q32866) and the Qubit® dsDNA HS Assay Kit. For detailed instructions, see the *Qubit® dsDNA HS Assay Kits User Guide*.

Amplified libraries typically have concentrations of 300–1500 ng/mL. This quantification method may not be appropriate for low-yield libraries, for example from low-input or FFPE samples.

1. Determine the amplified library concentration:
   a. Make a 1:200 working dilution of Qubit® dsDNA HS reagent using the Qubit® dsDNA HS Buffer.
   b. Mix the amplified library with the diluted dye reagent:
      - For high-quality input RNA, prepare a 1:10 dilution of the amplified library in Nuclease-free Water, and add 10 µL of the diluted library to 190 µL of diluted dye reagent.
      - For FFPE or low-input samples, mix 3 µL of the (undiluted) amplified library with 197 µL of diluted dye reagent.

      Several library dilutions may be necessary to get an accurate reading.
   c. Prepare each Qubit® standard as directed in the user guide.
   d. Measure the concentration on the Qubit® 2.0 Fluorometer.
   e. Calculate the concentration of the undiluted library by multiplying by 20.

      This can be calculated automatically using the "Calculate Stock Concentration" button.
2. Determine the library dilution that gives a concentration of approximately 15 ng/mL.

   For example:

   - The library concentration is 450 ng/mL.
   - The library dilution is 450 ng/mL divided by 15 ng/mL = 30.
   - Therefore, 1 µL of library mixed with 29 µL of Low TE (1:30 dilution) yields approximately 15 ng/mL.
3. Prior to combining libraries or template preparation, dilute an aliquot of each library to a concentration of approximately 15 ng/mL.

   Store diluted libraries stored at 4–8°C and use within 48 hours. Store undiluted libraries at 4–8°C for 48 hours; store at –20°C for long-term storage.

# (*Optional*) Combine barcoded libraries from different samples

You can prepare barcoded libraries from different samples using Ion Xpress™ Barcode Adapters 1–96 Kits. Multiple uniquely barcoded libraries can be combined as shown in the following figure and loaded on a single Ion Chip to minimize the sequencing run time and cost and to allow for accurate sample-to-sample comparisons.

Store diluted combined libraries at 4–8°C and use within 48 hours.

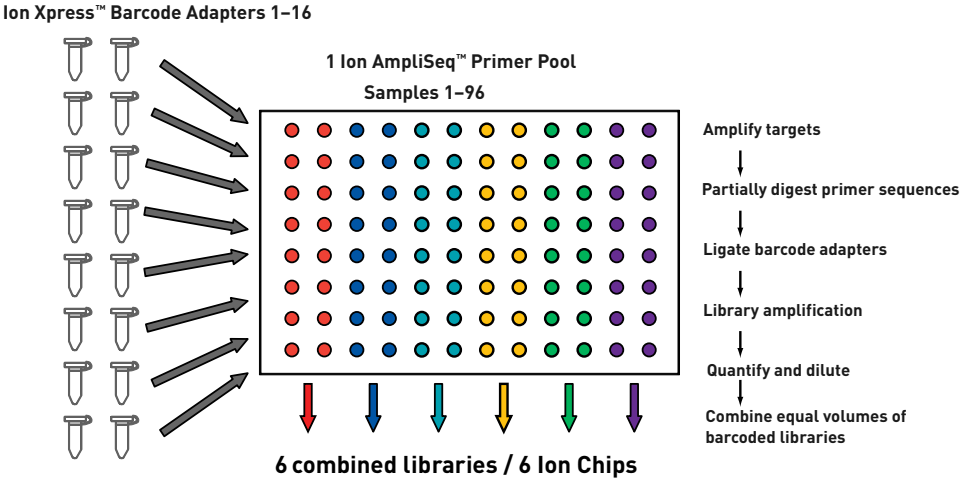



**Figure 1** Multiple barcoded libraries prepared from different samples using the same primer pool.

Example library combination algorithm: 96 samples and 16 barcodes. In this example, 1 barcoded library is generated from each sample using the same Ion AmpliSeq™ Primer Pool. After quantifying and diluting the libraries according to their Template Dilution Factors, equal volumes of the barcoded libraries are combined in sets of 16 barcodes. In this example, one 96-well plate yields 6 combined libraries (each combined library contains 16 barcoded libraries from 16 samples), for sequencing on 6 Ion Chips.

# Proceed to template preparation

The diluted library or combined libraries are ready for downstream template preparation using an appropriate Ion template preparation kit. Dilute the library immediately before template preparation, or store the dilution in a sealed plate at 4–8°C for up to 48 hours.

In general, Ion AmpliSeq™ libraries at 100 pM (or 15 ng/mL) yield sufficient monoclonal Ion Sphere™ Particles (ISPs).

Template preparation documentation is available on the Ion Torrent™ user community at **ioncommunity.lifetechnologies.com**. Follow the links under **Systems & Software > Ion PGM System > User Guides and Bulletins > Prepare Template**.

A

# Troubleshooting

## Library yield and quantification

| Observation | Possible cause | Recommended action |
|---|---|---|
| Low library yield | Mis-quantification of input RNA | Requantify input RNA using NanoDrop®, Agilent® RNA LabChip® Kit, or Qubit® 2.0 Fluorometer. |
| | Less than 10 ng of input RNA | Add more RNA or increase target amplification cycles. |
| | Use of incompatible beads | Use only the Dynabeads® Magnetic Beads provided with this kit. Other RNA magnetic bead cleanup kits are not compatible. |
| | Low expression of genes of interest | Increase the 42°C incubation in the reverse transcription reaction from 30 minutes to a maximum of 120 minutes. |
| | Inefficient PCR, digestion, or ligation | Ensure proper dispensing and mixing of viscous components at each step. |
| | Library discarded during two-round bead purification of the unamplified library | Be sure to save the supernatant during first-round purification, and save the bead pellet during the second round ("Purify the unamplified library (two-round purification)" on page 16). |
| | Overdrying of Dynabeads® Magnetic Beads | Do not dry the Dynabeads® Magnetic Beads more than 5 minutes. |
| High library yield | Mis-quantification of input RNA | Requantify input RNA using NanoDrop®, Agilent® RNA LabChip® Kit, or Qubit® 2.0 Fluorometer. |
| | More than 10 ng of input RNA | Add less RNA or decrease target amplification cycles.<br>Do not use more than 100 ng of input RNA in the reverse transcription reaction; this can cause non-linear target amplification. |
| High library yield on the Bioanalyzer® instrument | Marker mis-assignment (Figure 2) | Ensure that markers are assigned correctly. |
| High molecular weight material on the Bioanalyzer® instrument<br>High library yield on Qubit® Fluorometer | High molecular weight DNA was not removed during purification of the library (Figure 3) | Be sure to follow the protocol exactly as described for bead purification.<br>100% ethanol is difficult to pipet accurately; it is essential to pre-wet pipette tips. |

# Bias in amplicon representation

| Observation | Possible cause | Recommended action |
|---|---|---|
| Loss of short amplicons | Poor purification | Be sure to follow the protocol exactly as described for bead purification.<br>100% ethanol is difficult to pipet accurately; it is essential to pre-wet pipette tips. |
| | Denaturation of digested amplicon | Use the 60°C/20minute temperature incubation during the primer digestion step ("Partially digest primer sequences" on page 15). |
| Loss of long amplicons | Inefficient PCR | Use the 8-minute anneal and extend step for target amplification, and follow clean-up steps exactly. |
| | Too few nucleotide flows | Use an appropriate number of flows on the Ion PGM™ Sequencer. |
| Loss of AT-rich amplicons | Denaturation of digested amplicon | Use the 60°C/20 minute temperature incubation during the primer digestion step. |
| | Unknown | Amplicons with >80% AT often exhibit low representation. |
| Loss of GC-rich amplicons | Inadequate denaturation | Use a calibrated thermal cycler. |
| | Inefficient library amplification | Do not amplify the library (not required for qPCR quantitation). |
| | Unknown | Amplicons with >80% GC often exhibit low representation. |

# Other

| Observation | Possible cause | Recommended action |
|---|---|---|
| Adapter-dimer on the Bioanalyzer® instrument at 90–105 bp (Figure 4)<br>Adapter dimer on the Ion PGM™ Sequencer | Inefficient purification | Be sure to follow the protocol exactly as described for bead purification.<br>100% ethanol is difficult to pipet accurately; it is essential to pre-wet pipette tips. |
| | Adapter dimer formation | Do not combine Adapters, DNA Ligase, and Switch Solution prior to addition. |
| | | Use a 65°C temperature incubation instead of 60°C during the primer digestion step. |
| | Adapter concentration too high | Ensure that barcode adapters are diluted properly. |
| Lower-than-expected number of on-target reads | Unknown | Increase the number of target amplification cycles by two |

| Observation | Possible cause | Recommended action |
|---|---|---|
| Uneven barcode representation | Inaccurate library quantification | Use the Ion Library Quantitation Kit for the most specific and accurate library quantification. |
| | Inaccurate library combination | Dilute libraries to 100 pM, then combine equal volumes. Re-quantify the library pool to confirm the expected concentration. |
| High polyclonal ISPs (>40%) | Overseeding of library | Decrease amount of library added to the template preparation reaction by 50%. |
| | Library mis-quantification | Ensure that library was quantified accurately. |
| | Other | Check the appropriate template preparation user guide for more information. |
| High percentage of low quality ISPs (>15%) | Underseeding of library | Double the volume of library used in template preparation. |
| | | Use a fresh dilution of library prepared in a low-bind tube. |
| | Library mis-quantification | Ensure that library was quantified accurately. |
| | Other | Check the appropriate template preparation user guide for more information. |
| Inconsistent library yields from replicate RNA samples | Sample evaporation in the thermal cycler<br>8-strip PCR tubes must be sealed tightly to prevent evaporation. | Switch to 96-well MicroAmp® plates. |

# Example Bioanalyzer® instrument images

The following traces are from the Agilent® 2100 Bioanalyzer® instrument.

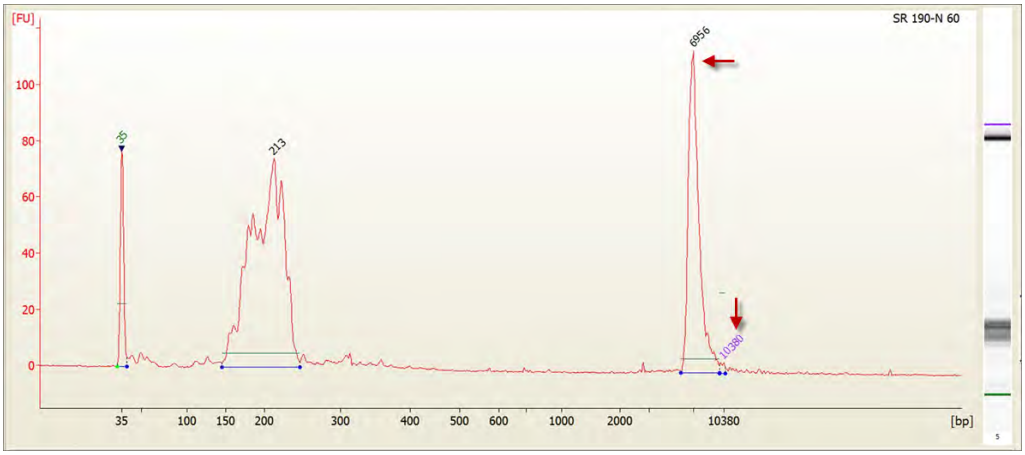



**Figure 2** Marker mis-assignment.

When the upper marker is assigned to the incorrect peak, library yield may be overestimated by more than 10-fold.

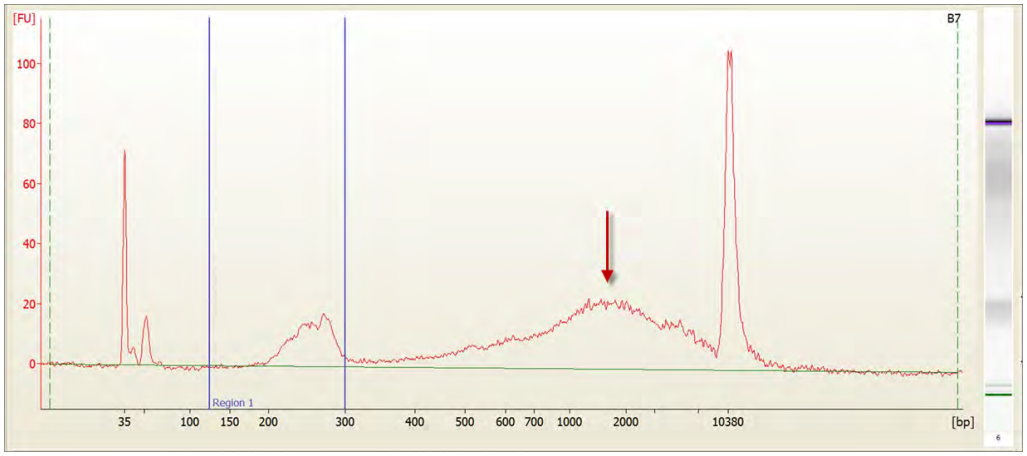



**Figure 3** High molecular weight material.
DNA outside the region window will not interfere with template preparation or sequencing.

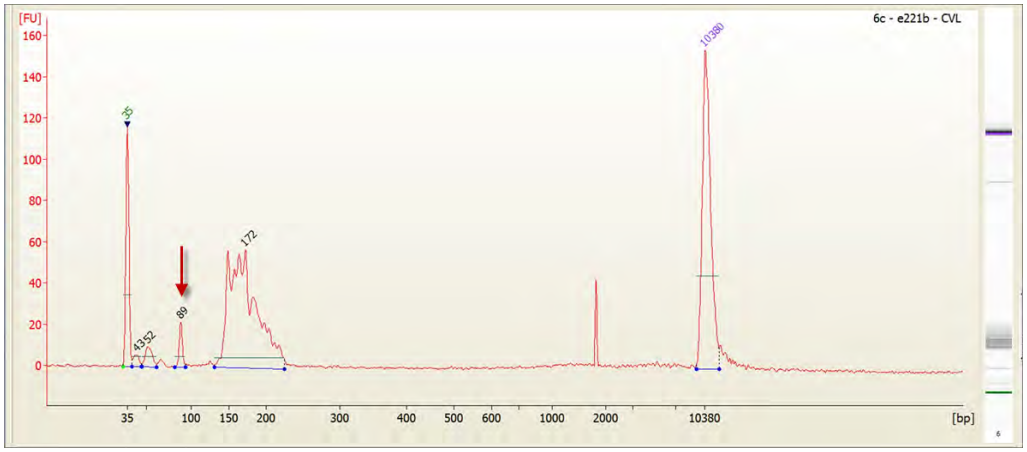



**Figure 4** Adapter dimers.
Standard adapters produce peaks at ~43 and ~53 bp, while adapter dimers run at ~90 bp. Barcode adapters run at ~53 bp, and barcode adapter dimers run at ~105 bp.

# B Supplemental Information

## Tips for the standard workflow

- Arrange samples in columns on the plate for easier pipetting with multichannel pipettes during purification with the magnet.
- Plate seals can be firmly applied using the applicator in the MicroAmp® Optical Adhesive Film Kit. Plate seals can be removed with much less effort when hot. Try removing seals right after taking the plate out of thermal cycler.
- If you intend to store combined libraries, prepare them at a higher concentration (i.e., 100 pM) and store in a MicroAmp® or Eppendorf LoBind® tube at –20°C. This will help prevent significant loss of the libraries on the surface of the tube.
- Combine and dilute barcode adapters in large batches and aliquot into 96-well plates.
- When making many Ion AmpliSeq™ libraries in a plate, it may be easier to mix by sealing, gently vortexing, and spinning the plate, rather than pipetting each sample up and down.

## Modifications to the standard workflow

The following modifications to the standard protocol are designed to allow advanced users to successfully modify and customize the standard Ion AmpliSeq™ protocol. These modifications are unsupported and in some cases may decrease performance.

### Modifications for limited samples

- Ion AmpliSeq™ target amplification is extremely sensitive and efficient. For RNA inputs that are less than 1 ng, add 3 cycles to the target amplification.
- If the sample concentration is estimated or unknown, it may still be possible to generate an Ion AmpliSeq™ library. Perform library amplification as described in "Amplify the library" on page 18, except split the library amplification reaction (50 µL) into three 15-µL reactions and perform 5, 7, and 10 library amplification cycles. Adjust the volumes in the subsequent purification procedure accordingly. After quantification, select the library with the lowest measurable yield and proceed to template preparation.
- Amplified libraries with little or no detectable product on the Bioanalyzer® instrument may still be quantified by qPCR.

## Considerations for optimal gene detection sensitivity

When barcoded adapters are used, combined Ion AmpliSeq™ libraries can be generated from multiple primer pools and the same RNA sample, or from multiple

Ion AmpliSeq™ RNA libraries. The number of libraries that can be accommodated in a single sequencing run depends on the number of wells per chip, the ability to reliably quantify and combine barcoded libraries, and the gene detection sensitivity required.

The following conditions can potentially lower sensitivity for detecting less abundant gene targets in a given sample, that is, reducing the number of sequence reads which map to an expected gene target. In other words, since the number of beads on a chip is finite, if the number of sequence reads falls below the mapped reads threshold (detection threshold), gene detection sensitivity is reduced.

- Very highly expressed genes in a sample.
  The highest expressing genes have the highest probability of being sampled in a sequencing library, and thus more reads are mapped to the highly expressed genes. Highly expressed genes occupy the most wells on the chip, which lowers the probability of detecting genes expressed at a much lower level.
- Barcoding multiple samples across one chip.
- Adding additional targets to the primer pool.
- Using a lower well-density chip.

For this reason, we recommend performing a non-barcoded 'pilot run' to assess the dynamic range of expression in your sample, prior to choosing the number of barcodes to use on a single chip.

1. Upon pilot run completion, analyze your data using the coverageAnalysis plugin, then download the coverage summary in the provided link at the bottom of the plugin results. The resulting table has a column called 'total_reads' representing the number of sequences which are detected (mapped) to expected targets.
2. To determine the number of barcodes which can be used and still achieve the required sensitivity, divide the 'total_reads' per gene by the desired number of barcodes. If this falls below your mapped reads threshold (we typically use 10), then reduce the number of barcodes until these genes are above the mapped reads threshold.

# Data analysis

Visit the Ion Community at **ioncommunity.lifetechnologies.com** and select **Products ▸ Torrent Suite** to access the latest user guides and information for Torrent Suite™ software, including the *Torrent Browser User Interface Guide*. Go to **ioncommunity.lifetechnologies.com/docs/DOC-7202** for more information on TaqMan® assays corresponding to the same or similar gene regions interrogated by Ion AmpliSeq™ RNA fixed or custom panels.

To enable analysis of Ion AmpliSeq™ RNA libraries:

- Upload the BED files for the target regions to your Torrent Server.
- Install the Human Canonical Transcripts mapping reference, hg19_RNA_CanTran.
- Install the latest coverageAnalysis plugin, which is required for analysis of Ion AmpliSeq™ RNA Custom as well as Ready-to-use Panels.
  The coverageAnalysis plugin supports Torrent Suite version 3.4 and higher.

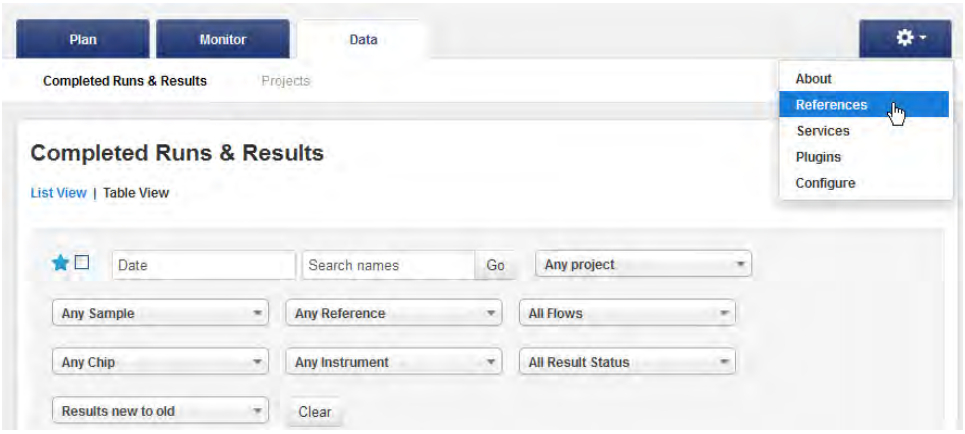
## Install the BED files on your Torrent Server

1. Download the BED file for the target regions of each Ready-to-use Panel from the panel's product page at **www.lifetechnologies.com** or from the Ion Community at **http://ioncommunity.lifetechnologies.com/community/protocols-home/pgm-protocols/library**.
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 **http://ioncommunity.lifetechnologies.com/community/products/torrent_suite**.

## Install the Ion AmpliSeq™ RNA mapping reference

Brief instructions for installing the hg19_RNA_CanTran mapping reference on your Torrent Server are provided below. Detailed instructions are provided under "Adding a Reference Sequence" in the *Torrent Browser User Interface Guide*.

1. Download the hg19_RNA_CanTran FASTA file from this link: **hg19_RNA_CanTran**.
2. In the Torrent Browser, click the **Settings** button on the right side of the screen and select **References**.




- Click on **Add Reference Sequence** and enter the information in the fields. In the **Short Name** field, enter the short form of the genome name **hg19_RNA_CanTran**.
- The transcript index creation takes a few minutes.

## Install the latest coverageAnalysis plugin

1. At **ioncommunity.lifetechnologies.com**, select **Products ▸ Torrent Browser Plugin Store**.
2. In the Overview tab, search using “coverageanalysis” in the SearchPlugins field.
3. Click the link for **coverageAnalysis** in the search results, and follow the instructions at the coverageAnalysis plugin page to install or update the plugin in your Torrent Browser.

## Run the coverageAnalysis plugin

- In the sequencing run setup, select the following:

| Tab | Action |
|---|---|
| Plan | Select the appropriate **AmpliSeq RNA** application. |
| References | Select **hg19_RNA_CanTran** for the Reference Library. |
| | Select your panel-specific BED file for the Targeted Regions BED file.[1] |
| Plugins | Check the box next to the coverageAnalysis plugin.[2] |

[1] The Hotspot Regions BED file is not needed for this application.

[2] Ensure that the latest coverageAnalysis plugin is installed.

- Complete all other run setup parameters as appropriate.
- If the run is completed without making a 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 on **Select plugins to run** at the bottom of the page, and select the **coverageAnalysis** plugin.

To execute the plugin:

1. On the coverageAnalysis set up page, confirm that hg19_RNA_CanTran is selected as the Reference Genome.
   If hg19_RNA_CanTran was not selected, reanalyze the sequencing run using this reference.

2. Select **Ion AmpliSeq RNA** as the Library Type.
   If **Ion AmpliSeq RNA** is not present in the drop-down menu, update the plugin following the instructions in the previous section.

3. Select the panel-specific name which is associated with your BED file for Targeted Regions.

4. Click on the **Submit** button.
   The run report is displayed. Click on **Refresh plugin status** to monitor the analysis.

5. Upon completion, click on the **coverageAnalysis_rna.html** link to view the results.
   An example report is shown in the following figure.

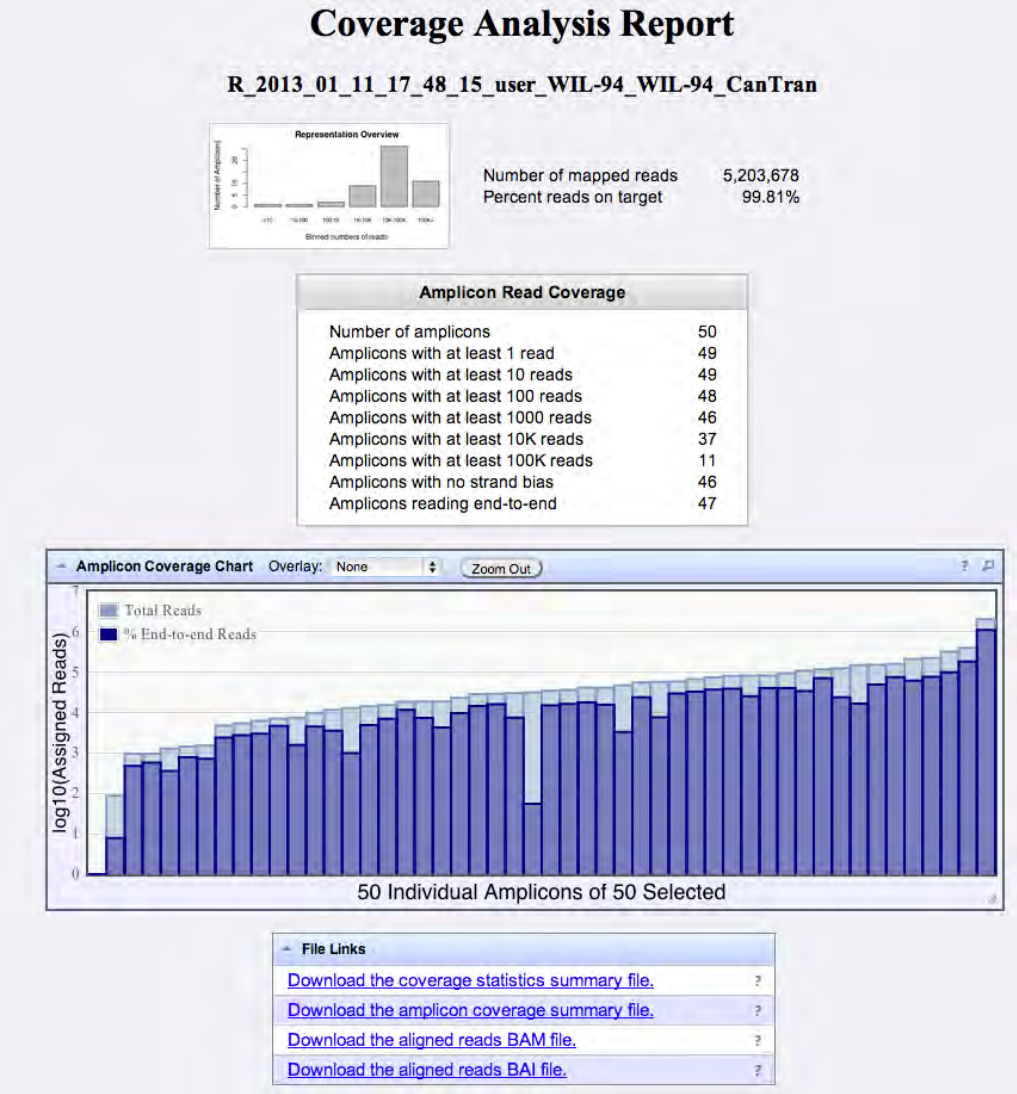



**Figure 5** Example Coverage Analysis Report

C

# 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, and 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.

## Specific chemical handling

| CAS | Chemical information | |
|---|---|---|
| 593-84-0 | Guanidine Isothiocyanate | Contact with acids or bleach liberates toxic gases. DO NOT ADD acids or bleach to any liquid wastes containing this product. |

# 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. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Safety equipment also may 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**

# Documentation and support

## Obtaining Certificates of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

## Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**.

**Note:** For the SDSs of chemicals not distributed by Life Technologies Corporation, contact the chemical manufacturer.

## Obtaining support

For the latest services and support information for all locations, go to:

**www.lifetechnologies.com/support**

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**ionsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

## Ion contact information

**Web site: lifetechnologies.com/iontorrent**

**Ion community: ioncommunity.lifetechnologies.com**

**Support email: ionsupport@lifetech.com**

**Phone numbers**
In North America: 1-87-SEQUENCE (1-877-378-3623)
Outside of North America: +1-203-458-8552

# Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

**Headquarters**
5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288
For support visit **lifetechnologies.com/support** or email **techsupport@lifetech.com**
lifetechnologies.com

15 January 2014