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Ion AmpliSeq™ DNA and RNA Library Preparation

USER GUIDE

for use with:
Ion AmpliSeq™ Library Kits 2.0
Ion AmpliSeq™ Ready-to-use Panels
Ion AmpliSeq™ Community Panels
Ion AmpliSeq™ Custom Panels
Ion AmpliSeq™ Sample ID Panel
Ion Library Equalizer™ Kit
Ion AmpliSeq™ Direct FFPE DNA Kit
IonCode™ Barcode Adapters
Ion Xpress™ Barcode Adapters

Catalog Number 4475345, 4480441, 4480442, 4479790, 4482298, A31133, A31136, and A29751
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ThermoFisher SCIENTIFIC

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Revision history: Pub. No. MAN0006735

Revision	Date	Description
C.0	3 June 2016	• Added support for the Ion AmpliSeq™ Direct FFPE DNA Kit • Added support for DNA/RNA input in range of 1–100 ng • Instructions for setting up amplification reactions for Ion AmpliSeq™ panels with more than one primer pool clarified • Added support for IonCode™ Barcode Adaptors • Ligation reaction temperature conditions updated • Added option for combined Equalizer™ procedure, and increased Equalizer™ library amplification cycle number from 7 to 9 • Table of PCR programs for qPCR library quantification updated • Tables for Ion chip capacities for Ion AmpliSeq™ DNA and RNA libraries updated to include Ion 520™, Ion 530™, and Ion 540™ chips • Graphics enhanced • Available Ready-to-use Ion AmpliSeq™ panels updated • Added support for the Qubit™ 3.0 Fluorometer • Added support for use of companion panels
B.0	15 July 2014	• Added instructions for preparing libraries from RNA.

Corporate entity: Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288

Contents

1 Product information

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

Product description

This guide covers the following products:

- Ion AmpliSeq™ Library Kits:
  - Ion AmpliSeq™ Library Kit 2.0 (Cat. No. 4475345)
  - Ion AmpliSeq™ Library Kit 2.0-96LV (Cat. No. 4480441)
  - Ion AmpliSeq™ Library Kit 2.0-384LV (Cat. No. 4480442)
- Ion Library Equalizer™ Kit (Cat. No. 4482298)
- Ion AmpliSeq™ Direct FFPE DNA Kit (Cat. Nos. A31133, A31136)
- Ion AmpliSeq™ Ready-to-use Panels (various Cat. Nos.)
- Ion AmpliSeq™ Custom and Community Panels (ordered at **www.ampliseq.com**)
- Ion AmpliSeq™ Sample ID Panel (Cat. No. 4479790)
- IonCode™ Barcode Adapters (Cat. No. A29751)
- Ion Xpress™ Barcode Adapters (various Cat. Nos.)

Ion AmpliSeq™ products include reagents to prepare targeted libraries from DNA or RNA for sequencing on the Ion PGM™, Ion Proton™, and Ion S5™/Ion S5™ XL Systems. The kits are designed for use with ready-to-use or custom panels and incorporate barcoded adapters so that multiple libraries can be combined and loaded onto a single Ion Chip to minimize the per-sample sequencing cost.

Ion AmpliSeq™ Library Kits

Ion AmpliSeq™ Library Kit 2.0 contains reagents for the rapid preparation of libraries with 12–24,576 primer pairs per target amplification reaction. These library kits use a plate-based protocol for easy sample handling, and for compatibility with automation in high throughput laboratories. Nucleic acid from a variety of sources—including formalin-fixed, paraffin-embedded (FFPE) tissue, and cell-free DNA (cfDNA)—can be used as the starting material.

Ion AmpliSeq™ Library Kit 2.0

The Ion AmpliSeq™ Library Kit 2.0 (Cat. No. 4475345) provides reagents for preparing 8 libraries for 1- or 2-pool panels.

Contents	Cap color	Amount	Storage
5X Ion AmpliSeq™ HiFi Mix	Red	32 µL	–30ºC to –10ºC
FuPa Reagent	Brown	16 µL	
Switch Solution	Yellow	32 µL	
DNA Ligase	Blue	16 µL	
Ion AmpliSeq™ Adapters	Green	16 µL	
Platinum™ PCR SuperMix HiFi	Black	400 µL	
Library Amplification Primer Mix	White	16 µL	
Low TE	Clear	1.7 mL	15°C to 30°C[1]

[1] Can be stored at –30ºC to –10ºC for convenience.

Ion AmpliSeq™ Library Kit 2.0-96LV or -384LV

The Ion AmpliSeq™ Library Kit 2.0-96LV or -384LV (Cat. No. 4480441 or 4480442) provides reagents for preparing 96 and 384 libraries, respectively, for 1- or 2-pool panels.

Contents	Cap color	Amount		Storage
		96LV	384LV	
5X Ion AmpliSeq™ HiFi Mix	Red	384 µL	4 × 384 µL	–30ºC to –10ºC
FuPa Reagent	Brown	192 µL	4 × 192 µL	
Switch Solution	Yellow	384 µL	4 × 384 µL	
DNA Ligase	Blue	192 µL	4 × 192 µL	
Ion AmpliSeq™ Adapters	Green	192 µL	4 × 192 µL	
Platinum™ PCR SuperMix HiFi	Black	3 × 1.6 mL	12 × 1.6 mL	
Library Amplification Primer Mix	White	192 µL	4 × 192 µL	
Low TE	Clear	12 mL	4 × 12 mL	15°C to 30°C[1]

[1] Can be stored at –30ºC to –10ºC for convenience.

Ion Library Equalizer™ Kit

The Ion Library Equalizer™ Kit provides an optional, streamlined method for normalizing library concentration without quantification. This kit should be used when library yields are consistently above the minimum expected concentration.

The Ion Library Equalizer™ Kit (Cat. No. 4482298) contains reagents for 96 libraries.

Component	Cap color	Amount	Storage
Ion Library Equalizer™ Primers	Pink	200 µL	2ºC to 8ºC
Ion Library Equalizer™ Capture	Purple	1 mL	
Ion Library Equalizer™ Elution Buffer	Clear	10 mL	
Ion Library Equalizer™ Beads	Orange	300 µL	
Ion Library Equalizer™ Wash Buffer	Clear	35 mL	

Ion AmpliSeq™ Direct FFPE DNA Kit

The Ion AmpliSeq™ Direct FFPE DNA Kit provides sufficient reagents for direct analysis of DNA from 8 (Cat. No. A31133) or 96 (Cat. No. A31136) unstained, slide-mounted, formalin-fixed, paraffin-embedded (FFPE) tissue samples for downstream library preparation without nucleic acid isolation or quantification.

Component	Cat. No. A31133 (8 rxns)	Cat. No. A31136 (96 rxns)	Storage
Transfer Solution (purple cap)	240 µL	3 × 960 µL	2°C to 8°C
Direct Reagent (orange cap)	170 µL	3 × 675 µL	

LiquidBiopsy™ Platform for isolating circulating tumor cells and cell-free DNA

The LiquidBiopsy™ Platform for rare cell isolation is a comprehensive, semi-automated, sample-to-sequence solution for isolating and analyzing circulating tumor cells (CTCs), cell-free DNA (cfDNA), and germline DNA from blood samples in less than 48 hours. By leveraging Ion AmpliSeq™ technology and the Ion sequencing systems, the workflow enables researchers to conduct highly multiplexed analyses on serially collected blood samples. See the *LiquidBiopsy™ Reagents and Consumables Kit User Guide* (Pub. No. MAN0013466) and *LiquidBiopsy™ Instrument User Guide* (Pub. No. MAN0013431) for more information.

Ion AmpliSeq™ Panels

Ion AmpliSeq™ Ready-to-use, Custom, and Community Panels provide pools of primers for amplification of target regions. The primers contain proprietary modifications that enable removal of primer sequences during library preparation for efficient target assessment during sequencing. Multiple primer pools can be used to create overlapping amplicons that enable complete coverage of large targets.

- Ion AmpliSeq™ Ready-to-use, Custom and Community Panels are available at **www.ampliseq.com**.
- Ion AmpliSeq™ Ready-to-use Panels may also be ordered from **www.thermofisher.com**.
- Visit **www.ampliseq.com** to design and order Ion AmpliSeq™ Custom Panels using Ion AmpliSeq™ Designer.

See Appendix C, "Ion AmpliSeq™ Custom Panels" for further information.

Ion AmpliSeq™ Ready-to-use Panels

Panel	Conc.	Approx. library size (with adapters)	Quantity	No. of primer pairs	Storage[1]
gDNA panels (human)					
Ion AmpliSeq™ Cancer Hotspot Panel v2 (Cat. No. 4475346)	5X	150–220 bp	1 tube (8 rxns)	207	–30°C to –10°C
Ion AmpliSeq™ Pharmacogenomics Panel (Cat. Nos. A29250, A29251)	5X	150–220 bp	1 tube (16 or 96 rxns)	119	
Ion AmpliSeq™ Comprehensive Cancer Panel (Cat. No. 4477685)	2X	150–250 bp	4 tubes (8 rxns)	~4,000/tube (~16,000 total)	
Ion AmpliSeq™ Inherited Disease Panel (Cat. No. 4477686)	2X	150–300 bp	3 tubes (8 rxns)	~3,500/tube (~10,500 total)	
RNA panels (human)					
Ion AmpliSeq™ RNA Apoptosis Panel (Cat. No. 4482571)	5X	150–220 bp	1 tube (24 rxns)	267	–30°C to –10°C
Ion AmpliSeq™ RNA Cancer Panel (Cat. No. 4482572)	5X	150–220 bp	1 tube (24 rxns)	50	

[1] Shipped at ambient temperature. Store as indicated.

Ion AmpliSeq™ Custom and Community Panels

Visit **www.ampliseq.com** to design and order Ion AmpliSeq™ Custom Panels using the Ion AmpliSeq™ Designer, and to order Ion AmpliSeq™ Community Panels. Each order includes one or more pre-made pools of primer pairs at 2X or 5X concentration for use with standard Ion AmpliSeq™ Library Kits.

Most panels include 1 or 2 primer pools. In some cases, 384-well plates with individual primer pairs are available at an additional charge.

Ion AmpliSeq™ Custom and Community DNA Panels

Average primer pairs per pool in a Custom or Community Panel	2X primer pools		Storage[1]
	Panels with 1 primer pool	Panels with 2 primer pools	
≤96 primer pairs	5 tubes, ~1500 µL each	10 tubes, ~1500 µL each	–30°C to –10°C
>96 primer pairs	20 tubes, ~1500 µL each	40 tubes, ~1500 µL each	

[1] Shipped at ambient temperature. Store as indicated.

Ion AmpliSeq™ Custom and Community RNA Panels

Number of primer pairs per pool in a Custom or Community Panel	5X primer pools		Storage[1]
	Panels with 1 primer pool	Panels with 2 primer pools	
12–1,200	3 tubes, ~1500 µL each	6 tubes, ~1500 µL each	–30°C to –10°C

[1] Shipped at ambient temperature. Store as indicated.

Ion AmpliSeq™ Sample ID Panel

Ion AmpliSeq™ Sample ID Panel (Cat. No. 4479790) is a companion panel of nine primer pairs that can be added to any Ion AmpliSeq™ human gDNA panel during target amplification to generate a unique identification tag for research samples.

Panel	Conc.	Approx. library size (with adapters)	Quantity	No. of primer pairs	Storage[1]
Ion AmpliSeq™ Sample ID Panel	20X	150–220 bp	1 tube (96 rxns)	9	–30°C to –10°C

[1] Shipped at ambient temperature. Store as indicated.

Ion AmpliSeq™ Chef-ready kits

Ion AmpliSeq™ Chef-ready kits provide primer panels at the appropriate volume and concentration ready to load into an Ion Chef™ instrument for automated Ion AmpliSeq™ library preparation. Each kit also includes the reagents and supplies supplied in the Ion AmpliSeq™ Kit for Chef DL8 sufficient for 4 library preparation runs. See the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432) for further details and a list of available Chef-ready kits.

Ion AmpliSeq™ Transcriptome Human Gene Expression Kit

For information and specific protocols for using the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (Cat. Nos. A26325, A26326, A26327) to generate transcriptome libraries from RNA, refer to the *Ion AmpliSeq™ Transcriptome Human Gene Expression Kit User Guide* (Pub. No. MAN0010742).

Ion AmpliSeq™ Exome RDY Kits

For information and specific protocols for using the Ion AmpliSeq™ Exome RDY and Exome RDY S5 Kits (Cat. Nos. A27192, A27193, A29854, A29855) to generate exome libraries, refer to the *Ion AmpliSeq™ Exome RDY Library Preparation User Guide* (Pub. No. MAN0010084).

IonCode™ Barcode Adapter Kit

The IonCode™ Barcode Adapters 1–384 Kit (Cat. No. A29751) provides 384 different pre-mixed adapters in a convenient 96-well plate format.

Component	Quantity	No. of reactions	Storage
IonCode™ Barcode Adapters 1–384 Kit	4 × 96-well plates (20 µL/well)	3,840 (10 reactions per barcode)	–30ºC to –10ºC

Ion Xpress™ Barcode Adapters

Each kit provides 16 different barcode adapters, sufficient for ~640 Ion AmpliSeq™ libraries.

Component	Cap color	Quantity	Volume per tube	Storage
Ion Xpress™ P1 Adapter	Violet	1 tube	320 µL	–30ºC to –10ºC
Ion Xpress™ Barcode X	White	16 tubes (1 per barcode)	20 µL each	

The following Ion Xpress™ Barcode Adapters Kits are available:

- Ion Xpress™ Barcode Adapters 1–16 (Cat. No. 4471250)
- Ion Xpress™ Barcode Adapters 17–32 (Cat. No. 4474009)
- Ion Xpress™ Barcode Adapters 33–48 (Cat. No. 4474518)
- Ion Xpress™ Barcode Adapters 49–64 (Cat. No. 4474519)
- Ion Xpress™ Barcode Adapters 65–80 (Cat. No. 4474520)
- Ion Xpress™ Barcode Adapters 81–96 (Cat. No. 4474521)
- Ion Xpress™ Barcode Adapters 1–96 (Cat. No. 4474517; Complete set of adapters)

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**www.fisherscientific.com**) or other major laboratory supplier.

Item	Source
One of the following: • GeneAmp™ PCR System 9700 or Dual 96-well Thermal Cycler • AB™ 2720 Thermal Cycler • Veriti™ 96-well Thermal Cycler • ProFlex™ 96-Well PCR System	See web product pages
MicroAmp™ Optical 96-well Reaction Plate	N8010560 4306737 (with barcode)
MicroAmp™ Adhesive Film	4306311
MicroAmp™ Compression Pad	4312639
Agencourt™ AMPure™ XP Kit	Beckman Coulter, A63880 or A63881
DynaMag™-96 Side Magnet, or other plate magnet	12331D
Nuclease-free Water	AM9932
Absolute ethanol	MLS
Pipettors, 2–200 µL, and low-retention filtered pipette tips	MLS
(RNA only) SuperScript™ VILO™ cDNA Synthesis Kit	11754-050

Recommended materials and equipment (optional)

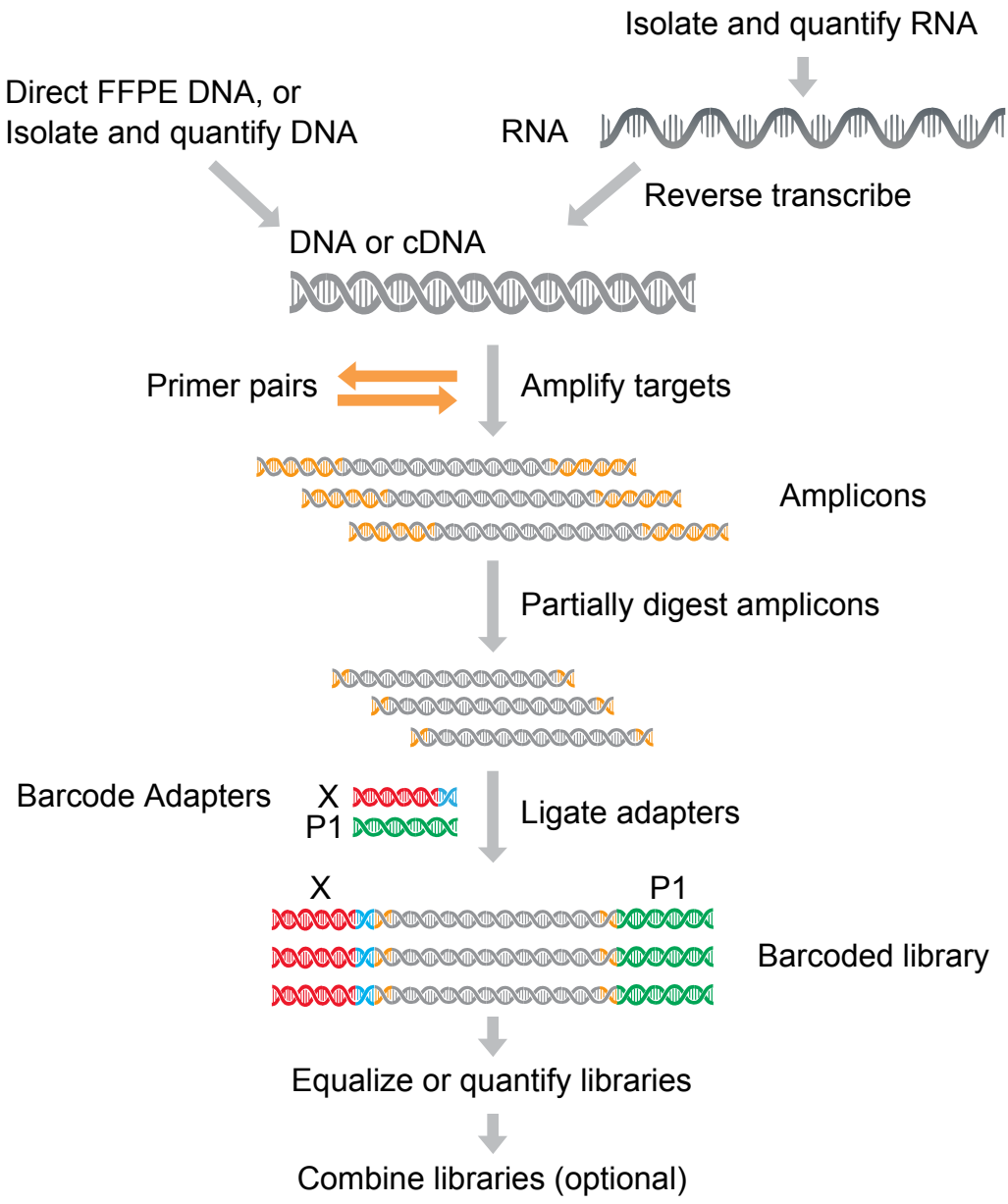
Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**www.fisherscientific.com**) or other major laboratory supplier.

Item	Source
Recommended additional equipment	
Real-time PCR instrument (e.g.,Applied Biosystems™ 7900HT, 7500, StepOne™, StepOnePlus™, ViiA™ 7 Systems, or QuantStudio™ 12K Flex Real-Time PCR System)	See web product pages
96-well plate centrifuge	MLS
Recommended for nucleic acid isolation	
Ion AmpliSeq™ Direct FFPE DNA Kit	A31133, A31136
RecoverAll™ Total Nucleic Acid Isolation Kit	AM1975

Item	Source
MagMAX™ FFPE Total Nucleic Acid Isolation Kit	4463365
PureLink™ Genomic DNA Mini Kit	K182000
Recommended for nucleic acid quantification	
TaqMan® RNase P Detection Reagents Kit *(Recommended for DNA only)*	4316831
Qubit™ 3.0 Fluorometer or Qubit™ 2.0 Fluorometer[1], Qubit™ dsDNA HS Assay Kit (DNA), or Qubit™ RNA HS Assay Kit (RNA)	Q33216, Q32851/Q32854, Q32852/Q32855
Recommended for library quantification *(If you are NOT using the Ion Library Equalizer™ Kit for library normalization, select one of the following:)*	
Ion Library TaqMan® Quantitation Kit	4468802
Qubit™ 3.0 Fluorometer or Qubit™ 2.0 Fluorometer[1] and Qubit™ dsDNA HS Assay Kit (DNA)	Q33216, Q32851/Q32854
Agilent™ 2100 Bioanalyzer™ and Agilent™ High Sensitivity DNA Kit	Agilent G2939AA, 5067-4626
Additional material recommended for the Ion AmpliSeq™ Direct FFPE DNA Kit	
Eppendorf LoBind™ tubes (0.5-mL or 1.5-mL, if not using 96-well reaction plate)	MLS
Recommended controls	
AcroMetrix™ MultiMixC FFPE Control	957186
AcroMetrix™ KRAS FFPE Process Controls	950450
AcroMetrix™ Oncology Hotspot Control	969056
Control DNA (CEPH 1347-02)	403062

[1] Supported but no longer available for purchase.

Ion AmpliSeq™ workflow starting from genomic DNA or RNA



2 Methods

Procedure overview

1. Use the Ion AmpliSeq™ Direct FFPE DNA Kit, or isolate genomic DNA or RNA.
2. If starting with RNA, reverse-transcribe to make cDNA.
3. Amplify target regions from DNA or cDNA with the Ion AmpliSeq™ Library Kit 2.0 and an Ion AmpliSeq™ Ready-to-use, Custom, or Community panel. For panels consisting of multiple primer pools, combine target amplification reactions after thermal cycling.
4. Partially digest amplicons with FuPa Reagent.
5. Ligate adapters with Switch Solution and DNA Ligase, then purify.
6. Normalize or quantify the libraries using one of three options:
   - Normalize libraries to ~100 pM without the need for quantification or dilution using the Ion Library Equalizer™ Kit.
   - Without further amplification, quantify libraries by qPCR and dilute to 100 pM.
   - Quantify libraries using the Qubit™ Fluorometer or the Agilent™ 2100 Bioanalyzer™ instrument and dilute to 100 pM. If you use one of these methods, which do not specifically detect amplifiable molecules, library amplification and purification are required before quantification.

   Note: The Ion Library Equalizer™ Kit offers the greatest convenience, but can result in low library yield when sample quality is low. qPCR typically provides the best sensitivity and barcode balance, and is recommended for libraries from RNA. Qubit™ fluorometry is the most economical, but lacks specificity. Agilent™ 2100 Bioanalyzer™ quantification generates the most information for troubleshooting.
7. When barcode adapters are used, libraries can be combined in various ways before sequencing. Combining libraries maximizes chip use while minimizing cost and labor. See Appendix B, "Strategies for combining Ion AmpliSeq™ libraries" for more information.

Automation protocols

For automated Ion AmpliSeq™ library preparation on the Ion Chef™ System, see the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432).

For information about preparing Ion AmpliSeq™ libraries on the Tecan Freedom EVO™ 150 liquid handling platform, see *Prepare Ion AmpliSeq™ 2.0 Libraries using the Tecan Freedom EVO™ NGS Workstation*, available on the Ion Community at **ioncommunity.thermofisher.com/docs/DOC-6536**.

Procedural guidelines

- Minimize freeze-thawing of Ion AmpliSeq™ Panels by aliquoting as needed for your experiments. Panels can be stored at 4°C for one year.
- 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 not supplied" on page 13.
- Pipet viscous solutions slowly and ensure complete mixing by vigorous vortexing or pipetting up and down several times.

Before you begin

- Thaw components that contain enzymes—such as 5X Ion AmpliSeq™ HiFi Mix, FuPa Reagent, DNA Ligase, and Platinum™ PCR SuperMix HiFi—on ice, and keep on ice during procedure. All other components, including primer pools, can be thawed at room temperature. Gently vortex and centrifuge before use.
- If there is visible precipitate in the 5X VILO™ Reaction Mix, or Switch Solution after thawing, vortex or pipet up and down at room temperature to resuspend.

DNA: Set up DNA target amplification

Guidelines for DNA isolation and quantification

- See "Recommended materials and equipment (optional)" on page 13 for recommended kits for isolating gDNA.
- We recommend the TaqMan® RNase P Detection Reagents Kit (Cat. No. 4316831) for quantifying amplifiable human genomic DNA (see "**Demonstrated Protocol: Sample Quantification for Ion AmpliSeq™ Library Preparation Using the TaqMan® RNAse P Detection Reagents Kit**"). The Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) can also be used.
- Quantification methods such as densitometry (for example, using a NanoDrop™ spectrophotometer) are not recommended, because they are not specific for DNA. Use of these methods can lead to gross overestimation of the concentration of sample DNA, under-seeding of the target amplification reaction, and low library yields.
- The Ion AmpliSeq™ Direct FFPE DNA Kit bypasses nucleic acid isolation when preparing libraries from FFPE sections on slides. See "Prepare DNA with Ion AmpliSeq™ Direct FFPE DNA Kit" on page 19 for a protocol for using this kit to prepare gDNA from FFPE tissue.

Guidelines for the amount of DNA needed per target amplification reaction

- For each target amplification reaction, use 300–30,000 copies of DNA (1–100 ng of mammalian gDNA) from normal or FFPE tissue, or cfDNA.
- Increasing the amount of DNA results in higher-quality libraries, especially when DNA quality or quantity is unknown. We recommend using 1 ng gDNA (300 copies) only with high-quality, well-quantified samples.
- Some Ion AmpliSeq™ Ready-to-use, Custom, and Community Panels for DNA are provided as multiple primer pools to create overlapping amplicons to cover large target regions. Panels with three or four primer pools require additional DNA compared with single- and two-primer pool panels.
- The maximum volume of DNA per reaction depends on the concentration of the Ion AmpliSeq™ primer pool you are using, the number of primer pools in the panel, and whether you are using a companion panel such as the Ion AmpliSeq™ Sample ID Panel. See the following "Prepare DNA target amplification reaction" topics on pages 21–25 for the maximum volume of DNA in target amplification reactions.
- For Ion AmpliSeq™ Direct FFPE DNA samples, library yield can be lower for a small tissue area or for degraded samples. Also, inhibitors such as high melanin content can reduce the efficiency of target amplification.

Prepare DNA with Ion AmpliSeq™ Direct FFPE DNA Kit

Use the following protocol with the Ion AmpliSeq™ Direct FFPE DNA Kit (Cat. Nos. A31133, A31136) to prepare DNA from FFPE tissue sections suitable for use in Ion AmpliSeq™ library preparation.

Prepare reagents

- Equilibrate Transfer Solution to room temperature (15–30°C) before use.
- Keep Direct Reagent on ice prior to use.

Prepare Direct FFPE DNA

The recommended tissue area to be used for this protocol is 4–100 mm^2 from a 5–10-µm thick unstained section mounted on a slide. Deparaffinization is not required. If desired, scrape unwanted tissue from the slide before transfer.

1. For each sample, pipet 30 µL of Transfer Solution into a single well of a 96-well PCR plate.
2. Using a single 20-µL pipette tip for each sample:
   a. Pipet 2–10 µL of the Transfer Solution from the well onto the region of interest of the FFPE tissue section mounted on a slide.
   b. Using the same 20-µL pipette tip, spread the Transfer Solution to ensure complete coverage of the region of interest, then scrape and break up the tissue with the pipette tip. The tissue should be a slurry of fine particles in the Transfer Solution.
3. Pipet the slurry from the slide back into the same well of the 96-well plate containing Transfer Solution.
4. Pipette the slurry up and down at least five times, leaving as much tissue as possible in the 96-well plate.

5. If needed, use the same tip to repeat steps 2–4, transferring as much of the region of interest as possible into the 96-well plate.

   Note: The final volume of Transfer Solution remaining in the 96-well plate can vary, but no volumetric adjustment is required.

6. Add 21 µL of Direct Reagent to each well containing sample in the 96-well plate.

7. Set a pipette to 30 µL, then mix the Direct Reagent and slurry by pipetting up and down ten times.

8. Seal the plate with a MicroAmp™ Adhesive Film, then verify that the contents are at the bottom of each well of the 96-well plate.

   Note: If necessary, gently tap the plate on a hard flat surface to collect the contents at the bottom of the wells.

9. Place a compression pad on the plate, load the plate into the thermal cycler, then run the following program:

Temperature	Time
65°C	15 minutes
20°C	Hold (for up to 30 minutes)

Proceed to "Remove an aliquot for library preparation".

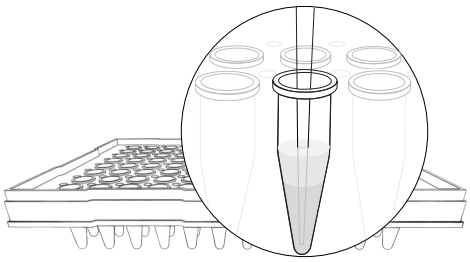
Note: See "(Optional) Removal of deaminated bases from Direct FFPE DNA" on page 47 for an optional Uracil DNA Glycosylase (UDG) treatment procedure.

Note: The Direct FFPE DNA preparation can be stored for up to 3 months at –20°C before library preparation.

Note: Sample DNA concentration can be evaluated using an optional Qubit™ fluorometry protocol. See "(Optional) Qubit™ Fluorometer: Quantify the FFPE DNA" on page 47.

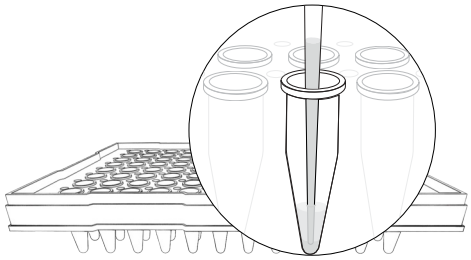
Remove an aliquot for library preparation

1. Set a 20-µL pipette to 15 µL, depress the plunger to the first stop and insert the pipet tip below the interface between phases, then pipet the lower (aqueous) phase up and down to mix the sample.



   Note: Mixing the sample before removal ensures a homogeneous sample before removing aliquots. Avoid pipetting the upper phase and interface while mixing.

2. Aspirate 6–20 µL —depending on the number of primer pools and reaction size— of the lower phase, then transfer the sample to the appropriate well of a 96-well PCR plate, or, if using a panel with multiple primer pools, to the Sample Master Mix tube.



Note: Use the maximum volume of DNA indicated in the appropriate target amplification reaction setup table.

IMPORTANT! Avoid pipetting the upper phase that contains the Transfer Solution. Carefully inspect each transferred sample aliquot for air bubbles. Remove any air bubbles by gently pipetting up and down.

Proceed to the "Prepare DNA target amplification reactions" protocol appropriate to the panel you are using to complete the assembly of the target amplification reactions.

Prepare DNA target amplification reactions — single primer pool

IMPORTANT! Primer pools and 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly. We recommend PCR setup on ice or a cold block.

1. For DNA panels with one primer pool, select the appropriate table below based on whether you are using a 2X primer pool or a 5X primer pool. Add the following components to a single well of a 96-well PCR plate. Prepare a master mix without sample DNA for multiple reactions.

   Note: If using the Ion AmpliSeq™ Direct FFPE DNA Kit, remove up to the maximum volume indicated in the table from the lower aqueous phase of the well and add to the target amplification reaction.

Component	Volume
2X Primer Pool	
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
2X Ion AmpliSeq™ Primer Pool	10 µL
DNA (1–100 ng), or Direct FFPE DNA preparation	≤6 µL
Nuclease-free Water	to 20 µL
5X Primer Pool	
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
5X Ion AmpliSeq™ Primer Pool	4 µL
DNA (1–100 ng), or Direct FFPE DNA preparation	≤12 µL
Nuclease-free Water	to 20 µL

2. Seal the plate with a MicroAmp™ Adhesive Film, then place a MicroAmp™ Compression Pad on the plate.

Proceed to “Amplify the targets“ on page 30.

Prepare DNA target amplification reactions — two primer pools

If you are using a DNA panel with two primer pools, 10-µL target amplification reaction volumes can be used, then combined after target amplification to yield a total volume of 20 µL.

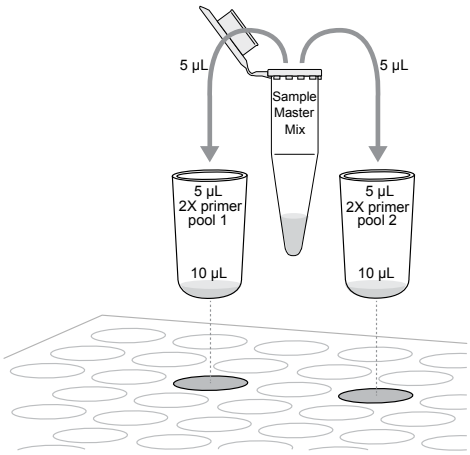
1. For panels with two primer pools, use the following table to prepare for each sample a target amplification master mix without primers in a 1.5-mL tube.

   Note: If using the Ion AmpliSeq™ Direct FFPE DNA Kit, remove up to the maximum volume indicated in the table from the lower aqueous phase of the well and add to the target amplification reaction master mix.

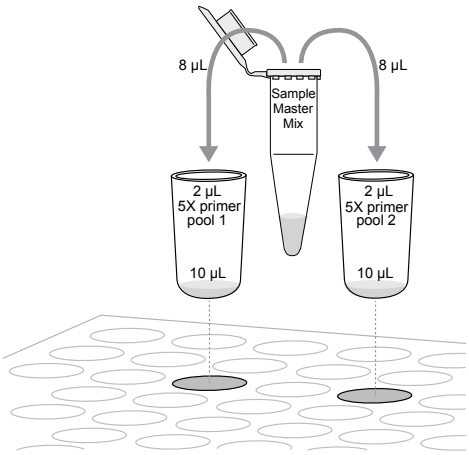
Component	Volume
Two-pool panels at 2X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	5 µL
DNA (1–100 ng), or Direct FFPE DNA preparation	⩽7.5 µL
Nuclease-free Water	to 12.5 µL
Two-pool panels at 5X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.5 µL
DNA (1–100 ng), or Direct FFPE DNA preparation	⩽13.5 µL
Nuclease-free Water	to 18 µL

2. Mix thoroughly by pipetting up and down five times, then transfer sample-specific master mixes to two wells of a 96-well PCR plate:
   - For 2X primer pools, transfer 5 µL of master mix into 2 wells. Add 5 µL of primer pool 1 into the first well, and 5 µL of primer pool 2 to the second well.
   - For 5X primer pools, transfer 8 µL of master mix into 2 wells. Add 2 µL of primer pool 1 into the first well, and 2 µL of primer pool 2 to the second well.

Two-pool panels at 2X concentration:



Two-pool panels at 5X concentration:



Note: If using Direct FFPE DNA preparations, distribute any remaining particulate tissue in the master mix evenly between the wells.

3. Seal the plate with a MicroAmp™ Adhesive Film, then place a MicroAmp™ Compression Pad on the plate.

Proceed to "Amplify the targets" on page 30.

Prepare DNA target amplification reactions — three primer pools

If you are using a DNA panel with three primer pools, 10-µL target amplification reaction volumes can be set up similar to panels with two primer pools, then combined after target amplification to yield a total volume of 30 µL.

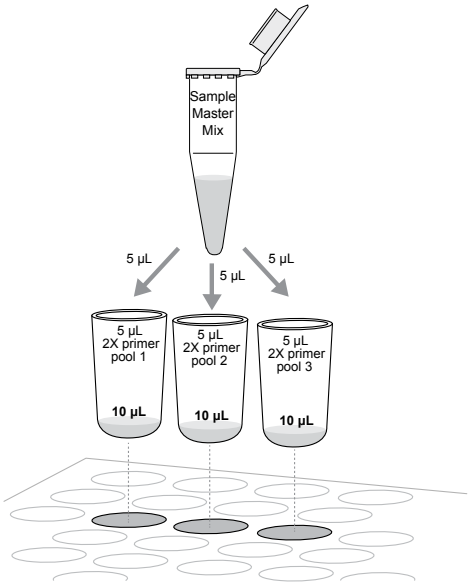
1. For panels with three primer pools, use the following table to prepare for each sample a target amplification master mix without primers in a 1.5-mL tube.

   Note: If using the Ion AmpliSeq™ Direct FFPE DNA Kit, remove up to the maximum volume indicated in the table, or 20 µL, from the lower aqueous phase of the well and add to the target amplification reaction master mix.

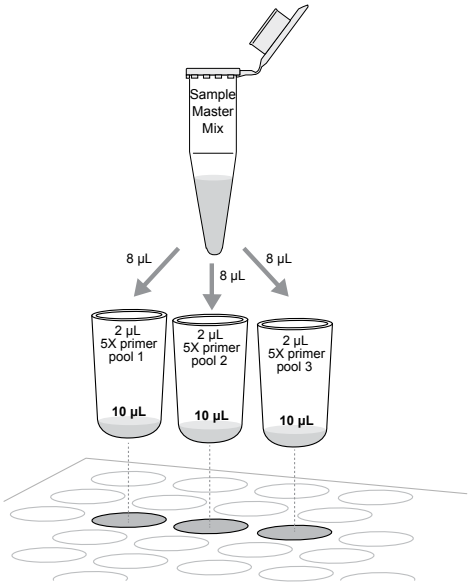
Component	Volume
Three-pool panels at 2X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	7 µL
DNA (1–100 ng)	⩽10.5 µL
Nuclease-free Water	to 17.5 µL
Three-pool panels at 5X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	7 µL
DNA (1–100 ng)	⩽21 µL
Nuclease-free Water	to 28 µL

2. Mix thoroughly by pipetting up and down five times, then transfer sample-specific master mixes to three wells of a 96-well PCR plate:
   - For 2X primer pools, transfer 5 µL of master mix into 3 wells. To each of these wells, add 5 µL of one of the three primer pools.
   - For 5X primer pools, transfer 8 µL of master mix into 3 wells. To each of these wells, add 2 µL of one of the three primer pools.

Three-pool panels at 2X concentration:



Three-pool panels at 5X concentration:



Note: If using Direct FFPE DNA preparations, distribute any remaining particulate tissue in the master mix evenly between the wells.

3. Seal the plate with a MicroAmp™ Adhesive Film, then place a MicroAmp™ Compression Pad on the plate.

Proceed to "Amplify the targets" on page 30.

Prepare DNA target amplification reactions — four primer pools

If you are using a DNA panel with four primer pools, 10-µL target amplification reaction volumes can be set up similar to panels with two primer pools, then combined after target amplification to yield a total volume of 40 µL.

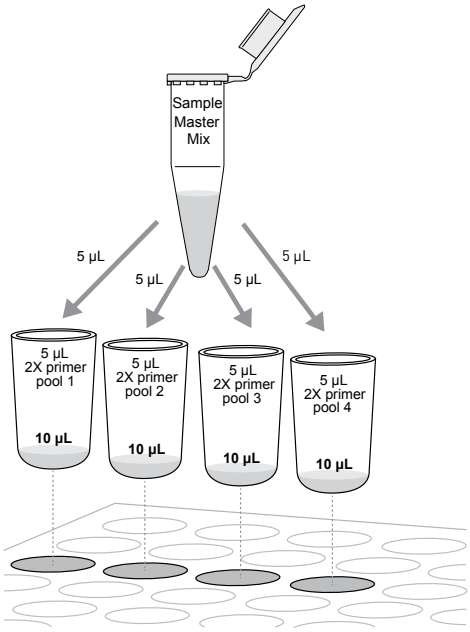
1. For panels with four primer pools, use the following table to prepare for each sample a target amplification master mix without primers in a 1.5-mL tube.

   Note: If using the Ion AmpliSeq™ Direct FFPE DNA Kit, remove up to the maximum volume indicated in the table, or 20 µL, from the lower aqueous phase of the well and add to the target amplification reaction master mix.

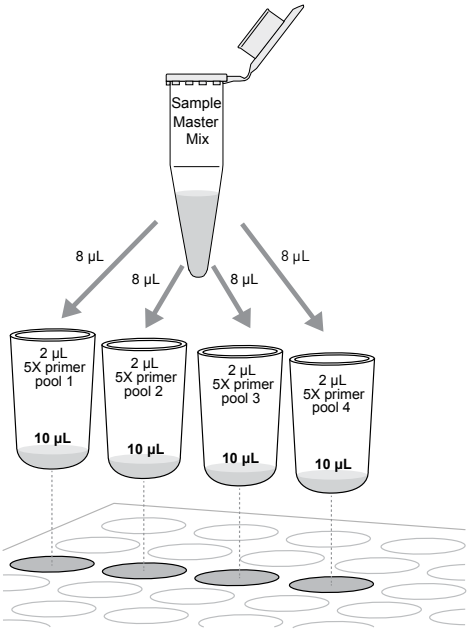
Component	Volume
Four-pool panels at 2X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	9 µL
DNA (1–100 ng)	≤13.5 µL
Nuclease-free Water	to 22.5 µL
Four-pool panels at 5X concentration	
5X Ion AmpliSeq™ HiFi Mix (red cap)	9 µL
DNA (1–100 ng)	≤27 µL
Nuclease-free Water	to 36 µL

2. Mix thoroughly by pipetting up and down five times, then transfer sample-specific master mixes to four wells of a 96-well PCR plate:
   - For 2X primer pools, transfer 5 µL of master mix into 4 wells. To each of these wells, add 5 µL of one of the four primer pools.
   - For 5X primer pools, transfer 8 µL of master mix into 4 wells. To each of these wells, add 2 µL of one of the four primer pools.

Four-pool panels at 2X concentration:



Four-pool panels at 5X concentration:



Note: If using Direct FFPE DNA preparations, distribute any remaining particulate tissue in the master mix evenly between the wells.

3. Seal the plate with a MicroAmp™ Adhesive Film, then place a MicroAmp™ Compression Pad on the plate.

Proceed to "Amplify the targets" on page 30.

RNA: Reverse transcribe and set up target amplification

Guidelines for RNA isolation, quantification, and input

- See "Required materials not supplied" on page 13 for kits recommended for isolating total RNA.
- We recommend the Qubit™ RNA HS Assay Kit (Cat. No. Q32855) for quantifying RNA.
- Each reverse transcription reaction requires 1–100 ng of DNase-treated total RNA (≥0.14 ng/µL).
- In general, library yield from high quality RNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.
- Increasing the amount of RNA will usually result in higher quality libraries, especially when RNA quality or quantity is unknown. We recommend using 1 ng total RNA only with high-quality, well-quantified samples.

Reverse transcribe RNA

If you are starting from genomic DNA, go to "DNA: Set up DNA target amplification" on page 18.

If you are starting from RNA, you must first reverse transcribe to cDNA.

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

2. For each sample, add the following components into a single well of a 96-well PCR plate. Prepare a master mix without sample RNA for multiple reactions.

Component	Volume
5X VILO™ Reaction Mix	2 µL
10X SuperScript™ Enzyme Mix	1 µL
Total RNA (1–100 ng)	⩽ 7 µL
Nuclease-free Water	to 10 µL
Total volume per well	**10 µL**

3. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least five times before sealing the plate.

4. Place a MicroAmp™ Compression Pad on the plate, 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
10°C	Hold

STOPPING POINT Samples can be stored at 10°C for up to 16 hours in the thermal cycler. For longer periods, store at –20°C.

5. Gently tap the plate on the bench to ensure reactions are at the bottom of the wells, or if possible, centrifuge the plate to collect any droplets. Proceed to the next step:
   - If you are using an RNA panel with one primer pool, proceed to "Prepare cDNA target amplification reactions — single primer pool" below
   - If you are using an RNA panel with two primer pools, proceed to “Prepare cDNA target amplification reactions — two primer pools“ on page 28

Prepare cDNA target amplification reactions — single primer pool

IMPORTANT! 5X Ion AmpliSeq™ primer pools and HiFi Mix are viscous. Pipet slowly and mix thoroughly. We recommend PCR setup on ice or a cold block.

1. For RNA panels with one pool, remove the seal from the plate and add the following components to each cDNA synthesis reaction. Prepare a master mix for multiple reactions.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
5X Ion AmpliSeq™ RNA Panel	4 µL
Nuclease-free Water	2 µL
Total volume per well (includes 10 µL from cDNA synthesis)	**~20 µL**

2. Seal the plate with a new MicroAmp™ Adhesive Film, then place a MicroAmp™ Compression Pad on the plate.

Proceed to “Amplify the targets“ on page 30.

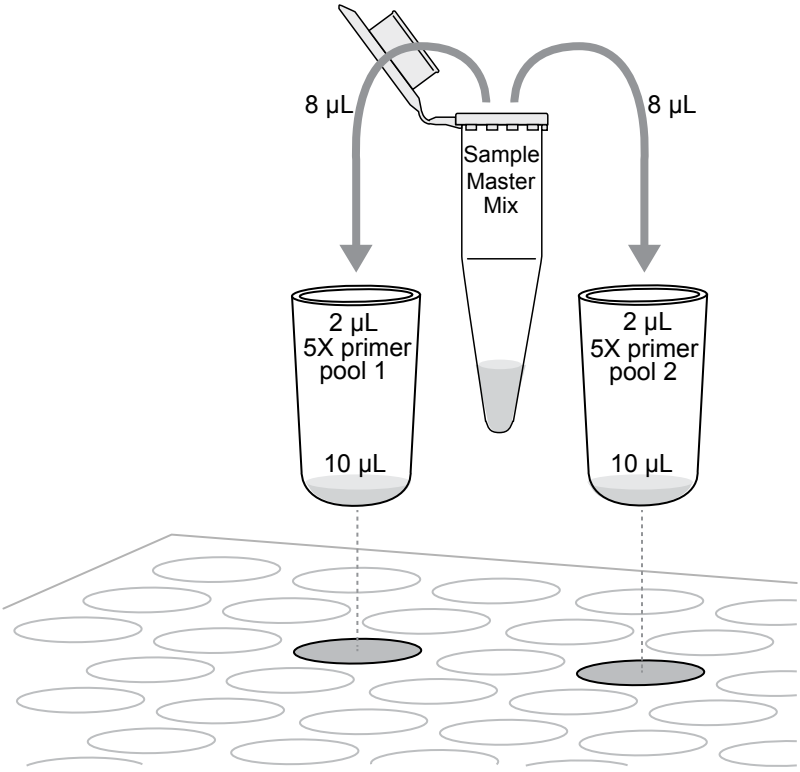
Prepare cDNA target amplification reactions — two primer pools

If you are using an RNA panel with two primer pools, 10-µL target amplification reaction volumes can be used, then combined after target amplification to yield a total volume of 20 µL.

1. For panels with two primer pools at 5X concentration, use the following table to prepare for each sample a target amplification master mix without primers in a 1.5-mL tube. Use the entire volume of the reverse transcription reaction in the amplification master mix.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.5 µL
cDNA	10 µL
Nuclease-free Water	3.5 µL
Total volume	18 µL

2. Mix thoroughly by pipetting up and down five times, then transfer 8 µL of each sample-specific master mixes into 2 wells of a 96-well PCR plate.



3. Add 2 µL of primer pool 1 into the first well, and 2 µL of primer pool 2 to the second well.

4. Seal the plate with a MicroAmp™ Adhesive Film, then place a MicroAmp™ Compression Pad on the plate.

Proceed to "Amplify the targets" on page 30.

Amplify the targets

To amplify target regions, run the following program.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	2 min
Cycle; set number according to the following tables	Denature	99°C	15 sec
	Anneal and extend	60°C	4/8/16 min[1]
Hold	—	10°C	Hold

[1] Use 4 minutes for ≤1536 primer pairs per pool; 8 minutes for 1,537–6,144 primer pairs per pool; 16 minutes for 6,145–24,576 primer pairs per pool.

Primer pairs per pool (excluding gene fusion primer pairs)[1]	Recommended number of amplification cycles (10 ng DNA/RNA, 3,000 copies)	
	High quality DNA/RNA	Low quality nucleic acid (FFPE DNA/RNA or cfDNA/RNA)
Panels for gene fusion detection only	27	30
12–24	21	24
25–48	20	23
49–96	19	22
97–192	18	21
193–384	17	20
385–768	16	19
769–1,536	15	18
1,537–3,072	14	17
3,073–6,144	13	16
6,145–12,288	12	15
12,289–24,576	11	14

[1] For RNA panels with both gene expression primer pairs and gene fusion primer pairs, use the number of gene expression primer pairs only to determine the appropriate number of amplification cycles.

Note: Cycle number can be increased when input material quality or quantity is questionable. Cycle number does not need adjustment when using the Ion AmpliSeq™ Sample ID Panel. If two primer pools for a single panel fall into different cycling categories, use the greater number of cycles.

Cycle number recommendations in the preceding table are based on 10-ng DNA/RNA input. Adjust cycle number from the preceding table for lower or higher DNA/RNA input:

Amount of DNA/RNA starting material	Adjustment to cycle number
1 ng (300 copies)	+3
10 ng (3,000 copies)	0
100 ng (30,000 copies)	–3

Note: We recommend that you run the Ion AmpliSeq™ Pharmacogenomics Research Panel (Cat. No. A29251), which has 119 primer pairs, using 20 amplification cycles instead of 18 cycles indicated in the table above.

IMPORTANT! When amplifying multiple samples in a single PCR plate, ensure that the input across the samples is roughly equivalent so that the selected cycle number for target amplification is optimal for all the samples in the run.

STOPPING POINT Target amplification reactions can be stored at 10°C overnight on the thermal cycler. For longer term, store at -20°C.

Combine target amplification reactions (for DNA and RNA panels with multiple primer pools)

1. Carefully remove the plate seal.
2. For each sample, combine the 10-µL target amplification reactions. The total volume for each sample should be:

Number of primer pools	Total volume of combined sample
2	20 µL
3	30 µL
4	40 µL

IMPORTANT!

- For 3-pool panels (30 µL total), increase the volumes of FuPa reagent, ligation components, and AMPure™ XP Reagent volumes by 50%.
- For 4-pool panels (40 µL total), double the volumes of FuPa reagent, ligation components, and AMPure™ XP Reagent.

Partially digest amplicons

1. Remove the plate seal and **add 2 µL of FuPa Reagent** (brown cap) to each amplified sample. The total volume is ~22 µL.
2. Seal the plate with a clear adhesive film, vortex thoroughly, then centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least five times before sealing the plate.
3. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

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

[1] Increase to 20 minutes for panels over 1,536 primer pairs/pool.

STOPPING POINT Store plate at –20°C for longer periods.

Ligate adapters to the amplicons and purify

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

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

Ion Xpress™ adapters require handling and dilution as described below.

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

Ion Xpress™ adapters only: Combine and dilute adapters

For each barcode X selected, prepare a mix of Ion P1 Adapter and Ion Xpress™ Barcode X at a final dilution of 1:4 for each adapter. Store diluted adapters at –20°C.

Substitute 2 µL of this barcode adapter mix for the Ion AmpliSeq™ Adapters in step 1 below. For example, combine the volumes indicated in the following table. Scale volumes as necessary.

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

[1] X = barcode chosen

Perform the 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 in the order listed to each well containing digested amplicons. If preparing multiple non-barcoded libraries, a master mix of Switch Solution and Ion AmpliSeq™ Adapters can be combined before addition.

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

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	IonCode™ Adapters *or* diluted Ion Xpress™ barcode adapter mix (for barcoded libraries) *or* Ion AmpliSeq Adapters (green cap, for non-barcoded libraries)	2 µL
3	DNA Ligase (blue cap)	2 µL
—	**Total volume** (including ~22 µL of digested amplicon)	**~30 µL**

2. Seal the plate with a new MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least five times before sealing the plate.

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

Temperature	Time
22°C	30 minutes
68°C	5 minutes
72°C	5 minutes
10°C	Hold (for up to 24 hours)

STOPPING POINT Samples can be stored overnight at 10°C on the thermal cycler. For longer periods, store at –20°C.

Purify the library

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

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

   Note: Visually inspect each well to ensure that the mixture is homogeneous.

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

3. Place the plate in a magnetic rack such as the DynaMag™-96 Side Magnet, then incubate for 2 minutes or until solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.

   IMPORTANT! If you are running a 3- or 4-pool panel that was combined after target amplification, you do NOT need to scale up volumes beyond this point.

4. Add 150 µL of freshly prepared 70% ethanol, then 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 five 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. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes. Do not overdry.

   IMPORTANT! Residual ethanol drops inhibit library amplification. If needed, centrifuge the plate and remove remaining ethanol before air-drying the beads.

Proceed immediately to one of the following:

- Option 1: Equalize the library.
- Option 2: Quantify the library by qPCR.
- Option 3: Quantify the amplified library with the Qubit™ 2.0 or 3.0 Fluorometer, or with the Agilent™ 2100 Bioanalyzer™ instrument.

Option 1: Equalize the library

IMPORTANT! We recommend the Ion Library Equalizer™ Kit when library yield is consistently above the minimum concentration outlined in this user guide. If sample quality or quantity is variable or unknown (such as RNA from FFPE tissue, or Direct FFPE DNA), we recommend using the qPCR method (see "Option 2: Quantify the library by qPCR" on page 39).

The Ion Library Equalizer™ Kit (Cat. No. 4482298) provides a method for normalizing library concentration at ~100 pM without the need for special instrumentation for quantification. First amplify the Ion AmpliSeq™ library, then capture the library on Equalizer™ Beads. After elution of the equalized library, proceed directly to combining libraries and/or template preparation.

Alternatively, for libraries that are run on the same chip, libraries can be combined during the equalization process.

Before you begin

Warm all the reagents in the Ion Library Equalizer™ Kit to room temperature. Vortex and centrifuge all reagents before use.

Amplify the library

1. Remove the plate from the magnet, then add 50 µL of Platinum™ PCR SuperMix HiFi (black cap) and 2 µL of Equalizer™ Primers (pink cap) to each bead pellet. The SuperMix and primers can be combined before addition.

   Note: Do NOT use the Library Amplification Primer Mix (white cap) provided in the Ion AmpliSeq™ Library Kit 2.0.

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

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

4. Seal the plate with a new clear adhesive film, place a compression pad on the plate, and load in the thermal cycler. Run the following program. During cycling, wash the Equalizer™ Beads, if they have not been previously washed.

Stage	Temperature	Time
Hold	98°C	2 minutes
9 cycles	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold (up to 1 hour)

Note: The concentration of the amplified library can be confirmed by removing 2 µL of the reaction and evaluating with the Ion Library TaqMan® Quantitation Kit. The Ion Library Equalizer™ Kit should only be used when library concentrations are routinely >4 nM after library amplification.

Wash the Equalizer™ Beads *(if not previously performed)*

1. Bring the Equalizer™ Beads to room temperature and mix thoroughly.

   Note: Beads for multiple reactions can be prepared in bulk, and stored in Equalizer™ Wash Buffer at 4°C for up to 12 months until use. After 12 months, re-wash beads with an equal volume of Equalizer™ Wash Buffer.

2. For each reaction, pipet 3 µL of beads into a clean 1.5-mL tube and add 6 µL/reaction of Equalizer™ Wash Buffer. For example, if you have four reactions, add 12 µL of beads and 24 µL of wash buffer.

3. Place the tube in a magnetic rack for 3 minutes or until the solution is completely clear.

4. Carefully remove and discard the supernatant without disturbing the pellet.

5. Remove the tube from the magnet, add 6 µL per reaction of Equalizer™ Wash Buffer, and pipet up and down to resuspend.

Add Equalizer™ Capture to the amplified library

1. (Optional) If possible after thermal cycling, centrifuge the plate at 100 × *g* for 30 seconds in a plate centrifuge to collect contents at the bottom of the wells.

2. Carefully remove the seal from the plate, then add 10 µL of Equalizer™ Capture to each library amplification reaction.

3. Seal the plate with a clear adhesive film, vortex thoroughly, then centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least five times before sealing the plate.

4. Incubate at room temperature for 5 minutes.

(Optional) Combine captured libraries

1. Determine the number of samples to be combined based on the coverage depth tables (see "Ion Chip capacities for Ion AmpliSeq™ DNA libraries sequenced at equal depths" on page 60 or "Ion Chip capacities for Ion AmpliSeq™ RNA libraries" on page 61).

2. Remove and combine an equal volume (5–10 µL) of each sample into a single well or tube. Mix the combined libraries thoroughly, then transfer 60 µL to a new well. Treat the combined libraries as a single sample and proceed to the next section.

   Example 1: If 8 libraries will be combined in a single templating and sequencing reaction, remove 7.5 µL of each library and combine them together in a new position on the 96-well plate.

   Example 2: If 384 libraries will be combined in a single templating and sequencing reaction, remove 5 µL of each library and combine them in a 2-mL tube. Mix thoroughly, then transfer 60 µL to a new position on the 96-well plate.

   Note: Save uncombined individual libraries for repeat analysis, if needed.

Add Equalizer™ Beads and wash

1. Mix the washed Equalizer™ Beads by gentle vortexing or pipetting up and down.

2. Add 6 µL of washed beads to each plate well containing the captured library (either combined or individual).

3. Set the pipette volume to 40 µL, then pipet the mixture up and down at least five times to mix thoroughly.

4. Incubate at room temperature for 5 minutes. Briefly centrifuge the plate to collect all the liquid to the bottom of the plate wells.

5. Place the plate in the magnet, then incubate for 2 minutes or until the solution is clear.

6. Carefully remove the supernatant without disturbing the pellet.

   Note: Check for droplets on the sides of the plate wells. If droplets are observed, seal the plate, then gently tap the plate on a hard, flat surface, or briefly centrifuge to collect all the liquid to the bottom of the plate wells.

   Note: For uncombined libraries, save the supernatant for repeat analysis if needed.

7. Add 150 µL of Equalizer™ Wash Buffer to each reaction.

8. Move the plate side-to-side in the two positions of the magnet to wash the beads.

   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 five times (with the pipettor set to at least half the total volume), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.

9. With the plate still in the magnet, carefully remove, then discard the supernatant without disturbing the pellet.

10. Repeat the bead wash as described in steps 7–9.

    Note: Ensure that as much wash buffer as possible is removed without disturbing the pellet.

Elute the Equalized library

1. Remove the plate from the magnet, then add 100 µL of Equalizer™ Elution Buffer to each pellet.
2. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least five times before sealing the plate.

   Note: Centrifuge with enough force to collect droplets, but not pellet beads. If beads are pelleted, vortex again and centrifuge more gently.
3. Elute the library by incubating in a thermal cycler at 32°C for 5 minutes.
4. Place the plate in the magnet, then incubate at room temperature for 5 minutes or until the solution is clear.
   The supernatant contains the Equalized library at ~100 pM, which can be stored with beads for up to 1 month at 4°–8°C.

Proceed to template preparation, or combine or store libraries as described below.

(Optional) Combine Equalized libraries

Multiple strategies for combining Ion AmpliSeq™ libraries are available. See Appendix B, "Strategies for combining Ion AmpliSeq™ libraries".

Store libraries

Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at –20°C.

Option 2: Quantify the library by qPCR

Elute the library, then determine the concentration by qPCR with the Ion Library TaqMan® Quantitation Kit (Cat. No. 4468802). Libraries that have not undergone a second round of amplification typically have yields of 100–500 pM. However, yield is not indicative of library quality. After quantification, determine the dilution factor that results in a concentration of ~100 pM, which is suitable for template preparation using an Ion template kit.

Elute the library

1. Remove the plate containing the Ion AmpliSeq™ library from the magnet, then add 50 µL of Low TE to the pellet to disperse the beads.
2. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least five times before sealing the plate.
3. Place the plate on the magnet for at least 2 minutes.
4. Prepare a 100-fold dilution for quantification. Remove 2 µL of supernatant, then combine with 198 µL of Nuclease-free Water.

Quantify library by qPCR and calculate the dilution factor

Determine the concentration of each Ion AmpliSeq™ library by qPCR with the Ion Library TaqMan® Quantitation Kit using the following steps. Analyze each sample, standard, and negative control in duplicate 20-µL reactions.

1. Prepare three 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, and 0.068 pM. Mark these tubes as standards, then use these concentrations in the qPCR instrument software.
2. Prepare reaction mixtures. For each sample, control, and standard, combine 20 µL of 2X TaqMan® MasterMix and 2 µL of 20X Ion TaqMan® Assay, then mix thoroughly. Dispense 11-µL aliquots into the wells of a PCR plate.
3. Add 9 µL of the diluted (1:100) Ion AmpliSeq™ library 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.
4. Program your real-time instrument as follows:
    a. Enter the concentrations of the control library standards.
    b. Select ROX™ Reference Dye as the passive reference dye.
    c. Select a reaction volume of 20 µL.
    d. Select FAM™ dye/MGB as the TaqMan® probe reporter/quencher.
    e. The Ion Library TaqMan® qPCR Mix can be used on various instruments, as listed in the following table. The fast cycling program was developed using the StepOnePlus™ System in Fast mode.

IMPORTANT! When quantifying libraries made from panels with 275-bp or 375-bp designs, use standard qPCR cycling. Fast cycling can result in inaccurate quantification.

Real-time PCR System	Reaction plate	Run mode	Stage	Temperature	Time
7500 Fast	96-well Fast	Fast	Hold (UDG incubation)	50°C	2 min
7900 HT 7900 HT Fast	96-well Fast 384-well Standard		Hold (polymerase activation)	95°C	20 sec
ViiA™ 7			Cycle (40 cycles)	95°C	1 sec
StepOne™ StepOnePlus™	48-/96-well Fast			60°C	20 sec
7300	96-well Standard	Standard	Hold (UDG incubation)	50°C	2 min
7500			Hold (polymerase activation)	95°C	2 min
7900 HT 7900 HT Fast			Cycle (40 cycles)	95°C	15 sec
ViiA™ 7				60°C	1 min

5. Following qPCR, calculate the average concentration of the undiluted Ion AmpliSeq™ library by multiplying the concentration determined with qPCR by 100.

6. Based on the calculated library concentration, determine the dilution that results in a concentration of ~100 pM.
   For example:
   - The undiluted library concentration is 300 pM.
   - The dilution factor is 300 pM/100 pM = 3.
   - Therefore, 10 µL of library mixed with 20 µL of Low TE (1:3 dilution) yields approximately 100 pM.

7. Dilute library to ~100 pM as described, then proceed to template preparation, or combine or store libraries as described below.

   Note: A library that yields less than 100 pM can be rescued with library amplification. Combine 25 µL of unamplified library with 72 µL of Platinum™ PCR SuperMix HiFi and 3 µL of Library Amplification Primer Mix. Perform 5–10 library amplification cycles (see step 4 of "Amplify the library" on page 35 or "Amplify the library" on page 41 for cycling conditions).

(Optional) Combine amplicon libraries

Multiple strategies for combining Ion AmpliSeq™ libraries are available. See Appendix B, "Strategies for combining Ion AmpliSeq™ libraries".

Store libraries

Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at –20°C.

Option 3: Quantify the amplified library with the Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ instrument

Ion AmpliSeq™ libraries must be amplified before quantification to enrich amplifiable material and obtain sufficient material for accurate quantification. Amplify the library using Platinum™ PCR SuperMix High Fidelity, then purify. Quantify the library using the Qubit™ 2.0 or 3.0 Fluorometer, or the Agilent™ 2100 Bioanalyzer™ instrument. Amplified libraries typically have yields of 2,000–10,000 pM. Yield is not indicative of library quality. After quantification, determine the dilution factor that results in a concentration of ~100 pM, which is appropriate for template preparation using an Ion template kit.

Alternatively, the Ion Library TaqMan® Quantitation Kit can be used to quantify amplified libraries.

Amplify the library

1. Remove the plate containing the Ion AmpliSeq™ library from the magnet, then add 50 µL of Platinum™ PCR SuperMix HiFi and 2 µL of Library Amplification Primer Mix to each bead pellet. The SuperMix and Primer Mix can be combined before addition.
2. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge briefly to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least five times before sealing the plate.
3. Place the plate back on the magnet for at least 2 minutes, then carefully transfer ~50 µL of supernatant from each well to a new plate without disturbing the pellet.
4. Place a MicroAmp™ Compression Pad on the plate, load in the thermal cycler, then run the following program:

Stage	Temperature	Time
Hold	98°C	2 minutes
5 cycles	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold

STOPPING POINT Samples can be stored at –20°C.

Purify the amplified library

Perform a two-round purification process with the Agencourt™ AMPure™ XP Kit:

- **First round at 0.5X bead-to-sample-volume ratio:** High molecular-weight DNA is bound to beads, while amplicons and primers remain in solution. **Save the supernatant.**
- **Second round at 1.2X bead-to-original-sample-volume ratio:** Amplicons are bound to beads, and primers remain in solution. **Save the bead pellet, and elute the amplicons from the beads.**

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

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

Do **NOT** substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP reagent.

First-round purification

1. Add 25 µL (0.5X sample volume) of Agencourt™ AMPure™ XP Reagent to each plate well containing ~50 µL of sample. Pipet up and down five times to mix the bead suspension with the DNA thoroughly, then seal the plate with MicroAmp™ Adhesive Film.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the plate in a magnet such as the DynaMag™ Side Magnet for at least 5 minutes or until the solution is clear.
4. Carefully transfer the supernatant from each well to a new well of the 96-well PCR plate without disturbing the pellet.

IMPORTANT! The **supernatant** contains the desired amplicons. Do not discard!

Second-round purification

1. To the supernatant from step 4 above, add 60 µL (1.2X original sample volume) of Agencourt™ AMPure™ XP Reagent. Pipet up and down five times to mix the bead suspension with the DNA thoroughly, then seal the plate with MicroAmp™ Adhesive Film.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the plate in the magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.

IMPORTANT! The amplicons are bound to the **beads. Save the bead pellet.**

4. 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 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 five 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. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes. **Do not overdry.**

7. Remove the plate from the magnet, then add 50 µL of Low TE to the pellet to disperse the beads.

8. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge to collect droplets. Alternatively, mix by setting a pipettor to 40 µL and pipet the mixture up and down at least five times before sealing the plate.

9. Place the plate in the magnet for at least 2 minutes and analyze an aliquot of the supernatant as described in:
   - "Qubit™ Fluorometer: Quantify the library and calculate the dilution factor" or
   - "Agilent™ 2100 Bioanalyzer™: Quantify the library and calculate the dilution factor".

IMPORTANT! The **supernatant** contains the desired amplicons. **Do not discard!**

Qubit™ Fluorometer: Quantify the library and calculate the dilution factor

Analyze 10 µL of each amplified library using the Qubit™ 2.0 or 3.0 Fluorometer and the Qubit™ dsDNA HS Assay Kit. Amplified libraries typically have concentrations of 300–1500 ng/mL. Libraries below 300 ng/mL can still provide good quality sequences. For more information, see the *Qubit™ dsDNA HS Assay Kits User Guide* (Pub. No. MAN0002326).

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. Combine 10 µL of the amplified Ion AmpliSeq™ library with 190 µL of dye reagent, mix well, then incubate for at least 2 minutes.
   c. Prepare each Qubit™ standard as directed in the user guide.
   d. Measure the concentration on the Qubit™ 2.0 or Qubit™ 3.0 Fluorometer.
   e. Calculate the concentration of the undiluted library by multiplying by 20.

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

Average amplicon size	Concentration in ng/mL (~100 pM)
140 bp	9
175 bp	11
225 bp	15
275 bp	18
375 bp	24

For example, with a FFPE-compatible 125–175 bp design (ave. 225 bp with adapters):

- The library concentration is 450 ng/mL.
- The dilution factor is 450 ng/mL divided by 15 ng/mL = 30.
- Therefore, 10 µL of library mixed with 290 µL of Low TE (1:30 dilution) yields approximately 15 ng/mL (~100 pM).

3. Dilute library to ~100 pM as described, then proceed to template preparation, or combine or store libraries as described below.

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

Analyze 1 µL of amplified library on the Agilent™ 2,100 Bioanalyzer™ instrument with the Agilent™ High Sensitivity DNA Kit (Cat. No. 5067-4626). Amplicon libraries should have multiple peaks in the 120–400 bp size range. Amplified libraries typically have concentrations of 2000–10,000 pM. Yield is not indicative of library quality, and libraries below 1,000 pM can still provide good quality sequences. If the library concentration is over 20,000 pM, dilute the library 1:10 and repeat the quantification to obtain a more accurate measurement.

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, then view the electropherogram of the sample to be quantified.
   b. Select the **Region Table** tab below, then 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 pmol/L (pM).
2. Based on the calculated library concentration, determine the dilution that results in a concentration of ~100 pM.
   For example:
   - The library concentration is 3,000 pM.
   - The dilution factor is 3,000 pM/100 pM = 30.
   - Therefore, 10 µL of library mixed with 290 µL of Low TE (1:30 dilution) yields approximately 100 pM.
3. Dilute library to ~100 pM as described, then proceed to template preparation, or combine or store libraries as described below.

(Optional) Combine amplicon libraries

Multiple strategies for combining Ion AmpliSeq™ libraries are available. See Appendix B, "Strategies for combining Ion AmpliSeq™ libraries".

Store libraries

Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at –20°C.

A

Tips and troubleshooting

Tips

- Arrange samples in columns on the plate for easier pipetting with multichannel pipettes during purification with the DynaMag™ Side 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 the thermal cycler.
- Use IonCode™ adapters to avoid handling and diluting adapters. Alternatively, combine and dilute Ion Xpress™ adapters in large batches and carefully aliquot into 96-well plates.
- If you are performing qPCR quantification, library amplification is unnecessary and the tube of Platinum™ PCR SuperMix HiFi supplied in the kit can be used for other applications. See the *Platinum™ PCR SuperMix High Fidelity User Guide* for instructions.
- When using the Qubit™ 2.0 or 3.0 Fluorometer, or the Agilent™ 2100 Bioanalyzer™ instrument, amplified libraries with little or no detectable product can still be quantified with qPCR.
- When transfer to a new plate is specified, solutions can be transferred to a clean well in the same plate instead, if desired.
- If library yield is below 100 pM, libraries can still be sequenced by using a proportionally larger volume into a combined library or into template preparation.
- When amplifying multiple samples in a single PCR plate, ensure that the input across the samples is roughly equivalent so that the selected cycle number is optimal for all the samples in the run.
- If you combine aliquots of captured libraries before adding Equalizer™ Beads, save the unused portions of 9-cycle amplified libraries for repeat analysis if needed.
- When trying the Ion Library Equalizer™ Kit for the first time, quantify the amplified libraries by qPCR to assure that libraries are routinely >4 nM in concentration.
- When setting up sample-specific master mixes for panels with 2 or more primer pools, master mixes can be set up in 96-well plates instead of tubes.
- When using the Ion AmpliSeq™ Direct FFPE DNA Kit, samples can be processed in 1.5-mL Eppendorf LoBind™ tubes before target amplification.

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.

Shortcuts

- Libraries can be created directly from whole blood collected in EDTA by adding 1 µL to a 20-µL target amplification reaction.
- If library yields are consistent and library balance is not critical, an equal volume of each library can be combined after barcode adapter ligation without quantification of individual libraries.
- When using qPCR quantification, careful removal of ethanol after the final wash eliminates the need for drying AMPure™ XP Beads. However, any remaining ethanol inhibits the library amplification reaction.
- When using qPCR quantification, the purification step after adapter ligation ("Purify the unamplified library") can be eliminated. Two additional target amplification cycles help reduce the amount of relative by-products that can go into template preparation.
- Library amplification before quantification with a Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ instrument can be carried out in the presence of the AMPure™ XP Beads without transfer to a new well.
- When using the Agilent™ 2100 Bioanalyzer™ instrument for quantification (but not the Qubit™ 2.0 or 3.0 Fluorometer), a single round of purification at 1.7X volume (85 µL) can be substituted for the two-round purification following library amplification ("Purify the amplified library"). High molecular weight material does not interfere with sequencing, but be sure that the markers are assigned correctly.

Limited samples

- Degraded samples with fragment sizes that are shorter than amplicon sizes can still yield Ion AmpliSeq™ libraries. For these samples, add up to 5 additional cycles to target amplification. Only primer pairs designed for cfDNA or FFPE samples are recommended for degraded samples.
- DNA from high-quality FFPE tissue can be used with longer amplicon designs. Uniformity and representation of longer amplicons can decrease.
- Ion AmpliSeq™ libraries prepared using single primer pools can be made from cells using a direct lysis method:
    a. Collect cells into a PCR plate containing 4–11 µL (depending on concentration of primer pool and use of Sample ID Panel) of Single Cell Lysis Solution (Ambion™ Single Cell Lysis Kit, Cat. No. 4458235; use 1 µL less buffer than the volume of input DNA specified in "Guidelines for the amount of DNA needed per target amplification reaction" on page 19).
    b. Incubate for 5 minutes at room temperature to lyse cells.
    c. Add 1 µL of Stop Solution (from the Ambion™ Cell Lysis Kit) without pipetting up and down, then incubate for 2 minutes.
    d. Proceed to target amplification by adding 4 µL 5X Ion AmpliSeq™ HiFi Mix and 2X or 5X primer pool for a total volume of 20 µL.

    Note: Increase the number of PCR cycles, using the guidelines for normal or FFPE DNA, by adding 4 cycles for 100 cells or 8 cycles for 10 cells. Longer anneal/extend times can improve uniformity.

(Optional) Removal of deaminated bases from Direct FFPE DNA

FFPE preservation methods can lead to significant cytosine deamination within the isolated DNA, and result in decreased sequencing quality. When using the Ion AmpliSeq™ Direct FFPE DNA Kit, deaminated cytosines (uracil) can be enzymatically removed by treatment with Uracil DNA Glycosylase (UDG) prior to target amplification.

1. Add 1–2 units of UDG to the Direct FFPE DNA aqueous phase of each sample after the 65°C/15 minute incubation.
2. Set a 20-µL pipette to 15 µL, then mix the lower (aqueous) phase by pipetting up and down ten times.
3. *(Optional)* If necessary, centrifuge briefly to collect contents and re-separate the liquid phases in the bottom of the tube.
4. Seal the plate and incubate at 37°C for 5 minutes, then incubate at 65°C for 5 minutes.

Proceed to “Remove an aliquot for library preparation“ on page 20.

(Optional) Qubit™ Fluorometer: Quantify the FFPE DNA

When using the Ion AmpliSeq™ Direct FFPE DNA Kit, the DNA concentration can be estimated using the Qubit™ 2.0 or Qubit™ 3.0 Fluorometer and the Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851). See the *Qubit™ dsDNA HS Assay Kits User Guide* (Pub. No. MAN0002326) for more information.

1. Set up the required number of 0.5-mL Qubit™ Assay tubes for standards and samples. The Qubit™ dsDNA HS Assay requires 2 standards.
2. Prepare sufficient Qubit™ working solution by diluting Qubit™ dsDNA HS Reagent 1:200 in Qubit™ dsDNA HS Buffer for all samples and standards required.
3. Combine 2 µL of the FFPE DNA sample with 198 µL (200-µL final volume) of working solution, mix well, then incubate for at least 2 minutes.
4. Prepare each Qubit™ standard as directed in the user guide.
5. Measure the concentration on the Qubit™ Fluorometer.
6. Calculate the concentration of the undiluted sample by multiplying by the dilution factor.

Proceed to the "Prepare DNA target amplification reactions" protocol appropriate to the panel you are using, adjusting the volume down to achieve the desired DNA input.

Troubleshooting

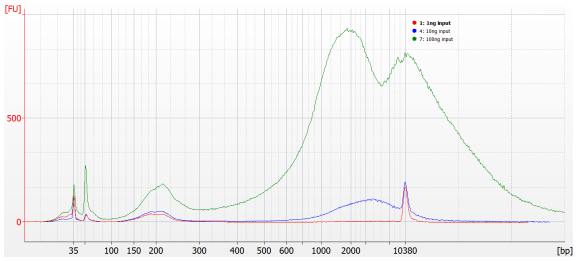
Ion AmpliSeq™ Direct FFPE DNA Kit troubleshooting

Observation	Possible cause	Recommended action
Tissue does not transfer from slide	Not enough Transfer Solution.	Hold the slide at a 45° angle and pipet extra Transfer Solution to the top of the slide allowing the tissue to flow towards the bottom. Remove collected tissue from the bottom, repeat as needed.
	Tissue is clumpy.	Transfer the mass of tissue to a collection tube, then continue breaking it up with a pipet tip.
		Pre-incubate with Transfer Solution on slide for 5 minutes then proceed to scraping.
Difficulty scraping tissue off the slide	Tissue is fibrous.	Scrape with 200-µL tip prior to transfer, using a circular motion then continue with a 20-µL tip.
		Scrape and homogenize tissue with razor blade then continue breaking up tissue with a 20-µL tip.
		Repeat transfer process with a larger volume of Transfer Solution.
	Target tissue area is surrounded by undesired tissue.	Use a scalpel blade to scrape away undesired tissue or paraffin, then use Transfer Solution to collect the desired tissue.
Excess undissolved tissue in Direct Reagent	Too much tissue in reaction.	Use 4–100 mm^2 tissue section. Tissue sections should be 5–10 µm thick.
	Digest may be incomplete.	Incubate for an additional 5–15 minutes at 65°C. After digestion sample may still be cloudy, this will not affect performance. Ensure homogenous mixing of the sample prior to removing an aliquot for Target Amplification. Undissolved tissue that can be aspirated with a pipet tip may still be added to the Target Amplification reaction.
		Centrifuge at ≥1,000 × *g* for 1 minute, then transfer 15 µL to a fresh tube, avoiding the fibrous pellet.

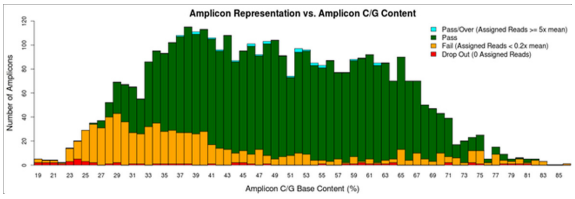
Observation	Possible cause	Recommended action
Transfer Solution and Direct Reagent do not separate into two phases	Too much paraffin in sample.	Use a scalpel blade to scrape away undesired paraffin prior to adding Transfer Solution to the desired tissue area.
		Centrifuge at ≥1,000 × *g* for 1 minute, then transfer 15 µL to a fresh tube, avoiding the fibrous pellet and tube walls.
		Perform partial deparaffinization before adding Transfer Solution to tissue on the slide: 1. Submerge slide in 100% xylene for 30 seconds. 2. Remove the slide and drain any excess xylene. 3. Submerge slide in 100% ethanol for 30 seconds. 4. Remove the slide and allow to air dry.
Difficulty transferring lower (aqueous) phase to Target Amplification reaction	Transfer Solution is in pipet tip.	Return tip contents to reaction tube, then centrifuge at ≥1,000 × *g* for 1 minute to separate phases. Move pipet quickly through the upper phase when transferring. **Note:** Transfer Solution will not interfere with Target Amplification.
Low AmpliSeq™ library yield (see page 50 for more low library yield troubleshooting)	Insufficient tissue was used.	Use 25–100 mm^2 tissue section of 5–10 µm thickness. If necessary, use multiple slides to obtain 25–100 mm^2 tissue.
	Insufficient amplifiable DNA was used.	FFPE DNA quality may vary due to tissue fixation methods, length of storage time, and storage conditions. Although the Qubit™ assay may detect the presence of DNA, the DNA may not be of sufficient quality to generate an AmpliSeq™ library. Re-prepare FFPE DNA from 100 mm^2 tissue section of 5–10 µm thickness. If necessary, use multiple slides to obtain 100 mm^2 tissue.
	Inhibitors are present in the tissue.	Inhibitors such as high melanin content can affect PCR, reduce the volume of input sample going into the target amplification reaction.
Qubit result indicates high yield (>10 ng/µL)	FFPE tissue has high DNA content.	Reduce the volume of input sample going into the Target Amplification reaction by one half to one quarter.
Qubit result indicates low yield (<0.5 ng/µL) Samples with low DNA yield may still be sufficient to generate an AmpliSeq™ library.	FFPE tissue has low DNA content.	Increase the number of Target Amplification cycles by 2 or 3.

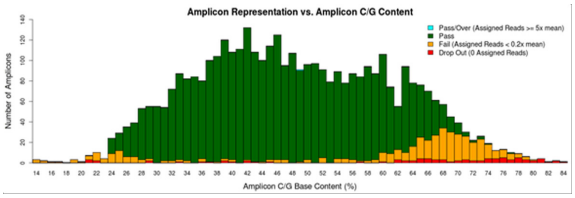
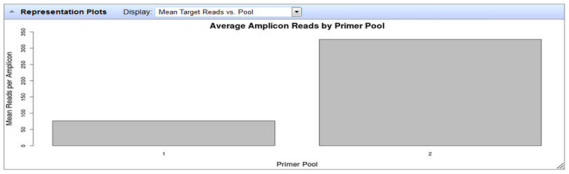
Library yield and quantification

Observation	Possible cause	Recommended action
Library yield is low—general (Library yield is NOT indicative of quality.)	Input DNA or RNA was mis-quantified.	Requantify input DNA using the TaqMan® RNase P Detection Reagents Kit; quantify RNA with Qubit™ 2.0 or 3.0 Fluorometer.
	Residual ethanol in sample DNA or RNA inhibited target amplification.	Carefully remove all drops, using an additional centrifugation and removal step, if necessary.
		Incubate uncapped tube in hood for 1 hour.
		Speed-vac tube at room temperature for 5 minutes.
	Less than expected input DNA/RNA was used.	Add more DNA/RNA or increase target amplification cycles.
	PCR, digestion, or ligation was inefficient.	Ensure proper dispensing and mixing of viscous components at each step.
	Library was 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.
	AMPure™ XP Beads were over-dried.	Do not dry the AMPure™ XP Beads more than 5 minutes.
	AMPure™ XP Beads inhibited library amplification.	Transfer library off of beads prior to amplification.
	qPCR cycling time is too short.	Use standard qPCR cycling for library designs >175 bp instead of Fast cycling.

Observation	Possible cause	Recommended action
Library yield with the Ion Library Equalizer™ Kit is less than expected	Quantity of library prior to equalization was inadequate.	Use the Ion Library Equalizer™ Kit only when library yield is consistently above the minimum expected concentration listed in this user guide. This can be assessed with qPCR, by removing 2 µL after library amplification.
	Equalizer™ Beads were not washed.	Be sure to wash Equalizer™ Beads before use.
	Wrong library amplification primers were used.	Use the Equalizer™ Primers provided in the Ion Library Equalizer™ Kit.
	Residual Equalizer™ Wash Buffer was present after wash.	Carefully remove all of the Equalizer™ Wash Buffer before elution.
Library yield is high	Input DNA or RNA was mis-quantified.	Requantify input DNA using the TaqMan® RNase P Detection Reagents Kit; quantify RNA with Qubit™ Fluorometer.
	More than 100 ng of input DNA/RNA was used.	Add less DNA/RNA, or decrease target amplification cycles.
Library yield is high as measured on the Agilent™ 2100 Bioanalyzer™ instrument	Markers are mis-assigned.	Ensure that markers are assigned correctly.
High molecular weight material is present on the Agilent™ 2100 Bioanalyzer™ instrument or library yield is high on the Qubit™ Fluorometer  **Example Agilent™ 2100 Bioanalyzer™ analysis** showing presence of high molecular weight material.	High molecular weight DNA was not removed during purification of the amplified library (does not interfere with sequencing).	Remove less supernatant in the first-round (0.5X) purification and be sure not to disturb bead pellet.
		Increase AMPure™ XP Reagent volume from 25 µL (0.5X) to 35 µL (0.7X) in the first-round purification.
	Inserts are concatamerizing during the ligation step.	Reduce nucleic acid input amount.
		Requantify sample(s) with Qubit™ 2.0 or Qubit™ 3.0 Fluorometer.
		Reduce target amplification cycle number.

Low amplicon uniformity (DNA only)

Observation	Possible cause	Recommended action
Short amplicons are under-represented	Purification was poor.	Vortex AMPure™ XP Reagent thoroughly before use, and be sure to dispense the full volume 100% ethanol is difficult to pipet accurately; it is essential to pre-wet pipette tips.
		In unamplified library purification, increase AMPure™ XP Reagent volume from 45 µL (1.5X) to 50 µL (1.7X).
		In amplified library purification, increase AMPure™ XP Reagent volume in second round from 60 µL (1.2X) to 70 µL (1.4X).
	Digested amplicons were denatured.	Use the 60°C/20 minute temperature incubation during the amplicon digestion step.
Long amplicons are under-represented	Samples are severely degraded.	Use an FFPE assay design for degraded or low quality samples.
	PCR was inefficient.	Double the anneal and extend time.
	Too few nucleotide flows were used.	Use an appropriate number of flows to sequence through amplicons.
AT-rich amplicons are under-represented  **Example of loss of AT-rich amplicons.** Within the Coverage Analysis Plugin, amplicon representation is plotted by GC content for an Ion AmpliSeq™ Panel. Amplicons with 23-50% GC show an excess failure rate (less than 20% of the mean read depth).	Target amplification was inefficient.	Double the anneal/extend time in the target amplification reaction.
		Decrease the anneal/extend temperature of the target amplification reaction from 60°C to 58°C.
	Digested amplicons were denatured.	Use the 60°C/20 minute temperature incubation during the amplicon digestion step.
	Digestion was inefficient.	Increase amplicon digestion times to 20 minutes for each step.

Observation	Possible cause	Recommended action
GC-rich amplicons are under-represented  **Example of loss of GC-rich amplicons.** Within the Coverage Analysis Plugin, amplicon representation is plotted by GC content for an Ion AmpliSeq™ Panel. Amplicons with 60-80% GC show an excess failure rate (less than 20% of the mean read depth).	Denaturation was inadequate during target amplification.	Use a calibrated thermal cycler.
	Target amplification was inefficient.	Increase the anneal/extend temperature of the target amplification reaction from 60°C to 62°C for the first two cycles of the target amplification reaction
	Library amplification was inefficient.	Do not amplify the library (not required for qPCR quantification).
Pool representation has low or uneven uniformity  **Example of pool imbalance.** Within the Coverage Analysis Plugin, mean read depth per primer pool is plotted for a two-pool Ion AmpliSeq™ Panel. In this example, Primer Pool 1 has approximately one quarter the reads of Primer Pool 2.	Amount of Direct FFPE DNA sample in target amplification reactions was variable.	Perform thorough mixing of the sample in Direct Reagent before removing an aliquot for target amplification and before splitting the sample master mix between wells.
	Amount of DNA in target amplification reactions varied.	Make a master mix for each sample DNA.
	Pipetting is inaccurate when pools are combined after target amplification.	Centrifuge the plate after target amplification. Make sure that all the volume of each pool is removed and combined into a single pool.
Uniformity is low (without bias)	Amplification was inadequate.	Double the recommended anneal/extend time for target amplification.

Other

Observation	Possible cause	Recommended action
Adapter dimers are present on the Agilent™ 2100 Bioanalyzer™ instrument at 90–105 bp or Adapter dimers are present during sequencing	Purification was inefficient.	In unamplified library purification, decrease AMPure™ XP Reagent volume from 45 µL (1.5X) to 30 µL (1X).
		In amplified library purification, decrease AMPure™ XP Reagent volume in second round from 60 µL (1.2X) to 50 µL (1.0X).
	Adapter dimers formed during reaction setup or during digestion.	Do not combine Adapters, DNA Ligase, and Switch Solution prior to addition.
		Use a 65°C temperature incubation instead of 60°C during the amplicon digestion step.
	Adapter concentration was too high.	Ensure that barcode adapters are diluted properly.
The number of on-target reads is lower than expected	Unknown.	Increase the number of target amplification cycles by two, or increase the anneal/extend temperature of the target amplification reaction from 60°C to 62°C for the first two cycles of the target amplification reaction.
	Sample ID Panel targets are counted as off-target reads.	Add back the on-target reads from the Sample ID Panel.
Barcode representation is uneven (Equalizer™ kit not used)	Library quantification was inaccurate.	Use the Ion Library TaqMan® Quantitation Kit for the most specific and accurate library quantification.
	Library combination was inaccurate.	Dilute libraries to 100 pM, then combine equal volumes.
Barcode representation is uneven (Ion Library Equalizer™ Kit used)	Yield of library amplification was inadequate.	When trying the Ion Library Equalizer™ Kit for the first time, quantify with qPCR to ensure libraries are >4 nM. If not the first time, increase input nucleic acid or target amplification cycles.

Observation	Possible cause	Recommended action
Percentage of polyclonal ISPs is high (>40%)	Library input was too high.	Decrease amount of library added to the template preparation reaction by 50%.
	Library was mis-quantified.	Ensure that library was quantified accurately.
	Other.	Check the appropriate template preparation user guide for more information.
Low quality ISPs are present at high percentage (> -15%)	Library input was too low.	Double the volume of library used in template preparation.
		Use a fresh dilution of library prepared in a low-bind tube.
	Other.	Check the appropriate template preparation user guide for more information.

B

Strategies for combining Ion AmpliSeq™ libraries

This section describes various strategies for combining Ion AmpliSeq™ libraries with unique barcodes for sequencing together on a chip. See "Ion Chip capacities for Ion AmpliSeq™ DNA libraries sequenced at equal depths" on page 60, and "Ion Chip capacities for Ion AmpliSeq™ RNA libraries" on page 61 for more information on the numbers of libraries that can be combined on a single chip.

Combine libraries prepared with one panel for equal depth of coverage

You can prepare barcoded libraries from different samples using IonCode™ or Ion Xpress™ Barcode Adapters. Multiple uniquely barcoded libraries can be combined during the Equalizer™ process, or after diluting each individual library to a 100-pM concentration.

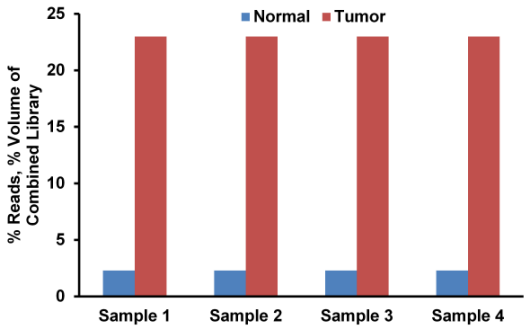
For example, if 16 libraries prepared with the same Ion AmpliSeq™ DNA or RNA panel is combined in a single templating and sequencing reaction:

1. Dilute all individual libraries to 100-pM concentration.
2. Combine 10 µL of each of the 16 libraries in a single tube.
3. Mix the combined libraries and proceed to templating and sequencing.

Combine libraries prepared from one panel to vary depth of coverage

When Ion AmpliSeq™ libraries with unique barcodes have been diluted to 100 pM each, unequal volumes of libraries can be combined to produce disproportionate numbers of reads for each barcode.

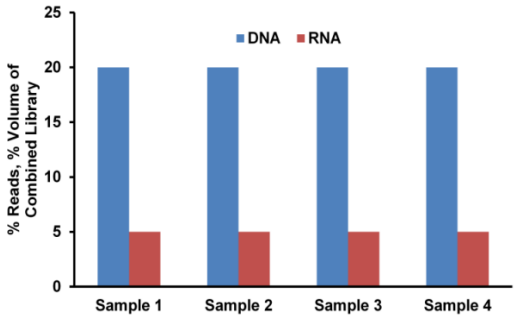
For example, when comparing tumor and normal sample pairs with the same panel, an average depth of coverage at ~250X may be preferred to type the germline single nucleotide polymorphisms (SNPs) in the normal sample, while an average depth of coverage at ~2,500X may be preferred to type somatic mutations in the tumor sample. In this case, barcoded tumor and normal libraries can be diluted and combined at a 1:10 (normal:tumor) ratio. If the chip capacity is sufficient, multiple tumor/normal sample-pair libraries can be combined in a single chip, as described in the following table:

Sample	Barcode	Fractional volume/ reads	
Sample 1 Normal	BC_0101	0.023	
Sample 1 Tumor	BC_0102	0.23	
Sample 2 Normal	BC_0103	0.023	
Sample 2 Tumor	BC_0104	0.23	
Sample 3 Normal	BC_0105	0.023	
Sample 3 Tumor	BC_0106	0.23	
Sample 4 Normal	BC_0107	0.023	
Sample 4 Tumor	BC_0108	0.23	
Sum	—	**1.0**	

Combine DNA and RNA libraries to obtain different numbers of reads

When Ion AmpliSeq™ libraries with unique barcodes prepared from DNA and RNA from multiple samples have been diluted to 100 pM each, unequal volumes of libraries can be combined to produce disproportionate numbers of reads. Use this strategy for combining DNA and RNA libraries prepared from the same sample to adjust the number of reads as desired.

For example, when comparing libraries generated from genomic DNA and RNA, one million reads may be desired for the DNA sample, while only 250,000 reads may be preferred to evaluate RNA fusions in the same tissue sample. In this case, barcoded DNA and RNA libraries can be diluted and combined at an 80:20 (DNA:RNA) ratio. If the chip capacity is sufficient, multiple DNA/RNA sample-pair libraries can be combined in a single chip, as described in the following table:

Sample	Barcode	Fractional volume/ reads	
Sample 1 DNA	BC_0101	0.2	
Sample 1 RNA	BC_0102	0.05	
Sample 2 DNA	BC_0103	0.2	
Sample 2 RNA	BC_0104	0.05	
Sample 3 DNA	BC_0105	0.2	
Sample 3 RNA	BC_0106	0.05	
Sample 4 DNA	BC_0107	0.2	
Sample 4 RNA	BC_0108	0.05	
Sum	—	**1.0**	

Combine libraries prepared using different panels for equal coverage

Use this method to combine libraries from different panels to be run on a single chip and sequenced at approximately the same depth. To prepare 100 µL of combined library from libraries prepared from two different panels, dilute the libraries to ~100 pM each and combine them using the following formula:

- Volume (µL) of library from panel 1 =
  100 µL × (number of primer pairs in panel 1/total number of primer pairs in panels 1 and 2)
- Volume (µL) of library from panel 2 =
  100 µL × (number of primer pairs in panel 2/total number of primer pairs in panels 1 and 2)

Example:

Number of primer pairs in panel 1 = 207

Number of primer pairs in panel 2 = 20

Volume of library from panel 1 = 100 µL × (207/227) = 91 µL

Volume of library from panel 2 = 100 µL × (20/227) = 9 µL

Total volume of combined libraries = 100 µL

Ion Chip capacities for Ion AmpliSeq™ DNA libraries sequenced at equal depths

As described in the previous section, combined libraries can be generated from multiple primer pools and the same sample, or from multiple samples when barcoded adapters are used. The number of combined libraries that can be accommodated in a single sequencing run depends on the size of the chip, the balance of barcoded library concentration, and the coverage required.

For a given chip, as the number of amplicons increases, the number of libraries that can be accommodated per sequencing run decreases. This relationship is shown in the following table. The numbers in the following table serve as a guide for approximate capacities. We suggest initially combining fewer libraries and determining real limits empirically.

Multiple chips can be run to increase coverage depth. Run data can be combined by using the CombineAlignments plugin. For detailed instructions on running the CombineAlignments plugin, see "Run the Installed Plugins" in the latest version of the *Torrent Suite™ Analysis Report Guide*.

	Ion 314™ Chip		Ion 316™ Chip		Ion 318™ Chip/ Ion 520™ Chip		Ion 530™ Chip		Ion PI™ Chip/ Ion 540™ Chip	
Coverage depth for a genomic DNA library with:	**>95% of bases at 30X**	**>95% of bases at 500X**	**>95% of bases at 30X**	**>95% of bases at 500X**	**>95% of bases at 30X**	**>95% of bases at 500X**	**>95% of bases at 30X**	**>95% of bases at 500X**	**>95% of bases at 30X**	**>95% of bases at 500X**
No. of amplicons per library	**Approximate number of libraries per chip OR (chips per library)[1]**									
12–24	128	8	>384	32	>384	64	>384	192	>384	>384
25–48	64	4	256	16	>384	32	>384	96	>384	320
49–96	32	2	128	8	256	16	>384	48	>384	160
97–192	16	1	64	4	128	8	384	24	>384	80
193–384	8	(2)	32	2	64	4	192	12	>384	40
385–768	4	—	16	1	32	2	96	6	320	20
769–1,536	2	—	8	(2)	16	1	48	3	160	10
1,537–3,072	1	—	4	—	8	(2)	24	1	80	5
3,073–6,144	(2)	—	2	—	4	—	12	(2)	40	2
6,145–12,288	—	—	1	—	2	—	6	—	20	1
12,289–24,576	—	—	(2)	—	1	—	3	—	10	(2)
24,577–49,152	—	—	—	—	(2)	—	1	—	5	—

[1] Values in parentheses indicate multiple chips per library. (2) = 1 library per 2 chips.

Ion Chip capacities for Ion AmpliSeq™ RNA libraries

We recommend that you plan for an average of 5,000 reads per amplicon for an Ion AmpliSeq™ RNA library targeting from 1–200 genes. The actual sequencing depth required depends on the expression levels of the gene targets in your sample RNA, so scale the sequencing depth up and down if needed to accommodate your sample type and research needs. For panels containing fusion detection assays, higher library multiplexing is possible as most targets will not be present and therefore will not create library molecules.

Use the following formula and the chip capacity following table to provide initial guidance on the capacity to multiplex RNA derived gene expression libraries on Ion Torrent™ sequencing chips.

$$\text{Number of libraries sequenced per chip} = \frac{\text{Chip capacity in reads}}{\text{(Sequencing depth) x (number of amplicons targeted)}}$$

	Ion Chip				
	Ion 314™ Chip	**Ion 316™ Chip**	**Ion 318™/ Ion 520™ Chip**	**Ion 530™ Chip**	**Ion PI™/ Ion 540™ Chip**
Chip capacity in reads (M)	0.3–0.5	1–2	3–5	15–20	60–80

Example:

Chip capacity of Ion 540™ Chip = 60,000,000 reads

Sequencing depth = 5000 reads per amplicon

Number of amplicons targeted = 100

60,000,000 / (5,000 × 100) = 120 libraries that can be sequenced per Ion 540™ Chip

These recommendations serve as suggestions only and the actual capacity to multiplex libraries is determined by the expression levels of the genes included in your Ion AmpliSeq™ RNA panel. The expression levels of the individual genes can vary by input RNA type as well. We suggest using the formula for new panels and determining actual multiplexing limits empirically.

You can choose to sequence libraries from panels with large numbers of amplicons at lower sequencing depth. We recommend sequencing 8–12 libraries prepared with the Ion AmpliSeq™ Transcriptome Human Gene Expression panel per Ion PI™ or Ion 540™ Chip. Most samples express 12,000–16,000 genes from this panel of 20,802 amplicons.

C

Ion AmpliSeq™ Custom Panels

Each Ion AmpliSeq™ Custom Panel order includes primer pools at the standard 2X or 5X concentration, in some cases, 384-well plates containing all the individual primer pairs. Each plate well contains the forward and reverse primer in Low TE at a concentration of 307 µM per primer. You can use the plates to remake the entire panel, a smaller panel containing a subset of primer pairs, to combine two panels, or to make a 50X companion panel to spike into other panels. Primer pairs from different panels can be combined as long as the amplicons within a pool do not overlap. When a panel with a new footprint is created, the designed BED file should be modified accordingly.

Prepare primer pools from plates

To create a primer pool from 384-well plates, combine and dilute the desired primer pairs to the appropriate concentration:

- For panels with up to 96 primer pairs per pool, combine and dilute to 1000 nM (5X, 1000 nM).
- For panels with 97–1,228 primer pairs per pool, combine and dilute to 250 nM (5X, 250 nM).
- For panels with 1,229–3,072 primer pairs per pool, combine and dilute to 100 nM (2X, 100 nM).

For example, to create a primer pool with 2 µL of each primer pair:

1. Vortex the primer plate, then centrifuge.
2. Remove the plate seal and transfer 2 µL of each desired primer pair into a tube.

3. Add Low TE (10 mM Tris, pH 8.0; 0.1 mM EDTA) to the tube to the appropriate final volume shown in the following table:

To make primer pools...	Action
5X, 1000 nM (12–96 primer pairs)	Add Low TE to a final volume of 0.614 mL[1]
5X, 250 nM (97–1,228 primer pairs)	Add Low TE to a final volume of 2.456 mL [2]
2X, 100 nM (1,229–3,072 primer pairs)	Add Low TE to a final volume of 6.140 mL
50X companion panel, 2500 nM (1–123 primer pairs)	Add Low TE to a final volume of 0.246 mL

[1] Can be prepared at 2X, 400 nM by increasing the final volume to 1.535 mL.
[2] Can be prepared at 2X, 100 nM by increasing the final volume to 6.140 mL.

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

Expand a panel by adding a companion panel

Any Ion AmpliSeq™ panel can be modified through the use of a companion panel. Companion panels enable addition of new content, positive control assays, and improvements in uniformity (for example by adding the same primer pair to two or more primer pools).

1. Prepare a companion panel as described above, then use it to spike another panel before use:

If a panel is:	Action
5X, 250 nM	Add 150 µL of 50X, 2500 nM companion panel to 1.5 mL of 5X primer pool
2X, 100 nM	Add 60 µL of 50X, 2500 nM companion panel to 1.5 mL of 2X primer pool

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

D Ion AmpliSeq™ Sample ID Panel

Using the Sample ID Panel

1. If you are using Ready-to-use, Custom, or Community Panel in a manual Ion AmpliSeq™ protocol, create a sample signature by adding 1 µL of the Ion AmpliSeq™ Sample ID Panel to the target amplification reaction.

   Note: The Ion AmpliSeq™ Sample ID Panel can be used to match a tumor and normal sample. However, copy number variations in the tumor sample can distort the allele balance in the fingerprint.

   Note: The Ion AmpliSeq™ Sample ID Panel can be added to custom panels at the design stage at **www.ampliseq.com**.

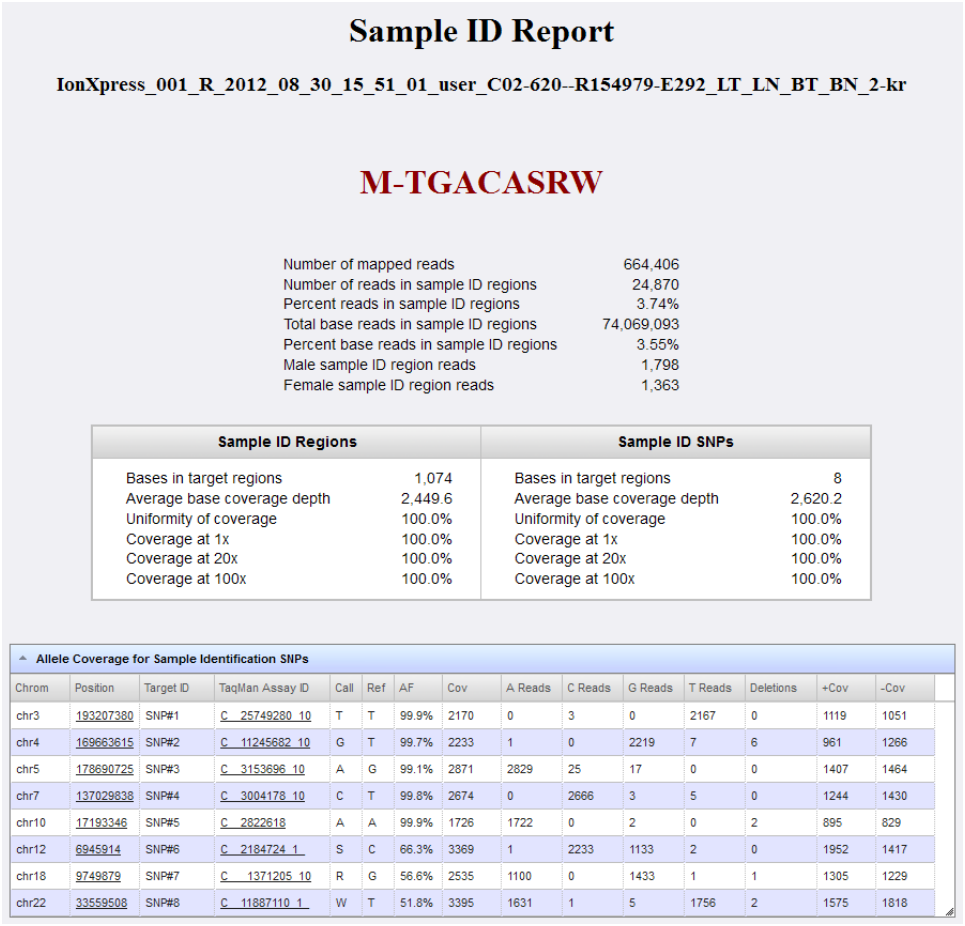
2. Select the following settings when creating a new Planned Run in the Torrent Browser Planned Run Wizard:
   a. In the **Kits** screen, select the **Control Sequence**, then choose **Ion AmpliSeq Sample ID panel** from the dropdown menu.

   b. In the **Plugins** screen, select the **coverageAnalysis** plugin checkbox, then click **Configure** (see "Configure and run the Torrent Coverage Analysis Plugin" on page 69). In the configuration dialog, select the **Sample Tracking** checkbox. This enables the analysis to produce a statistic for reads mapped to Sample ID targets so that the level of off-target reads is accurately represented in the Coverage Analysis Report.

      Note: If **Sample Tracking** is not selected, Sample ID reads are counted as off-target reads.

   c. In the **Plugins** screen, select the **sampleID plugin**. A Sample ID Report is then automatically generated after the run.

3. Following sequencing, select the **Data** tab in the Torrent Browser, then select **Completed Runs and Results**. Open the report for the run, then scroll down to the Plugin Summary section to find the sampleID plugin results.



Sample ID Report

IonXpress_001_R_2012_08_30_15_51_01_user_C02-620--R154979-E292_LT_LN_BT_BN_2-kr

M-TGACASRW

Number of mapped reads	664,406
Number of reads in sample ID regions	24,870
Percent reads in sample ID regions	3.74%
Total base reads in sample ID regions	74,069,093
Percent base reads in sample ID regions	3.55%
Male sample ID region reads	1,798
Female sample ID region reads	1,363

Sample ID Regions		Sample ID SNPs	
Bases in target regions	1,074	Bases in target regions	8
Average base coverage depth	2,449.6	Average base coverage depth	2,620.2
Uniformity of coverage	100.0%	Uniformity of coverage	100.0%
Coverage at 1x	100.0%	Coverage at 1x	100.0%
Coverage at 20x	100.0%	Coverage at 20x	100.0%
Coverage at 100x	100.0%	Coverage at 100x	100.0%

Allele Coverage for Sample Identification SNPs

Chrom	Position	Target ID	TaqMan Assay ID	Call	Ref	AF	Cov	A Reads	C Reads	G Reads	T Reads	Deletions	+Cov	-Cov
chr3	193207380	SNP#1	C__25749280_10	T	T	99.9%	2170	0	3	0	2167	0	1119	1051
chr4	169663615	SNP#2	C__11245682_10	G	T	99.7%	2233	1	0	2219	7	6	961	1266
chr5	178690725	SNP#3	C__3153696_10	A	G	99.1%	2871	2829	25	17	0	0	1407	1464
chr7	137029838	SNP#4	C__3004178_10	C	T	99.8%	2674	0	2666	3	5	0	1244	1430
chr10	17193346	SNP#5	C__2822618	A	A	99.9%	1726	1722	0	2	0	2	895	829
chr12	6945914	SNP#6	C__2184724_1	S	C	66.3%	3369	1	2233	1133	2	0	1952	1417
chr18	9749879	SNP#7	C__1371205_10	R	G	56.6%	2535	1100	0	1433	1	1	1305	1229
chr22	33559508	SNP#8	C__11887110_1	W	T	51.8%	3395	1631	1	5	1756	2	1575	1818

Data analysis

Getting started

Complete the steps summarized on this page, and described in more detail on the following pages, to enable variant calling analysis of your sequencing data.

Access Torrent Suite™ documentation

Visit the Ion Community at **ioncommunity.thermofisher.com** and select **Products ▸ Ion Software ▸ Torrent Suite** to access the latest user guides and information for Torrent Suite™ software.

Enable variant calling in Torrent Suite™ Software

Torrent Suite™ Software is required for sequence analysis of libraries prepared with Ion AmpliSeq™ primer panels. The software includes the Torrent Variant Caller Plugin, which can be used to call single nucleotide polymorphism (SNP) and insertion/ deletion (InDel) variants within the genomic regions covered by the Ion AmpliSeq™ panels. To enable variant calling:

1. Install the hg19 human genome reference on your Torrent Server, if it is not already installed.
2. Import the Browser Extensible Data (BED) files for the Ion AmpliSeq™ panel target regions to your Torrent Server, or use the analysis plan available at **www.ampliseq.com**.

Enable analysis of Ion AmpliSeq™ RNA libraries

Follow these steps to enable analysis of Ion AmpliSeq™ RNA libraries:

1. Upload the BED files for the target regions to your Torrent Server.
2. Install the Human Canonical Transcripts mapping reference, hg19 AmpliSeq Transcriptome ERCC v1.
3. In the Torrent Browser Planned Run Wizard, select and configure the Torrent ampliSeqRNA Plugin, which is recommended for analysis of sequence data from libraries generated from Ion AmpliSeq™ RNA Custom and Ready-to-use Panels.

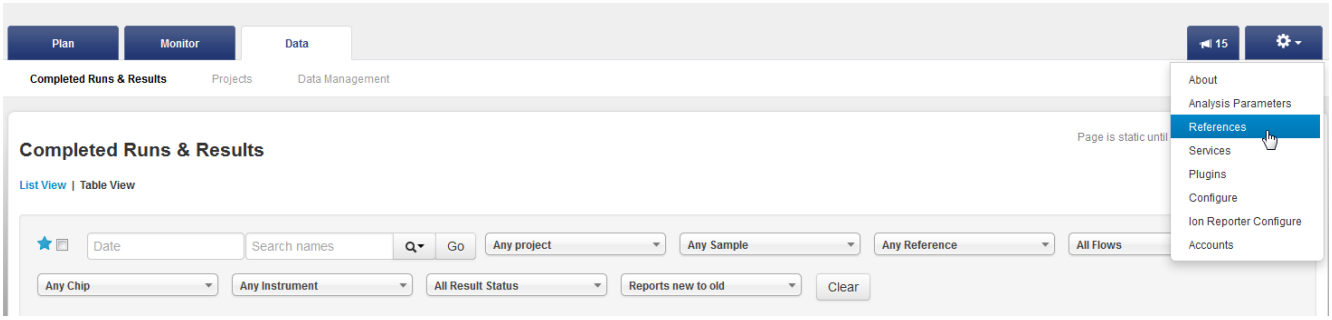
   Note: The Torrent Coverage Analysis Plugin can also be used to analyze results from Ion AmpliSeq™ RNA libraries for (unfiltered) target coverage statistics.

   Note: Go to the **Torrent Browser Plugin Store** on Ion Community to download plugins not installed on your Torrent Server.

Install the hg19 reference

Brief instructions for installing the hg19 reference on your Torrent Server are provided below. Detailed instructions are provided in the *Torrent Suite™ Software User Interface Guide*.

1. Download the FASTA file for the Ion AmpliSeq™ Primer Pool target regions from this link: **hg19.zip**. Download the FASTA file for the hg19_AmpliSeq Transcriptome ERCC v1 file from this link: **hg19_ampliseq_transcriptome_ercc_v1.zip**.
2. In the Torrent Browser, click the Settings button on the right side of the screen and select **References** from the pull down menu.



3. Click **Import Preloaded Ion References**, then click **Import** to the right of the reference file of interest.

   Note: The genome index creation will take a few hours; the transcript index creation will take a few minutes.

Import BED files into your Torrent Server

BED files supply chromosome regions and restrict analysis to the regions in the designated Ion AmpliSeq™ panel.

Two types of BED files are frequently used:

- **Designed BED file—** Specifies the amplified regions that are used with targeted sequencing. It is also known as a Target Regions file.
- **Hotspot BED file—** Specifies regions of known mutations, for example from COSMIC or dbSNP databases or from customer-defined regions. A hotspot file is optional. Positions in a hotspot file will always be present in the VCF file and in the variant table in Torrent Suite™ and Ion Reporter™ software. They may have a variant present, or they may be reference, or no-call. All alleles present at this position, with the number of reads of each allele, will always be shown at Hotspot positions. In addition, Hotspots can be called with different thresholds (for example, lower allele frequency). It is also known as a Hotspots Regions file.

Download the BED file for the target regions of each Ready-to-use Panel from the panel's product page at **www.thermofisher.com** or from the Ion AmpliSeq™ Designer site at **www.ampliseq.com**.

Upload the files to your Torrent Server by following instructions for BED file upload in the latest Torrent Suite™ software documentation, available at **ioncommunity.thermofisher.com**.

Torrent Coverage Analysis Plugin

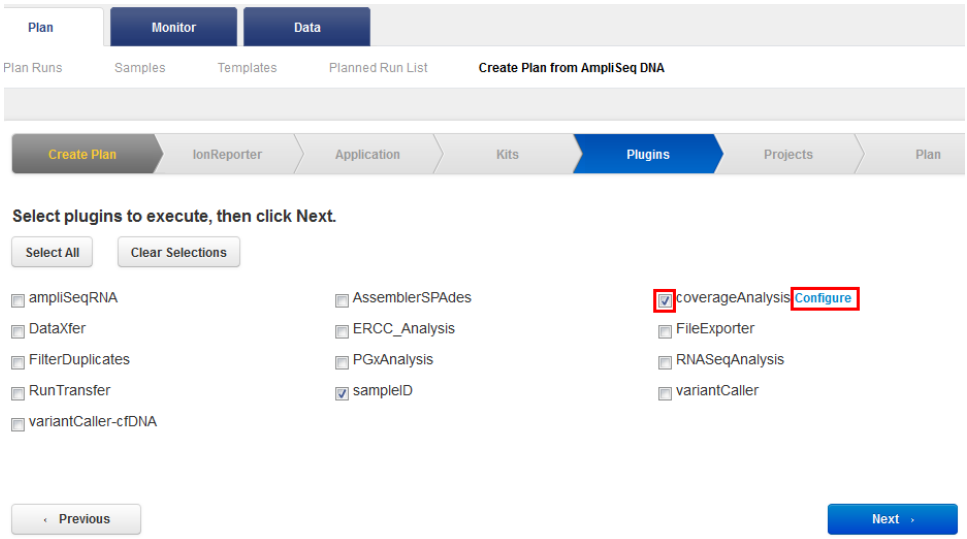
The Torrent Coverage Analysis Plugin provides statistics and graphs describing the level of sequence coverage produced for targeted genomic regions.

Configure and run the Torrent Coverage Analysis Plugin

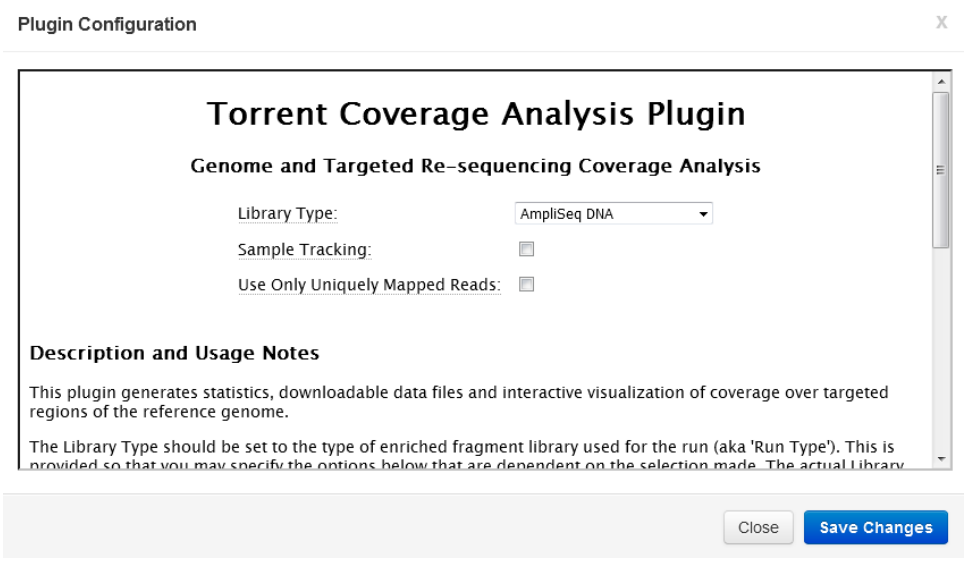
There are two ways to run the Torrent Coverage Analysis Plugin:

- Automatically, by selecting and configuring the plugin during run planning or
- Manually, allowing you to run the plugin at any time from a completed run report.

1. To run the Coverage Analysis Plugin automatically during run analysis, select the **coverageAnalysis** checkbox in the **Plugins** chevron in the template or Planned Run Wizard. After selecting the checkbox, a **Configure** link appears:



2. Click **Configure** to open the Torrent Coverage Analysis Plugin configuration dialog:



3. Select the settings appropriate to your run, then click **Save Changes**.

Coverage Analysis Report

The run report will include basic run metrics, but more detail can be found by clicking on the **coverageAnalysis.html** link, including options to download a barcode summary report, and an amplicon coverage matrix.

Coverage Analysis Report

R_1970_02_24_15_47_34_user_C41-52-BRCA

Barcode Summary

Library type: AmpliSeq DNA
Target regions: BRCA

Barcode Name	Sample	Mapped Reads	On Target	Mean Depth	Uniformity
IonCode_0101	Colon 113408A2	436,853	96.51%	1,863	99.72%
IonCode_0102	Colon 120777A2	360,847	96.52%	1,516	99.27%
IonCode_0103	Colon 1187375B	386,278	95.85%	1,696	99.78%
IonCode_0104	Colon 1185856B	378,090	95.94%	1,650	100.00%
IonCode_0105	Colon 1212186B	147,593	96.15%	628.2	95.11%
IonCode_0106	Colon 1196055B	401,636	96.23%	1,762	100.00%
IonCode_0107	Colon 406489B2	360,969	96.40%	1,571	100.00%
IonCode_0108	Colon 405312B2	81,324	91.65%	308.4	85.25%

1 | 20 items per page | 1 - 8 of 8 items

- Download Barcode Summary Report
- Download barcode/amplicon coverage matrix
- Download a ZIP report summary.

More detailed information can be obtained for each barcoded library by clicking the Barcode ID.

Coverage Analysis Report

Sample Name: Colon 113408A2

IonCode_0101_R_1970_02_24_15_47_34_user_C41-52-BRCA

Library type: AmpliSeq DNA
Reference: hg19 (DNA)
Target regions: BRCA_v10_NoSampleID_20160201

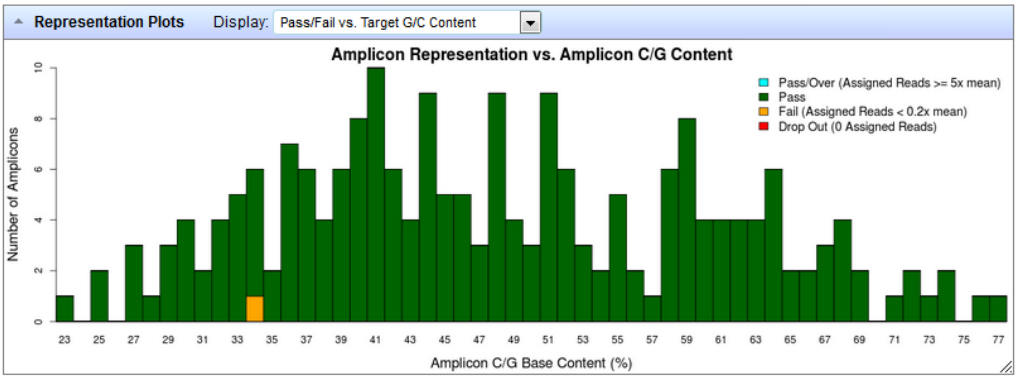
Number of mapped reads	436,853
Percent reads on target	96.51%
Average base coverage depth	1,863
Uniformity of base coverage	99.72%

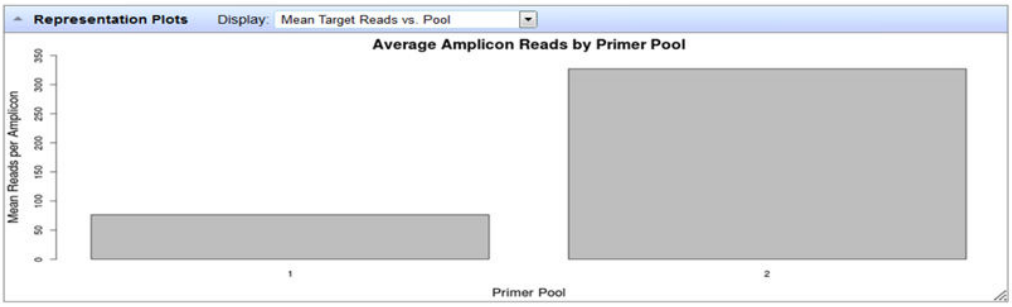
Amplicon Read Coverage		Target Base Coverage	
Number of amplicons	265	Bases in target regions	22,404
Percent assigned amplicon reads	96.51%	Percent base reads on target	94.67%
Average reads per amplicon	1,591	Average base coverage depth	1,863
Uniformity of amplicon coverage	99.62%	Uniformity of base coverage	99.72%
Amplicons with at least 1 read	100.00%	Target base coverage at 1x	100.00%
Amplicons with at least 20 reads	100.00%	Target base coverage at 20x	100.00%
Amplicons with at least 100 reads	100.00%	Target base coverage at 100x	100.00%
Amplicons with at least 500 reads	99.25%	Target base coverage at 500x	99.26%
Amplicons with no strand bias	99.25%	Target bases with no strand bias	99.02%
Amplicons reading end-to-end	97.36%	Percent end-to-end reads	91.28%

At the top of the Coverage Analysis Report you will find a table that shows amplicon statistics on the left side and base statistics on the right side. All metrics are defined with popup explanations. The plugin run options can be reviewed as popup help on the report title "Coverage Analysis Report."

The Representation Plots (which are revealed by clicking on the triangle in the blue bar) allow you to visualize amplicon representation by GC content and length. If the designed BED file defines multiple primer pools, a mean target depth per primer pool representation plot is also available.

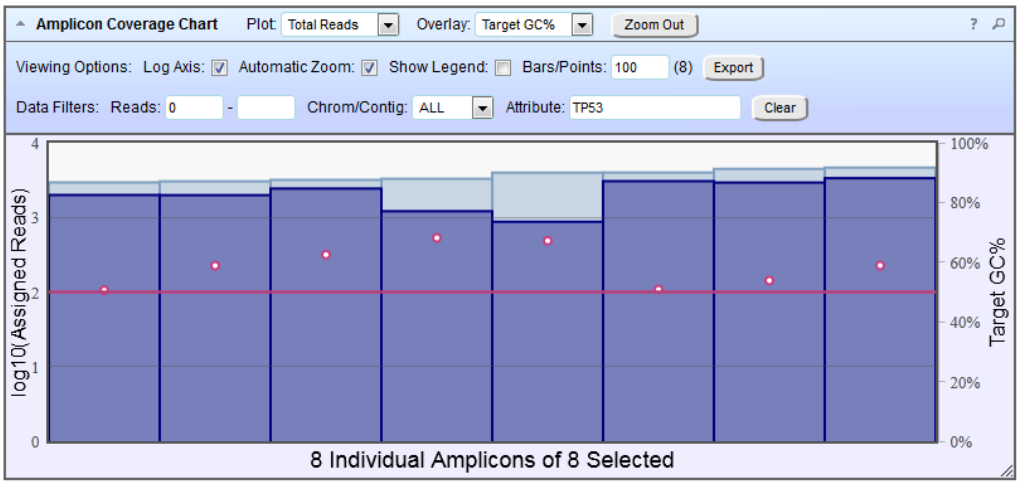
In the example below, pool 1 is under-represented:





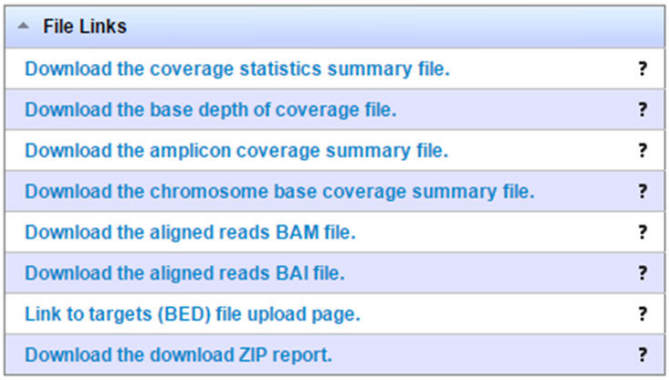
The Amplicon Coverage Chart shows amplicons binned by representation, low to high, with a variety of overlays. You can zoom into this graph to see how many amplicons are in each bin, and click on a bar to get more information about that group of amplicons.

By clicking on the magnifying glass in the upper right corner of the Amplicon Coverage Chart, you can change and filter what is shown on the chart by number of reads, chromosome, or gene.



The Reference Coverage Chart shows the strand-specific coverage in red and green for each amplicon. The example below shows the coverage for amplicons in the *TP53* gene:

Finally, at the bottom of the Coverage Analysis Report you will find links to download the data in this report as well as the BAM and BAI files required for IGV. Read numbers reported in the amplicon coverage summary file can be used to estimate RNA expression levels in RNA Ion AmpliSeq™ libraries.



The following figure shows an example RNA Coverage Analysis Report:

Coverage Analysis Report

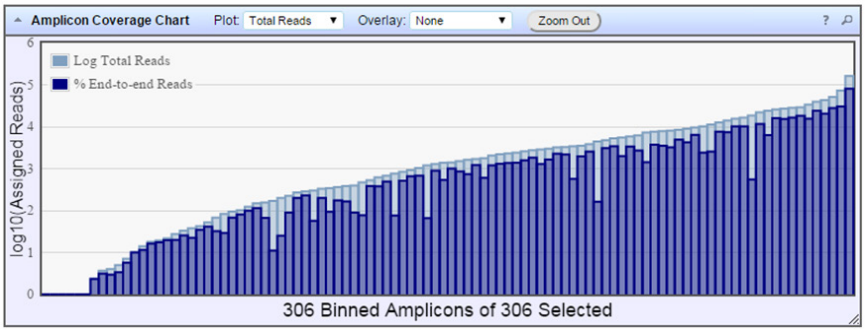
Sample Name: UHR_21cycles

IonCode_0117_R_2016_03_29_11_29_03_user_Megalosaurus-68

Library type: AmpliSeq RNA
Reference: hg19_rna_ImmuneResponsePanelv2 (RNA)
Target regions: IOpanel2_2016_01.05

Amplicon Read Coverage

Number of amplicons	306
Amplicons with at least 1 read	287
Amplicons with at least 10 reads	270
Amplicons with at least 100 reads	230
Amplicons with at least 1000 reads	165
Amplicons with at least 10K reads	57
Amplicons with at least 100K reads	2
Amplicons with no strand bias	304
Amplicons reading end-to-end	263



Torrent Variant Caller Plugin

For detailed instructions on running the Torrent Variant Caller, refer to "Run the Torrent Variant Caller Plugin" in the *Torrent Suite™ Analysis Report Guide*. The Torrent Variant Caller (TVC) Plugin calls single-nucleotide polymorphisms (SNPs), multinucleotide polymorphisms (MNPs), insertions, and deletions in a sample across a reference or within a targeted subset of that reference.

This plugin provides optimized pre-set parameters for many experiment types, but is also very customizable. After you find a parameter combination that works well on your data and that has the balance of specificity and sensitivity that you want, you can save that parameter set and reuse it in your research. This is supported on both manual launches of the plugin and in automatic launches through the run plan template wizard.

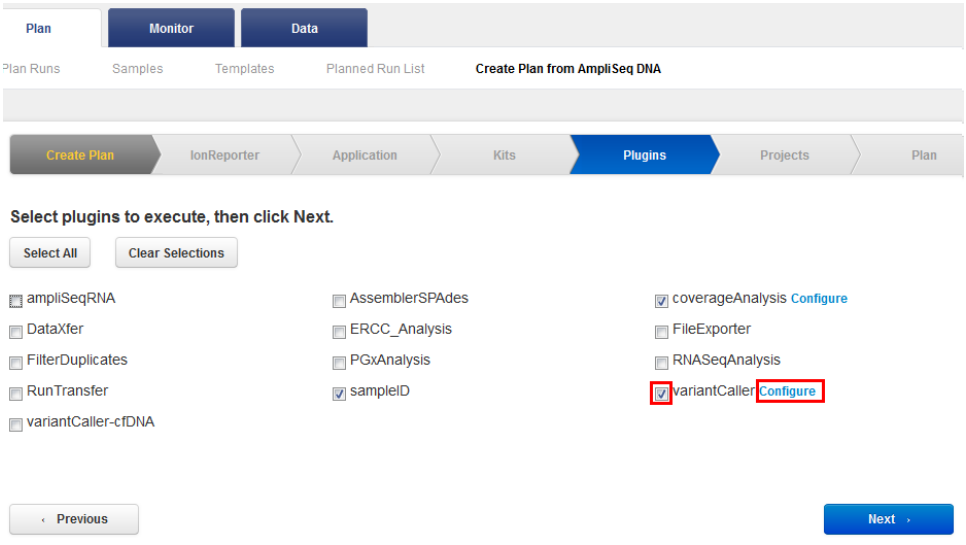
Note: The TVC plugin run uses the same target regions file and hotspots file as the main Torrent Suite™ software analysis (if those files are present in the main analysis). Through the run plan wizard there is no facility in the TVC configuration to change the target regions file or hotspots file. You can use a different target regions file and hotspots file with a manual TVC launch from a completed run report.

Configure and run the Torrent Variant Caller Plugin

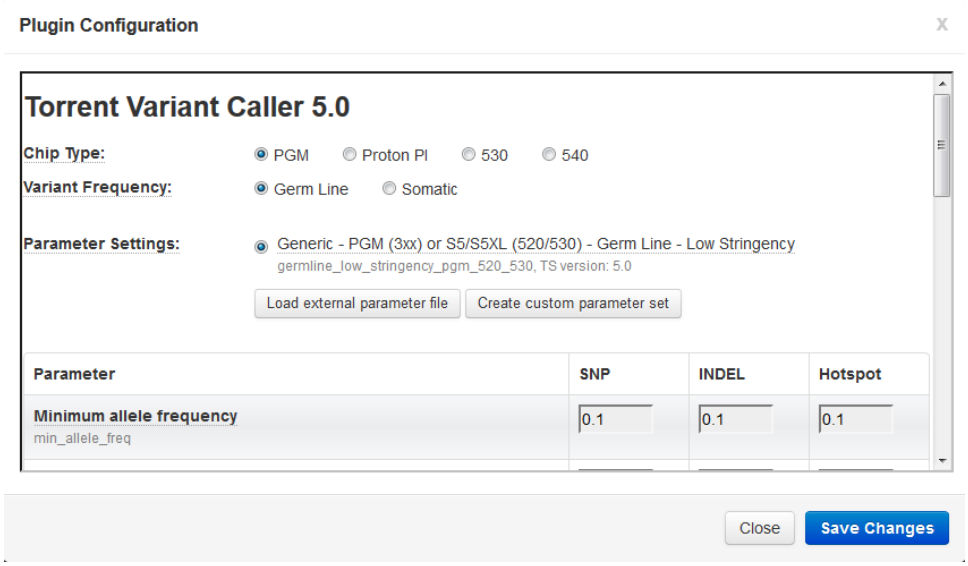
There are two ways to run the TVC Plugin:

- Automatically, by selecting and configuring the plugin during run planning or
- Manually, allowing you to run the plugin at any time from a completed run report.

1. To run the TVC plugin automatically during run analysis, select the **variantCaller** checkbox in the **Plugins** chevron in the template or Create Plan Wizard. After selecting the checkbox, a **Configure** link appears:



2. Click **Configure** to open the Torrent Variant Caller configuration dialog:



3. Select one of the pre-defined parameter sets, create a custom parameter set, or load an external parameter file optimized for your Ready-to-use or community Ion AmpliSeq™ panel, which can be downloaded from the panel page on **www.ampliseq.com**. Click **Save Changes**.

   TVC's default parameter setting groups are organized according to these attributes:

   - **Variant frequency—** Somatic settings are optimized to detect low frequency variants. Germ-line settings are optimized for high frequency settings.
   - **Sequencing instrument—** The Ion PGM™, Ion Proton™, Ion S5™, or Ion S5™ XL sequencers. Parameter defaults are different for the different sequencing platforms.

Torrent ampliSeqRNA Plugin

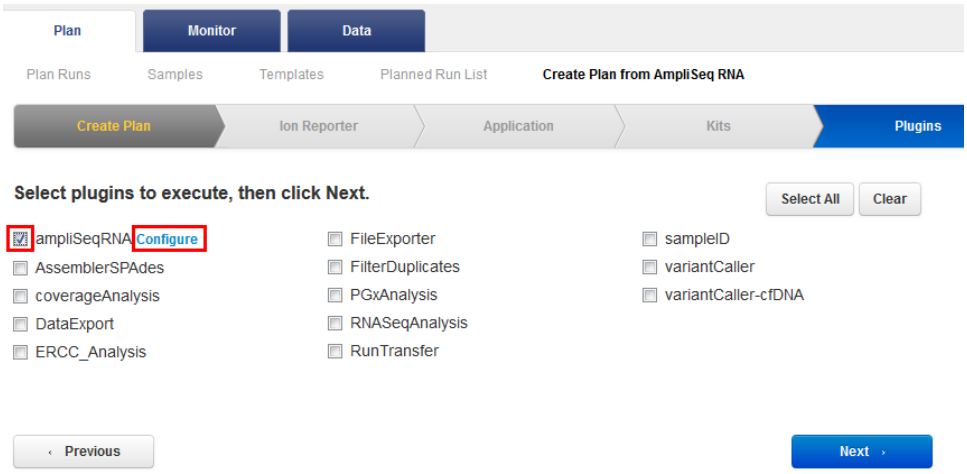
The Torrent ampliSeqRNA Plugin generates statistics, downloadable data files, and interactive visualization of targeted RNA amplicons.

Configure and run the Torrent ampliSeqRNA Plugin

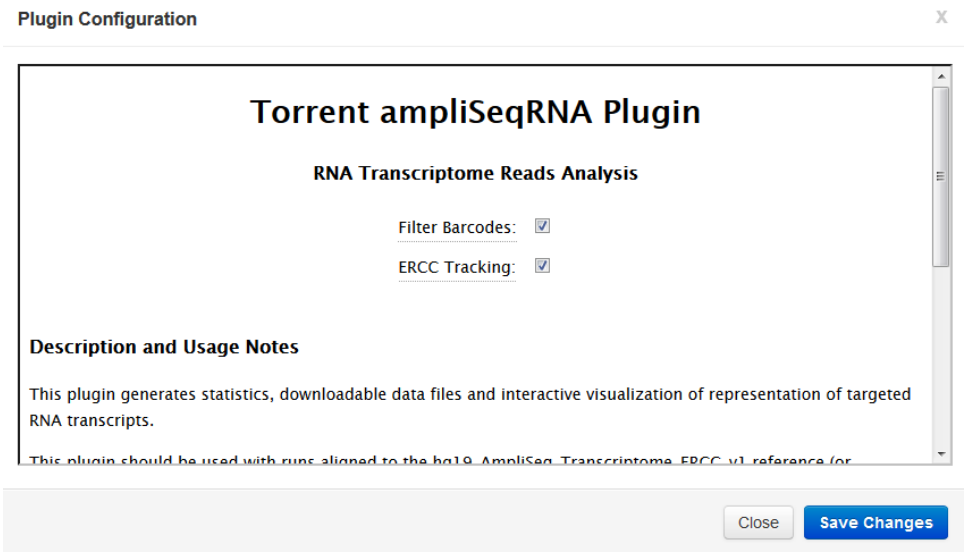
There are two ways to run the Torrent ampliSeqRNA Plugin:

- Automatically, by selecting and configuring the plugin during run planning or
- Manually, allowing you to run the plugin at any time from a completed run report.

1. To run the ampliSeqRNA plugin automatically during run analysis, select the **ampliSeqRNA** checkbox in the **Plugins** chevron in the template or Create Plan Wizard. After selecting the checkbox, a **Configure** link appears:



2. Click **Configure** to open the Torrent ampliSeqRNA Plugin configuration dialog:



3. Select the checkbox for one or both Filter Barcodes and ERCC Tracking.
    - **Filter Barcodes** filters low frequency barcode reads, which might result from barcode contamination, from subsequent analysis.
    - **ERCC Tracking** is recommended if you spiked your RNA amplicon library with ERCC tracking targets. The analysis produces an extra statistic for reads mapped to these targets so that the true level of "off-target" reads is accurately represented. Your reference should include ERCC references for ERCC reads to be included in the fraction of reads considered "on-target".
4. Click **Save Changes**.

Variant analysis using Ion Reporter™ software

Ion Reporter™ software comprises a suite of bioinformatics tools that streamlines and simplifies data analysis, annotation, and reporting of Ion semiconductor sequencing data. Ion Reporter™ software helps you to interpret DNA variants faster and more consistently.

The software allows you to integrate your variants with comprehensive public and curated annotations, along with your own lab-specific content. Additionally, analysis modules and parameters are configurable, allowing you to customize workflows as needed.

All the steps, from data import to annotating variants, are automated in the Ion Reporter™ software workflow. Key features include the following:

- Detection of SNPs and Indels
- Detection of Copy Number Variants (CNVs) on fixed Ion AmpliSeq™ panels and on custom Ion AmpliSeq™ panels that have been designed for copy number detection.
- Tumor : normal analyses
- Multi-sample analyses, including:
    - Tumor : normal workflow to detect somatic variants present only in a Tumor sample and not in its matched Normal sample
    - Genetic Trio workflow, to identify, annotate, and filter variants of special interest in Trio analysis (Sample, Father and Mother).

Visit **ioncommunity.thermofisher.com/community/products/software/ion_reporter** for more information about Ion Reporter™ software.

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.

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

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  - Certificates of Analysis
  - 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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